Research of nitroxynil residues in bovine milk following a single administration in the dry period by ultra-performance liquid chromatography tandem mass spectrometry

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Abstract

Nitroxynil (NIT) is a halogenated phenol used to control fascioliasis in cattle and sheep. The Commission Regulation EU No 37/2010 has established maximum residue limits for NIT in bovine and ovine muscle (400 µg kg^{-1}), fat (200 µg kg^{-1}), liver (20 µg kg^{-1}) and kidney (400 µg kg^{-1}), and more recently in bovine and ovine milk (20 µg kg^{-1}). Thirty-five pregnant dairy cows were treated in this study with nitroxynil (340 mg/mL solution for injection) at the recommended dose of 10 mg/kg body weight at the start of the dry period, *i.e.* 53 to 74 days before the expected calving. Calving occurred between 43 days and 79 days after treatment. The concentrations of NIT in the milk were monitored for up to 120 days after calving. NIT residues were extracted using acetonitrile; magnesium sulfate and sodium chloride were added to induce liquid-liquid partitioning and purified by dispersive solid phase extraction for clean-up. NIT was detected by ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) in negative ionization mode. The highest concentrations of this drug were found in two animals at the first milking, 48 and 53 day post treatment with levels of 362 and 657 µg kg^{-1}, respectively. NIT residues were below the limit of detection of the method (0.24 µg/kg^{-1}) between 67 and 106 day post-treatment. Following calving, residues rapidly depleted in animals and were non-detectable from 10 to 38 days post-calving. In particular, in all animals milk resulted compliant (<20 µg/kg^{-1}) three days *post partum*.

Introduction

Nitroxynil (NIT) (3-iodo-4-hydroxy-5-nitrobenzonitrile) is a phenol derivative (Devis *et al*., 1966) used in cattle and sheep for the control of fascioliasis (McKellar and Kinabo, 1991). When compared with other fasciolicides, this compound shows high activity against both adult and immature liver flukes (Martínez-Valladares *et al*., 2010; McKinstry *et al*., 2009; Hutchinson *et al*., 2009). Recently the Commission Regulation (EU) No 201/2012 (European Commission, 2012) has amended the Annex of the Regulation (EU) No 37/2010 (European Commission, 2010) on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin and maximum residue limits (MRLs) for the marker residue NIT in bovine and ovine milk was established at 20 g/kg^{-1}. MRLs set for muscle, fat, liver and kidney have not been modified and amounted to 400 , 200 , 20 and 400 µg kg^{-1}, respectively.

NIT residues, compared to other classes of anthelmintics, have the longest half-life in the body (McKellar and Kinabo, 1991). This is due to its strong plasma protein binding which is more than 98% for this compound (Alvinerie *et al*., 1995). The selective mode of action of this highly protein-bound anthelmintic may be explained, in part, by its effect against blood-sucking parasites, concentrating the anthelmintic in the parasite (without the high tissues levels being produced in the host). The high level of protein binding may explain the selective effect of these agents, and the fact that well bled out carcasses have low tissues residue levels. Thus, the mode of action of this group of anthelmintic involves the selective delivery of the proton ionophores to the parasite because of the high level of plasma-protein binding. This study was conducted in pregnant dairy cows (n=35) at the beginning of the dry period because they are the target animal population for the investigated treatment. The occurrence and the depletion of NIT in bovine milk after calving was investigated.

