Investigation of the Persistence of Closantel Residues in Bovine Milk Following Lactating-Cow and Dry-Cow Treatments and Its Migration into Dairy Products

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ABSTRACT: Closantel is a veterinary drug used to treat liver fluke in cattle and sheep. A provisional maximum residue limit (MRL) of 45 μg/kg in milk has been set by the European Union. The purpose of this study was to investigate the persistence of closantel residues in milk and the migration of residues into milk products. Following dry-cow treatment, residues ranged from undetectable to 8.7 μg/kg at the first milking. Following lactating-cow treatment, residues detected ranged from 278 to 482 μg/kg at day 1 post-treatment and were detectable above the MRL for 52 days and detectable for 198 days. At day 2 and day 23 post-treatment, the milk was collected and dairy products manufactured. Closantel residues concentrated in the cheese, butter, and skim milk powder. The results indicate that closantel is best used as a dry-cow treatment.

KEYWORDS: closantel, residues, milk, dairy products, butter, buttermilk, cheese, whey, skim milk, powders, flukicide, UHPLC-MS/MS

■ INTRODUCTION

Closantel is a broad spectrum salicylanilide anthelmintic that is commonly used in cattle and sheep to treat parasitic infections.[1] It is effective against both mature and development stages of a number of hematophagous nematodes, trematodes, and arthropods in sheep, goats, and cattle.[2,3] It binds strongly to plasma proteins, which serves to prolong anthelmintic activity for up to 28 days.[4,5] One of the most important applications of closantel is the control of fascioliasis, which impacts animal health and productivity by reducing weight gain and milk yield and results in suboptimal fertility. Liver fluke infection in some species such as sheep often leads to mortality.[6−9] The effective control of liver fluke, therefore, is important from both animal welfare and economic perspectives. Closantel can be used as a stand alone product or can be used in combination with other anthelmintic active ingredients, namely, ivermectin, mebendazole, and oxfendazole, which extend the spectrum of anthelmintic activity.

Because of the recurrent nature of parasitic infestations, flukicides are routinely administered to food-producing animals, including dairy cows.[10,11] Occasionally, to achieve optimal fluke control, it is sometimes necessary to treat cows during lactation. As with the administration of many veterinary medicines, the use of flukicides in dairy animals can result in veterinary drug residues in food products destined for the human food chain, including milk and dairy products,[12,13] leading to regulatory concern in Europe and elsewhere.[14] In Ireland, the most common and practical time to dose milk-producing animals for fluke is during the period of time the animal is not producing milk, which is typically 60 days prior to calving, known as the dry period.[15] During this time, cows are not contributing milk

to the human food chain and the opportunity for residues to enter the food chain is limited.

Prior to 2010, closantel was licensed for the control of liver fluke in Irish dairy cows. Its licensed use in dairy cows, however, was limited to the dry period similar to a number of additional flukicide products. At that time, a 60 day withdrawal period was thought to be sufficient to ensure that milk from treated cows was residue free. However, following the implementation of a new sensitive test for anthelmintic drug residues in Irish foodstuffs, closantel residues were detected in milk for longer than 60 days.[16] The concentration of closantel residue in milk samples was low (ca. 70 μg/kg), but samples were declared noncompliant due to the lack of a maximum residue limit (MRL) for closantel. Subsequently, veterinary medicinal products containing flukicides having no MRL for milk (namely, clorsulon, triclabendazole, nitroxynil, rafoxanide, and closantel) were prohibited from use in Irish dairy cows during lactating or dry periods.[6] Following a scientific review by the European Medicinal Agency, Committee on Veterinary Medicinal Products (CVMP), a provisional MRL of 45 μg/kg (until January 1, 2014) has been set for closantel in milk.[17] However, closantel continues to be prohibited for use in dairy cows, as satisfactory data to support the establishment of a withdrawal period has yet to be produced. Withdrawal periods can vary depending on animal species and food being produced and are required to support licensing of the use of closantel in
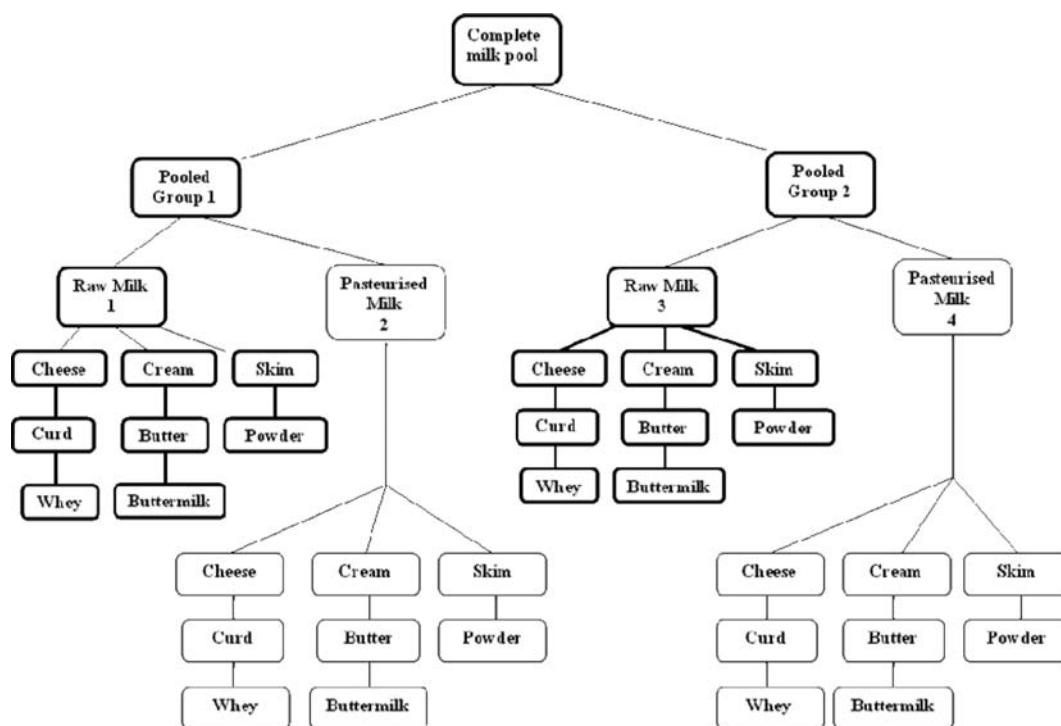
Figure 1. Flowchart of the process showing the milk products manufactured from milk containing closantel. This process was repeated with high and low levels of residue.

