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Depletion of metronidazole in brook trout (*Salvelinus fontinalis*)

Lynn Watson[a,b]*, James D. MacNeil[a,b], Ross Potter[a] and Jocelyne Hellou[c,d]

[a]*Canadian Food Inspection Agency, Dartmouth Laboratories, Dartmouth, NS, Canada;* [b]*Department of Chemistry, St. Mary's University, Halifax, NS, Canada;* [c]*Marine Environmental Sciences Division, Fisheries and Oceans Canada, Bedford Institute of Oceanography, Dartmouth, NS, Canada;* [d]*Departments of Chemistry and of Oceanography, Dalhousie University, Halifax, NS, Canada*

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Metronidazole (MNZ), which is effective in the treatment of intestinal infections in fish, is also a suspected carcinogen and has been banned in numerous jurisdictions for use in any food-producing animal, including fish. Few reports have been published on the depletion of MNZ in fish. A depletion study was therefore undertaken using MNZ in feed provided to trout under controlled conditions. The water was maintained at 17.5 ± 0.9°C throughout the medication and depletion periods in the study. Following a 20-day acclimatisation period in the holding tanks, the trout (approximately 150–200 g bodyweight at the start of the study) were subjected to two separate medication and withdrawal periods: (A) 5 day medication/5 day withdrawal and (B) 5 day medication/16 day withdrawal. This simulated a potential multiple dosing in an aquaculture setting. In both medication periods, the trout were dosed with medicated feed containing 3 g MNZ kg^{-1} fish. Fish were sacrificed in accordance with accepted animal care protocols and tissue samples were analysed by UPLC-MS/MS. Analyte concentrations in trout muscle ranged from a high of 27 000 ± 10 000 ng g^{-1} for MNZ and 830 ± 570 ng g^{-1} for MNZ-OH on day 1 of withdrawal period A to a low of 1.8 ± 2.3 ng g^{-1} for MNZ and < 0.4 ng g^{-1} for MNZ-OH on day 16 of withdrawal period B. The results demonstrate that when using the UPLC-MS/MS method, residues of MNZ may be detected in fish treated with MNZ after 16 days of withdrawal.

Keywords: metronidazole; fish; veterinary drug residues; feed; LC-MS/MS; depletion; metabolism

Introduction

Compounds of the nitroimidazole class have demonstrated antiprotozoal and antibacterial activities, leading to their use in both human and veterinary medicine (Mahugo-Santana et al. 2010). Metronidazole (MNZ), a member of the nitroimidazole class of compounds, has been reported to be effective in the treatment of disease in aquaculture and aquarium fish (Tojo & Santamarina 1998a, 1998b; Sangmaneedet & Smith 1999; Sorensen & Hansen 2000). Although the use of nitroimidazoles in aquaculture is not approved in the European Union (Mahugo-Santana et al. 2010), residues of MNZ have been detected in water samples collected in a study at a marine fish farm. However, the source of the residues was not determined (Muňoz et al. 2010). MNZ has also been detected in hospital sewage water and, more recently, in river waters (Kasprzyk-Hordern et al. 2008), indicating the potential for various sources of environmental residues resulting from both human and veterinary uses (Lindberg et al. 2004). Inclusion of nitroimidazole residues in a monitoring programme for veterinary drug residues in aquaculture products is ranked as highly desirable by the European Union (2012).

It was reported in an early review that MNZ was the nitroimidazole compound that was most investigated for mutagenicity to bacteria (Voogd 1981). The hydroxy metabolite of MNZ has been shown to have approximately 65% of the activity of the parent drug on bacteria and the activity of MNZ was enhanced when combined with its hydroxy metabolite (Pendland et al. 1994). A study of the genotoxic activity of MNZ *in vitro* using human lymphocyte cells and *in vivo* using mouse bone marrow cells showed a genotoxic effect, indicating that MNZ is a direct mutagenic and chromosome-damaging agent (Mudry et al. 1994). Genotoxic effects of MNZ were also evaluated in hamster embryo and it was concluded that MNZ is capable of the transmission of potential genotoxic effects to the foetus (Garry & Nelson 1987). Concerns about the potential adverse effects of residues of nitroimidazoles in foods have led to a ban on their use in food-producing animals, including fish, in various jurisdictions, including the European Union, the United States, Canada and China (Tarbin et al. 2012).

