EXCRETION OF TILMICOSIN IN MILK OF RABBITS AFTER SUBCUTANEOUS ADMINISTRATION

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ABSTRACT

To check the pattern of tilmicosin excretion in milk and to determine the best therapeutic schedule for the treatment of mastitis in does, 3 groups of does were administered one single subcutaneous dose (20 mg/kg) before parturition, a single dose after parturition, or two doses, one before and one after parturition. Milk concentrations of tilmicosin were determined by a validated HPLC method and were compared to the minimal inhibitory concentrations (MIC) for *Staphylococcus aureus* strains isolated from clinically mastitic does. Treatments were well tolerated and no adverse reactions were observed. Tilmicosin peak concentrations in milk were attained on the first day in does treated once before parturition (1.51±1.30 µg/g), and in those treated twice, before and after parturition (3.05±1.88 µg/g). When tilmicosin was administered once the day after parturition, peak concentrations in milk were detected on the 2nd experimental day (group B: 2.33±1.47 µg/g). The administration of two doses produced the highest concentrations of tilmicosin at all sampling times, with differences from the other therapeutic schedules being significant on the first 3 days. Close to 50% of the *S.aureus* isolates would be sensitive to the milk concentrations of tilmicosin obtained in does treated twice, before and after parturition. As the intensive use in rabbitries of antibiotics has been associated with an overall increase of pathogen resistance, these results indicate that tilmicosin is a potential tool for the therapy of rabbit mastitis, and that it could represent an alternative to the usual approaches for the therapy of this disease.

Key words: tilmicosin, mastitis, milk, rabbit.

INTRODUCTION

Mastitis associated with *Staphylococcus aureus* or *Pasteurella multocida* is, besides podal plagues, one of the most important pathologies affecting does. Late therapy is often unsuccessful, so the control of economic losses is achieved by prophylaxis using effective antibacterial agents and by the elimination of does with chronic forms of the disease (ROSELL *et al.*, 2000).
Tilmicosin (Figure 1), a broad-spectrum macrolide antibiotic effective against *Pasteurella multocida, Bordetella bronchiseptica, Staphylococcus aureus and Mycoplasma* spp. (EMEA/MRL/736/00-Final, 2000), is a potential alternative to the commonly used penicillins or tetracyclines. In fact, clinical experiments demonstrated that a single subcutaneous injection of tilmicosin at 20 mg/kg b.w. was more effective on rabbit mastitis than a single subcutaneous injection of long-acting tetracycline at the same dosage (CHRISTODOULOPOULOS *et al.,* 2001). The therapeutic efficacy of tilmicosin against mastitis is probably related to effective concentrations attained by the drug in milk and in the mammary tissues. In cattle and sheep, tilmicosin is excreted in milk for many days after single subcutaneous administration.
This study was designed to evaluate the pattern and duration of tilmicosin excretion in milk from rabbits treated subcutaneously and to determine the best therapeutic scheme for the treatment of mastitis. Milk concentrations of tilmicosin were determined by a validated HPLC method and were compared to the minimal inhibitory concentrations (MIC) for *Staphylococcus aureus* strains isolated from mastitic does.

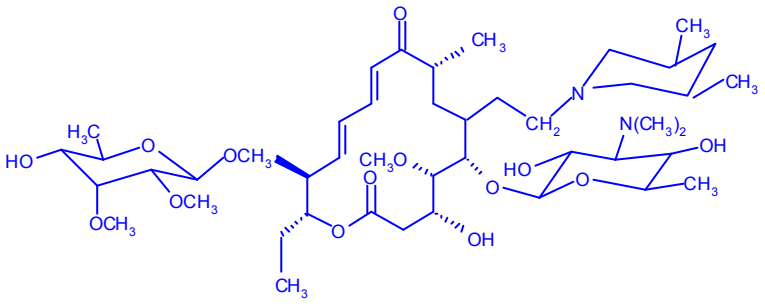

Figure 1. Tilmicosin structural formula

MATERIAL AND METHODS

Animals, treatments and sampling

Thirthy healthy does (Hyla hybrid), multiparous and pregnant, weighing 4.0-5.9 kg, were used. The rabbits were maintained in individual cages and were fed the standard pellet diet throughout the experimental period. Tap water was provided *ad libitum*.
Tilmicosin (Micotil 300®) was administered subcutaneously at 20 mg/kg body weight.
The animals were divided into 3 groups of 10 rabbits each and treated as follows: group A was treated once, 2 days before parturition; group B was treated once, the day after parturition; group C was treated twice, the first time 2 days before parturition and the

second the day after parturition. According to normal doe management, parturition was induced by a single intramuscular injection of oxytocin (5 UI).
Milk samples (about 2 mL) were manually collected every 24 h for 7 days starting from the day after parturition. Sampling was always performed before allowing the does to feed the nest. The samples were immediately frozen and transported to the laboratory, where they were kept at –20°C until analysis.

