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ARTICLE

Investigation of the Persistence of Levamisole and Oxyclozanide in Milk and Fate in Cheese

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In this study, dairy cows (n = six) were treated with an oral combination product containing levamisole (5 mg/kg body weight, (bw)) and oxyclozanide (10 mg/kg bw). Animals were milked twice daily up to day 16 post-treatment. Milk samples were subsequently analyzed by ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC−MS/MS). The highest levels of levamisole (<600 μg/kg) and oxyclozanide (<25 μg/kg) were determined at first and third milking, respectively. Residues of levamisole and oxyclozanide were typically below reporting limits of 0.83 and 1 μg/kg respectively at the 11th and 13th milking, respectively. Soft (3 days ripening), hard (35 days ripening) and whey cheeses were produced from the milk samples collected from the first two milkings. Levamisole residues were found to concentrate in all cheese types. There was a 3-fold concentration effect for levamisole in mature cheese. Oxyclozanide residues were found to occur at lower levels in soft and hard cheese than milk with a 10-fold concentration in whey cheese compared to milk. The results of this study demonstrate that levamisole and oxyclozanide residues are rapidly excreted in dairy cows and milk is compliant after a few days. Oxyclozanide and levamisole residues were shown to be stable during the fermentation process and the whey heat treatment to persist in cheese.

KEYWORDS: Levamisole; oxyclozanide; anthelmintic drugs; depletion; fate; milk; cheese; QuEChERS; UPLC−MS/MS

INTRODUCTION

Anthelmintic drugs are widely used in veterinary medicine for protecting or treating animals mainly against gastrointestinal nematodes and trematodes. Oxyclozanide is mainly used in the treatment of adult stages of liver fluke, and levamisole is a broad spectrum anthelmintic used for the treatment of nematode parasites at both larval and adult stages. In the case of oxyclozanide, a number of more potent drugs are on the market with greater efficacy against liver fluke such as triclabendazole, clorsulon, nitroxynil and rafoxanide (*1*). However, it continues to be an important drug because of its reported efficacy against stomach fluke, which is becoming an emerging problem for herds (*2*, *3*). Similarly, while levamisole resistance has developed in many herds, it continues to be an important drug because of its proposed immunostimulant properties (*4*) and treatment of respiratory infection (*5*). The milk yield of dairy cows with parasitic infections is substantially reduced, and it has been reported that treating animals with anthelmintic drugs increases milk yield. Block et al. (*6*) treated cows with levamisole at calving which resulted in an increased milk production. Spence et al. (*7*) treated infected cows with broad spectrum anthelmintics increasing milk production by 4.8% per lactation.

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Little information has been published on the fate of levamisole and oxyclozanide residues in milk. Until recently there were no analytical methods available that could allow the simultaneous analysis of both residues (*8*, *9*). A number of groups have reported on the persistence of levamisole residues in cows' milk, however many of these use less sensitive HPLC based methods compared to recent MS based methods. Some groups have used a limited number of animals (*10*−*14*). Only two papers have been reported on the persistence of oxyclozanide in milk (*15*, *16*).

Several groups have reported on the fate of anthelmintic drug residues in cheese, but these have largely focused on macrocyclic lactones and the benzimidazoles (*17*−*23*). Anastasio et al., Imperiale et al. and Cerkvenik et al. found higher levels of ivermectin in fat products like cheese than in milk (*19*−*22*). Anastasio et al. found higher levels of eprinomectin in cheese than milk (*23*). Production of this type of data is important to develop complex exposure models to estimate drug exposure from dairy products.

The purpose of this study was to investigate the persistence of oxyclozanide and levamisole residues in bovine milk after treatment with a combination product and their subsequent fate during cheese production. No data has been published in peer reviewed literature concerning the fate of these residues during cheese production. The knowledge generated from this study is particularly useful should these drugs be accidentally administered to dairy cows.

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MATERIALS AND METHODS

Animal Studies. Six Friesian cows weighing between 400 and 500 kg were selected for the study. The six cows were treated with the maximum dose (150 mL for animals 300 kg and over) of TOLOXAN. The first milk sample was taking at 6 p.m., 9.5 h after administration. Milk samples were subsequently taken from the animals twice daily, morning (6 a.m.) and evening (6 p.m.) over a 16 day period, and the samples were frozen (−20 °C) until analysis.

