Determination of Tilmicosin Residues in Cow and Sheep Milk by Liquid Chromatography

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This method was developed and validated to detect and quantitate tilmicosin residues in cow milk over a range of 0.010–10 µg/mL, and in sheep milk over a range of 0.025–0.5 µg/mL. The procedure involves extracting the milk sample with acetonitrile and using a C18 cartridge to perform a solid-phase extraction cleanup of the extract. A reversed-phase gradient liquid chromatography method with detection at 280 nm is used to separate the tilmicosin from matrix components in a 30 min run time. The limit of quantitation of the method is 0.010 µg/mL for cow milk, and 0.025 µg/mL for sheep milk. Average percentage recoveries for milk samples ranged from 82 to 94%. Percentage relative standard deviation values ranged from 3.1 to 17.2%.

Tilmicosin (20-deoxo-20-(3,5-dimethyl piperidin-1-yl)-desmycosin) is a semisynthetic macrolide antibiotic (1) that has a spectrum of microbiological activity against Pasteurella spp., Mycoplasma spp., and a variety of Gram-positive organisms (2). Figure 1 shows the structure of tilmicosin. Tilmicosin is composed of cis- and trans-piperidinyl isomers in a ratio of approximately 85 to 15. A bovine injection formulation of tilmicosin (Micotil®, Eli Lilly and Co., Greenfield, IN) has been approved in several countries to treat Pasteurella haemolytica-caused bovine respiratory disease in beef cattle (3–5). Micotil is also approved in some countries outside the United States for use in sheep (6). A Type A medicated article (premix) formulation of tilmicosin (Pulmotil®, Eli Lilly and Co.) has been developed as a feed additive to control porcine bacterial pneumonia caused by Actinobacillus pleuropneumoniae and Pasteurella multocida.

Methods for tilmicosin in milk (7–9) have been previously reported. This study investigates a method to determine tilmicosin in cow and sheep milk. Compared to previously published methods, this method is more sensitive and specific, and can be used with both cow and sheep milk. Previous methods reported limits of quantitation (LOQs) of 0.020 µg/mL and higher for bovine milk and 0.050 µg/mL for sheep milk, with limits of detection (LODs) of 0.0125 µg/mL and higher. The method reported here, which uses extraction and cleanup conditions based on a previously published tilmicosin tissue method (10), has an LOQ for bovine milk of 0.010 µg/mL, an LOQ for sheep milk of 0.025 µg/mL, and an LOD of 0.0036 µg/mL. This method also offers greater specificity for tilmicosin over matrix components than previously reported methods. In addition, specificity of the chromatographic conditions for tilmicosin over a wide variety of mastitis drugs is presented. The method is validated for cow and sheep milk with respect to linearity, accuracy, precision, sensitivity, and specificity.

Experimental

Reagents

Reagents should be prepared in an approved fume hood with appropriate personal protective equipment.

(a) Water (H2O).—LC grade, Milli-Q filtered, or distilled deionized are acceptable.

(b) Solvents.—Methanol and acetonitrile, LC grade.

(c) Acids.—Phosphoric acid and glacial acetic acid, ACS grade.

(d) Dibutylamine.—99+% pure (Aldrich Chemical Co., Milwaukee, WI; Cat. No. D4, 495-2). Caution: Toxic, possible mutagen.

(e) Tilmicosin reference standard.—The total tilmicosin potency is the sum of potency from the cis- and trans-isomers (Eli Lilly and Co.).

(f) Drugs for method specificity testing.—Hydrocortisone, oxytetracycline, neomycin sulfate, prednisolone, sulfadiazine, nafcinil, cloxacillin, erythromycin, chlorotetra-cycline, penicillin-G, dihydrostreptomycin, cephalirin, streptomycin, cefuroxime, cefoperazone, amoxicillin, ampicillin, novobiocin, and trimethoprim (Sigma Chemical Co., St. Louis, MO).

(g) 25% Acetonitrile in water (v/v).—If stoppered tightly, this solution is stable for 2 weeks.

(h) 5% Acetic acid in methanol (v/v).—If stoppered tightly, this solution is stable for as long as 2 weeks.

