Short communication: Determination of withdrawal time for oxytetracycline in different types of goats for milk consumption

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ABSTRACT

Antibiotics are widely used in animal husbandry and the presence of antibiotics in milk is a health hazard. The objective of this study was to determine residual amounts of oxytetracycline in fresh, aged, and pasteurized milk of 3 breeds of goats using HPLC analysis. It was also essential to determine the safe withdrawal period of oxytetracycline in lactating goats. The quantitative results obtained using the HPLC system were compared with the tolerance limit of oxytetracycline in milk in the United States. Fifteen milking does, 5 Nubians, 5 Alpines, and 5 LaManchas were randomly selected from the milking herd at the International Goat Research Center at Prairie View A&M University. A simple sample preparation and isocratic HPLC method using ultraviolet detection was used for analysis of milk samples. The HPLC results indicated that the withdrawal period of oxytetracycline in treated Alpine does was 82 h (7 milking), whereas for Nubian does the period was 58 h (5 milking), and for LaManchas the period was 72 h (6 milking) after drug administration. The overall withdrawal period for all the treated goats of 3 breeds was 72 h. Although these results indicated that the depletion rate of this antibiotic was faster in goats than the reported data for cows, the 96-h withdrawal period that is currently used for lactating cows is still necessary for these 3 breeds of goats. Additionally, our results indicated that oxytetracycline is not stable in goat milk at refrigeration temperature or during pasteurization and will decrease significantly.

Key words: oxytetracycline, residues, goat milk, withdrawal period

Short Communication

Milk has been an important food for humans since the domestication of dairy animals. It is a common component of the animal-derived food products that comprise many diets. Methods to ensure the safety and

quality of goat milk and milk products are necessary with the expansion of goat dairy industry and use of goat milk in various sectors of food processing. Therefore, it is important to make sure that marketed goat milk is unadulterated and safe for human consumption.

Antibiotics are widely used in animal husbandry for the treatment of diseases, health maintenance, and, in some countries, at subtherapeutic levels as feed additives to suppress undesirable bacteria (Pharmacia and Upjohn, 1998; Kelly et al., 2004; Sawant et al., 2005). The improper use of antibiotics for the control of diseases such as mastitis is the major source of drug residues found in milk (Sawant et al., 2005; HHS, 2010). Concern exists that antibiotic residues in foods could significantly shift the resistance patterns in the microbial population in the human intestinal tract (Kelly et al., 2004; Jones 2009). The concerns about antibiotic resistance exist from food-borne pathogens such as *Salmonella*, *Campylobacter*, and enterococci (Pharmacia and Upjohn, 1998; HHS, 2010).

The presence of antibiotic residues in milk is known to interfere with the manufacture of several fermented dairy products by inhibiting starter activity, which can lead to monetary losses (Mayra-Makinen, 1995; Hays, 2003; Jones, 2009). Milk supplies containing antibiotics above certain concentrations are illegal. The maximum concentrations of residues are set for different antibiotics so that no unintended harmful effects are likely to occur from these drugs (Hays, 2003; FDA, 2013).

Tetracyclines are a group of broad-spectrum antibiotics that are active against gram-positive and gram-negative bacteria such as chlamydia, mycoplasmas, rickettsiae, and protozoan parasites. The tetracycline group is currently used to treat goats for diseases such as mastitis, pink eye, and urinary and enteric infections (Pharmacia and Upjohn, 1998). They are generally regarded as relatively nontoxic, but they could produce a large number of adverse effects, some of which can be life threatening under certain circumstances. Those effects include superinfection, diarrhea, ingestion, direct toxicity, irritation, dizziness, antianabolic effects, photosensitivity, and allergic symptoms in humans.