Materials and Methods

No. 35 healthy pregnant Friesian-Holstein dairy cows from Animal & Grassland Research and Innovation Centre, Fermoy, Ireland were selected for the experiment. The cows used in this study, with an average body weight of 600 kg, had not been treated with products containing NIT within 12 months before the start of this study, as documented by the farm records. The cows were treated at the start of the dry period, from 53 to 74 days before the expected calving with TRODAX 340 mg/mL (Merial Animal Health, Harlow, UK). This corresponds to 1.5 mL solution for injection per 50 kg body weight. The administered dose was calculated on the basis of the estimated average body weight of the cows. After calving, milk samples were taken daily for the first 4 days and then every 3 days for up to the 120th day and stored at -20°C until analysis. Ultra-pure water (18.2 MOhm) was generated in-house using a Millipore (Cork, Ireland) water purification system. HPLC-grade methanol (MeOH) and acetonitrile (MeCN), 99.5% deuterated methanol (MeOH-d), and ammonium formate (puriss *pro analysi*) were sourced from Sigma-Aldrich (Dublin, Ireland). Dimethyl sulfoxide (DMSO), isopropyl-alcohol (IPA), and glacial acetic acid (HOAc) were obtained from BDH Chemicals Ltd. (Poole, UK). Pre-weighed mixtures of 4 g anhydrous magnesium sulphate ($MgSO_4$) and 1 g sodium chloride (NaCl) in 50 mL centrifuge tubes, and 1.5 g $MgSO_4$ and 0.5 g C_{18} in 50 mL centrifuge tubes were obtained from UCT Inc. (Bristol, PA, USA). Organic milk, for control samples, was purchased in supermarkets and tested for residues prior to analysis.

Nitroxynil and 13C Nitroxynil were purchased from Sigma-Aldrich and Witega (Berlin, Germany) laboratories, respectively. Primary stock standard solution was prepared in MeOH at concentrations of 4000 and 1000 µg mL^{-1} for nitroxynil and 13C nitroxynil, respectively.

Intermediate working standard solution was prepared at a concentration of 50 µg mL^{-1} for nitroxynil in MeOH. A working internal standard solution was prepared at 4 µg mL^{-1} for 13C nitroxynil in MeOH-d.

Extracted matrix calibrants were prepared by fortifying negative milk samples prior to

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extraction with a working standard mix, prepared at the following concentrations: 0.1, 0.25, 0.5, 1, 2.5, and 5 µg mL^{-1}, standard 1 to 6, respectively. 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 50 µg kg^{-1}. Samples with values above the concentration of 50 µg kg^{-1} were diluted with control milk. 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.

A glass dispenser (Dispensette® lll; Brand, Wertheim, Germany) was used for aliquoting MeCN extraction solvent, a Mistral 3000i centrifuge, micro centrifuge (Eppendorf, Hamburg, Germany), a multi-vortexer, a Caliper Life Sciences (Runcorn, UK) Turbovap LV evaporator, and a Transsonic 780LH ultrasonic bath were used for the extraction.

Chromatographic separations were performed using a Waters Acquity UHPLC system (Waters; Milford, MA, USA) comprising of a stainless steel HSS T3 analytical column (100×2.1 mm, particle size=1.8 mm) equipped with an in-line filter unit containing a 0.2 µm stainless steel replacement filter maintained at a temperature of 60°C and the pump was operated at a flow rate of 0.6 mL min^{-1}. Analytes were separated using a binary gradient elution containing a mobile phase A water:MeCN (90:10, v/v) with 0.01% HOAc and mobile phase B MeOH:MeCN (75:25, v/v) with 5mM ammonium formate. The gradient profile was as follows: i) 0-0.5 min, 100% A; ii) 5 min, 50% A; iii) 7 min, 10% A; iv) 8.5 min, 10% A; v) 8.51 min, 0% A; vi) 9.5 min, 0% A; vii) 9.51 min, 100% A; viii) 13 min 100% A. Injection volume was 5 µL.

NIT residues were quantified using a Waters Quattro Premier XE triple-quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) interface operating in negative mode (Waters). The UPLC-MS/MS system was controlled by MassLynx™ software and data was processed using TargetLynx™ Software (Waters).

