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Cyano Metabolite as a Biomarker of Nitrofurazone in Channel Catfish

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The use of nitrofuran drugs in food-producing animals continues to attract international concern as a food safety issue. Methods for monitoring nitrofuran residues have been directed to the intact side chain of tissue-bound metabolites. Semicarbazide, the side chain of nitrofurazone (NFZ), can enter food products from non-NFZ sources, suggesting the need for an alternative biomarker for confirmatory purposes. We characterized a cyano derivative as a major metabolite of NFZ in channel catfish (*Ictalurus punctatus*). The depletion of cyano metabolite was examined in the muscle of channel catfish after oral dosing (10 mg of NFZ/kg of body weight). Parent NFZ was rapidly eliminated in muscle, with a half-life of 6.3 h. The cyano metabolite was detected for up to 2 weeks, with an elimination half-life of 81 h. The cyano metabolite represents an alternative biomarker for confirming the use of NFZ in channel catfish.

KEYWORDS: Nitrofurazone; channel catfish; LC−MS; biomarker; cyano metabolite

INTRODUCTION

Nitrofurans are a class of antibacterial drugs that have been widely used in food animal production (*1*, *2*). Because of their toxicological properties (*3*, *4*), nitrofuran drugs are prohibited from use in food animals in many countries, including the United States. Nonetheless, monitoring programs continue to find violative residues in animal products, including those derived from aquaculture.

Nitrofurans are rapidly metabolized *in vivo*, principally through nitroreduction and formation of tissue-bound metabolites (*5*). Current methodology for monitoring nitrofuran drug residues is based on measurement of the intact side chains of these metabolites. Liquid chromatography−tandem mass spectrometry (LC−MS/MS) methods incorporate mild acid hydrolysis (to release the side chain) and 2-nitrobenzaldehyde (NBA) derivatization *in situ* (*6*, *7*). These methods are very sensitive and effective in the determination of the side chains of tissue-bound residues, specifically semicarbazide (SEM), 3-amino-2-oxazolidone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), and 1-amino-hydantoin (AHD) from nitrofurazone (NFZ), furazolidone, furaltadone, and nitrofurantoin, respectively. Residues of these major nitrofuran drugs can be measured simultaneously on a single LC−MS run. Limitations of this approach include a long derivatization time and complicated sample preparation.

Previous studies (*8*−*11*) demonstrate that SEM in food products could be misinterpreted because of possible contamination from non-NFZ sources. SEM has been found in some foods through contact with foamed seals and packaging materials, originating from the thermal degradation of azodicarbonamide (ADC), a foaming agent (*8*). SEM contamination of chicken products has occurred from contact with ADC-treated flour (*9*). In addition, SEM can be formed from nitrogen-containing compounds with amidino or ureido moieties (e.g., arginine, creatine, creatinine, and urea) through hypochlorite treatment, as observed in shrimp, chicken, and other food substances (*10*, *11*). A confirmatory biomarker is needed for monitoring the use of NFZ in food animals (*12*).

The open-chain cyano metabolite of nitrofuran drugs, formed through reduction of the nitro group (**Figure 1**), is a major metabolite in animals, including fish (*13*−*17*). The cyano metabolite possesses structural features confirmatory for nitrofuran use. Herein, we report the characterization of a cyano derivative as a major metabolite and potential biomarker of NFZ in the channel catfish (*Ictalurus punctatus*).

MATERIALS AND METHODS

Chemicals and Reagents. 2-(5-Nitro-2-furanyl)-[^{14}C]methylene hydrazine carboxamide (^{14}C-NFZ) was obtained from Dupont-NEN (Boston, MA). Acetonitrile and water were HPLC-grade purchased from Burdick and Jackson (Muskegon, MI), and methanol, hexane, and glacial acetic acid were purchased from J.T. Baker (Phillipsburg, PA). Dimethyl sulfoxide (DMSO), magnesium chloride ($MgCl_2$), sodium phosphate monobasic, potassium phosphate dibasic, sodium chloride, 5-nitro-2-furaldehyde, β-nicotinamide adenine dinucleotide phosphate (β-NADP), heparin lithium salt, reduced tetra(cyclohexylammonium) salt (NADPH), and semicarbazide-^{13}C-$^{15}N_2$ hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes were from BD Biosciences (Franklin Lakes, NJ).