Current methods for detection of antibiotic residues in milk include tests such as microbiological inhibition

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tests, immunoassay tests, and chemical or physical methods such as spectrophotometric and chromatographic methods (Podhoriak et al., 1999; Sierra et al., 2009; Beltrán et al., 2013). The available microbiological tests are nonspecific for some antibiotics, such as tetracyclines and penicillins, whereas immunoassays are usually quite expensive. A quantitatively accurate chemical method for detecting antibiotics in milk is HPLC. Considerable progress has been reported in the development of HPLC methods for determination of tetracyclines and other antibiotics in both milk and meat tissues (Furusawa, 2003; Andersen et al., 2005; Fritz and Zuo, 2007; Fletouris and Papapanagiotou, 2008).

The Food and Drug Administration (FDA) established guidelines for the use of oxytetracycline (Liquamycin LA-200; Pfizer, Brazil) injections in lactating dairy cows in 1998; prior to that, oxytetracycline was only approved for beef animals (FDA, 1998). With this approval, oxytetracycline is labeled for administration to beef and dairy animals for treatment of pneumonia, shipping fever complexes associated with *Pasteurella* spp., *Haemophilus* spp., pink eye, foot rot, bacterial enteritis, leptospirosis, wound infections, and acute metritis. The FDA and dairy industry set a new tolerance level of 300 ng/mL for tetracyclines in milk when the approval notice for oxytetracycline was published in the FDA (2013). In the Codex Alimentarius Commission (2009), the European Union, and other regulatory organizations in some countries, the maximum residue level for oxytetracycline in milk is 100 ng/mL (Veterinary Drug MRL Database; www.mrldatabase.com).

The withdrawal period is the waiting time that must elapse before treated animals or their products can be processed for human consumption (Pharmacia and Upjohn, 1998). The withdrawal period for this antibiotic for lactating cows is 96 h after the last treatment. However, the withdrawal period for this antibiotic has not been determined for milking goats. Goat milk producers currently use the withdrawal time recommended for lactating cows. Currently, considerable variation exists in the management practices associated with antibiotic use on dairy farms (Sawant et al., 2005). Goats are considered minor species and no FDA-accepted screening test exists for tetracyclines in goat milk (FDA, 1998). It is important to quantitatively determine the exact residual amounts of these antibiotics in goat milk and to calculate the safe withdrawal period of treated lactating goats. Information on the safe withdrawal periods for antibiotic residues in goat milk is limited and to our knowledge the depletion rate of oxytetracycline in the Alpine, Nubian, and LaMancha breeds of goats has not been previously determined. Therefore, the objectives of our study were (1) to determine the residual

amounts of oxytetracycline in the milks of Alpine, Nubian, and LaMancha goats for a period of time with a quantitative technique (HPLC), (2) to determine the safe withdrawal periods of oxytetracycline residues in milk of Alpine, Nubian, and LaMancha breeds of goats treated intramuscularly with this antibiotic, and (3) to determine the levels of oxytetracycline residues in goat milk treated differently, such as fresh, 72-h raw milk, and pasteurized milk.

Animal Selection and Treatment

Fifteen milking does, 5 Nubian, 5 Alpine, and 5 LaMancha, were randomly selected from the herd at the International Goat Research Center at Prairie View A&M University. The selected does were average milk producers with BW ranging from 55 to 75 kg and were in mid lactation. Each goat was intramuscularly injected with oxytetracycline (Liquamycin LA-200; Pfizer Inc., New York, NY) in the hind leg according to animal BW. A dose of 8 mg of oxytetracycline per pound of BW was applied for all injections (1 mL/11.4 kg of BW) according to the manufacturer's recommendation. One milliliter of oxytetracycline contained 200 mg of amphoteric oxytetracycline base in the aqueous solution (manufacturer's certified concentration). Each doe was given 2 antibiotic injections, 1 in the evening before milking and the second injection 48 h later according to the recommended therapeutic practice.