Milk samples (10±0.1 g) were weighed into centrifuge tubes (50 mL) and fortified with internal standard and left to sit for 15 min. Acetonitrile (12 mL) was added to tube one containing $MgSO_4$ (4 g) and NaCl (1 g). The contents of tube one was added to the sample and shaken immediately to extract the residues into the MeCN layer. The sample was centrifuged for 12 min at 3500 RPM (959 g). A dispersive-SPE cleanup step was performed by pouring the supernatant from tube one into tube two (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 2500 RPM (489 g). The supernatant (6 mL) and DMSO (0.25 mL) were added to a starstedt tube (15 mL) and vortexed for one min. The MeCN layer was evaporated under nitrogen at 50°C to 0.25 mL. Extracts were filtered through 0.2 µm polytetrafluoroethylene (PTFE) syringe filters (Whatman Rezist®; Whatman, Maidstone, UK) and injected onto the UPLC-MS/MS system. Any samples that fell outside the calibration range were diluted in negative milk and reanalysed.

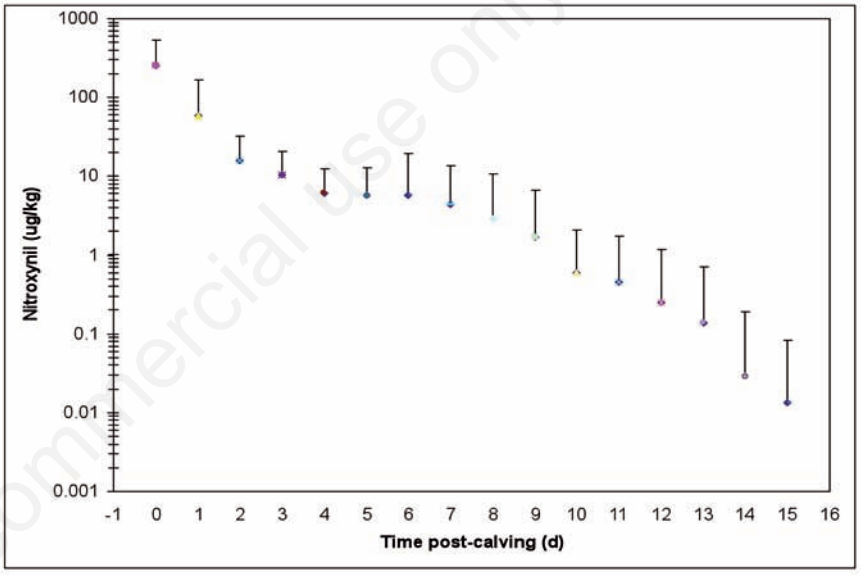
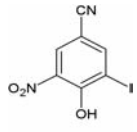
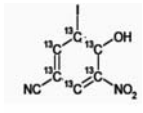


Figure 1. Concentration of Nitroxynil (average of 35 cows) in post-calving period (days) and standard deviation (whiskers).

Table 1. Multiple reaction monitoring window.

Analyte	tR (min)	Transition (m/z)	Cone (V)	CE (V)	MRM window	ESI Polarity	IS
Nitroxynil	03.46	288→126	36	24	3	-	13C
		288→161	36	20	3	-	
13C 6 Nitroxynil	03.46	295→126	40	25	3	-	IS
							

t_R, retention time; CE, collision energy; MRM, multiple reaction monitoring; ESI, electrospray ionisation; IS, internal standard.

Results

The method used for the detection of NIT has been developed previously (Whelan *et al*., 2010) for 38 anthelmintic residues in milk. In this study the samples were only monitored for NIT. The multiple reaction monitoring (MRM) window (Table 1) only contains two transitions, one for nitroxynil and one for 13C6 nitroxynil.

The following transitions were input into MRM windows: 288à126 (*m/z*) and 288à161 for nitroxynil; 295à126.69 for the 13C6 nitroxynil internal standard. The retention time, 3.46 min, was the same for both nitroxynil and 13C6 nitroxynil that had been detected in negative ion mode.

In the method developed by Whelan *et al*., the incurred samples were mostly highly positive and the sensitivity for negative ionization mode was not so acceptable. NIT was linear in the range 1-50 µg kg^{-1}. Maximum concentration of the drug was 657 µg kg^{-1}, which is outside the linear range of the calibration curve. Samples were typically 10 time more concentrated then the range of the curve so, after the first quantification, samples were re-extracted with a dilution in organic milk (analysed previously) by a factor of 1 in 10 or 1 in 5 depending on the initial concentration found in the first extraction.