Mature, soft and whey cheese were produced from the first two milkings collected 9.5 and 21.5 h after administration by the following procedure. Pooled raw milk from the six animals was collected (60 L) and heated to 38 °C, a curdling agent (cagliodoro) was added and kept for 30 min. The curdle was then broken and kept for 15 min, after which the curdle and liquid whey were separated. The curdle was then placed in a mold, and the surface was salted to produce soft cheese after 3 days of maturation. Half of the soft cheese was refrigerated for 35 days to produce mature cheese. The liquid whey was boiled and placed immediately in a mold to obtain the whey cheese. All samples were frozen (−20 °C) until analysis.

Materials and Reagents. Preweighed 50 mL polystyrene centrifuge tubes containing 4 g of anhydrous (anh) magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl) (tube 1), and 1.5 g of anh $MgSO_4$ and 0.5 g of C_{18} (tube 2) were obtained from UCT, Inc. (Bristol, PA).

Extraction and Cleanup. As described in Whelan et al. (*9*), milk samples (10 g ± 0.1 g) were weighed into centrifuge tubes (50 mL) and fortified with internal standard and left to sit for 15 min. Cheese samples (4 g ± 0.04 g) and Millipore water (6 g ± 0.06 g) were weighed into centrifuge tubes (50 mL) and placed in a water bath at 50 °C until the cheese and water become homogeneous; they were then fortified with internal standard and left to sit for 15 min. MeCN (12 mL) was added to tube 1 containing $MgSO_4$ (4 g) and NaCl (1 g). The contents of tube 1 were added to the sample and shaken immediately to extract the residues into the MeCN layer. The sample was centrifuged for 12 min at 3,500 rpm (959*g*). A dispersive-SPE cleanup step was performed by pouring the supernatant from tube 1 into tube 2 (50 mL) containing $MgSO_4$ (1.5 g) and C_{18} (0.5 g). The samples were vortexed for 30 s and centrifuged for 10 min at 2,500 rpm (489*g*). The supernatant (6 mL) and DMSO (0.25 mL) were added to a starstedt tube (15 mL) and vortexed for 1 min. The MeCN layer was evaporated under nitrogen at 50 °C to 0.25 mL. Extracts were filtered through 0.2 μm PTFE syringe filters (Whatman Rezist) and injected onto the UPLC−MS/MS system. Any samples that fell outside the calibration range were diluted in negative milk and reanalyzed.

UPLC−MS/MS Analysis. As previously described in Whelan et al. (*9*), chromatographic separations were performed using a Waters (Milford, MA) Acquity UPLC system, the column used was a 100 mm × 2.1 mm i.d., 1.8 μm, Acquity HSS T3, with an in-line filter unit with 0.2 μm stainless steel replacement filters (Waters). The column oven was maintained at a temperature of 60 °C, and the Acquity pump was maintained at a flow rate of 0.6 mL/min. Analytes were separated using the following gradient elution comprising mobile phase A, 0.01% HOAc in water:MeCN (90:10 v/v), and mobile phase B, 5 mM ammonium formate in MeOH:MeCN (75:25, v/v). The gradient profile was as follows: 0−0.5 min, 100% A; 5 min, 50% A; 7 min, 10% A; 8.5 min, 10% A; 8.51 min, 0% A; 9.5 min, 0% A; 9.51 min, 100% A; 13 min 100% A.

A Waters Quattro Premier XE mass spectrometer was used to quantify the veterinary drug residues found in the milk samples The electrospray ionization (ESI) UPLC−MS/MS system was controlled by MassLynx software and chromatographic data was processed using TargetLynx Software (both from Waters). Injection volume was 2 μL. The analytes and internal standards (**Figure 1**) were tuned on the UPLC−MS/MS, and the optimum conditions were obtained during tuning and were input into the MS settings. The following transitions were input into multiple reaction monitoring (MRM) windows: 210.10 → 183.08 (*m*/*z*), d_5-levamisole; 204.93 → 122.89 (*m*/*z*) and 204.93 → 177.94 (*m*/*z*), levamisole; 212.05 → 92.00 (*m*/*z*), salicylanilide; 397.80 → 175.75 (*m*/*z*) and 397.80 → 201.80 (*m*/*z*), oxyclozanide. d_5-Levamisole retention time 2.82 min and levamisole retention time 2.83 min were input to MRM channel 1 and detected in positive ion mode; salicylanilide retention time 5.95 min and oxyclozanide retention time 6.83 min (**Figure 2**) were input to MRM channel 2. The MRMs were time-sectored; dwell time, interscan delay and interchannel delays were set to get maximum response from the instrument.