(i) 1M Dibutylammonium phosphate buffer (DBAP).—To 700 ± 10 mL LC grade H2O, add 168 ± 2 mL dibutylamine. (One mole dibutylamine is equal to 168.5 mL.) Add about 70 mL phosphoric acid slowly while stirring to completely dis-
solve the dibutylamine. It is critical to allow the solution to cool to room temperature after adding the acid and before adjusting the pH. Adjust the pH to 2.5–2.6 by adding about 18 mL H$_3$PO$_4$ in 1–2 mL increments. Transfer the solution to a 1 L graduated cylinder. Dilute to 1000 ± 10 mL with H$_2$O, mix, and filter under vacuum. If stopped tightly, this solution is stable for as long as 6 months when stored at ambient temperature. Caution: This solution should be prepared in a chemical fume hood; use appropriate eye protection, impervious gloves, and a lab coat.

(j) Mobile phase A: acetonitrile.—Filter and degas the acetonitrile with the appropriate apparatus. If stopped tightly, this solution is stable for 12 months when stored at ambient temperature.

(k) Mobile phase B: H$_2$O.—Filter and degas with the appropriate apparatus. If stopped tightly, this solution is stable for 2 weeks when stored at ambient temperature.

(l) Mobile phase C: 0.02M DBAP solution.—Dilute 20 ± 1 mL 1M DBAP solution (refer to reagent [i]) to 1000 ± 10 mL with H$_2$O. Filter and degas with the appropriate apparatus. If stopped tightly, this solution is stable for 1 week when stored at ambient temperature. Caution: This solution should be prepared in a chemical fume hood; use appropriate eye protection, impervious gloves, and a lab coat.

Apparatus

(a) Analytical balance ±0.0001 g.
(b) pH meter.
(c) Magnetic stirrer and stir bars.
(d) Vacuum oven.—Capable of maintaining a temperature of 60° ± 5°C and a minimum vacuum of –26 in. Hg.
(e) Actinic glassware or glassware covered with aluminum foil.
(f) Ultrasonic generator and probe.—Such as Branson Sonifier 450 with a 1/4 or 1/8 in. microtip (Branson Ultrasonics, Danbury, CT).
(g) Centrifuge.—Capable of providing at least 2000 rpm (relative centrifugal force, about 500–600 × g) with 50 mL disposable plastic centrifuge tubes.
(h) Solid-phase extraction (SPE) cartridges.—The following SPE cartridges gave acceptable precision and accuracy with this method:

- Waters Sep-Pak Plus C$_{18}$, Part No. WAT020515
- Varian Bond Elut C$_{18}$/OH, Part No. 1211-3045. Chan et al. (10) performed their validation with these cartridges.
- Supelco Supelclean LC$_{18}$, Part No. 57054
- Fisher PrepSep R-C$_{18}$, Part No. P463R

The following SPE cartridges did not give acceptable precision and accuracy with this method:

(i) Large volume plastic reservoirs.—To fit SPE cartridges.
(j) Frits.—20 µm pore size, to fit SPE cartridges (for example, Varian Part No. 1213-1024).
(k) Vacuum manifold system.—Capable of processing several samples at once.
(l) Centrifuge tubes.—50 mL disposable plastic.
(m) PTFE filters.—0.45–0.50 µm (e.g., Gelman Acrodisc CR, Product No. 4422).
(n) Volumetric flasks.—2 mL.
(o) Assorted laboratory glassware and pipets.—Volumetric pipets and volumetric flasks of nonstandard sizes are available through Sun Brokers, Inc., Wilmington, NC.
(p) Micropipettor.
(q) Filtering and degassing apparatus.—0.45 µm Nylon-66 filters.
(r) LC autosampler or manual injector.
(s) LC pump.—Capable of ternary gradient elution solvent delivery.
(t) LC variable wavelength UV detector.
(u) LC detector recording device.
(v) LC column.—25 cm × 4.6 mm id Spherisorb Phenyl, 5 µm packing or equivalent.

Standard Preparation

Standards should be prepared in an approved fume hood or ventilated balance enclosure with appropriate personal protective equipment. Standards may be prepared in different volumes, provided the ratio of components remains the same.
Perform these steps under conditions that protect the samples from bright light. Actinic glassware or glassware covered with aluminum foil should be used for all standard preparations.