Preparation of the Internal Standard. 5-Nitro-2-furaldehyde (0.036 mol in 310 μL DMSO) was combined with 2.5 mL of semicarbazide-^{13}C0$^{15}N_2$ hydrochloride (0.036 mol) solution in 0.1 M HCl,

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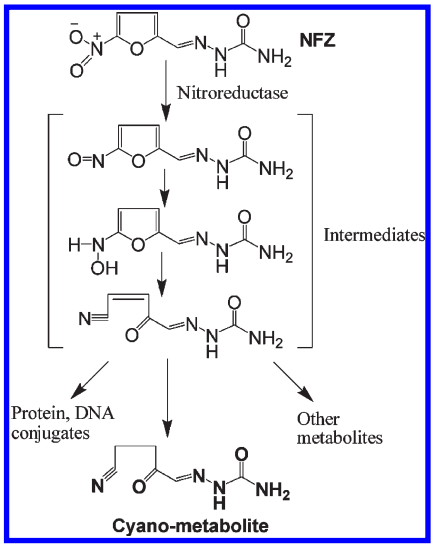

Figure 1. Pathway for metabolic reduction of NFZ.

in a 25 mL beaker. The reaction mixture was stirred at 45 °C for 2 h. The liquid portion was removed by filtration through qualitative filter paper (Whatman International, Maidstone, England), and the solid was washed with 20 mL of cold LC-grade water. The concentration of $^{13}C,^{15}N_2$-NFZ was determined by LC−MS/MS using NFZ as the reference material. The ionization responses for NFZ and isotopic-labeled NFZ were assumed to be the same.

***In Vitro* Microsomal Incubation.** A total of 100 μL of phosphate buffer (0.5 M, pH 7.4) was placed in a 2.0 mL microcentrifuge tube along with 25 μL of $MgCl_2$ (60 mM), 50 μL of NFZ (1 mM) or 250 μL of ^{14}C-NFZ (0.1 mM), and 50 μL of human microsomes (50 mg/mL). The reaction was started by adding 50 μL of NADPH (10 mM). The final volume was adjusted to 500 μL with water, and the solution was incubated in a water bath at 37 °C for 2 h. After incubation, the solution was centrifuged through a Millipore 10-K centrifugal concentrator (Millipore, Billerica, MA) to remove microsomal protein and larger particles. The ultrafiltrate was analyzed by LC−radiometric and LC−MS/MS methods. The major metabolite identified in human microsomal incubations was the same as that found with fish liver slices while providing a cleaner extract for analysis.

Animal Dosing and Sampling. Channel catfish, mean body weight of 0.55 kg, were obtained from a local farm. Fish were orally dosed with ^{14}C-NFZ at 1 mg/kg body weight, as previously described (*7*), and sampled at 18 h after dosing for metabolic profiling. For metabolite elimination studies, fish were dosed with unlabeled NFZ (10 mg/kg body weight) by mixing solid NFZ with 0.25 g of ground catfish feed (Purina Mills, Inc., St. Louis, MO) in gelatin capsules (size number 00, Eli Lilly and Co., Indianapolis, IN). Fish (n = 5) were sampled at 2, 4, 8, 12, 96, 168, 192, 240, and 336 h after dosing. Muscle tissues were collected, homogenized, and stored at −80 °C in 50 mL polypropylene centrifuge tubes.

Extraction of Tissues. For radiometric analyses, 2 g of muscle homogenate (fish dosed with ^{14}C-NFZ) was shaken with 2 mL of 50 mM phosphate buffer (pH 7.4) for 2 h in a 50 mL polypropylene centrifuge tube. The mixture was centrifuged at 3000*g* (Beckman Coulter, Allegra 6R centrifuge, Fullerton, CA) at 15 °C for 10 min. The supernatant was transferred to a 2.0 mL polypropylene microcentrifuge tube, capped, and centrifuged at 14000*g* (Eppendorf Centrifuge 5415D, Westbury, NY) for 10 min to remove larger particles. The supernatant was centrifuged at 14000*g* for 30 min through a Millipore 10-K centrifugal concentrator (Millipore, Billerica, MA). The ultrafiltrate was analyzed by the LC−radiometric method.