The 5-nitroimidazoles are rapidly metabolised with the primary hydroxy metabolite of dimetridazole, ipronidazole and MNZ being formed by the oxidation of the side-chain in the C-2 position of the imidazole ring (Rose et al. 1999). Information on the depletion and distribution of MNZ and MNZ-OH in fish is, however, limited. In rainbow trout (*Oncorhynchus mykiss*) given feed containing MNZ in an aquaculture pilot plant, residues of MNZ

*Corresponding author. Email: lynn.watson@inspection.gc.ca

and MNZ-OH were detected in muscle and skin tissues shortly after the administration period, but not 3 weeks later using an LC-UV analysis with an LOD of 3 ng g^{-1} for MNZ and 5 ng g^{-1} for MNZ-OH (Sorensen & Hansen 2000). At the first day of withdrawal following the administration period, MNZ residues were 2690 ± 1080 ng g^{-1} in muscle and 2360 ± 810 ng g^{-1} in skin, while hydroxy MNZ concentrations were 44 ± 22 ng g^{-1} in muscle and 31 ± 11 ng g^{-1} in skin. No residues were detected at the second time point, which was after 3 weeks of withdrawal. The fraction of MNZ-OH to MNZ was less than 2% on the first day after the administration period. This ratio of MNZ-OH to MNZ was also observed in tilapia treated with MNZ for 5 days, where residues were detected in muscle tissue at days 1, 3 and 5 of withdrawal using an LC-UV method with an LOD of 2 ng g^{-1} for MNZ and 5 ng g^{-1} for MNZ-OH (Maher et al. 2008). MNZ concentrations in muscle declined in tilapia from 2933 ng g^{-1} at day 1 of withdrawal to 524 ng g^{-1} at day 5, while residues of MNZ-OH were 59 and 15 ng g^{-1} at days 1 and 5 of withdrawal.

As MNZ is banned for use in several jurisdictions, the objective of this study was to investigate the persistence of MNZ residues in the tissue of fish that have been dosed, as well as to gain insight into the appropriate residues for monitoring (i.e. MNZ or MNZ-OH). The two depletion studies available in the literature were taken into consideration when planning the current investigation conducted to obtain additional information on the depletion profile of MNZ in brook (speckled) trout (*Salvelinus fontinalis*), a typical species representative of a high lipid content fish raised in aquaculture, and to provide known incurred material for use in method validation and quality assurance. It was also anticipated that the use of a newly developed analytical method using UPLC-MS/MS (Watson et al. 2014) could provide a better opportunity to detect trace residues of MNZ than the two earlier studies using LC-UV for analysis. The multiple dosing and withdrawal in the current study also provided information about the persistence of residues, as a result of frequent dosing in an aquaculture environment.

Materials and methods

Reagents and chemicals

The analytical standards metronidazole (MNZ) and hydroxy metronidazole (MNZ-OH) were obtained from Sigma Aldrich (St. Louis, MO, USA). All were of analytical grade (> 99%). HPLC-grade dichloromethane, acetonitrile, hexane (distilled in glass), acetone and laboratory-grade formic acid (90%) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Perchloric acid (60%) was from SEASTAR Chemicals, Inc. (Sidney, BC, Canada). Glacial acetic acid came from J.T. Baker (Phillipsburg, NJ, USA). Water was purified using a Milli-Q water system from Millipore (Bedford, MA, USA). Sep-Pak C-18 SPE cartridges were purchased from Waters (Milford, MA, USA). The 0.2 µm nylon membrane syringe filters were purchased from Pall Corporation (Ann Arbor, MI, USA).