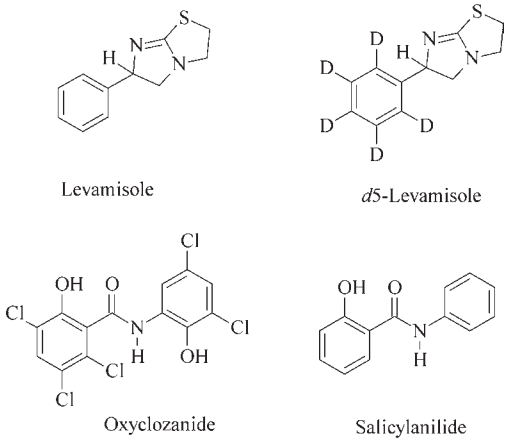

Figure 1. Chemical structures of levamisole, oxyclozanide and their internal standards (d_5-levamisole and salicylanilide).

Calibration. Levamisole, oxyclozanide and salicylanilide were purchased from Sigma-Aldrich. d_5-Levamisole was purchased from Witega Laboratories. Primary stock standard solutions were prepared at concentrations of 4 and 2 mg/mL for oxyclozanide and levamisole respectively. Internal standards (salicylanilide and d_5-levamisole) were prepared at a concentration of 1 mg/mL. d_5-Levamisole was prepared in deuterated methanol (in case of deuterium exchange in solution), and the remaining powders were prepared in methanol.

Extracted matrix calibrants were prepared by fortifying negative milk samples prior to extraction with working standard mixes, prepared at the following concentrations (in μg/mL): 10, 5 (std 7) 5, 4 (std 6), for levamisole and oxyclozanide respectively and 2 (std 5), 1 (std 4), 0.5 (std 3), 0.2 (std 2), and 0.1 (std 1) for both analytes. Matrix-matched calibration curves were prepared by fortifying matrix blanks before extraction with 100 μL of the standards to give working standard curves in the sample equivalent range of 1 to 100 and 1 to 50 μg/kg for levamisole and oxyclozanide respectively. An additional four blank matrix samples (recovery controls) were fortified after extraction, two with std 2 (50 μL) and two with std 5 (50 μL) to monitor for loss of analytes during extraction.

RESULTS AND DISCUSSION

Extraction, Cleanup and MS Analysis. The extraction, cleanup procedure and UPLC−MS/MS method used in this investigation were developed previously (*9*). The extraction and cleanup method did not require modifications as the method performance was satisfactory. This was demonstrated for levamisole by participating in a proficiency study. As this was not a multi method and the sensitivity of levamisole and oxyclozanide was not critical, it was possible to reduce the injection volume from 5 to 2 μL and still detect sub μg/kg levels. This resulted in an extended linear range from 1−50 μg/kg to 1−100 μg/kg for levamisole, and oxyclozanide remained 1−50 μg/kg with an improvement in the r^2 value due to the reduced amount of matrix injected onto the system. A preliminary run was carried out to determine the concentration of the analytes in the samples. The samples were then diluted in negative milk samples to fit the calibration range and the samples were re-extracted.

The method was extended to cheese samples. The only modification was to the initial sample, 4 g of cheese and 6 g of water was added to the centrifuge and the sample was heated to 50 °C. This resulted in a liquid suspension of cheese and water. The cheese suspension was then treated the same as the milk samples.

Persistence of Levamisole and Oxyclozanide Residues in Bovine Milk. There are surprising reports in literature of the persistence of levamisole residues in the milk of dairy cows after oral administration. Archambault et al. (*10*) investigated the persistence of levamisole in dairy cows after a subcutaneous (sc) injection (10 mg/kg bw).