Sample Collection

The treated goats were hand milked and a sample from each goat was placed on ice in a container and brought to the laboratory for analysis. Milk samples were collected twice daily in the mornings and evenings after the second antibiotic injection for up to 138 h. Each milk sample was divided into 3 portions. One portion of raw milk sample was aged in the refrigerator (at 4°C) for 72 h before analysis, whereas the second portion of sample was pasteurized (at 63°C for 30 min) and frozen until analysis. The third portion of the sample was analyzed the day they were collected. All fresh milk samples from treated animals were analyzed for oxytetracycline residues until depletion using the HPLC system.

Sample Extraction

Five milliliters of milk were deproteinized by mixing with 1 mL of 1 M HCl and then 15 mL of acetonitrile was added to the mixture according to the method of Moats and Harik-Khan (1995) that was modified. The modifications included filtering the supernatant (filter

paper #541, Whatman International Ltd., Maidstone, UK) and collecting approximately 12 mL of filtrate. The filtrate was placed into the Rotavapor concentrator (Buchi, RE-200, Flawil, Switzerland) flask for evaporation under reduced pressure. The sample flask of the Rotavapor was placed in a distilled water bath at 36°C and then the vacuum to the Rotavapor concentrator was applied by an aspirator pump (Cole-Parmer Instrument, model 7049-00, Vernon Hills, IL). The contents were evaporated to approximately 1 mL or slightly less (but not to dryness) and were quantitatively transferred into a graduated evaporator receiver tube. The final volume of the extract was adjusted exactly to 1 mL with small rinses of distilled water. The extract was filtered through a 25-mm Acrodisc (0.45 µm HT Tuffryn membrane, Life Sciences, NY). The filtered samples were loaded into HPLC vials for analysis.

Standard Preparation and Spiking of Milk

Antibiotic-free milk (control) was collected from the selected does before drug injection and used for preparation of standards with oxytetracycline at known concentrations of 100, 200, 300, 400, and at 500 ng/mL according to the referenced method (Moats and Harik-Khan, 1995). The previous standards were prepared from oxytetracycline with certified concentration (200 mg/mL) by dilution in mobile phase. Five milliliters of control milk samples were spiked at concentrations of 100, 200, 300, 400, and 500 ng/mL and then were extracted and analyzed by the following HPLC method.

HPLC Analysis

The Waters HPLC system (Waters, Milford, MA) with 515 pump, 2489 UV detector, 717 autosampler, and Empower 2 software were used for analyses. A ProteCol-GP C18 125 (150 × 4.6 mm ID, 5 µm 120A; SGE Analytical Sci, Austin, TX) analytical column was used. The mobile phase was prepared by mixing 65% 0.02 M H₃PO₄ and 35% 0.01 M Na decanesulfonate-acetonitrile according to the reference method. The mobile phase was equilibrated for 30 min before sample injection. The flow rate was 1 mL/min with the UV detection set at 380 nm. Oxytetracycline standards were analyzed at the beginning and at the end of each day to get accurate quantification. A 50-µL portion of each standard and sample was injected into the system. Sample analyses were performed after duplicate standards with known concentrations were analyzed. A linear regression equation was established for each day of analysis using the software and peak areas and concentrations of standards. The concentrations of oxytetracycline residues in samples were calculated using

the linear regression equation for each day of analysis. The correlation coefficient (*r*) of the curve for each day of analysis ranged from 0.88 to 0.99 and could slightly differ due to HPLC response sensitivity and variability in sample extraction.

Statistical Analysis

The following model was used to analyze data by PROC MIXED procedure of SAS (2010) for repeated measures and LSMEANS for differences between the concentrations of residual oxytetracycline in the fresh milk of 3 breeds of goats (Table 1):

$$Y_{ijk} = \mu + B_i + E_{ik} + H_j + (B \times H)_{ij} + E_{ijk},$$

where Y_{ijk} = mean concentration of antibiotic residues, μ = population mean, B_i = breed effect with 3 categories ($i = A, N, L$; number of animals $k = 5/\text{breed}$), H_j = hours after injection of antibiotic ($j = 10, 24, 34, 48, 58, 72, 82, \text{ and } 96$), $(B \times H)_{ij}$ = interaction effect of breed and hours after antibiotic injections, and E_{ijk} = random error term.