Experimental design

The depletion study was conducted at the Department of Fisheries and Oceans, Bedford Institute of Oceanography (BIO) with speckled (brook) trout (*Salvelinus fontinalis)* provided by the Nova Scotia Provincial Government, McGowan Lake Hatchery, Caledonia, Nova Scotia. Fish feed pellets, provided by the fish hatchery, were manufactured at Corey Feed Mills Ltd (Fredericton, NB, Canada).

Seventy-three brook trout (weight approximately 200 g) were transported from the Caledonia, NS, fish hatchery to BIO fish laboratories by truck using aerated tanks. Once relocated to BIO, the fish were housed in two separate indoor tanks of 2700 L with a continuous flow capable of holding 40 fish each. City water filtered through fine gravel, charcoal and ultraviolet (UV) light was used and the water temperature was recorded daily at approximately 09.00 hours by BIO staff for the entire study, including the acclimatisation period (Table 1). The mean temperature with standard deviation (SD) from the day the fish arrived to the end of the depletion study was 18.2 ± 1.3°C. The mean temperature from the first day of medication to the last day of withdrawal was 17.5 ± 0.9°C.

Since nitroimidazoles are banned substances, it was necessary to avoid external contamination of the water system. Faeces and any residues at the bottom of the tank were vacuumed out and placed in buckets to settle, then transferred to jars and disposed of along with other laboratory waste chemicals. Incurred tissues not required for analysis in the depletion study were maintained in frozen storage for use as analyst training and quality control materials.

Fish were not fed on the first day of arrival as they were expected not to eat when stressed from travel. After that they were fed once daily with the commercial food pellets used at the hatchery at 1% of their body weight per day prior to exposure to the prepared medicated feed and also during the withdrawal periods. Fish were acclimatised for 2 weeks at BIO prior to commencement of the study to ascertain the amount of feed that would be consumed by the fish under new holding conditions.

The trout underwent two subsequent medication and withdrawal periods: (A) 5 day medication/5 day withdrawal and (B) 5 day medication/16 day withdrawal. Fish were fed a prepared medicated diet (3 mg MNZ kg^{-1} fish), once daily during two medicated periods and the drug-free diet during the acclimation and withdrawal periods at a daily rate equivalent to 1% body weight. Immediately prior to the first feeding of the medicated diet, seven fish

Table 1. Mean weight and length of trout for each sampling time point.

Time point	Mean weight (g)	SD	Mean length (cm)	SD
Control	334.3	100.1	28	2
Day 3 medication A	158.8	46.0	22	2
Day 5 medication A	187.7	57.4	24	3
Day 1 withdrawal A	212.6	31.7	25	2
Day 3 withdrawal A	169.1	37.8	22	2
Day 5 withdrawal A	218.1	77.3	25	3
Day 1 withdrawal B	202.3	48.3	24	3
Day 6 withdrawal B	165.5	45.4	24	1
Day 9 withdrawal B	199.0	47.5	25	2
Day 12 withdrawal B	212.6	80.3	25	2
Day 16 withdrawal B	234.1	84.3	26	4

were sampled as controls. The fish were humanely sacrificed, without chemicals, to avoid potential interferences during analysis. The initial sampling was followed by samplings of seven fish each at days 3 and 5 during medication period A, followed by samplings at days 1, 3 and 5 of withdrawal period A. The remaining fish were then subjected to a subsequent 5-day medication period B, followed by a 16-day withdrawal period B. Samples (five fish per day) were taken on days 1, 6, 9, 12 and 16 of withdrawal B. Fish were sacrificed at BIO, placed on ice and transported immediately to the adjacent CFIA facility (1–2 min between facilities). All fish sampled were weighed and measured prior to being filleted and frozen at −30ºC pending analysis (Table 1).