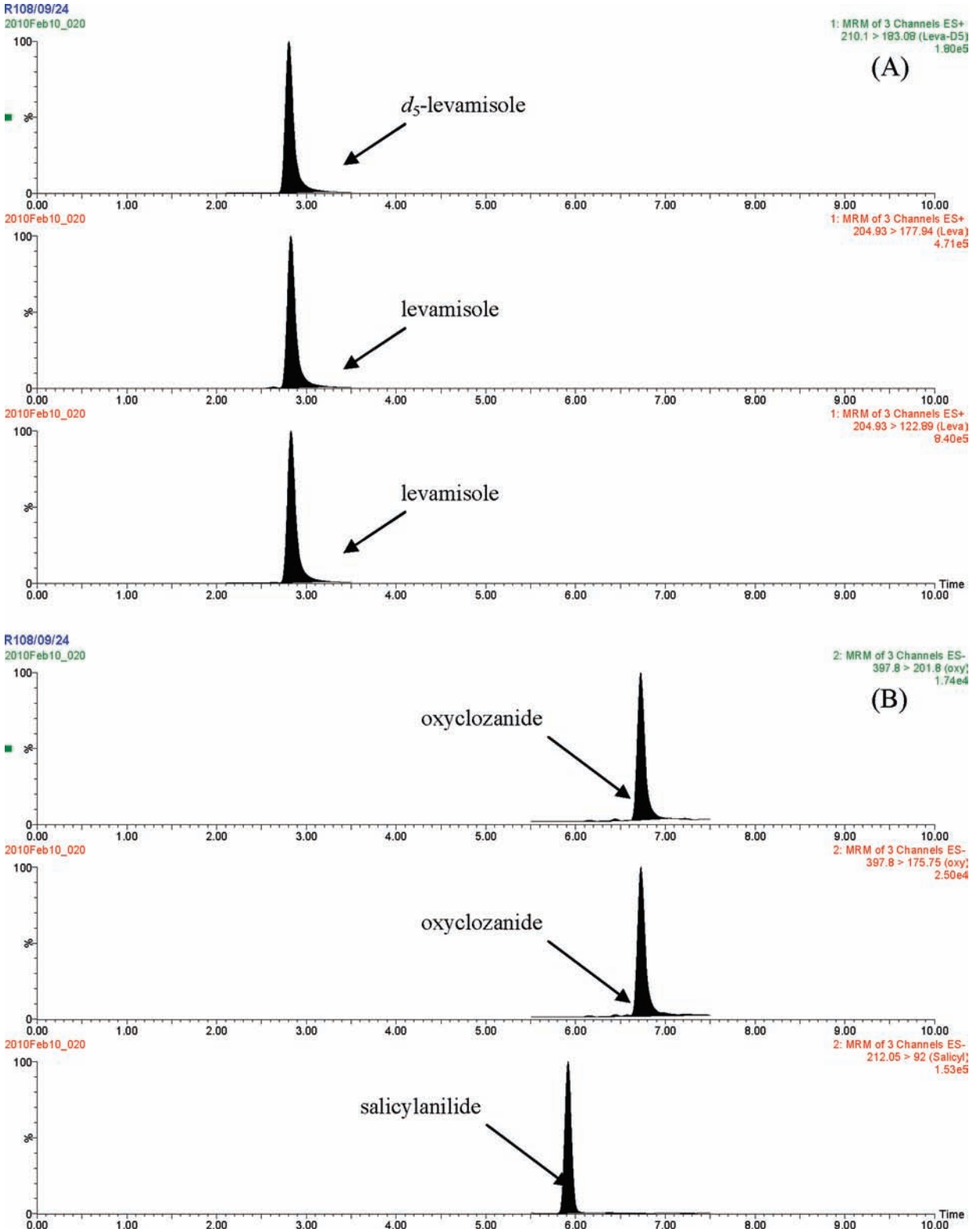

Figure 2. LC−MS/MS traces of an incurred milk sample containing levamisole (**A**) and oxyclozanide residues (**B**) at concentrations of 56.2 and 7.4 μg/kg, respectively.

The analytical method employed was only to measure levamisole residues to the limit of detection (LOD) of the assay, 100 μg/kg. Levamisole residues persisted for approximately 12 days above the LOD post-treatment. Osterdahl et al. (*12*) treated cows (n = 42) infected with lungworm by an intramuscular injection (7 mg/kg bw). Levamisole residues were less than the LOD, 40 μg/L in all animals by 29 h post-treatment. De Ruyck et al. (*14*) treated cows (n = 4 each dose) topically with 10 and 20 mg/kg bw levamisole, and levels declined to 21 and 83 μg/L respectively by 79 h post-treatment. At 7 h post-treatment, mean concentrations of levamisole residues were 1896 (low dose) and 6027 μg/kg (high dose). Paulson and Feil (*13*) treated dairy cows with oral (n = 1) and sc injectable doses (n = 1) with radiolabeled levamisole (8 mg/kg bw). Highest concentrations after oral and subcutaneous doses were