dairy cows, and withholding times must be respected to avoid MRL violations in milk.[10] The depletion of closantel residues in milk following its intramuscular administration at 5 mg/kg to lactating dairy cows peaked at a concentration of 1070 μg/kg^{-1} in the milk at day 7 and decreased to 220 μg kg^{-1} in the milk at day 35.[2] It was also reported that, following administration of an oral dose (5 mg/kg body weight), residues of oxyclozanide, which belongs to the same salicylanilide group as closantel, were below the reporting limits of 1 μg/kg in the milk of lactating dairy cows by day 7.[16] Rafoxanide, also a salicylanilide, could be detected in milk for 33 and 68 days (in separate studies), following administration to dairy cows during lactation.[18,19] Similarly, studies have been carried out on the persistence of additional flukicides in milk, namely, nitroxynil which persisted for at least 58 days in four of the six lactating cows.[20] In studies on triclabendazole (TCB), residues were monitored for up to 10 days postadministration[13,21] and 23 days postadministration, where residues were no longer detected.[22] However, no studies have been reported on the persistence of closantel in milk sourced from cows returning to lactation following closantel administration during the previous dry period, i.e., the period between the end of one lactation cycle and the beginning of another, when cows are in-calf.

In the interest of accumulating knowledge on the potential safe use of the flukicide closantel, it is important to establish the residence time and appropriate withdrawal periods in both lactating and dry cows in licensed trials. Therefore, the primary objective of this study was to investigate the persistence of closantel residues in bovine milk following its administration to cows during both lactation and dry periods and to study the transfer of residues from the milk of lactating cows to dairy products (cheese, cream, curd, whey, butter, skim milk, and buttermilk). In addition, the stability of closantel residues in products during prolonged storage was studied.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile, methanol, ammonium formate (all LC-MS grade), NaCl, and anhydrous MgSO$_4$ (both analytical grade) were sourced from Sigma-Aldrich (Dublin, Ireland) and VWR (Leicester, UK). Acetonitrile (pesticide grade), formic acid, and dimethyl sulfoxide (both analytical grade) were sourced from BDH Chemicals Ltd. (Poole, UK). Dispersive solid-phase extraction tubes containing C$_{18}$ (0.5 g) and MgSO$_4$ (1.5 g) were supplied by Agilent Technologies (Santa Clara, CA). Ultrapure water (18.2 MΩ) was generated in-house using a Millipore water purification system. Closantel (Vetranal grade) and closantel-^{13}C6 (internal standard) were purchased from Sigma Aldrich and Witega Laboratories (Berlin, Germany), respectively. Stock solutions were prepared for closantel and closantel-^{13}C6 at concentrations of 2 and 1 mg/mL in MeOH and denatured methanol (MeOD), respectively.

Animal Studies. Trial Conducted during the Dry Period. In this licensed study (licensed by both the Department of Health & Children, Ireland, ref: B100/4375, and by the Department of Agriculture Food and Marine, license no. RL/10/03A), six dairy cows of differing breeds (three Holstein Friesian, two Montbeliard, and one Norwegian Red) were weighed (animals weighed between 453 and 596 kg), and closantel was administered subcutaneously using 10 mL of Flukiver, 50 mg/mL per 100 kg live weight. A single dose was administered by subcutaneous injection at drying off. Flukiver 50 mg/mL solution (Janssen Cilag Ltd.) (VPA: 10545/010/001) contains closantel as closantel sodium 50 mg/mL. The animals were isolated in a paddock on pasture and were milked after other cows in the herd were milked. A 50 mL milk sample was collected from each cow prior to administration of Flukiver to act as a control. Study animals were bred using artificial insemination, which was not synchronized. Differences in individual cow reproductive cycles, therefore, lead to cows being at different stages of gestation at administration of closantel. Following calving (at 61 to 117 days postadministration), milk samples were taken at day 3.5 of subsequent lactation and following that every seven days for up to 77 days postcalving. Samples were labeled on collection, stored at −20 °C, and analyzed within one week of collection.