(a) Dried tilmicosin reference standard.—Place at least 50 mg tilmicosin reference standard in an actinic glass bottle. Cover the mouth of the open bottle with a piece of filter paper and secure it with a rubber band. Place in a vacuum oven at 60° ± 5°C and at a gauge pressure of at least 26 in. Hg (660 mm Hg) for at least 3 h. Remove the reference standard from the oven and allow to cool to room temperature in a desiccator (about 30 min). Tilmicosin reference standard should be dried immediately before use in preparation of stock standard solution.

(b) Stock standard solution, 250 µg/mL.—Accurately weigh an amount of dried tilmicosin reference standard equivalent to about 25 mg when compensated for purity and transfer to a 100 mL volumetric flask. Dissolve and dilute to volume with methanol. Stopper the flask tightly. Tilmicosin standard solutions of this concentration are stable when stored at 4°C in a light-protected environment for 3 months. Discard any unused portion of the dried reference standard.

(c) Fortifying standard solution, 0.5 µg/mL.—Remove the 250 µg/mL stock standard solution from the refrigerator and allow to warm to room temperature. Pipet 4.00 mL 250 µg/mL stock standard solution into a 20 mL volumetric flask and dilute to volume with methanol. The concentration of this intermediate standard solution is 50 µg/mL. Using a volumetric pipet, add 1.00 mL 50 µg/mL intermediate standard solution to a 100 mL volumetric flask, and dilute to volume with methanol. This fortifying standard solution (concentration, 0.5 µg/mL) will be used to fortify the milk standard recovery sample. Use the actual concentration for the stock standard in calculating the concentration of the fortification standard. This solution is stable for 8 days when stored in a light-protected environment at room temperature.

(d) Working standard solutions, 0.025, 0.0375, 0.05, 0.25, 0.5, 2.5, and 5.0 µg/mL.—Using volumetric pipets and flasks, make dilutions with 5% acetic acid in methanol as outlined in Table 1. Use the actual concentration for the stock standard in calculating the concentration of the working standards. Tilmicosin standard solutions of these concentrations are stable when stored in a light-protected environment for 8 days at room temperature.

### Milk Samples

(a) Milk sample storage.—Store all test milk at freezer temperatures (about −20°C) until it is ready to be defrosted for assay.

(b) Preparation of recovery and negative control samples.—On the day of analysis, prepare one recovery sample of milk to be analyzed. Fortify untreated control milk with 0.5 µg/mL fortifying standard solution to obtain a 0.040 µg/mL sample. Fortify with a micropipettor at a rate of 800 µL solution/10 mL milk. Include an untreated (negative) control milk sample with each set of treated samples.

(c) Sample preparation.—Perform all steps from this point on under conditions that protect the samples from bright light.

Defrost the test milk. Vigorously shake or stir the completely defrosted milk in the container to homogenize the sample. Immediately pipet 10.0 mL milk into a 50 mL centrifuge tube by volumetric or mechanical pipet. Add 30 ± 1 mL acetonitrile to the sample. Sonicate using an ultrasonic probe for about 30 s to adequately homogenize the sample. (Eliminating the sonication step leads to lower recovery.) Take care not to overheat the sample during sonication at this step; the sample should become only slightly warm to the touch. Centrifuge at 2000 rpm (relative centrifugal force about 500–600 x g) for 10 min. Exact speed, centrifugal force, size, or spin time is not critical and may be adjusted, provided the sample is sedimented to give a clear supernate. Decant any unused portion of the dried reference standard.

Following application of the sample extract to the SPE cartridge, wash the cartridge with about 10 mL water as an interference elution solvent. Flow rate is not important for this step. Then wash the cartridge with about 10 mL 25% acetonitrile in water no faster than about 5 mL/min.

