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Investigation of the Persistence of Nitroxynil Residues in Milk from Lactating Dairy Cows by Ultra Performance Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT: Nitroxynil is an anthelmintic used in the treatment of liver fluke. In this study, six dairy cows were treated during lactation with Trodax, a 34% solution containing nitroxynil as its N-ethylglucamine salt, indicated for the treatment of fascioliasis in cattle and sheep. Samples were collected twice daily for 16 days and later at weekly intervals up to 58 days post-treatment. Nitroxynil residues were extracted from milk samples using acetonitrile; magnesium sulfate and sodium chloride were added to induce liquid—liquid partitioning and purified by dispersive solid phase extraction for clean-up. Nitroxynil was determined by ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) in negative ionization mode. The limit of detection (CCα) of the method is 0.24 μg/kg. Maximum concentration of nitroxynil in the samples was in the range of 688−1358 μg/kg, with levels persisting for 58 days in four of the six lactating cows. Incurred nitroxynil samples were treated with sulfatase and β-glucuronidase from *Helix pomatia*; the results indicated the presence of glucuronide conjugates in samples at early withdrawal times. At later withdrawal times the concentration of free nitroxynil was lower than the concentration in the control samples, indicating potential degradation during enzymatic treatment.

KEYWORDS: nitroxynil, anthelmintic, flukicide, fate, dairy cows, milk, QuEChERS, enzyme hydrolysis, UPLC-MS/MS

■ INTRODUCTION

Nitroxynil is a halogenated phenol (Figure 1) that is active against both mature and immature stages of liver fluke and is used in the treatment of fasciolosis and hemonchosis in sheep and cattle. However, nitroxynil has been found to have lower efficacy against immature fluke, which could be due to high protein binding of nitroxynil in blood.[1−3] It has been reported that nitroxynil has undesirable toxic effects at high doses in laboratory animals, namely, goitrogenic effects.[4]

Several anthelmintic drugs are licensed for treating parasitic infections in food-producing animals. However, only a limited number of products are licensed for treatment of animals during the lactating period and have a maximum residue limit (MRL) listed under European Council Regulation 2377/90. As a result, veterinary products containing nitroxynil were recently prohibited for the treatment of fluke in dairy cows in the Republic of Ireland.[5,6]

Few papers have been published to date on the persistence of nitroxynil in dairy cows. These studies were carried out using polarography and gas chromatography coupled to electron capture detector (GC-ECD) or mass spectrometry (GC-MS).[7−10] Takeba and Matsumoto[7] treated three cows by subcutaneous injection, resulting in a maximum concentration of 0.26 mg/kg; residues were below 0.1 μg/kg by week 8. Heeschen et al.[8] treated three animals subcutaneously with 10 mg/kg of body weight (bw) nitroxynil, resulting in a maximum concentration of 1.5 mg/kg, and residues persisted in milk for 14 days. Bluthgen et al.[9] treated three animals subcutaneously with 10 mg/kg of bw with a maximum concentration of 146 mg/kg. Takeshita et al.[10]

treated two animals subcutaneously with 10 mg/kg of bw nitroxynil and analyzed samples using a GLC, GC-MS, and polarographic techniques. Nitroxynil residues were nondetectable in samples after 45 days.

Recently, a sensitive UPLC-MS/MS method was developed by our group for the detection of a wide range of anthelmintic residues in milk.[11] The residues were extracted from milk using a modified QuEChERs (quick, easy, cheap, effective, rugged, and safe) extraction method, which is a quick and easy extraction procedure for isolating a wide range of residues. The method allows the detection of nitroxynil residues to <1 μg/kg.[11] The objective of this work was to apply this method to milk from lactating dairy cows treated with nitroxynil to determine the persistence of nitroxynil in milk. In addition, enzymatic hydrolysis was used to investigate the presence of conjugated forms of nitroxynil residues in milk. This information can be utilized to identify withdrawal periods for nitroxynil in dairy cows in the event that a MRL is defined.