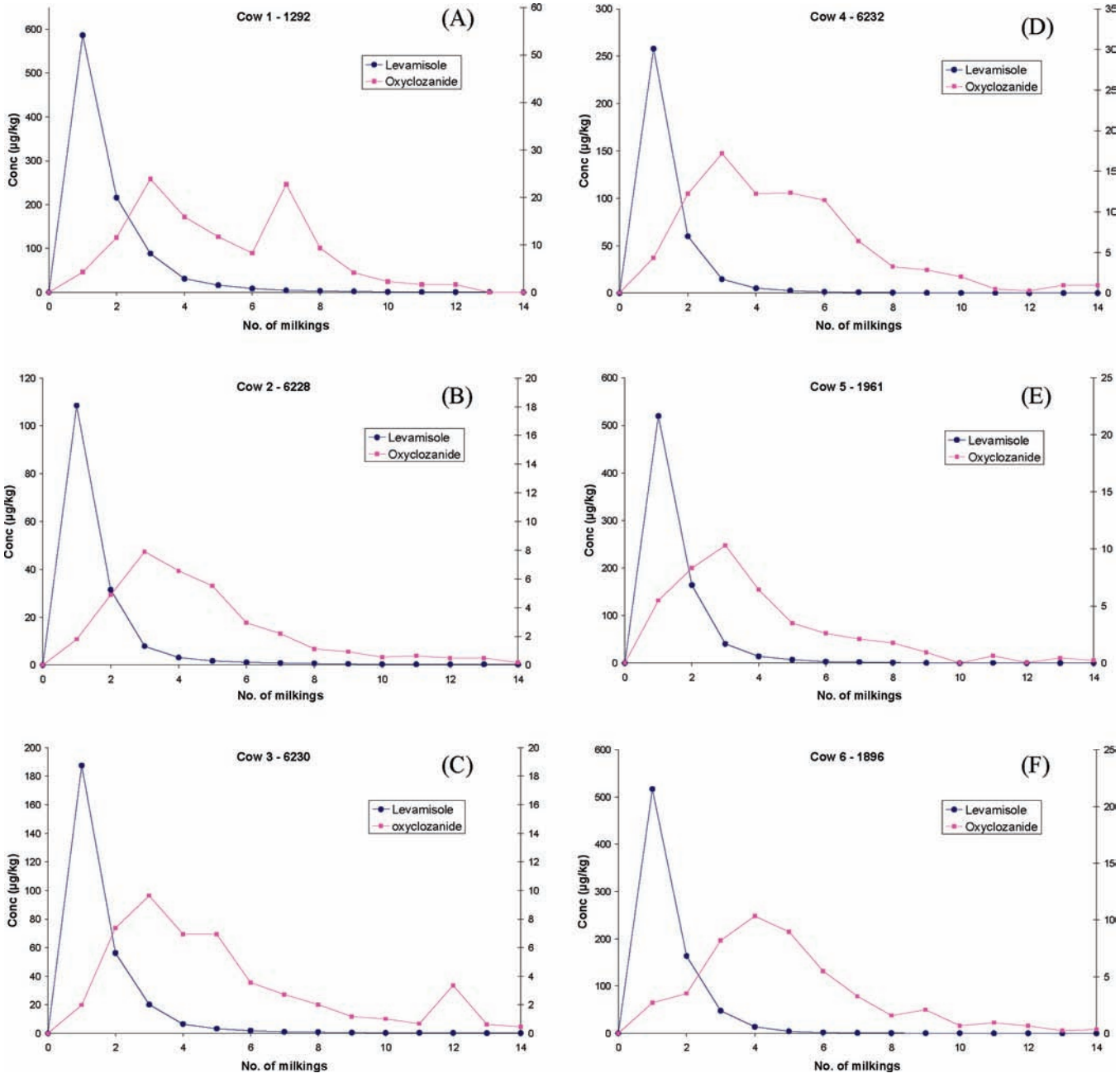

Figure 3. Plot of levamisole (left *Y*-axis) and oxyclozanide (right *Y*-axis) concentrations (μg/kg) in milk as a function of withdrawal time (h) for animals 1−6 respectively (**A**−**F**).