After the 25% acetonitrile in water has drained from the SPE cartridge, allow the SPE cartridge to dry at high vacuum (at least 5 in./130 mm Hg) for at least 1 min. Once the SPE cartridge has dried, turn off the vacuum and elute the tilmicosin with 5% glacial acetic acid in methanol. Fill a small syringe with about 3 mL elution solution. Remove the SPE cartridge from the manifold and connect it to the syringe. Hold the outlet of the SPE cartridge over the mouth of a 2 mL volumetric flask and slowly depress the plunger on the syringe to push the eluent through the cartridge. Collect the eluent in the flask until the liquid level just reaches the vol-

### Table 1. Tilmicosin working standard dilutions

<table>
<thead>
<tr>
<th>Final concn, µg/mL</th>
<th>Curve for which std. is used</th>
<th>Concн std. added, µg/mL</th>
<th>Volume std. added, mL</th>
<th>Final vol. of std., mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>High</td>
<td>250</td>
<td>1.00</td>
<td>50</td>
</tr>
<tr>
<td>2.50</td>
<td>High</td>
<td>5.00</td>
<td>10.0</td>
<td>20</td>
</tr>
<tr>
<td>0.500</td>
<td>High</td>
<td>5.00</td>
<td>2.00</td>
<td>20</td>
</tr>
<tr>
<td>0.250</td>
<td>Low, high</td>
<td>5.00</td>
<td>1.00</td>
<td>20</td>
</tr>
<tr>
<td>0.050</td>
<td>Low</td>
<td>5.00</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>0.0375</td>
<td>Low</td>
<td>0.500</td>
<td>3.00</td>
<td>40</td>
</tr>
<tr>
<td>0.0250</td>
<td>Low</td>
<td>0.500</td>
<td>1.00</td>
<td>20</td>
</tr>
</tbody>
</table>
ume marker. (Not all of the elution solution in the syringe is needed to elute to the 2 mL mark.) The elution should take at least 10 s. The resulting sample may be colored or cloudy. Stopper the flask and invert it several times to mix the sample. Filter the sample with a 0.45 µm PTFE filter into an LC autosampler vial for assay. These extracts are stable for at least 7 days when stored at room temperature in a light-protected environment.

**Liquid Chromatography**

(a) **Operating conditions.**—Column: 25 cm × 4.6 mm id Sigma-Aldrich Spherisorb Phenyl 5 µm packing, or equivalent (Jones Apex Phenyl, 5 µm packing, is also suitable for this procedure). A zero volume in-line frit has been used to extend the life of the analytical column. The in-line frit can easily be changed if the back pressure becomes too high. Normal operating pressures of 2–3 kpsi can be expected with this method. If column is new, flush it before initial use. The recommended flushing procedure is 30 min with 100% acetonitrile at 2 mL/min followed by 30 min with 100% water at 2 mL/min.

Wavelength: 280 nm; flow rate: 1.5 mL/min; injection volume: 100 µL; column temperature: ambient; run time: 30 min; mobile phase A: acetonitrile; mobile phase B: water; mobile phase C: 0.02M DBAP solution; gradient conditions: See Table 2.

Presamples: At least 1 methanol injection should be used to equilibrate the column to the gradient conditions.

Initial instrument setup: The 0.02M DBAP solution will desorb compounds previously trapped in the system or column, producing a large shift in the baseline after the 0.02M DBAP solution is introduced. To correct this, flush the system and column with a mobile phase that is 25% acetonitrile and 75% 0.02M DBAP or repeat the gradient cycle until a satisfactory baseline is obtained. Approximately 45 min of flushing the system and column, or about 3 gradient cycles should be sufficient to clear up this problem.

Column storage: Short term or long term, the column can be stored with acetonitrile–water (1+1).

(b) **System suitability.**—System suitability should be checked at least daily or when any significant changes are made to the instrument set-up. As a test of system suitability, observe the retention time of the tilmicosin peak in a 100 µL injection of one of the standard curve solutions. To be acceptable, the retention time of the tilmicosin peak must fall in the range of 13–19 min. When the tilmicosin peak is eluted in the 13–19 min range, it is free of interference from sample matrix, related substances, degradation products, and other potential interfering compounds. Once the system suitability criteria are passed, the retention time of the tilmicosin peak should not fluctuate by more than about 0.2 min. See Figure 2 for an example chromatogram of a tilmicosin standard.