The data for concentrations of residual oxytetracycline in fresh, pasteurized, and 72-h raw milk were analyzed by factorial design using the PROC GLM procedure of SAS (2010) and the LSMEANS for detecting differences between means of the residual oxytetracycline in fresh, 72-h aged, and pasteurized milks of the 3 breeds of goats (Table 2):

$$Y_{itk} = \mu + B_i + M_t + (B \times M)_{it} + E_{itk},$$

where Y_{itk} = mean concentration of antibiotic residues, μ = population mean, B_i = breed effect with 3 categories ($i = A, N, L$; number of animals $k = 5/\text{breed}$), M_t = milk type ($t = \text{fresh, pasteurized, or 72-h raw milk}$), $(B \times M)_{it}$ = interaction effect of breed and milk type, and E_{itk} = random error term.

The residual concentrations of oxytetracycline in goat milk up to 96 h after treatment were determined using the modified HPLC technique (Moats and Harik-Khan, 1995) that was sensitive to detect concentrations below the requirement of regulatory agencies. The results indicated that the withdrawal period of oxytetracycline in treated Alpine does is 82 h (7 milking), whereas for Nubian goats it is 58 h (5 milking) after drug treatment (Table 1). The LaMancha does treated at 72 h after injection (6 milking) had residual antibiotics lower than the tolerance level. The mean concentration of oxytetracycline for the Alpine breed was 273 ng/mL after 82 h of drug administration, which is below the tolerance level. Whereas the mean concentration of oxytetracycline in the milk of the Nubian breed was 181 ng/mL

Table 1. Concentrations (mean $\pm$ SD) of residual oxytetracycline (ng/mL) in the milk of 3 breeds of goats separately and all treated goats of 3 breeds at different milking intervals ($n = 3$ replicates)

Time (h)	Breeds of goat			
	Alpine	Nubian	LaMancha	All breeds
10	1,567 ^a $\pm$ 209	1,406 ^a $\pm$ 330	1,732 ^a $\pm$ 209	1,569 ^a $\pm$ 148
24	1,537 ^a $\pm$ 209	1,576 ^a $\pm$ 382	1,329 ^b $\pm$ 209	1,481 ^a $\pm$ 163
34	1,315 ^a $\pm$ 209	1,080 ^a $\pm$ 330	1,019 ^b $\pm$ 209	1,138 ^b $\pm$ 148
48	599 ^b $\pm$ 209	431 ^b $\pm$ 330	379 ^{cd} $\pm$ 209	469 ^c $\pm$ 148
58	305 ^{bc} $\pm$ 209	181 ^b $\pm$ 330	558 ^c $\pm$ 209	348 ^c $\pm$ 148
72	333 ^{bc} $\pm$ 209	245 ^b $\pm$ 330	281 ^{cd} $\pm$ 209	286 ^{cd} $\pm$ 148
82	273 ^{bc} $\pm$ 209	204 ^b $\pm$ 330	271 ^{cd} $\pm$ 209	249 ^{cd} $\pm$ 148
96	93 ^c $\pm$ 270	81 ^b $\pm$ 330	87 ^d $\pm$ 270	87 ^d $\pm$ 169

^{a-f}Means within each column with different superscripts are different ($P < 0.05$).

after 58 h of injection, that for the LaMancha breed was 281 ng/mL after 72 h of injection. A difference was noted between the withdrawal periods for Alpine, LaMancha, and Nubian breeds of goats. According to our study, the withdrawal period for the Alpine goats is longer than the other 2 breeds by 1 or 2 milking periods.