■ MATERIALS AND METHODS

Reagents and Samples. Preweighed tubes containing 4 g of anhydrous (anh) magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl) in 50 mL centrifuge tubes (tube 1) and 0.15 g of anh $MgSO_4$ and 0.05 g of end-capped octadecylsilane C_{18} in 2 mL
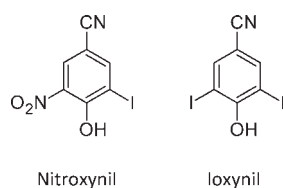
Figure 1. Chemical structures of nitroxynil and its internal standard ioxynil.

microcentrifuge tubes (tube 2) were obtained from UCT, Inc. (Bristol, PA). Sulfatase from *Helix pomatia* type H-1 and β-glucuronidase from *H. pomatia* type H-5 were purchased from Sigma-Aldrich (Ireland). Organic milk was purchased in supermarkets and tested for residues prior to analysis.

UPLC-MS/MS Analysis. As previously described,[11] chromatographic separations were performed using an 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, all from Waters (Milford, MA). The column temperature and the pump flow rate were maintained at 60 °C and 0.6 mL/min, respectively.

Analytes were separated using the following mobile phase conditions: mobile phase A, 0.01% acetic acid (HOAc) in water/acetonitrile (H_2O/MeCN) (90:10, v/v), and mobile phase B, 5 mM ammonium formate in methanol (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.

Nitroxynil and ioxynil were quantitated (Figure 2) using a Waters Quattro Premier XE triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. The UPLC-MS/MS system was controlled by MassLynx software, and data were processed using TargetLynx software (both from Waters). Injection volume was 5 μL. Nitroxynil and ioxynil were tuned on the UPLC-MS/MS, and the optimum conditions obtained were input into the multiple reaction monitoring (MRM) window. The following transitions were input into a MRM window: m/z 288.90 $\rightarrow$ 126.86 and m/z 288.90 $\rightarrow$ 161.94, nitroxynil (retention time of 3.13 min); and m/z 369.65 $\rightarrow$ 126.80, ioxynil (retention time of 4.55 min). The MRM was in negative ion mode with dwell time set to ensure 12−15 data points were obtained for each analyte.

Calibration. Nitroxynil and ioxynil were purchased from Sigma-Aldrich. Ioxynil (Figure 1) is a herbicide from the same structural group as nitroxynil; as this drug is not used in animals, it was chosen as a suitable internal standard.

Primary stock standard solution was prepared at concentrations of 4000 and 1000 μg/mL for nitroxynil and ioxynil in MeOH, respectively. Matrix calibrants were prepared by fortifying negative milk samples (10 g) with 100 μL of standard solutions containing nitroxynil at 0.1, 0.25, 0.5, 1, 2.5, and 5 μg/mL to give matrix concentrations of 1, 2.5, 5, 10, 25, and 50 μg/kg, respectively. The range of the matrix calibration curve was extended for the alternative method to 200 μg/kg by fortifying two negative samples with 200 and 400 μL of standard 6 (5 μg/mL) to give matrix concentrations of 100 and 200 μg/kg, respectively. Four blank matrix samples (recovery controls) were fortified after extraction, two with 50 μL of standard 2 (0.25 μg/mL) and two with 50 μL of standard 5 (2.5 μg/mL). Recovery controls were spiked with 50 μL rather than 100 μL as half the original extract was brought through to the final step. The recovery controls are used to monitor for loss of nitroxynil during extraction.

Sample Preparation. The method used for samples with concentrations >20 μg/kg was a modified version of the previous method.[11] The modifications were required to extend the linear range of the analytical method. Milk samples (10 ± 0.1 g) were weighed into centrifuge tubes (50 mL) and fortified with internal standard (10 μg/kg) and kept for
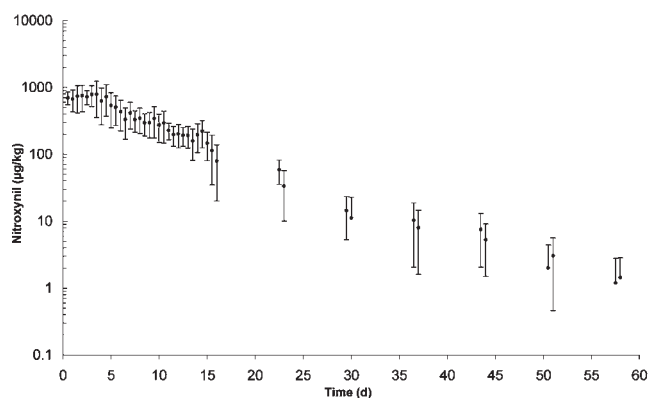

Figure 2. Mean nitroxynil depletion in milk of six animals treated subcutaneously with nitroxynil up to 58 days post-treatment.