To correct system unsuitability, modify the 5–25 min gradient conditions to move tilmicosin to an appropriate retention time (13–19 min). Tilmicosin is strongly adsorbed to residual silanols; therefore, the amine modifier (0.02M DBAP) is necessary to displace the tilmicosin from the residual silanols. The retention time of tilmicosin may be shortened or lengthened by increasing or decreasing the amount of acetonitrile in the 5–25 min time window (11).

The column and chromatographic conditions were chosen to elute both the cis- and trans-isomers of tilmicosin as a single peak. With some columns, peak broadening, fronting, or tailing may be observed. If the same distortion in peak shape is observed in both standard and sample peaks, and the tilmicosin peaks can be appropriately integrated, quantitation is not precluded. To correct peak distortion, a new column is usually required.

(c) **Analytical procedure.**—Inject 100 µL tilmicosin system suitability standard and adjust the gradient, if necessary, as described above. Next, inject 100 µL working standard solutions (5.0, 2.5, 0.50, 0.25, 0.05, 0.0375, and 0.025 µg/mL), followed by a 100 µL injection of the control sample, recovery sample, and samples for analysis. Finally, inject an additional 100 µL of each working standard solution. Note: If analyzing more than 30 samples at once, run a third standard curve in the middle of the run.

**Calculations**

Integrate the area of the tilmicosin peak for the standards and samples. All standard injections, both before and after the samples, should be used in linear regression calculations. However, in the case of a lengthy run using 3 or more sets of standard curve solutions, the analyst may calculate results for a set of samples using only the bracketing curves. This is a preferred approach if the LC system sensitivity or peak retention time has drifted significantly during the course of a lengthy run. When the linear response of the standard curve is calculated over the entire standard curve concentration range, deviations from linearity at the lowest levels begin to occur. Therefore, it is much better to use 2 standard curves, 1 over the range of 0.025–0.25 µg/mL and the other over the range of 0.25–5.0 µg/mL. From the area of the standard solutions, calculate the 2 standard curves for tilmicosin response. Using the appropriate standard curve, calculate the

### Table 2. LC gradient conditions

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A, %</td>
</tr>
<tr>
<td>0.0</td>
<td>50</td>
</tr>
<tr>
<td>3.0</td>
<td>50</td>
</tr>
<tr>
<td>4.0</td>
<td>15</td>
</tr>
<tr>
<td>5.0</td>
<td>15</td>
</tr>
<tr>
<td>25.0</td>
<td>25</td>
</tr>
<tr>
<td>25.1</td>
<td>50</td>
</tr>
<tr>
<td>30.0</td>
<td>50</td>
</tr>
</tbody>
</table>

* A linear, gradient ramp is maintained between each time point.
Tilmicosin concentration in µg/mL with the following formula:

\[ \text{Tilmicosin concn, µg tilmicosin/mL milk} = \frac{(A - B)}{C \times D \times E/F \times G} \]

where \( A \) = area of the tilmicosin peak from the chromatogram; \( B \) = intercept of the standard curve; \( C \) = slope of the standard curve; \( D \) = final volume of sample extract (2.0 mL); \( E \) = dilution factor (mL dilution volume/mL sample extract); this factor is used only when high concentration cow milk sample extracts are diluted and reassayed by LC as described below; \( F \) = volume of milk sample (10 mL); and \( G \) = purity of tilmicosin reference standard.

Note: Factor \( G \) may not be necessary if the purity of the tilmicosin standard is accounted for when the standard is prepared or by the computer system used to do the calculation.

Note: Report concentrations to 3 significant figures.

If the concentration of tilmicosin in a cow milk sample is greater than the high point on the standard curve, the sample should be appropriately diluted using a micropipettor and the sample diluent. The sample will then be reassayed by LC. The value generated by the reassy will be used as the final result for that sample. This dilution procedure may not be used for sheep milk samples. Currently, this method is validated to 0.5 µg/mL for sheep milk, and to 10 µg/mL for cow milk.

If the concentration of tilmicosin in the samples is less than the LOQ for the method, the sample results will be reported as such (i.e., <0.010 µg/mL for cow milk, and <0.025 µg/mL for sheep milk).