Animal Studies. Trial Conducted during the Lactating Period. In a licensed study (licensed by both the Department of Health & Children, Ireland, ref: B100/4375, and by the Department of Agriculture Food and Marine, license no. RL/10/03), the same six dairy cows of differing breeds (above) were weighed and during lactation were administered closantel subcutaneously using 10 mL of Flukiver, 50 mg/mL per 100 kg live weight, with a single dose by subcutaneous injection, on the same day. Prior to administration, a 50 mL milk sample was collected from each cow as a control. Following treatment, milk samples were taken twice daily (morning and evening) up to day 59, and subsequently samples were collected weekly until day 199 post-treatment. Samples were labeled on collection, stored at −20 °C, and analyzed within one week of collection.

Design of Dairy Processing Experiments. The cows were divided into two groups of three cows (balanced by breed as much as possible), and the total milk from each group was collected and pooled on days 2 and 25, which were representative of high and low closantel concentrations, respectively. Each milk pool was further subdivided and one portion was pasteurized at (72 °C × 15 s) while the second remained unpasteurized, resulting in eight portions of milk. From each portion, a semisoft laboratory scale cheese was manufactured, and the remainder of the portion was separated into skim milk and cream. After separation of the curd and whey during cheesemaking, both were analyzed in addition to the final cheese. Butter and buttermilk were manufactured from the cream, and skim milk powder was manufactured from the skim milk (Figure 1).

Cheesemaking. Milk (10 L; pasteurized and unpasteurized) was transferred into the vat and heated to 32 °C with constant gentle agitation, while periodically monitoring the pH. When the temperature reached 20 °C, 1 g of starter culture (direct vat set - EU RST 63; Chr. Hansen, Little Island, Cork 1) was added. The milk was ripened until the pH dropped below pH 6.55 (20−45 min), and rennet (Moorepark Technologies Ltd., Fermoy, Co. Cork) was added (1.7 mL diluted in 200 mL of sterile water). The curd was allowed to set for 75 min until it was firm enough to cut. The curd was cut into cubes (approximately 1 cm cubed) and stirred for 5 min, and the temperature was raised to a final temperature of 36 °C at a rate of 1 °C every 5 min, while stirring continuously. After cooking, the curd was placed into cheese molds when the pH was at or below pH 6.4. The cheeses were turned postfilling, every 30 min for 3 h and every 90 min thereafter until brining. The molds were maintained at ambient temperature until the pH dropped to 5.25−5.3 (8−10 h postmolding). The cheese was brined by immersing it in a 23% brine solution for 75 min, drained for 10 min postbrining, and analyzed for closantel residues.

Separation of Whole Milk To Manufacture Skim Milk and Cream. Milk was heated to 50 °C and separated using a Disc Bowl Centrifuge (Armfield, Hampshire, UK.). Cream and skim milk fractions were collected.

Manufacture of Butter. Following the separation of milk into skim milk and cream, the cream was chilled and whisked in a food blender until the cream separated into buttermilk and butter. Skim milk, cream, butter, and buttermilk samples were analyzed from fresh product immediately after manufacture.

Manufacture of Skim Milk Powder. A laboratory scale Buchi Mini Spray Dryer B-191 was used for the manufacture of skim milk powder. Prior to spray drying, the skim milk was heated to 50 °C. The spray drier used was a benchtop-laboratory-scale spray-drier (B-191; Buchi, Flawil, Switzerland). The skim milk suspension was pneumatically atomized into a vertical drying chamber using a two-fluid nozzle system. The inlet temperature was maintained at 185 °C throughout the analysis. The flow-rate of the skim milk suspension was varied by the adjustment of controls wherein this flow-rate controlled the outlet air-temperature which was maintained at 90 ± 2 °C. Spray-dried samples were collected at the base of the cyclone.

To prevent cross-contamination of residue between samples, the spray drier was dismantled, cleaned between each sample, and following thorough cleaning and drying was reassembled, and distilled water was pumped through for a minimum of 1 h.

Stability of Closantel Residues in Dairy Products. The stability of closantel residues was assessed in cheese and butter during cheese ripening and refrigerated butter storage, respectively. Samples of fresh product stored at 14 °C and 4 °C, respectively, were tested weekly over a 21-day period. Once each weekly sample had been taken, the remaining cheese or butter portion was frozen at −20 °C and further analyzed after 6 and 12 months. Skim milk, cream, buttermilk, curd, and whey samples were analyzed on the day of manufacture, frozen immediately at −20 °C, and stored at that temperature for further residue stability analysis at 6 and 12 months. Skim milk powder was stored in 50 mL centrifuge tubes in the dark at ambient temperature (18−22 °C). Samples of powder were analyzed at day 0, 6, and 12 months.