For LC−MS/MS analyses, 2 g of muscle homogenate (fish dosed with unlabeled NFZ) was shaken with 5 mL of 50 mM phosphate buffer (pH 7.4) and 75 μL of internal standard (37.5 pmol) for 2 h. After centrifugation at 3000*g* at 15 °C for 10 min, the supernatant was transferred to a 15 mL polypropylene centrifuge tube and washed with 5 mL of hexane. The solution was vortex-mixed followed by centrifugation at 3000*g* for 10 min. The hexane layer was discarded, and the aqueous solution (3 mL) was applied on a preconditioned Waters Oasis HLB cartridge (3 cc/60 mg, 30 μm, Milford, MA). The HLB column was washed with 3 mL of water twice, and analytes were eluted with 4 mL of methanol and dried under a nitrogen gas stream. The dried residues were dissolved in 180 μL of 0.1% acetic acid aqueous solution, prior to LC−MS/MS analysis.

LC−Radiometric Analysis. Extracts of muscle and microsomal incubation samples were fractionated on an Atlantis T3, 5 μm, 4.6 × 150 mm column (Waters, Milford, MA) and Agilent 1200 LC system (Santa Clara, CA). The mobile phase consisted of water (A) and acetonitrile (B), with a linear gradient of 30 min of 0−40% B and 5 min of 40−50% B and a flow rate of 1.0 mL/min. Fractions were collected in 6 mL vials (PerkinElmer, Meridan, CT) with a Gilson FC204 fraction collector (Middleton, WI) at 0.25 min/tube. To each vial was added 6 mL of Ultima Gold (PerkinElmer, Meridan, CT), and the vials were analyzed by liquid scintillation counting with a Tri-Carb 2500 TR liquid scintillation analyzer (PerkinElmer, Meridan, CT).

LC−MS/MS Analysis. LC−MS/MS analyses of microsomal incubation samples were performed on a Waters Alliance 2695 LC system (Waters, Milford, MA) coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionization probe (Micromass, Inc., U.K.) in positive- or negative-ion mode. MassLynx software was used with the following instrument settings: capillary voltage, 3.5 kV; cone voltage, 15 V for positive-ion mode and 10 V for negative-ion mode; extractor voltage, 3 V; rf lens, 0 V; source temperature, 120 °C; desolvation gas flow rate, 450 L/h; desolvation temperature, 350 °C; dwell time, 100 ms; collision gas, 0.21 L/h; and collision energy, 10 eV.

LC−MS/MS analyses of muscle extracts were performed on a HP1100 LC system (Agilent Technologies, Palo Alto, CA) coupled to a 4000 QTRAP triple quadrupole/linear ion trap hybrid mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). Ion scanning was performed with the Analyst software and the following instrument settings: ion spray voltage, 3.5 kV; rf, 0 V; dwell time, 100 ms; collision gas flow rate, 4 psi; and collision energy, 45 eV. LC separations were conducted on an Atlantis T3, 5 μm, 2.1 × 150 mm column (Waters, Milford, MA), with the column temperature at 30 °C. LC mobile-phase solvents were water (A) and acetonitrile (B) for negative-ion mode and included 0.1% acetic acid for positive-ion mode. The linear gradient was 0−40% B for 30 min, and the flow rate was 0.2 mL/min.

RESULTS AND DISCUSSION

Nitroreduction is a major metabolic pathway for nitrofuran drugs (*13*−*18*). Intermediates with active nitroso and hydroxylamino functional groups covalently bind to protein and DNA (**Figure 1**). A cyano metabolite is produced by rearrangement of the hydroxylamine derivative followed by reduction of the double bond. The cyano metabolite is a major extractable residue of nitrofuran drugs in the rat (*14*) and swine (*16*−*18*). In fish, a cyano metabolite of furazolidone was identified in the grouper (*15*).