UPLC-MS/MS

Chromatographic separation was performed on an Acquity HSS T3 C18 1.8 µm particle size, 2.1 mm i.d. × 50 mm column (Waters) maintained at 35°C using a gradient programme consisting of mobile phase A (0.1% acetic acid in water) and mobile phase B (0.1% acetic acid in acetonitrile) at a flow rate of 0.4 ml min^{-1}. Initial conditions (0–0.5 min) were 95% A–5% B, then 70% A–30% B (0.5–5 min), next 5% A–95% B (5–7 min), holding for 0.5 min at 0.6 ml min^{-1} and finally 95% A–5% B (7.5–8.5 min). An equilibration time of 1 min under initial conditions between sample injections was allowed (Watson et al. 2014). The volume of extract injected was 20 µl.

MS tune parameters for maximum intensity of precursor ions were: capillary voltage, 2 kV; extractor voltage, 5 V; source temperature, 130°C; desolvation temperature, 450°C; desolvation gas flow, 550 l h^{-1}. ESI-positive mode was used for all compounds and the data were acquired in SRM mode. SRM transitions were *m/z* 171.8 > 127.9 and 171.8 > 82.0 with a cone voltage of 25 V and collision energies 15 and 20 eV respectively for MNZ. Transitions for MNZ-OH were *m/z* 188.2 > 127.9 and 188.2 > 122.9 with a cone voltage of 20 V and collision energies of 15 eV for both. The calibration curve range for the method was 1–50 ng g^{-1}.

Method validation

The LOD and LOQ for the method were determined for MNZ and MNZ-OH using Atlantic salmon (*Salmo salar*) as a representative member of the Salmonidae family of fish, which includes trout (Watson et al. 2014). LOD was determined by evaluating the least sensitive SRM transition for five blank samples per run on 4 separate days (n = 20). It was then calculated as three times the average tissue concentration of the instrument noise. LOQ was calculated by multiplying the LOD by 3.

Method accuracy was assessed from recovery studies at three fortification levels, five samples per level, on 3 separate days (n = 45). Method repeatability was determined by analysing prepared pools of test sample material containing specific concentrations of analyte (n = 45) at the same levels used for recovery studies. Intermediate precision was determined from the results generated by a second analyst using the same pooled material (n = 45).

Preparation, extraction and analysis of food pellets

Medicated feed was prepared at the BIO facility using the same feed pellets the fish were fed prior to the medication period by immersing feed pellets in an acetone solution of MNZ, then evaporating the acetone by air drying for 24 h at RT to yield pellets containing a MNZ concentration of 3 g kg^{-1} of feed. To account for MNZ loss during preparation of the medicated feed, a 10% excess of MNZ was added. It was determined that 4 ml of acetone should be added per 10 g of pellets for optimal absorption by the pellets. Therefore, a 400 ml acetone solution containing 3.3 g of MNZ was prepared to obtain a final concentration of approximately 3 g kg^{-1} MNZ in 1 kg of feed. The medicated pellets were stored at −20°C until use; then small quantities were thawed and refrigerated in preparation for each daily feeding.

A sample of the pellets was extracted and analysed by LC-MS/MS to determine the concentration achieved using a modified version of the extraction and analysis previously developed for tissue samples (Watson et al. 2014).

Two separate dilutions, one of 10^5 and another 10^6 times, were done on the final extracts to avoid contamination and to keep concentrations within the standard curve. Non-medicated feed was spiked at the expected concentration (3 g kg^{-1}) of the medicated feed and treated in the same manner to correct for any losses during sample preparation and analysis. Samples were analysed and results calculated taking into consideration the change in sample weight and the dilutions.

Preparation, extraction and analysis of trout samples

Duplicate test portions of each homogenised muscle tissue sample were extracted with acidic acetonitrile followed by C-18 SPE column clean up, then analysed by UPLC-MS/MS as described in a previous paper (Watson et al. 2014). Quality assurance guidelines were maintained using spike recoveries and matrix-matched calibration curves. The mean, SD and %RSD were calculated for the results at each time point. The mean concentration results were plotted versus time using SD for error bars to show change in concentration with time. A Grubb's test was also performed to detect any outliers.