Analysis of Dairy Product Samples. Milk, skim milk, whey, and buttermilk samples were manually mixed end-over-end to ensure sample homogeneity prior to taking a sample aliquot. A 10 g (±0.1 g) quantity of milk buttermilk, skim milk, or whey samples was weighed into a 50 mL centrifuge tube. Butter, cheese, cream, curd, or powder samples were weighed (1 ± 0.01 g) into centrifuge tubes (50 mL), and ultrapure water (9 mL) was added where the samples were manually mixed end-over-end to ensure sample homogeneity. Cream samples were weighed (4 g ± 0.04 g) into centrifuge tubes (50 mL), and ultrapure water (6 mL) was added where the samples were manually mixed end-over-end to ensure sample homogeneity. Cheese and curd samples (4 ± 0.04 g) were weighed into centrifuge tubes (50 mL), and ultrapure water (6 mL) was added. The tubes containing cheese, curd, and butter were placed in a water bath at 50 °C until the product and water became homogeneous. Samples, extracted matrix calibrants, and recovery controls were fortified with 25 μL of internal standard. The recovery controls were further fortified with 100 μL of working standards 1−8 described in calibrants, and fortified samples were allowed to sit for 15 min.

Pesticide grade acetonitrile (12 mL) was added to each tube. $MgSO_4$ (4 g) and NaCl (1 g) were subsequently added to each tube in batches of six and shaken vigorously by hand (1 min). Tubes were centrifuged at 2876g for 12 min at 4 °C. The upper acetonitrile layer was decanted into 50 mL polypropylene tubes containing C_{18} (0.5 g) and $MgSO_4$ (1.5 g), which were vortexed (1 min) and centrifuged at 1467g for 10 min at 4 °C. A 6 mL portion of the supernatant was transferred into a 15 mL polypropylene tube containing 0.25 mL of dimethyl sulfoxide (DMSO). The tubes were placed in a Turbovap, and the acetonitrile was evaporated at 50 °C. Extracts were filtered through 0.2 μm PTFE syringe filters (Whatman Rezist) and injected onto the UHPLC-MS/MS system. UHPLC-MS/MS conditions are described elsewhere.[16]

Calibration and Controls. Extracted matrix calibrants were prepared by adding closantel working standard solutions to closantel negative milk samples prior to extraction. The following concentrations (in μg/mL) were used: 40 (standard 8), 20 (standard 7), 10 (standard 6), 5.0 (standard 5), 2.0 (standard 4), 1.0 (standard 3), 0.40 (standard 2), and 0.20 (standard 1). Calibration curves were prepared by fortifying matrix blanks before extraction with 100 μL of the standards to give a working milk standard curve in the range of 1.0 to 200 μg/kg. Additionally, four blank milk samples (recovery controls) were fortified after evaporation, two with standard 2 (50 μL) and two with standard 5 (50 μL) to monitor for loss of analytes during extraction.

Validation. The following analytical method performance parameters were investigated: within laboratory reproducibility (WLR), within laboratory repeatability (WLr), specificity, linearity, and accuracy.

The milk validation was carried out in bovine, caprine and ovine milk by fortifying the milks at 22.5, 45.0, and 67.5 μg/kg. In each case, the samples were analyzed using a standard curve prepared from the corresponding milk type. Each milk type validation run was carried out by a separate analyst, and the data generated were used to calculate the WLR. A WLr validation was carried out in bovine milk by the same analyst repeating the validation on an additional two occasions.

The dairy product validations were carried out by fortifying negative controls (butter, feta cheese, and skimmed milk powder) at 225, 450, and 675 μg/kg. Prior to the validation, a range of dairy products were analyzed by UHPLC-MS/MS to identify a suitable negative control

Table 1. Validation Results for Closantel in Milk (*n* = 7 for each of three different analyses on separate days) and Dairy Products (*n* = 7 for one analysis on different days)

	fortification level (μg/kg)								
	bovine milk			caprine milk			ovine milk		
	22.5	45	67.5	22.5	45	67.5	22.5	45	67.5
mean (μg/kg)	22.3	43.1	65.7	22.7	44.9	67.4	20.9	42.4	62.3
SD (μg/kg)	2.9	3.2	4.0	1.4	3.8	2.1	0.8	3.0	2.7
CV (%)	12.8	7.3	6.1	6.2	8.5	3.2	3.8	7.1	4.4
accuracy (%)	99	96	97	101	100	100	93	94	92
	fortification level (μg/kg)								
	butter			cheese			skim milk powder		
	225	450	675	225	450	675	225	450	675
mean (μg/kg)	223	452	679	236	454	680	226	455	643
SD (μg/kg)	8.3	9.5	17.3	6.8	13.5	20.9	5.0	23.5	56.8
CV (%)	3.7	2.1	2.5	2.9	3.0	3.1	2.2	5.2	8.8
accuracy (%)	99	100	101	105	101	101	101	101	95

that did not contain closantel residues and to evaluate the selectivity of the method. In each case, the samples were analyzed using a standard curve prepared in the corresponding dairy product matrix.

Statistical Analyses. All statistical analyses were carried out using SAS (Jmp version 10, SAS Institute, Cary, NC). A randomized design that incorporated the treatment (pasteurized and unpasteurized) and the effect of residue over time was used. The response variables are related to the cheese and butter manufactured using pasteurized and unpasteurized milk.

Small sample sizes impacted the assumptions of normality. Therefore, nonparametric statistical methods were used for data analysis, and both values for each three-cow group are shown. Statistically significant differences ($P < 0.05$) between treatments and time were determined by using Kruskal–Wallis nonparametric one-way analysis of variance and the Mann–Whitney–Wilcoxon test.