Calculate percentage recovery of fortified control sample with the following formula:

\[ \text{Recovery, %} = \left( \frac{\text{µg/mL detected}}{\text{µg/mL added}} \right) \times 100 \]

Quality Control

(a) Linearity evaluation.—The correlation coefficient (r) for the standard curve should be \( \geq 0.995 \). If r is not 0.995 or greater, attempt to determine the source of the problem. If the problem is instrument-related, fix the problem and run the extracts again. If it is not an instrument problem, the standards should be prepared again and the extracts reassyed with the new standards.

(b) Percentage recovery of fortified control sample.—If the percentage recovery of the fortified control sample for a set of samples is \( \geq 70\% \) and \( \leq 110\% \), the results for that set of samples will be deemed acceptable. This guideline was taken from the European guidelines for acceptable recovery for residue methods (12). If the recovery is outside the range of 70–110%, the incurred milk should be reassayed unless there is a valid justification for the low recovery and an assurance that this would not be replicated in the incurred samples (e.g., the loss of some of the acetic acid-methanol eluate for the recovery sample).

Note: If the criteria for linear regression or recovery are not met, and the samples are reassayed, the values from the initial assay of the samples will not be used in reporting the final assay results.

Results and Discussion

Validation data for the method have been collected with respect to linearity, accuracy, precision, sensitivity, and specificity.

Linearity of the standard curve was determined by preparing 5 solutions of tilmicosin reference standard, in duplicate, over the concentration range of 0.025–5.0 µg/mL. In practice, acceptable linearity is not achieved when such a large concentration range of standards is used. Alternatively, 2 standard curves were used: one ranging from 0.025 to 0.25 µg/mL, and the other ranging from 0.25 to 5.0 µg/mL. When the standard curve was split, a linear response in both sections of the curve was generated. The slope, intercept, r, and percentage of relative standard deviation (RSD) for the standard curve ranging from 0.025 to 0.25 µg/mL were 0.03872, 0.2092, 0.9991, and 4.8%, respectively. The slope, intercept, r, and percentage RSD for the standard curve ranging from 0.25 to 5.0 µg/mL were 0.04528, –1.47, 0.999, and 1.8%, respectively. Therefore, the method has acceptable linearity.

Method accuracy and precision were investigated by assaying 3 replicates of cow and sheep milk at 0.010, 0.025, and 1.0 µg/mL for 3 days by a single analyst. Additionally, cow milk was investigated at the 0.10 µg/mL level. The average concentration, percentage of RSD, and the percentage recovery from each day of analysis at each concentration level are summarized in Table 3. The acceptance criterion for method accuracy was an average percentage recovery for each group of samples between 70 and 110%. This criterion was met for fortified milk samples at all concentrations. The acceptance criterion for method precision was an RSD of 15% or less for each group of samples. This criterion was met for fortified milk samples at all concentrations, except at the 0.010 and 0.025 µg/mL levels for sheep milk, where the RSD exceeded 15%. Further consideration of method requirements for sheep milk led to consideration of the RSD of 17% for sheep milk at 0.025 µg/mL as acceptable. An Analytical Methods

<table>
<thead>
<tr>
<th>Species</th>
<th>Fortified tilmicosin concn, µg/mL</th>
<th>Avg. measured tilmicosin concn, µg/mL</th>
<th>RSD, %</th>
<th>Average recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>0.0101</td>
<td>0.0095</td>
<td>7.8</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>0.0252</td>
<td>0.0221</td>
<td>5.0</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>0.101</td>
<td>0.0824</td>
<td>9.1</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>0.505</td>
<td>0.428</td>
<td>3.8</td>
<td>85</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.0101</td>
<td>0.0089</td>
<td>28.2</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>0.0252</td>
<td>0.0230</td>
<td>17.2</td>
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</tr>
<tr>
<td></td>
<td>0.505</td>
<td>0.450</td>
<td>3.1</td>
<td>89</td>
</tr>
</tbody>
</table>
Validation Conference Report (13) supports this conclusion, recommending a coefficient of variation of $\leq 20\%$ for samples at the method LOQ. Thus, the LOQ for cow milk is 0.010 µg/mL and for sheep milk is 0.025 µg/mL.