The residual concentrations of oxytetracycline were not different ($P < 0.05$) between the 3 breeds of goats at each milking interval. The residual concentration of oxytetracycline in milk is at the highest level at 10 h after treatment and then decreases as time elapses. The mean concentrations of oxytetracycline in the milk of Nubian goats differed ($P < 0.05$) between 10 and 48 h after drug administration. Likewise, the mean concentrations of oxytetracycline in the milk of Alpines differed ($P < 0.05$) between 10 and 48 h after drug injection, whereas for the LaMancha does the concentrations of antibiotic differed ($P < 0.05$) between 10, 24, and 48 h after treatment. The rate of decrease of oxytetracycline in milk is higher at the initial milking times until 48 h elapses and then the rate of decrease slows down (Table 1). This difference in the depletion rate of oxytetracycline from milks of these animals could be due to the genetic variation of the 3 breeds or differences in the rates of metabolism of these breeds.

However, the mean concentration of drug residue in all of the treated doses of the 3 breeds of goats was 286 ng/mL at 72 h after injection, which is lower than the

tolerance level of this antibiotic in milk (Table 1). The overall results indicated that the withdrawal period for oxytetracycline in all the treated goats of 3 breeds is 72 h (6 milkings) after injection. Payne et al. (2002) intramuscularly injected 8 milking goats of mixed breed with the same formulation of oxytetracycline that was used in our study and determined the concentrations of drug residues in milk over time. They got similar results to ours using a different HPLC technique. Similarly, Rule et al. (2001) intramuscularly injected 5 lactating goats of Murciano-Granadina breed with long-acting formulation of oxytetracycline and based on a screening test (Delvotest SP, DSM Food Specialties, Delft, the Netherlands) concluded that milk of these animals should not be used for human consumption for 3 d (72 h). The mean concentrations of the residues were higher at the beginning of the study and then started decreasing significantly between 10, 34, and 48 h after drug administration (Table 1). The HPLC method is quantitative and can be used to check the performance of commercial screening tests as to whether or not violative residues are actually present in goat milk.

The concentrations of residual oxytetracycline in fresh, pasteurized, and raw aged milks were assessed for each breed of goats for the milk obtained 10 h after treatment (Table 2). The concentrations of residual antibiotic in fresh, pasteurized, and raw aged goat milks differed significantly ($P < 0.05$) for the Alpine and LaMancha breeds of goats, whereas the concentra-

Table 2. Concentrations (mean $\pm$ SD) of residual oxytetracycline (ng/mL) in fresh, pasteurized, and raw aged goat milks of 3 breeds separately and all treated goats¹

Milk type	Alpine	LaMancha	Nubian	All treated goats
Fresh	1,529 ^a $\pm$ 244	1,838 ^a $\pm$ 267	1,782 ^a $\pm$ 388	1,716 ^a $\pm$ 177
Pasteurized	860 ^b $\pm$ 244	806 ^b $\pm$ 244	587 ^b $\pm$ 414	751 ^b $\pm$ 180
Aged	341 ^c $\pm$ 244	368 ^c $\pm$ 244	549 ^b $\pm$ 414	419 ^c $\pm$ 180

^{a-c}Means with the same superscripts are not different ($P < 0.05$).

¹The samples came from the 10-h withdrawal period. Number of replicates = 3.

Figure 1. A representative chromatogram of control milk sample with no oxytetracycline.

Figure 2. A representative chromatogram of a milk standard with 300 ng/mL of oxytetracycline. The retention time for the oxytetracycline peak is 5.929 min.

Table 3. Recovery of oxytetracycline spiked in milk (ng/mL) as standards by the analytical system using ($n = 6$) replicates