■ **RESULTS**

Analytical Methodology. Samples were analyzed by adapting a method that was previously developed for the analysis of anthelmintic residues in milk samples.[12] In this work, closantel-[13]C6 was included as an internal standard to improve the accuracy and precision of the analytical method. The method was revalidated using bovine, caprine, and ovine milk samples. Accuracy and precision (expressed as the coefficient of variation) were in the ranges of 92–102% and 2.1–18.8%, respectively (Table 1). Recoveries of closantel from bovine, caprine, and ovine milk were calculated by comparing the concentrations in fortified samples with control samples spiked postevaporation to be 65%, 53%, and 45%, respectively. Further validations were carried out using butter, cheese, and skim milk powder samples. Accuracy and precision were in the acceptable ranges of 95–105% and 2.1–8.8%, respectively. The mean recovery of closantel from butter, cheese, and powder samples was 100%, 100%, and 99%, respectively. Initial experiments were carried out showing that accuracy was satisfactory using a milk calibration curve, and therefore samples from the different matrixes were measured against a milk calibration curve.

The method was specific, and other anthelmintic drug residues did not interfere with the results. More than 30 different milk and dairy product samples were used to evaluate the selectivity of the method, and the matrix did not impact the accuracy of results. The linearity of calibrations curves was satisfactory, with R^2 values of >0.99.

Closantel Residues in Milk. Dry Period. Control milk samples taken from each animal prior to administration resulted

in no detectable closantel residue levels. This implies that any potential closantel residues present in milk postcalving were due to the treatment.

In all cases, the first milk samples analyzed postcalving had residue levels less than the provisional MRL of 45 μg/kg. The values obtained ranged from nondetected to 8.7 μg/kg, for animals calving at 117 and 61 days postadministration, respectively (Figure 2). The limit of detection of the method

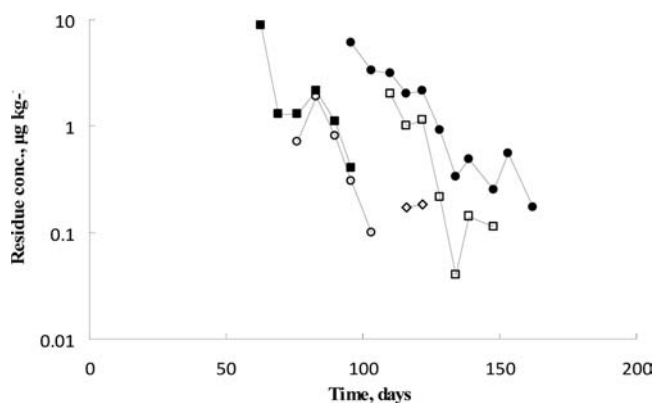**Figure 2.** Excretion of closantel (μg/kg) into milk as a function of time (days) postcalving, following administration of closantel at drying-off. The analysis of the milk from each individual cow is shown: cow no. 3429 (□), cow no. 2644 (◇), cow no. 4422 (●), cow no. 4686 (■), cow no. 4670 (○). Cow no. 4584 had no detectable residue in the milk postcalving.

(CCα) was 0.67 μg/kg. Even though closantel levels in the milk postcalving were well below the provisional MRL in the case of milk samples analyzed from all six cows, the milk was analyzed weekly until no residue was detected in any cow for three consecutive readings (Figure 2).

Calving occurred at between 61 and 117 days postadministration of the closantel. The period of time taken to obtain undetectable closantel residues in the milk postcalving was different for each individual cow. One cow had no detectable residue from the first sample analyzed postcalving (117 days postadministration), while another cow had no detectable residue after 14 days postcalving (114 days postadministration). Three cows took between 35 and 49 days postcalving to have no detectable residue in the milk (73 to 103 days

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postadministration) while one cow had detectable residue for up to 77 days postcalving (98 days postadministration).

Closantel Residues in Milk. Lactating Period. Similar to the dry cow study described previously, control milk samples taken from each animal prior to administration had no detectable closantel residues. The maximum concentration of closantel measured in the milk of treated animals was 356 ± 98 μg/kg ($n = 6$) which occurred at day 2 post-treatment (Figure 3). Closantel residues were detectable in milk for 199 days

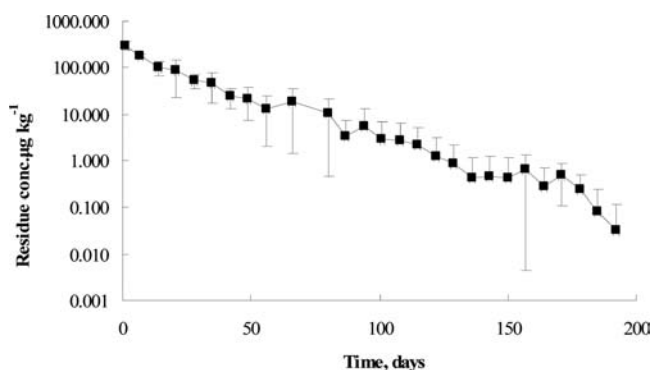**Figure 3.** Excretion of closantel (μg/kg) into milk as a function of time (days), following administration of closantel (as Flukiver, 50 mg/mL per 100 kg live weight) during the lactation period. The average and standard deviation of the excretion of closantel into the milk of six cows is shown.

post-treatment in one of the six cows, while the remaining five cows had no detectable residues in the milk, between 178 and 192 days. These results indicate that the closantel residues persisted for at least 199 days in milk. However, after a period of approximately 52 days postadministration, closantel residues in milk were below the provisional MRL of 45 μg/kg.