The highest fortification level tested in milk was 0.5 µg/mL. To attempt to extend the assayable concentration range of the method to 10 µg/mL, 3 samples each of cow and sheep milk were fortified at a 10 µg/mL level and assayed. The final sample extracts were diluted to be in the range of the standard curve. The percentage recovery and percentage of RSD results obtained for the cow milk samples were comparable to those of the other concentrations tested, extending the range of this method for cow milk to 10 µg/mL. The percentage recovery and percentage of RSD results obtained for the sheep milk samples were not comparable to those of the other concentrations tested; therefore the highest concentration for which this method is validated for sheep milk is 0.5 µg/mL.

No significant interference was observed around the retention time of tilmicosin in any of the control cow milk tested, which included milk from multiple animals at varying stages of lactation. Figure 2 shows representative chromatograms of a tilmicosin standard, a control cow milk sample, and a fortified cow milk sample. Sheep milk had more matrix peaks present, but the peaks did not interfere with the integration of tilmicosin in any of the samples tested. Milk from multiple animals was tested. Figure 3 shows representative

**Figure 2.** Chromatograms of a tilmicosin standard (0.25 µg/mL), control cow milk, and cow milk fortified at 0.010 µg/mL.

**Figure 3.** Chromatograms of a tilmicosin standard (0.25 µg/mL), control sheep milk, and sheep milk fortified at 0.025 µg/mL.
chromatograms of a tilmicosin standard, a control sheep milk sample, and a fortified sheep milk sample. Figure 4 shows example chromatograms of 3 incurred cow milk samples.

The chromatography of this method was tested with several currently available mastitis drugs, namely: hydrocortisone, oxytetracycline, neomycin sulfate, prednisolone, sulfadiazine, nafcillin, cloxacillin, erythromycin, chlorotetracycline, penicillin-G, dihydrostreptomycin, cephapirin, streptomycin, cefuroxime, cefoperazone, amoxicillin, ampicillin, novobiocin, and trimethoprim. These substances, tested at levels of about 3–8 ppm (µg/mL), either had retention times that differed from tilmicosin or were not detectable by this specific method. A few of the compounds had peaks that eluted within a few minutes of tilmicosin. Figure 5 shows a chromatogram of tilmicosin compared with trimethoprim, cephapirin, and penicillin-G. This chromatography offers a greater specificity for tilmicosin than other chromatography previously reported for tissue and milk methods (8, 10).

The LOD for the method was determined by measuring the peak heights of a pool of 20 control cow and sheep milk

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**Figure 4.** Chromatograms of incurred cow milk and a tilmicosin standard (0.25 µg/mL).

**Figure 5.** Method specificity: Tilmicosin (0.50 µg/mL) versus trimethoprim, cephapirin, and penicillin-G.
samples. The LOD was defined as the concentration of tilmicosin standard that corresponded to the average peak height of the control samples plus 3 times the standard deviation of the peak height. This concentration is 0.018 µg/mL for a tilmicosin standard, which corresponds to a concentration of 0.0036 µg/mL in milk.

Stability of the fortification solutions, the standard curve solutions, and the final LC sample extracts, and of tilmicosin in cow and sheep milk matrixes were investigated. The fortification and standard curve solutions are stable for at least 7 days at room temperature. LC sample extracts from cow and sheep milk were stable for at least 7 days at room temperature. The stability of tilmicosin in the cow and sheep milk matrixes was investigated. The fortified milk samples, and has adequate precision (RSD 3.1–17.2%) and recovery (82–94%). The method is specific with respect to milk matrixes, tilmicosin-related substances and degradation products, and potential interfering drugs. Finally, the method is demonstrably rugged and reliable.

Conclusions

The milk method presented is suitable for use in analysis of cow milk over a concentration range of 0.010–10 µg/mL, and for sheep milk over a concentration range of 0.025–0.5 µg/mL. The method has been tested with incurred and fortified milk samples, and has adequate precision (RSD 3.1–17.2%) and recovery (82–94%). The method is specific with respect to milk matrixes, tilmicosin-related substances and degradation products, and potential interfering drugs. Finally, the method is demonstrably rugged and reliable.

Acknowledgments

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References

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Table 4. Analyst-to-analyst method performance ruggedness data

<table>
<thead>
<tr>
<th>Analyst</th>
<th>Concentration, µg/mL</th>
<th>Average recovery, %</th>
<th>RSD, %</th>
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