We found parent ^{14}C-NFZ extensively metabolized *in vitro* by microsomal incubations and *in vivo* after oral dosing in channel catfish. Extractable metabolites were mostly of higher polarity compared to the parent drug. Representative LC−radiometric chromatograms of catfish muscle tissue and microsomal extracts are presented in **Figure 2**. Under our conditions of analysis, the parent drug eluted at 21 min. A major metabolite eluted at 12.5 min. This metabolite was later confirmed as the cyano metabolite of NFZ, using LC−MS/MS.

The cyano metabolite was identified by LC−MS/MS, having the same retention time as that of the radiolabeled compound by LC−radiometric analysis. MS/MS spectra were generated by electrospray ionization (ESI) in positive- and negative-ion modes (**Figure 3**). The parent ions of m/z 169 $[M + H]^+$ and 167 $[M - H]^-$ were consistent with the molecular weight of the cyano metabolite formed by nitroreduction of NFZ. The main fragment ions of m/z 126 (**Figure 3a**) and 124 (**Figure 3b**) in positive- and negative-ion modes, respectively, resulted from the neutral loss of HNCO (43 Da) from the side chain of NFZ. The loss of HCN (27 Da) from the opposite end of the molecule in negative-ion mode resulted in the fragment ion of m/z 140, in abundance.

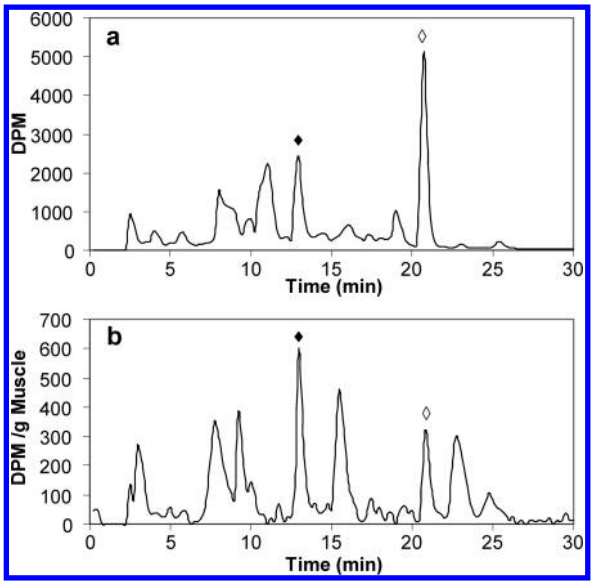

Figure 2. Radio-chromatograms: (a) microsomal incubations with ^{14}C-NFZ and (b) muscle of catfish dosed with ^{14}C-NFZ (♦, cyano metabolite; ◊, NFZ).

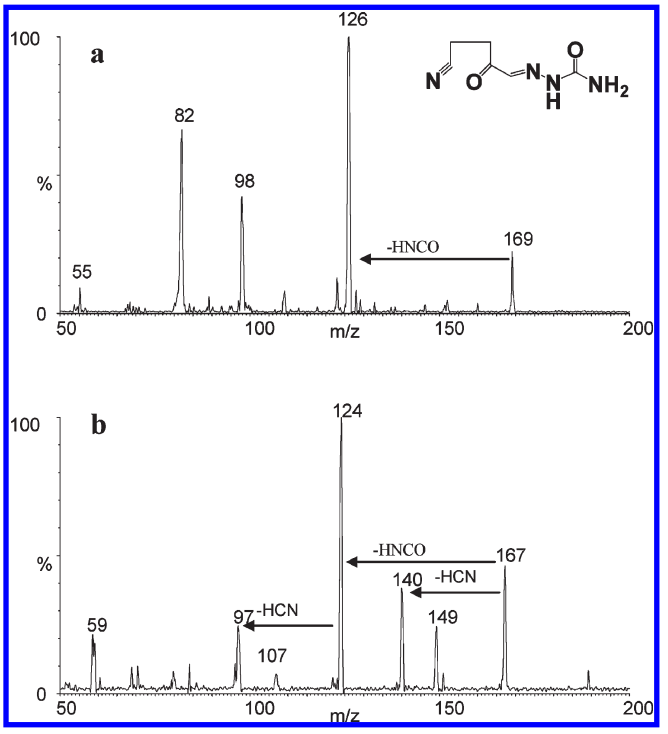

Figure 3. ESI−MS/MS spectra of cyano metabolite: (a) $(M + H)^+$ at *m*/*z* 169 and (b) $(M - H)^-$ at *m*/*z* 167.