Results and discussion

The fish ate at a consistent rate during the 20-day acclimatisation period. One mortality was found on day 8 after arrival of the fish at BIO, but otherwise all fish remained healthy during the study.

The LOD determined in the representative species, salmon, was 0.08 ng g^{-1} for MNZ and 0.40 ng g^{-1} for MNZ-OH. LOQs were 0.24 and 1.2 ng g^{-1} for MNZ and MNZ-OH, respectively. Mean recoveries determined during validation in salmon for MNZ were 114% at a concentration of 1 ng g^{-1}, 97% at 10 ng g^{-1} and 119% at 50 ng g^{-1}. The mean recoveries met the requirements of the Codex Alimentarius Guidelines of 50–120% for concentration ≤ 1 ng g^{-1}, 60–120% for 1–10 ng g^{-1}, and 70–120% for 10–100 ng g^{-1} (CAC 2009). Method repeatability results also met Codex requirements with no RSD exceeding 20%. RSD results for intermediate precision remained below 20% as well, except for MNZ-OH, which had an RSD of 26% at a level of 1 ng g^{-1}. However, this was below the RSD of 35% which is set for this level.

As described by Watson et al. (2014), method validation experiments were based on recommendations from the International Union of Pure and Applied Chemistry (Thompson et al. 2002) and the Codex Alimentarius Commission (CAC 2009). In addition, the fortified tissue samples used in validation met Commission Decision 2002/657/EC criteria for identification and confirmation (European Commission 2002). Analytes were confirmed to be positive based on their retention times being within 2.5% and ion ratios being within 20% as compared with standards with each confirmation ion having a signal-to-noise ratio of at least 3:1.

The medicated feed was analysed in triplicate, with three medicated pellet sample extracts being diluted 10^6 times and another three samples being diluted 10^5 times to bring concentrations within the analytical range of the calibration curve. Non-medicated pellet samples spiked at 3 g kg^{-1} as controls were also analysed in triplicate with 10^5 and 10^6 dilutions. The average concentration in the medicated pellets was determined to be 2.8 g MNZ kg^{-1} of feed (RSD 5.6%), with an average spike recovery of 104%.

Samples from each fish taken at each time point were analysed for MNZ and its metabolite MNZ-OH in duplicate and the mean, SD and %RSD calculated. If the % RSD for duplicates fell outside of typical method repeatability (> 25%), the analysis was repeated. The results for all samples at each time point were then averaged and the SD and %RSD calculated. The results obtained for MNZ and MNZ-OH for the first medication and withdrawal period (A) are given in Table 2. For the second medication and withdrawal period (B), MNZ and MNZ-OH results are in Table 3. There were large ranges with high %RSDs between samples from the same time point. A Grubb's test revealed one outlier result and the analysis was repeated. The range of results was anticipated due to the variability typically observed when working with live animals. The fish were the same age, but ranged in size and fed and metabolised the drug at different rates. Even though results from the same time points varied, an overall

Table 2. Mean concentrations of residues of MNZ and MNZ-OH in muscle of trout treated with MNZ during the initial medication and withdrawal period (A).

Time point	MNZ concentration (ng g^{-1}), mean ± SD	MNZ-OH concentration (ng g^{-1}), mean ± SD	Ratio of MNZ-OH to MNZ (%)
Control	< 0.08	< 0.40	
Day 3 medication	20 775 ± 2744	329 ± 82	1.6
Day 5 medication	21 205 ± 10 387	764 ± 464	3.6
Day 1 withdrawal	27 425 ± 10 263	833 ± 574	3.0
Day 3 withdrawal	9911 ± 4814	607 ± 315	6.1
Day 5 withdrawal	3618 ± 2963	246 ± 156	6.8

Table 3. Mean concentrations of MNZ and MNZ-OH in muscle of trout treated with MNZ during the second medication and withdrawal period (B).