Milk for Product Manufacture. Milk from both groups of three cows containing high (day 2) or low (day 23) residue concentrations of closantel (at days 2 and 23, respectively) was collected and used for dairy product manufacture.

There was no significant difference in the closantel residue levels between pasteurized and unpasteurized milk ($P > 0.05$) at both high and low residue levels (Tables 1 and 2). The residue concentration in the milk from each group was different,

ranging from 236 to 401 μg/kg at high residue levels and between 53 and 67 μg/kg at low residue levels. This could be explained by the fact that individual animals will metabolize the drug to varying degrees,[23] and this was reflected in the products manufactured from the milk. In each of the independent duplicates, the trends in the results between the different treatments and products were similar.

Transfer of Closantel Residues into Cheese. The migration of closantel during cheesemaking with milk containing both high and low concentrations of closantel residue in pasteurized and unpasteurized milk is shown in Table 2. During cheesemaking, curds and whey are separated; the whey is not used any further, and the curds continue to expel smaller amounts of whey to produce cheese. During the manufacture of cheese from milk with high residue concentrations, closantel residues were similar in curd and starting milk, with approximately half that amount in the whey. However, during the manufacture of cheese from milk with low residue concentrations, closantel residues were approximately six times higher in curd compared with the starting milk, with approximately 12% of the amount present in curd lost through the whey (Table 2).

The closantel concentrations continued to increase during ripening of the cheese as a result of further whey expulsion, except for the cheese made from pasteurized milk with high residue concentration. Generally, there was a higher concentration of residue in the cheese after manufacture than in the starting milk, although with lower residue concentrations the difference between milk and cheese was greater, i.e., 53−67 μg/kg in milk compared to 266−293 μg/kg in the cheese. During ripening of the cheese over a 3 week period, the residue concentration in the cheese made from high residue milk increased significantly ($P < 0.05$) while in the cheese made from low residue milk there was no significant difference ($P > 0.05$) in residue levels during cheese ripening.

Transfer of Closantel Residues into Butter, Buttermilk, Cream, Skim Milk, and Skim Milk Powder. The migration of closantel residues (from both high and low residue containing milk) to cream and skim milk during the separation of whole milk is shown in Table 3. In both high and low residue-containing milk, the residue was concentrated at least 10-fold in the cream.

Table 2. Concentrations (μg/kg) of Closantel Residues in Pasteurized and Unpasteurized Milk, Cheese, and Cheese Products during Manufacture and Cheese Ripening at Three Different Time-Points and during Frozen Storage at −20°C

| product | high residue μg/kg | | | | | | low residue μg/kg | | | | | |
	U^a	U 6 months[b]	U 12 months	P^c	P 6 months	P 12 months	U	U 6 months	U 12 months	P	P 6 months	P 12 months
milk	273; 236	452; 408	371; 428	401; 337	645; 404	499; 406	67; 55	106; 90	72; 30	67; 53	99; 96	93; 42
curd	283; 322	688; 583	1346; 1184	331; 613	482; 435	1220; 1680	173; 165	378; 334	242; 168	190; 143	447; 432	141; 219
whey	160; 130	181;196	228; 229	140; 135	218; 219	257; 228	21; 23	39; 42	23; 20	25; 24	42; 39	23; 22
cheese, week 0[d]	339; 634	898; 1237	1494; 1706	249; 502	1062; 1089	1182; 1902	266; 216	526; 464	295; 220	293; 255	503; 382	279; 228
cheese, week 1	1204; 1102	1508; 1620	2716; 2336	1092; 1064	1356; 1193	2474; 2066	420; 285	771; 602	332; 283	377; 265	805; 566	316; 263
cheese, week 2	1180; 866	1730, 1776	3278; 2350	1098; 676	1667; 1689	2954, 2620	554; 272	1072; 687	444; 264	465; 335	907; 607	354; 268
cheese, week 3	1466; 1094	2093; 1296	2934; 2328	1329; 1090	1982; 1409	3128; 2652	394; 276	1008; 609	449; 284	390; 328	956; 749	396; 333

[a]Refers to unpasteurized milk. [b]Refers to frozen (−20 °C) storage. [c]Refers to milk that has been heat treated (pasteurized) at 72 °C for 15 s. [d]Refers to ripening time of the cheese.