15 min. Samples at concentrations outside the calibration range were diluted 1 in 10 or 1 in 5 using negative control. The residues in the samples were extracted into MeCN (12 mL), in the presence of $MgSO_4$ (4 g) and NaCl (1 g) from tube 1. Samples were shaken immediately and centrifuged for 12 min at 3500 rpm (959g). A dispersive solid phase extraction (d-SPE) cleanup step was performed by adding 1 mL of the supernatant into tube 2, a microcentrifuge tube (2 mL) containing $MgSO_4$ (0.15 g) and C_{18} (0.05 g). The samples were vortexed for 30 s and centrifuged for 2 min at 16200g. The supernatant (0.5 mL) was added to a test tube containing DMSO (1 mL) and vortexed for 1 min. MeCN was evaporated under nitrogen at 50 °C using a Turbovap apparatus at a constant volume. Extracts were filtered through 0.2 μm PTFE 13 mm syringe filters (Whatman Rezist) and injected onto the UPLC-MS/MS system.

Milk samples at concentrations <20 μg/kg were tested using the method described.[11] This method used large-scale d-SPE tubes (samples and sorbents scaled up by a factor of 10); the supernatant was added to a centrifuge tube (50 mL) containing 1.5 g of $MgSO_4$ and 0.5 g of C_{18}, vortexed, and centrifuged for 10 min at 489g. The supernatant (6 mL) was added to a 15 mL test tube containing DMSO (0.25 mL) and concentrated.

Validation of Alternative Extraction Method. The alternative method was validated according to European Legislation 2002/657/EC.[12] Within-laboratory repeatability (WLr) was performed; this investigation resulted in the following parameters: linearity, precision, and recovery of the method. Milk samples were fortified at 1, 1.5, and 2 times the second lowest calibration level (LCL), 2 μg/kg.

Enzymatic Hydrolysis Studies. During the method development stage samples containing nitroxynil were treated with sulfatase and β-glucuronidase from *H. pomatia* (200 units) and buffered with 2 mL of sodium acetate (2 M, pH 5.2). Samples containing low, medium, and high concentrations of nitroxynil were subjected to enzymatic hydrolysis at 37 °C for 1, 2, 4, 6, and 16 h. Samples were cooled to ambient temperature after removal from the water bath. Cooled samples were spiked with internal standards (25 μL) and extracted as described in the previous (Sample Preparation) section.

Statistical Analysis. The results from the enzymatic hydrolysis studies were analyzed using a one-tailed t test. The null hypothesis was that enzymatically treated samples were not greater than the nontreated samples.

Animal Studies. Six cows weighing between 580 and 626 kg were selected for the study. The six cows were treated at 8.30 a.m. as per dosage regimen for Trodax 34% as described on the label (1.5 mL of Trodax 34% per 50 kg of bw). Animals were administered between 17.40 and 18.78 mL of Trodax 34% by means of subcutaneous injection. The first milking was taken 6.5 h after treatment at 3 p.m.; subsequent milk samples were taken from animals twice daily, morning (7 a.m.) and

Table 1. Results of Spiked Samples Buffered with 2 mL of Sodium Acetate (2 M, pH 5.2)[a]

nitroxynil	untreated sample, μg/kg (% CV)	buffer, 0 h, μg/kg (% CV)	buffer, 1 h, μg/kg (% CV)	buffer, 2 h, μg/kg (% CV)	buffer, 4 h, μg/kg (% CV)	buffer, 6 h, μg/kg (% CV)	buffer, 16 h, μg/kg (% CV)
low concn (n = 3)	23.75 (11.0)	24.87 (3.3)	24.83 (6.9)	25.73 (7.1)	26.7 (7.2)	25.33 (6.7)	25.7 (8.2)
mid concn (n = 3)	190.95 (2.5)	190.97 (1.9)	185.6 (7.8)	191.93 (1.4)	196.87 (2.5)	198.17 (2.6)	187.1 (5.5)
high concn (n = 3)	779.55 (3.4)	822.37 (1.7)	816.97 (1.3)	804.4 (1.5)	776.73 (4.6)	844.63 (5.5)	800.7 (2.6)

[a] Table contains the concentration of milk samples spiked with nitroxynil untreated with enzyme and spiked samples buffered with sodium acetate and incubated for 0, 1, 2, 4, 6 and 16 h. It also shows the % CV of the replicates.