Quantity added	Quantity calculated	Deviation (%)	Mean correlation coefficient
100	109.06	9.06	88.8
200	216.14	8.07	
300	275.65	8.11	
400	366.02	-8.50	
500	520.06	4.01	

tion of oxytetracycline for fresh milk was different from that of pasteurized or raw aged milk for the Nubians. The residual concentrations of oxytetracycline were not different between the 3 breeds of goats for fresh or raw aged milks. The pasteurized milk for Nubians differed from the Alpines or LaManchas. The concentrations of oxytetracycline in the fresh, pasteurized, and raw aged milks from the 10-h milking in all the treated goats of 3 breeds (Table 2) showed that they are all significantly different ($P < 0.05$). It is conceivable that the heat of pasteurization could have reduced the level of natural inhibitory enzymes in milk or the level of this antibiotic in pasteurized goat milk. Yamaki et al. (2004) and Molina et al. (2003) also noticed that the level of positive and doubtful responses to antibiotic residue screenings of Delvotest SP and BRT AiM (AiM-Analytik in Milch Produktions-und Vertriebs GmbH, Munchen, Germany) were lower in ewe milk heated at 82°C for 10 min compared with unheated milk. However, 72-h aging of raw goat milk in the refrigerator was not expected to have significantly lowered the level of oxytetracycline compared with fresh or pasteurized milks. We can only speculate that either raw milk natural enzymes or microbial population may have reduced the level of this antibiotic compared with other milks. Podhorniak et al. (1999) also noticed that tetracycline was not stable in raw cow milk under storage conditions in the refrigerator. This finding will have a profound implication on the level of oxytetracycline in marketed goat milk, as commercial goat milk is usually stored at refrigerated temperatures for several hours in the bulk tank of dairy farms before it is picked up and then stored at the milk cooperative collection point before it is processed. During this delay, which could be as long as 72 h, the concentration of oxytetracycline will go down considerably if present in raw goat milk.

The results of our study indicated that the concentrations of oxytetracycline residues in goat milk using the HPLC analytical system was under the US tolerance limit at 82, 72, and 58 h after injection for the Alpines, LaMancha, and Nubian breeds of goats, respectively. Likewise, the overall HPLC results for all the treated does of 3 breeds indicated that oxytetracycline residues

in the milk of lactating goats was lower than the US tolerance limit at 72 h after injection. The results of the present study clearly indicates that the 96-h withdrawal period that was originally designed for lactating cows is applicable to lactating goats and increases the margin of safety for human consumption.

Comparing the chromatograms of control milk with that of spiked standards indicated that oxytetracycline has a sharp peak at concentration of 300 ng/mL (Figures 1 and 2). The antibiotic peak was clear with a high degree of resolution, and our analytical system was sensitive in detecting oxytetracycline residues below the tolerance level up to 100 ng/mL. The repeatability of the technique was measured with 6 sets of spiked milk standards (Table 3). The range of correlation coefficient (r) values for the calculation of oxytetracycline in milk samples were from 0.88 to 0.99. At 110 h after drug administration (9 milkings), the residues in milk could no longer be detected by the HPLC method.

In conclusion, the modified HPLC method was able to determine the different withdrawal periods of the 3 breeds of goats. The results of our study indicated that the withdrawal period for oxytetracycline residues in milk was 58 h for the Nubian breed, 72 h for the LaMancha breed, and 82 h for the Alpine breed of lactating does. The results indicated that the withdrawal period for oxytetracycline residues in goat milk for all the treated does of 3 breeds was 72 h (6 milkings). The overall withdrawal period that was determined by this technique is shorter for lactating goats than the 96-h withdrawal period that has been practiced for lactating cows. Our results indicated that adoption of cow dairy regulation on oxytetracycline to dairy goats will meet the US regulatory requirement of less than 300 ng/mL that is safe for human consumption.

Additionally, the results of the current study indicated that oxytetracycline is not stable in goat milk at refrigerated temperatures and decreases significantly compared with fresh or pasteurized milk. Commercial goat milk is exposed to several hours of refrigerated storage at bulk tanks of milk-producing farms and then at the silo of milk cooperatives before it is processed. This usual delay in processing of commercial goat milk will significantly reduce the amount of oxytetracycline, if any is present in raw milk. If pasteurized milk is produced, heat of pasteurization will further reduce any oxytetracycline that may be present in raw goat milk.

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