Table 3. Concentrations (μg/kg) of Closantel Residues in Pasteurized and Unpasteurized Milk, Cream, Skim Milk, Butter, Buttermilk, and Skim Milk Powder during Manufacture and Storage at Three Different Time-Points and during Frozen Storage at −20 °C

product	high residue μg/kg						low residue μg/kg					
	U[a]	U 6 months[b]	U 12 months	P[c]	P 6 months	P 12 months	U	U 6 months	U 12 months	P	P 6 months	P 12 months
milk	306; 302	550; 436	414; 492	315; 273	587; 453	430; 434	66; 50	101; 88	43; 60	67; 52	107; 81	38; 55
skim milk	193; 144	228; 270	312; 308	172; 178	297; 254	319; 307	33; 11	52;18	26; <10	36; 31	48; 50	33; 36
cream	2407;1628	5054; 3122	6912; 3930	1400; 1892	2316; 3018	3404; 2820	455; 407	988; 851	475; 422	458; 349	874; 620	555; 302
skim powder	1571;1558	ND;[d] 3084	ND; 2984	1722;1458	2072; 3236	3588; 3200	378; 90	698; 152	ND; ND	373; 316	796; 632	ND; ND
butter, week 0[e]	3656; ND	7636; ND	8204; ND	2466; 2788	5666; 5632	5300; 6260	778; 1071	1836; 2173	799; 1032	793; 590	1581; 1182	956; 623
butter, week 1	3868; ND	8112; ND	8280; ND	2320; 2684	5128; 5158	4612; 5856	753; 991	1600; 1875	752; 977	772; 555	1502; 1239	874; 521
butter, week 2	3634; ND	7340; ND	7556; ND	2332; 2752	5008; 5362	4844; 5572	781; 1072	1419; 1765	527; 1131	859; 629	1659; 999	895; 599
butter, week 3	3704; ND	7162; ND	5452; ND	2468; 2722	4492; 4750	3768; 3944	694; 914	1474; 1768	709; 770	712; 537	1371; 1132	498; 429
buttermilk	1205; ND	680; ND	584; ND	304; 355	439; 529	437; 483	62; 17	119; 47	30; 12	82; 37	184; 69	60; 23

[a]Unpasteurized milk. [b]Refers to frozen (−20 °C) storage. [c]Milk that has been pasteurized (72 °C for 15 s). [d]Not determined. [e]Ripening time of the butter.

When cream from high residue-containing milk was converted to butter, it was found that approximately 90% of the residue migrated with the fat, resulting in further concentration in the butter; the remaining residue remained in the buttermilk. Similarly, with cream from low residue-containing milk, the majority of the residues migrated with the fat to the butter, with relatively little remaining in the buttermilk (Table 3).

When skim milk powder was manufactured from skim milk containing both high and low residue concentrations, the residue in the skim milk powder increased 8-fold at high residue level and 10-fold at the low residue level, compared with the starting skim milk.

Stability of Closantel Residues in Dairy Products. With the exception of the butter made from unpasteurized milk with high residue concentrations, where there was only one value, there was no significant difference ($P > 0.05$) in the residue concentrations of butter stored at 4 °C for 3 weeks.

In high or low residue-containing milk, cheese, butter, buttermilk, curd, whey, skim milk, and cream samples, most residue measurements were significantly different ($P < 0.05$) following storage at −20 °C for 6 and 12 months, generally with increased concentrations detected after 6 months storage and further increases after 12 months storage. For example, the concentration of closantel in butter increased from approximately 3700 μg/kg when measured in fresh product to approximately 7500 μg/kg after 6 months storage at −20 °C and in most cases increased further after 12 months storage (Table 3). On the other hand, the concentration detected in buttermilk decreased from 1205 μg/kg to 680 μg/kg after 6 months storage (Table 3). In powder manufactured from both pasteurized and unpasteurized milk, the residue concentrations obtained after 12 months storage were increased significantly ($P < 0.05$) at both high and low residue levels, compared to those obtained from freshly prepared samples, under the storage conditions used.

■ DISCUSSION

The results of this study show that, following closantel administration during the dry period, residues in the milk directly postcalving were well below the provisional European MRL of 45 μg/kg, even when the period between administration and calving was as short as 61 days This indicates that closantel may be suitable for use as a flukicide in the dry period, based on the provisional MRL of 45 μg/kg that has been set in milk. However, if the dry period was shorter, this would increase the risk of residue concentrations closer to the provisional MRL being detected in the milk. Further studies would need to be undertaken to determine the impact of a shorter dry period.

Administration of closantel in the lactating cow study, however, highlighted that residues were detected in milk up to day 199 but were below the current provisional MRL by day 52 postadministration. This study therefore demonstrates that the withdrawal time potentially required following administration of closantel during lactation is of such duration as to render its use as a flukicide in lactating cows producing milk for human consumption impractical.

There is minimal data available on the presence and withdrawal of closantel residue levels in bovine milk following administration to lactating or dry cows. A recent study was carried out on 402 animals exposed to *F. hepatica* to assess the effect of closantel treatment during the dry period on milk production.[24] In that study, the effectiveness of closantel as a flukicide in the drying off period was demonstrated, resulting in an increase in milk production in the subsequent lactation; however, the milk was not analyzed for the potential persistence of closantel residues in the milk. A further study in Iran showed a 91% efficacy of closantel against sheep gastrointestinal parasites.[25]

There have been previous studies on the bioavailability of closantel in sheep and cattle,[2,26] camels,[27] goats, and sheep.[5,28] However, there have been few previous studies on the presence and stability of closantel residue levels in the products manufactured from milk which contains residues.