Analytical method

Tilmicosin concentrations were determined by a HPLC method. Milk samples were extracted with acetonitrile and purified by solid phase extraction (SPE) on Sep-Pack C_{18} columns (Italy Waters S.p.A., Milano, Italy). The eluate was evaporated to dryness and the residue dissolved in 0.25 mL of 0.25% acetic acid in methanol. The HPLC system consisted of a LC Pump Series 200, a UV-Visible Detector LC 295 set at 282 nm, an autosampler Advanced LC Sample Processor ISS200 with a Reodyne valve (Perkin Elmer, Norwalk, CT, USA). Samples were chromatographed on a LUNA C_{18} column (250x4.6 mm, ID, 5 µm; Phenomenex, Torrence, CA, USA). The mobile phase was a mixture of methanol:acetonitrile:0.008M tetrabutyl ammonium bromide (TBA) pH 2.5 (11:8:81 v:v:v). The flow rate was set at 1 mL/min and the injection volume was 20 µL. The linearity of detector response, accuracy, repeatability and reproducibility as well as the limit of detection (LOD) and the limit of quantification (LOQ) were investigated.

Statistical analysis

Mean values and standard deviations of tilmicosin concentrations in milk were calculated. The values lower than the LOQ have been set to half of the LOQ. Data from all groups were analyzed by 2-way ANOVA followed by Bonferroni post tests.

Susceptibility of isolates

Thirty-three isolates of *S.aureus* were obtained from clinical cases of mastitis in does. Disk diffusion susceptibility testing was performed according to the NCCLS guidelines (2002). Zone diameters were read and MIC calculated by a Sirscam 2000 Automated Zone Reader (I2a, Montpellier, France) using the MIC breakpoints for veterinary pathogens reported in the NCCLS guidelines (2002).

RESULTS AND DISCUSSION

The HPLC method used for tilmicosin determination was adapted from a previous study on tilmicosin excretion in milk of dairy cows (FEDRIZZI *et al*., 2002) and proved to be robust, as a matrix from a different species could be easily analyzed without substantial modifications of the procedure. Representative chromatograms are presented in Figure 2 and the results on accuracy and precision are listed in Table 1. The calibration curves from spiked milk samples were linear from 0.025 µg/g to 0.200 µg/g (r^2 0.9937). The limit of detection (LOD) and the limit of quantification (LOQ) were 0.020 µg/g and 0.025 µg/g,

respectively. All samples with tilmicosin concentrations higher than 0.100 µg/g were diluted and reanalised.

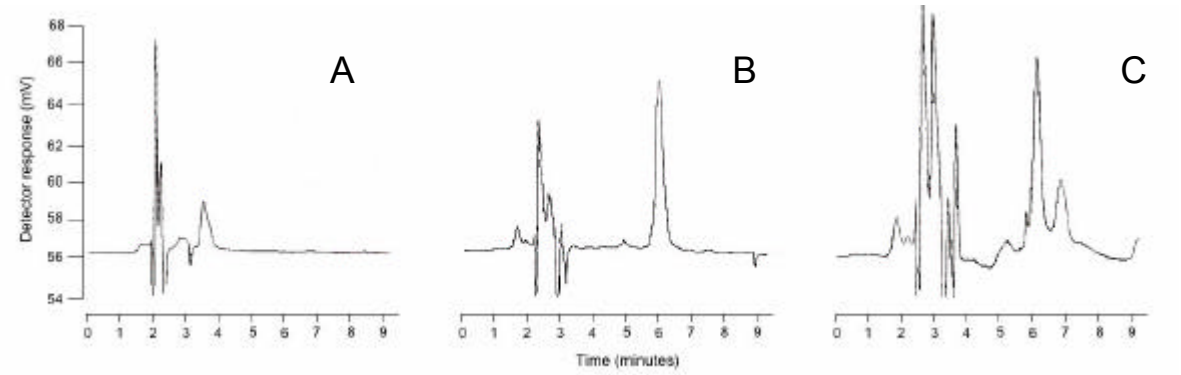

Figure 2. Chromatograms of blank milk sample (A), milk sample spiked with tilmicosin (0.050 µg/g)(B), and an experimental milk sample (C) collected 1 day after parturition of a does given tilmicosin subcutaneously at 20 mg/kg b.w.