Time point	MNZ concentration (ng g^{-1}), mean ± SD	MNZ-OH concentration (ng g^{-1}), mean ± SD	Percentage ratio of MNZ-OH to MNZ
Day 1 withdrawal	20 346 ± 5670	552 ± 242	2.7
Day 6 withdrawal	1058 ± 613	83 ± 65	7.8
Day 9 withdrawal	165 ± 188	23 ± 16	14
Day 12 withdrawal	51 ± 81	6.7 ± 10	13
Day 16 withdrawal	1.8 ± 2.3	< 0.40	–

increase in concentrations of MNZ and MNZ-OH was seen during the medication period and a decrease during the withdrawal periods.

No residues were detected before the initial medication period (A), but a concentration in the range of 20 000 ng g^{-1} was found in muscle tissue after 3 days of medication and again at 5 days of medication in medication/withdrawal period A. The concentrations of MNZ-OH approximately doubled between days 3 and 5 of medication in period A. Depletion was rapid and non-linear, with an estimated half-life ($t_{½}$) for MNZ in muscle of 1.7 days after medication/withdrawal period A and 1.3 days after medication period B. The $t_{½}$ for MNZ-OH in muscle was estimated to be 2.5 days after medication period A, but the estimate from the data for medication period B was approximately 2 days. Low concentrations of drug residues were still detectable after the second medication/withdrawal period (B) at day 16 of withdrawal. The mean MNZ and MNZ-OH residue concentrations peaked at the beginning of withdrawal period A at 27 000 ± 10 000 and 830 ± 570 ng g^{-1} respectively. On day 5 of withdrawal period A the MNZ residue level was 3600 ± 3000 ng g^{-1} and MNZ-OH was 240 ± 160 ng g^{-1}. At the beginning of withdrawal period B, the residue concentration was 20 000 ± 5700 ng g^{-1} for MNZ and 550 ± 240 ng g^{-1} MNZ-OH. By day 16 of the withdrawal period (B), the MNZ concentration was 1.8 ± 2.3 ng g^{-1} and MNZ-OH was below the LOD (< 0.4 ng g^{-1}).

Due to the relatively high concentrations of MNZ and MNZ-OH detected during the medication periods, extracts were diluted appropriately in order for the concentrations to fall within the range of the calibration curve for the method. The values reported were corrected for these dilutions. The highest results obtained were 5–10 times higher than detected in previous depletion studies (Sorensen & Hansen 2000; Maher et al. 2008). However, the trout in an earlier study were fed a much lower dose of MNZ (nearly six times lower; Sorensen & Hansen 2000).

The dose used in the present study is comparable with that reported in a second published study using a different fish species (tilapia) (Maher et al. 2008). Different results would be expected, especially considering the differences in typical fat content of trout and tilapia, 6% (Blanchet et al. 2005) and 1.7–1.9% (Erickson 1992), respectively. Depletion was rapid in tilapia, with residues of both MNZ and MNZ-OH being reduced by day 5 to approximately 10% of the residues observed after 1 day of withdrawal. In the present study during medication/withdrawal period A, depletion of MNZ from day 1 to day 5 withdrawal was similar to that observed in tilapia, but residues of MNZ-OH were more persistent, with concentrations at day 5 withdrawal approximately 25% of those observed after 1 day of withdrawal (Figures 1 and 2). Similar results were observed for medication/withdrawal period B, with residues of MNZ and MNZ-OH at day 12 of withdrawal still accounting for approximately 2% and 1%, respectively, of the residues present at day 1 of withdrawal. Quantifiable residues of MNZ were still present after 16 days of withdrawal, but residues of MNZ-OH were below the method LOD for representative salmonid species (0.40 ng g^{-1}).