191 and 208 μg/kg, respectively. Levamisole residues were measurable at approximately 1 μg/kg (levamisole parent drug) after both treatments. Simkins et al. (*11*) carried out an extensive study in four groups of animals (n = 5 animals per group), which were treated with different formulations, oral drench, pellets, bolus and subcutaneous injection containing 8 mg/kg bw levamisole. Levamisole residues were less than 10 μg/kg at 48 h for all formulations. Highest levamisole residues were detectable at 12 h post-treatment: oral dose (240 to 750 μg/kg), pellet (160 to 900 μg/kg), bolus (220 to 840 μg/kg) and sc injection (140 to 830 μg/kg).

In this study, dairy cows (n = 6) were treated with a combination product containing levamisole and oxyclozanide. Milk samples were collected at morning (6 a.m.) and evening (6 p.m.) milking for 16 days post-treatment, and analysis was carried out by UPLC−MS/MS. In agreement with previous studies, highest levels of levamisole residues were detected at the first milking and ranged from 108 to 586 μg/kg (**Figure 3**). Levamisole residues were found to be detectable at above the limit of reporting for levamisole (CCα = 0.83 μg/kg) (*9*). Residues of levamisole were < LOR on the 11th, 7th, 8th, 7th, 9th and 8th milking for cows 1−6 respectively. The results of the study show that, when LC−MS/MS technology was used, levamisole residues were compliant 130 h post-treatment.

Only two papers have been reported on the persistence of oxyclozanide in milk. Fujinuma et al. treated cows orally with 10 mg/kg bw oxyclozanide, and residues were detectable until 30−47 h above 1 μg/kg (*15*). Residues were typically at < 10 μg/kg in all animals at all time points. Bluthgen et al. treated cows orally with 10 mg/kg bw oxyclozanide, and residues were detected at 130 μg/kg at 48 h post-treatment and 10 μg/kg at the prescribed withholding period of 120 h (*16*).

In this study the highest levels of oxyclozanide ranged between 8 and 24 μg/kg and were determined at the third milking. Oxyclozanide residues were < 1 μg/kg at 13th, 8th, 11th, 11th, 9th,