Table 1. Accuracy and precision (repeatability, reproducibility) of the HPLC method adopted for tilmicosin analysis

Concentrations	*Accuracy (n=6)*	*Repeatability (n=6)*	*Reproducibility (6 replicates per 3 days)*
0.025 µg/g	103.05%	14.18%	18.81%
0.050 µg/g	100.13%	17.87%	17.88%
0.100 µg/g	88.65%	3.69%	11.69%

Treatments were well tolerated, with no adverse reactions observed except for local transient irritation at the site of injection. This observation was in agreement with previous results of clinical trials using the same dosage and route of administration (McKAY *et al*., 1996; PRADELLA *et al.*, 2000; CHRISTODOULOPOULOS *et al.,* 2001; ALTUNOK *et al*., 2002).

Tilmicosin concentrations in rabbit milk are presented in Figure 3 as mean ± standard deviation. Peak levels were attained on the first day in does treated once before parturition (1.51±1.30 µg/g), and in those treated twice, before and after parturition (3.05±1.88 µg/g). When tilmicosin was administered once the day after parturition, peak concentrations in milk were detected on the 2nd experimental day (group B: 2.33±1.47 µg/g). The treatment scheme of group C, based on two subcutaneous administrations, produced the highest concentrations of tilmicosin at all sampling times, with differences from the other therapeutic schemes being significant on the first 3 days. Previous studies demonstrated that the administration of a single subcutaneous dose of tilmicosin in cows (10 mg/kg) or tilmicosin administered via medicated feed (350 ppm) in sows produced milk antibiotic concentrations higher than those found in rabbits. In cows a mean peak concentration of 6.46±2.03 µg/mL (ZIV *et al.*, 1995) and in sows a range from 3 µg/g to 13 µg/g (BIANCOTTO *et al*., 2002) were attained.

MICs of tilmicosin against the isolated strains of *S.aureus* calculated by Sirscam 2000 are listed in Table 2. Although the MIC breakpoints given by NCCLS are not for milk, and a matrix effect on antibiotics efficacy is possible, we underline that close to 50% of the isolates would be sensitive to the milk concentrations of tilmicosin found in does. As the intensive use of antibiotics in rabbitries has been associated with an overall decrease of pathogen sensitivity, these results suggest that tilmicosin can be an additional tool for the treatment of mastitis associated with sensitive *S.aureus.*

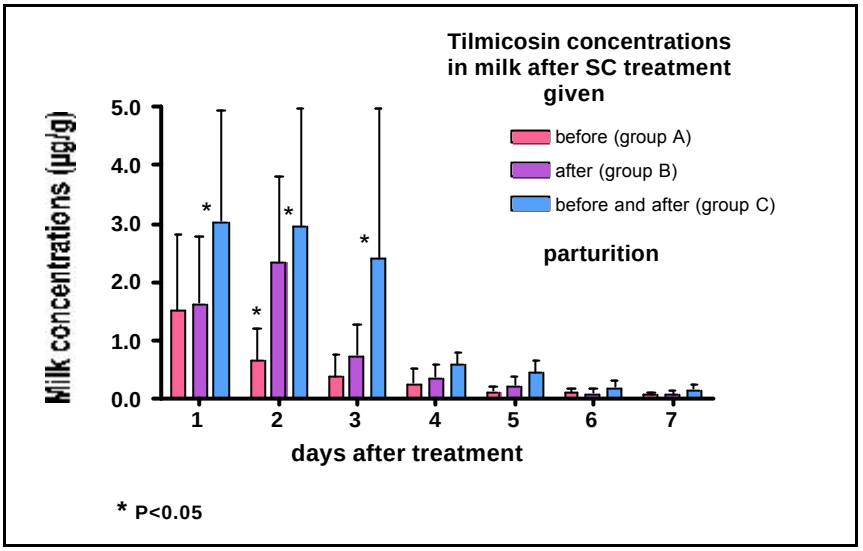

Figure 3. Tilmicosin excretion in milk of rabbits treated subcutaneously at 20 mg/kg b.w.

Table 2. Minimal inhibitory concentrations (MICs) of tilmicosin against strains of *S.aureus* isolated from rabbits (n=33)

*MIC (mg/mL)**	*0.03*	*0.06*	*0.25*	*1*	*2*	*4*	*8*	*16*	*32*	*128*	*>256*
N	2	1	5	2	4	4	3	1	2	3	5
Cumulative %	6	9	25	31	44	56	66	69	75	84	100

*serial dilutions that did not represent the MIC of any isolate were omitted

CONCLUSIONS

This study demonstrates that tilmicosin is excreted in does milk after subcutaneous administration at 20 mg/kg b.w. before and/or after parturition and that it attains the highest concentrations when given two times, once before and once after parturition. Although MICs of tilmicosin against *S.aureus* isolates from doe mastitis suggest that this antibiotic is a potential tool for the therapy of rabbit mastitis, and that it could represent

an alternative to the usual approaches for the therapy of this disease, further studies are needed to confirm the clinical relevance of these findings.

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