The fraction of MNZ-OH to MNZ was reported as less than 2% on the first day after the administration period in the earlier trout study (Sorensen & Hansen 2000) and the results observed with tilapia were similar (Maher et al. 2008). In the present study, on day 3 of medication period A, the fraction of MNZ-OH to MNZ was less than 2% as well, but it increased as the study continued and was 6.8% by day 5 of

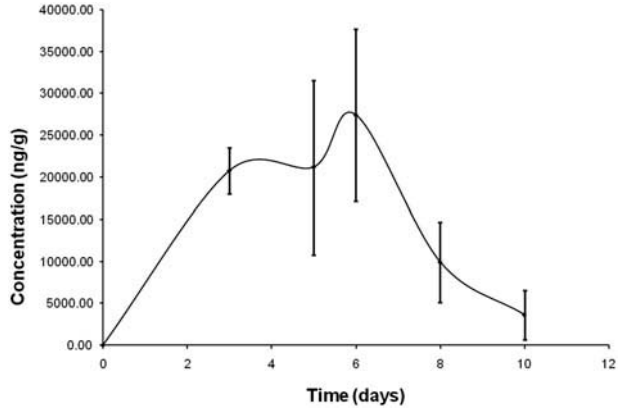

Figure 1. Depletion curve representing MNZ mean concentrations with error bars in trout tissue during the first MNZ medication period (days 1–5) and withdrawal period (days 6–10).

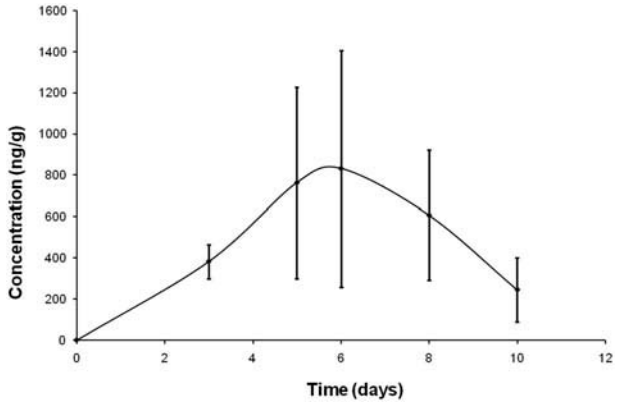

Figure 2. Depletion curve representing MNZ-OH mean concentrations in trout tissue during the first MNZ medication period (days 1–5) and withdrawal period (days 6–10).

withdrawal. During medication/withdrawal period B, the fraction of MNZ-OH started at 2.7% on day 1 withdrawal and increased reaching 14% on day 9 of withdrawal. Fractions of MNZ-OH to MNZ at each time point can be seen in Table 2 for medication/withdrawal period A and in Table 3 for medication/withdrawal period B.

The analysis of the incurred samples demonstrated the suitability of the UPLC-MS/MS method for the determination of residues of nitroimidazoles in fish muscle tissue, as recommended for validation of methods for veterinary drug residues (European Commission 2002; CAC, 2009). This enhanced the validation work reported by Watson et al. (2014), which was performed using fortified tissue. The data also provided a better understanding of the depletion profile for MNZ and its hydroxy metabolite in trout exposed to MNZ than the earlier study, which only provided data for two time points (1 day and 3 weeks). In addition, the study demonstrated the effects of multiple dosing of MNZ, which may occur in an aquaculture setting.

Conclusions

MNZ is rapidly metabolised in brook (speckled) trout after exposure through medicated feed, with residues of MNZ declining from > 20 000 to approximately 2 ng g^{-1} over a 16-day withdrawal period. Residues of the primary metabolite, MNZ-OH, account for 2–14% of the total detected residues, with the proportion of MNZ-OH increasing with increasing withdrawal time. The data indicate that quantifiable residues of MNZ are present after 16 days of withdrawal and demonstrate the suitability of the UPLC-MS/MS method for use in a routine monitoring programme for nitroimidazole residues in fish tissues.

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