The results of this study show that NIT residues are very persistent but at low levels in bovine milk of cows treated during the drying period. In two out of 35 animals, residues were found to be detectable up to 102 days after treatment with concentration of 2 µg kg^{-1}. The cc for this compound is 0.24 µg kg^{-1}.

At the first milking day, in 14 out of 35 animals, NIT residues were above the MRL of 20 µg kg^{-1} set by European Regulation and the highest levels of the drug were detected in two animals (Table 2) at the 48th and 53rd day post treatment with concentrations of 362 and 657 µg kg^{-1}, respectively.

Mean concentrations of NIT were <20 µg kg^{-1} three days post-calving (Figure 1).

Cow (n)	Day calved (PT) (µg/kg)	Cmax (day)	Cmax (day)	Tmax (PT day)	LOD (PC day)	LOD
1	56	06.05	57	67	71	15
2	50	15.03	53	74	78	28
3	73	06.05	81	99	102	29
4	73	20.04	74	88	92	19
5	47	362.04	48	78	81	34
6	62	04.04	64	74	78	16
7	77	06.04	78	102	106	29
8	58	16.02	60	67	71	13
9	71	6	74	88	92	21
10	57	86.09	57	88	92	25
11	62	07.08	64	81	85	23
12	68	04.05	74	88	92	24
13	53	657	53	81	85	32
14	80	14	81	102	106	26
15	76	03.01	81	88	92	16
16	60	02.05	67	78	81	21
17	46	122.04	47	78	81	35
18	47	35	47	64	67	20
19	73	06.09	81	88	92	19
20	55	19.07	57	81	85	30
21	50	22.06	53	81	85	35
22	50	39.07	53	85	88	38
23	43	81.07	49	78	81	38
24	52	13.03	57	81	85	33
25	56	26.03	57	81	85	29
26	64	07.01	67	71	74	10
27	71	05.06	74	81	85	14
28	64	24.01	67	81	85	21
29	53	07.01	57	74	78	25
30	70	01.06	74	78	81	11
31	62	28.04	67	94	99	37
32	70	08.06	71	88	92	22
33	58	27.04	60	92	94	36
34	51	11.06	53	74	78	27
35	48	237.04	48	71	74	26

PT, post-treatment; Cmax, concentration maximum; Tmax, time maximum; LOD, limit of detection; PC, post calving.

Discussion

NIT residues were found to be very persistent in milk compared to other anthelmintic drug residues (EMEA, 1998; Whelan *et al*., 2011). When dairy cows are treated with Trodax (340 mg/mL) at the highest recommended dose of 10 mg nitroxynil per kg body weight at the beginning of a dry period (about 53 to 80 days before calving – expected calving dates), residues of this flukicide were quantifiable in the milk of the 35 cows until at least 13 days after calving (decision limit, cc =0.24 µg kg^{-1}).

Highest concentrations of the drug were detected at the first milkings and ranged from 656 to 362 µg kg^{-1} at 53rd and 67th day after treatment, respectively, and were below the limit of detection of the method between 67th and 106th day post treatment.

However, there was considerable variation in levels among animals. This could be due to size difference in body compartments and the amount of fat of each animal. The age and health of the animal also can have an effect on the results, the drugs behave differently in animals with/without parasitic infections (Moreno *et al*., 2010).

Conclusions

According to the Commission Regulation 2010/37/EC, NIT residues in bovine milk cannot exceed the MRL of 20 ppb. In this study, levels above the MRLs were found only three day after calving.

In conclusion, highest concentrations of NIT occur in the milk produced shortly after calving. This colostrum milk is not suitable for human consumption and is collected separately from the milk that enters the bulk tank on the dairy farm. After the colostrum period, *i.e.* about 3 days after calving, the concentrations in milk decrease to 10 µg kg^{-1} or below and residues are non-detectable 10 to 38 days post-calving.

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