Table 2. Results of Enzymatic Hydrolysis of Nitroxynil Incurred Milk Samples[a]

	nitroxynil (μg/kg)					
incubation time (h)	β-glucuronidase (n = 3)			sulfatase (n = 3)		
0[b]	186	322.33	530.33	186	322.33	530.33
1	14.22	17.37	40.33	−14.67	−11.89	−8.04
2	1.08	29.27	40.04	−11.11	−4.86	11.63
4	13.62	32.26	34.63	−9.86	3.62	4.09
6	−1.48	−6.82	NC[c]	NC	NC	NC
16	−7.87	−0.39	NC	NC	NC	NC

[a] Table contains the concentration of nitroxynil in untreated milk samples and the % increase/decrease (±) in the concentration of nitroxynil after 1, 2, 4, 6 and 16 h of incubation of samples treated with enzymes. [b] Samples at 0 h were not treated with enzyme. [c] NC, no results collected.

evening (4 p.m.) over a 16 day period and then weekly until day 58. The samples were frozen (−20 °C) until analysis.

RESULTS AND DISCUSSION

Method Development. The method used for the detection of nitroxynil was developed previously.[11] The method was developed to detect 38 anthelmintic residues in milk; however, in this study the samples were monitored only for nitroxynil.

The MRM window contains three transitions, two for nitroxynil and one for ioxynil, both monitored in negative ion mode. Time sectoring was not important for this method as it contained only three transitions. The dwell time was set to obtain a total of 12−15 data points across a peak. The interscan and interchannel delays were set to 5 ms as no switching was required between successive MRM windows.

At early time points milk samples were mostly highly positive (maximum nitroxynil concentration = 1358 μg/kg), and the method was modified to extend the linear range. In the method developed by Whelan et al., nitroxynil was linear in the range of 1−25 μg/kg. Maximum concentration of nitroxynil in the samples was 956, 749, 1358, 1313, 688, and 803 μg/kg for cows 1−6, respectively, and these concentrations were outside the linear range of the calibration curve.

The samples were diluted 5−10-fold in negative control milk prior to extraction. The linearity of the method was further extended through dilution of sample extracts. This was achieved by extracting milk samples with MeCN (12 mL) and scaling down the d-SPE step by a factor of 10 to 1 mL of supernatant added to 50 mg of end-capped C_{18}. Samples were further diluted 1 in 2 by transferring the supernatant (0.5 mL) to test tubes containing DMSO (1 mL) and evaporated to a constant volume

of 1 mL. The DMSO acts as a keeper when added to the sample prior to extraction, ensuring analytes remain in solution. Another advantage of DMSO as injection solvent over MeCN is better peak shape for early-eluting analytes such as nitroxynil without reduction of injection volume (5 μL). Samples with levels <20 μg/kg were extracted according to the original method,[11] and samples with a concentration greater than the limit of detection (CCα) of 0.24 μg/kg were reported.

Method Validation. The method for measuring nitroxynil residues was validated at 1, 1.5, and 2 times the second lowest calibration level (LCL), 2 μg/kg according to 2002/657/EC. WLr was carried out by one analyst; milk samples (n = 6) were fortified at 2, 3, and 4 μg/kg. This was repeated on three separate days and was used to determine the accuracy, precision, and linearity of method. Mean recovery of nitroxynil was typically >98%, which is within the acceptable range of 70−110% as required by 2002/657/EC. The precision of the method was <5.0% relative standard deviations: 4.5, 5, and 2.9%, respectively, at 1, 1.5, and 2 times the second LCL.

Enzyme Hydrolysis of Conjugated Nitroxynil. The investigation of the effect of enzymatic hydrolysis on drug residues is important to measure the concentration of conjugated forms of the drug and define an MRL. In addition, it has been demonstrated that the inclusion of a hydrolysis step can result in the improved detection of a drug residue through liberation of the conjugates.[13,14] Prior to this study, no information was available concerning the presence of conjugated forms of nitroxynil in the milk of dairy cows.

Initial studies were carried out by treating an incurred sample (n = 2) containing of 339 μg/kg of nitroxynil with 2 mL of buffer and 200 units of enzyme. Samples were incubated for 0.5, 1, 2, 4, 6, and 16 h. This study showed that nitroxynil levels decreased from 0.5 to 6 h (decrease from −11.5 to −0.8% for β-glucuronidase and decrease from −27.3 to −4.6% for sulfatase). However, a 22% increase in measured nitroxynil levels was observed following incubation for 16 h.