There have been some studies on other veterinary drug residues in products manufactured from milk in which residues were detected. Triclabendazole and rafoxanide residues were detected in milk products manufactured from milk containing each analyte.[22,29] With triclabendazole, the residues migrated in

the fat, concentrating in the cream, butter, and cheese. However with skim milk powder, manufactured from skim milk, the triclabendazole residues concentrated in the powder, despite the relatively low levels of residue detected in the starting skim milk.[22] Similar results were found with rafoxanide regarding residues migrating in cream, butter, and cheese as well as skim milk powder, despite the relatively low residue levels in the starting skim milk.[29] For both triclabendazole and rafoxanide, there were no significant differences in residue levels between products manufactured from pasteurized and unpasteurized milk, nor were there any significant differences in residues in butter and cheese stored at −20 °C for up to 1 year.[22,29] In storage of skim milk powder at ambient temperature for up to 1 year, rafoxanide residues remained stable in skim milk powder manufactured from both pasteurized and unpasteurized milk.[29] However, triclabendazole residues increased in skim milk powder manufactured from both pasteurized and unpasteurized milk when stored for up to 1 year, under conditions similar to that of skim milk powder containing rafoxanide residues.[22] The results from the dairy product manufacture in this current study, demonstrated that, similar to triclabendazole and rafoxanide studies conducted on milk products, closantel residues also migrated with the fat and concentrated in the cheese, butter, and cream, with lower concentrations found in the whey, buttermilk, and skim milk. Another study was conducted on the persistence of ivermectin (IVM) and moxidectin (MXD) residues in lactating sheep and fate during cheese production and ripening.[30] Similar to closantel, IVM and MXD are lipophilic compounds and have similar retention times when separated by UHPLC-MS/MS.[12] In those studies, the concentrations of IVM and MXD gradually increased approximately 3-fold in cheese during the ripening period of up to 40 days. This is similar to the increase in closantel residues in cheese during ripening observed in the current study. The significance of this result indicates that cheese manufactured with milk containing low residue concentrations could result in higher residue concentrations in cheese during the ripening process than in the starting milk.

In further studies on the thermal stability of veterinary drug residues in products manufactured from milk containing such residues, the stability of IVM, MXD, and eprinomectin (EPM) was studied in deliberately contaminated ewe's milk.[31] The milk was heat treated to 65 °C for 30 min and 75 °C for 15 s, and all three residues were stable during the heat treatment processes. In a separate study investigating the stability of IVM, MXD, and EPM residues in ewes' milk, cheese, and yogurt, residues were stable during high heat treatment temperatures of up to 90 °C.[32]

In a study conducted on the thermal stability of sulfamethazine in spray-dried milk the level of sulfamethazine detected in the milk samples increased 10-fold in the powder compared with the starting milk samples.[33] Similar results on the concentration of residues in powder were found in the current study. Processing of skim milk to powder involves the evaporation of water during the manufacturing process, increasing the residue concentrations in the powder. This concentration of residues in skim milk powder was found in another study involving powder manufacture [19] which demonstrated that >90% of the residue of the flukicides, nitroxynil, oxyclosanide, and levamisole migrate with the skim milk and were transferred to the skim milk powder.

A previous study on the thermal stability of closantel in beef[34] showed that closantel residues were stable during roasting and frying of beef, at internal temperatures of 92.9 °C and 67.3 °C, respectively. In separate studies on clenbuterol, levamisole, and sulfamethazine, the residues were stable under a range of cooking processes, including, boiling, roasting, grilling, frying, pressure cooking, and microwaving, in meat.[35−38] However, oxytetracycline was unstable during these processes. This heat stability of closantel was also evident in the current study, as pasteurization and spray-drying temperatures had no effect on the presence of closantel residues in the milk products.

There are limited studies on the stability of closantel residue in milk or milk products in storage, whether ambient or frozen, over time. There has been a previous study on the stability of IVM residues in sheep milk during storage at −20 °C for up to 2 years.[39] In that study the milk was taken from 13 ewes administered with 0.2 mg/kg bw of IVM, and during lactation, the milk was collected, up to 9 days postadministration. A portion of each milk sample was frozen with no heat treatment, while the remainder of the samples collected were subjected to three separate heat treatments. One group of samples was heated at 74 °C for 40 s, the other group heated at 80 °C for 1 min, and the final group heated at 100 °C for 10 s. The results demonstrated no significant differences in the IVM residues present between the milk receiving different heat treatments and the raw milk. The findings on the stability of IVM residues over time in storage at −20 °C indicated that there was no significant change in residue concentrations within the first year of storage; however, after 2 years of freezer storage, the levels of IVM residues decreased significantly. This result differs from the current study, where the closantel residues generally increased considerably, in many cases by as much as 50%, during 6−12 months of freezer storage at −20 °C and during ambient temperature storage for powder for 1 year also. The change in closantel measurements during storage requires further research to understand the effect of storage on closantel and to determine if the increased values are real or just an artifact of methodology. The fact that the concentration of residue increased from undetectable in milk to detectable in powder is important information for infant food manufacturers and highlights the significance of developing very sensitive test methods to ensure milk is free of even low level residues.

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Notes

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