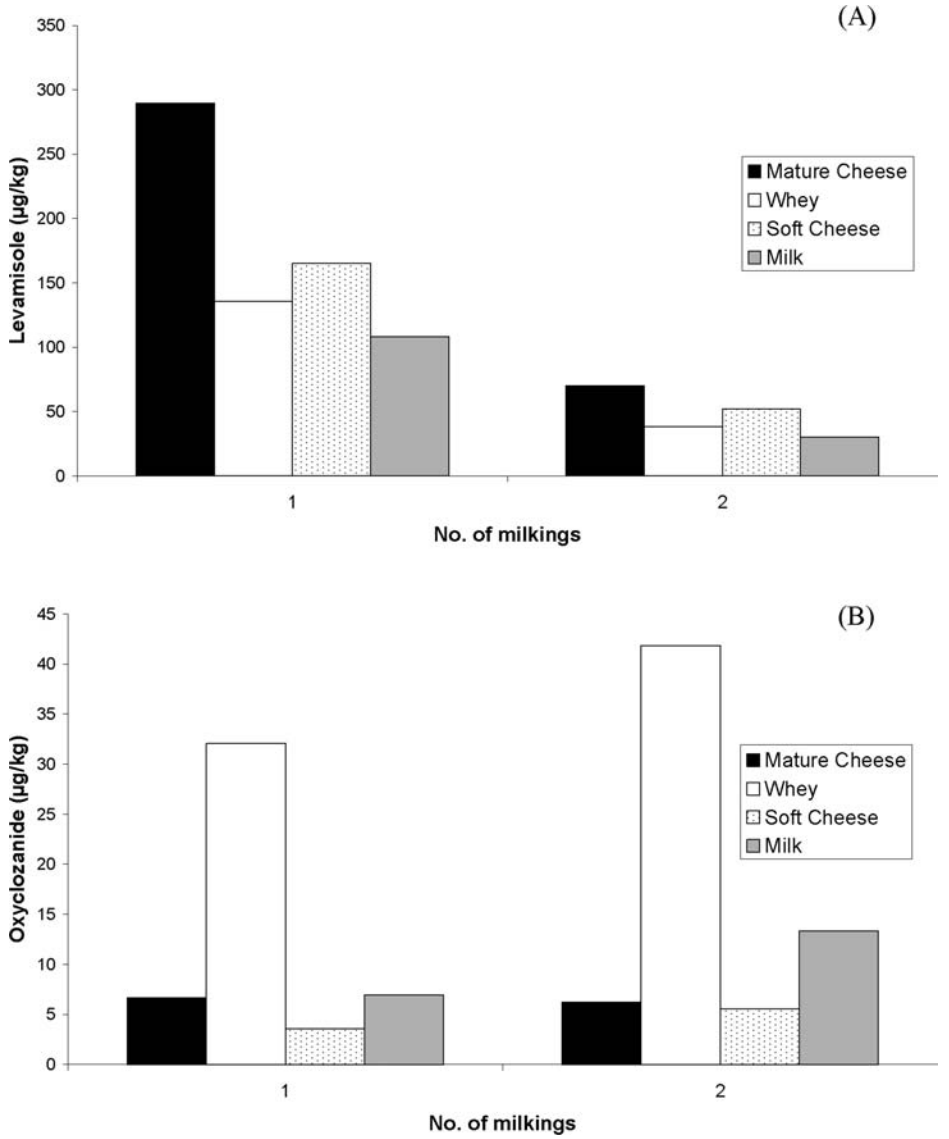

Figure 4. Profile of levamisole (**A**) and oxyclozanide (**B**) concentrations (μg/kg) in milk and cheese (soft, mature and whey) from treated animals.

and 10th milking for animals 1−6 respectively. Residues were below the MRL (10 μg/kg) at the 8th, 7th, 4th and 5th milking for cows 1, 4, 5 and 6, and below the MRL for all time points for cows 2 and 3.

The 7th and 12th milkings for animals 1 and 3 respectively were at higher concentrations than the previous samples in the depletion profile. These samples were reanalyzed to ensure results were correct and repeat analyses confirmed previous results. The results were further verified as the concentration of levamisole in the samples was as expected in the depletion profile.

Fate of Residues in Cheeses. Three types of cheese were produced from pooled milk of the six animals from the first two milkings 9.5 and 21.5 h after administration: soft, mature and whey cheese. The cheese samples were produced from milk incurred with levamisole and oxyclozanide.

Levamisole Residues in Cheese. In general, the amount of levamisole residues found in the soft, mature and whey cheeses produced from the first two milkings had similar patterns (**Figure 4**). The concentration of levamisole in the pooled milk was 108 and 30 μg/kg for the first and second milkings respectively. The cheeses produced from the first milking had the following concentrations: 290, 136, and 165 μg/kg for mature, whey and soft cheese respectively. In the cheese produced from the second milking 70, 38, and 52 μg/kg levamisole residues were detected in the mature, whey and soft cheese respectively. The values observed in cheese deserve careful attention as the concentration was higher than that found in milk. The results indicate that the levamisole residue binds more strongly to the fat in the curdle than the proteins in the whey. Levamisole residues concentrated in mature cheese samples as water content decreased. The results found indicate that levamisole residues survive the fermentation process and the whey heat treatment and are stable during storage.

Oxyclozanide Residues in Cheese. The levels of oxyclozanide found in mature, whey and soft cheese produced from the first two milkings indicate oxyclozanide is stable during storage and survives the fermentation process (**Figure 4**). The concentration of oxyclozanide in the pooled milk was 7.0 and 13.4 μg/kg for the first and second milking respectively. In the cheese produced from the first milking 6.7, 32.1, and 3.6 μg/kg oxyclozanide residues were detected in the mature, whey and soft cheese respectively. In the cheese produced from the second milking 6.2, 41.9, and 5.6 μg/kg oxyclozanide was detected in mature, whey and soft cheese respectively. Oxyclozanide residues were found to 10-fold concentrate in whey cheese, which is likely due to strong binding of acidic drug residue to the proteins in the whey.

In summary this study showed that levamisole and oxyclozanide are rapidly excreted in milk and residues are compliant after the 11th and 13th milkings, respectively. Combination products containing levamisole and oxyclozanide are currently not allowed for the treatment of dairy animals, and the results of this study show the consequences of hypothetical illegal use. Levamisole is well absorbed after oral administration as seen in **Figure 3**. However, the results indicate that a short withdrawal period would be needed to ensure milk is compliant in the event that a MRL is set for levamisole. Presently, no MRL is set for levamisole and detection of its residues in milk or dairy products will deem a sample noncompliant. This study also shows levamisole and oxyclozanide are stable during storage and survive the fermentation process with an increase in concentration compared to the concentration found in milk.

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