The presence of conjugated forms of nitroxynil was then investigated through enzymatic hydrolysis of milk from treated animals taken at 0.5, 1.5, 11, 15, 16, and 16 days post-treatment using a 16 h incubation time. These samples were determined to contain nonconjugated forms of nitroxynil at mean concentrations of 644, 714, 206, 176, 73, and 78 μg/kg (n = 4 replicate analyses), respectively. Samples containing high levels of nitroxynil (0.5 and 1.5 days post-treatment) were subjected to enzymatic hydrolysis with sulfatase and β-glucuronidase, which resulted in nitroxynil concentrations between −7 to +16% and +35 to +44% of the corresponding untreated control samples, respectively. Nitroxynil concentrations at medium levels (11 and 15 days post-treatment) treated with sulfatase and β-glucuronidase were at −18 to +14 and at +16 to +34% of the levels in untreated control samples, respectively. At the low levels (16 days
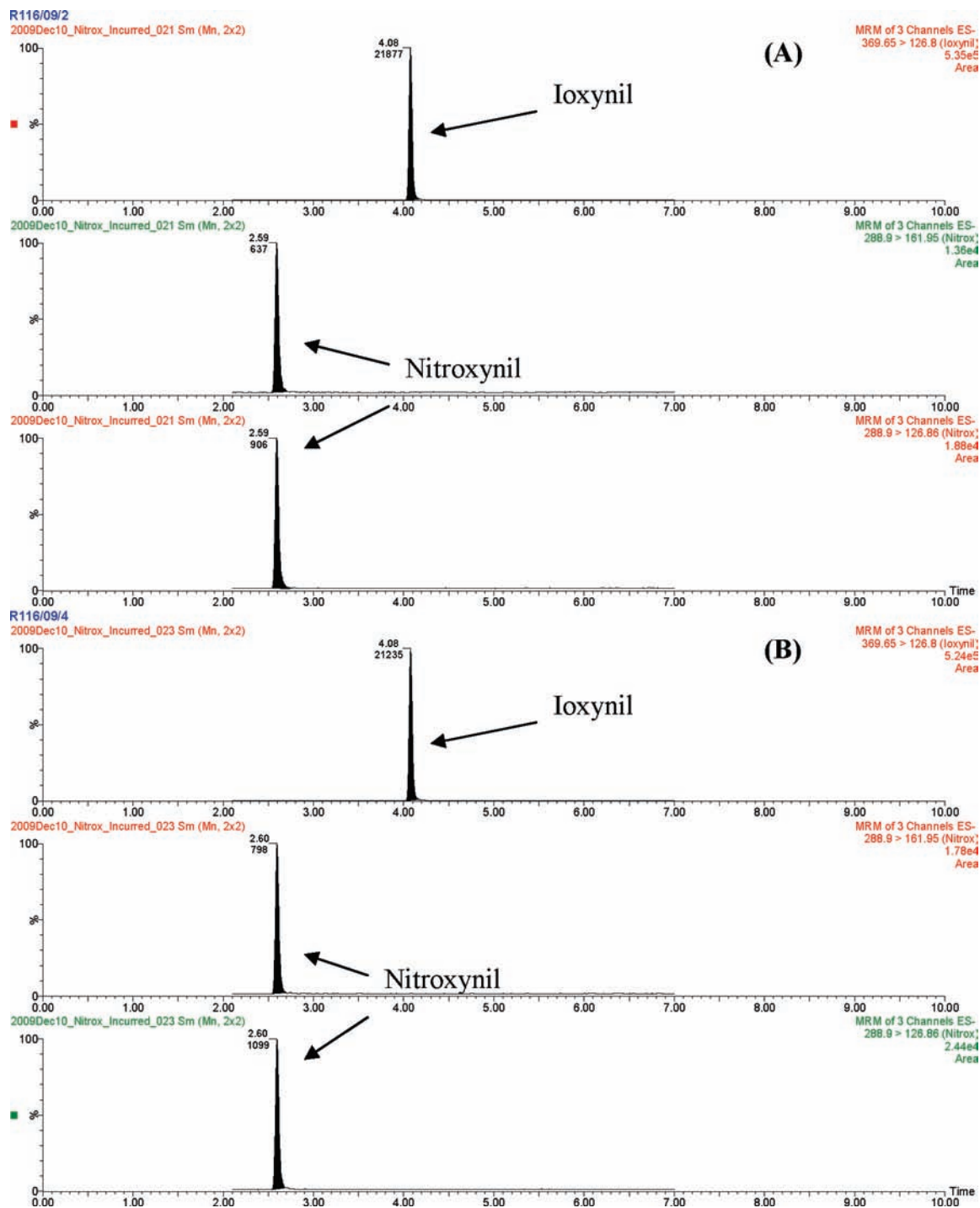
Figure 3. Chromatographic trace of incurred milk containing nitroxynil at (A) 9.7 μg/kg (animal 3) and (B) 12.4 μg/kg (animal 5), 36 days post-treatment, and ioxynil (I.S.).

post-treatment) reductions of nitroxynil concentration of -46 to -100% and -7 to -100% were observed following sulfatase and β-glucuronidase treatments, respectively.

Nitroxynil concentrations in milk samples taken at 0.5, 1.5, 11, and 15 days appeared to increase following enzymatic hydrolysis with β-glucuronidase. Some of these results were found to be significantly different at the 90% confidence level ($P < 0.1, < 0.1, < 0.1$, and > 0.1, respectively). At later withdrawal times significantly lower concentrations of nitroxynil residues were detected in

samples following enzymatic treatment, indicating potential degradation during the hydrolysis.

The study showed that nitroxynil residues degraded significantly at lower concentrations, and as the method development was carried out at a single concentration, this phenomenon was illustrated only after the study was complete for 16 h. Upon checking the literature, it was noted that Cooper et al.[15] carried out a cooking study on incurred liver, muscle, and kidney samples and found that nitroxynil was highly unstable in this cooking

study; the cooking study was carried out at a higher temperature but for a much shorter time. It was initially thought that this reduction may be indicative of poor thermal stability of nitroxynil.

Further experiments were designed to investigate the stability of nitroxynil in buffered milk samples at 37 °C. Milk samples were fortified at three different concentrations (25, 200, and 750 μg/kg), buffered and incubated for 1, 2, 4, 6, and 16 h. This study showed no significant increase or decrease in the concentration of nitroxynil in the samples (Table 1).

Further stability experiments were carried out using fortified control milk samples subjected to enzymatic hydrolysis, which showed that nitroxynil residues were stable. The cause of degradation of nitroxynil in incurred samples during enzymatic hydrolysis is unclear. Most instances of noncompliant concentrations of nitroxynil detected in milk are at low levels, and due to the potential for degradation during hydrolysis, it is proposed to monitor for the free form in milk as set out in Council Regulation 37/2010/EC[16] for animal tissue.

Additional enzymatic hydrolysis studies were carried out using incurred milk samples containing nitroxynil residues ranging between 186 and 530 μg/kg. The results of this identified that nitroxynil residues occur in the conjugate form in the milk of dairy cows, as the glucuronide form (Table 2). At early withdrawal periods, enzymatic hydrolysis with β-glucuronidase resulted in a 40% increase in nitroxynil residues. However, at later withdrawal times nitroxynil levels increased by <15% post-enzymatic hydrolysis.

Persistence of Nitroxynil Residues. The residues found in milk over the 58 day study illustrate the long persistence of nitroxynil in milk compared to other anthelmintic drug residues.[17−19] In two of the six animals, residues were found to be nondetectable 58 days post-treatment. However, residues were detectable in all other samples at levels ranging between 0.8 and 3.26 μg/kg. The highest levels of nitroxynil measured in the six cows ranged between 749 and 1358 μg/kg (Figure 3). Mean concentrations of nitroxynil were <20 μg/kg at 30 days post-treatment. Variation in levels between animals could be due to size difference in the body compartments of each animal. The age and health of the animal also can have an effect on the results, and the drug can behave differently in animals with/without parasitic infections.

To summarize, following administration of a single subcutaneous injection of nitroxynil to lactating dairy cows, residues were detectable in milk at 58 days post-treatment. The presence of conjugates was investigated in samples indicating the presence of the glucuronide form of the drug in milk. The results of this study justify the monitoring of nitroxynil as free residues in milk because of potential degradation during enzymatic hydrolysis; degradation appears to be more pronounced at lower concentrations, which may result in potential false-negative results.

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