The product ion *m*/*z* 97 in negative-ion mode was due to the combined loss of HNCO and HCN.

The cyano metabolite was monitored in muscle tissue by selected reaction monitoring (SRM) in negative-ion mode, with higher sensitivity and less matrix effects compared to the positive-ion mode. The cyano metabolite was quantified (NFZ equivalents) using the ion transition *m*/*z* 167 → 124 and confirmed by including the transitions *m*/*z* 167 → 140, 97. Isotope-labeled ^{13}C,^{15}N$_2$-NFZ served as the internal standard by monitoring the transition *m*/*z* 200 → 153 and confirmed by including *m*/*z* 200 → 126, 82. For parent NFZ, the transition *m*/*z* 197 → 150 was used, along with *m*/*z* 197 → 124, 80 for confirmation (**Figure 4**). Retention times of the cyano metabolite and parent NFZ, under

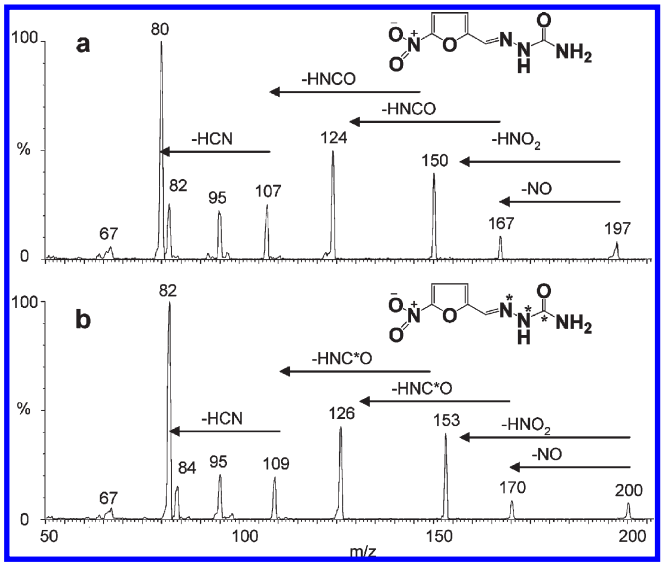

Figure 4. ESI−MS/MS spectra of (a) $(M - H)^-$ ion at *m*/*z* 197 for parent NFZ and (b) $(M - H)^-$ ion at *m*/*z* 200 for the internal standard ^{13}C, ^{15}N$_2$-NFZ (C* = ^{13}C and N* = ^{15}N).

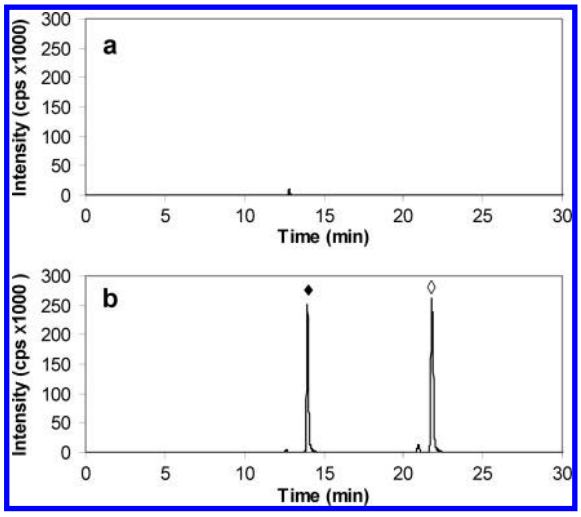

Figure 5. SRM sum-overlay chromatograms of cyano metabolite monitored at *m*/*z* 167 → 124 and parent NFZ at *m*/*z* 197 → 150 for extracts of muscle from (a) control catfish and (b) catfish dosed with 10 mg/kg NFZ, with a 12 h elimination (♦, cyano metabolite; ◊, parent NFZ).

our conditions of analysis, were 14.0 and 22 min, respectively (**Figure 5**). No interfering compounds were found in extracts of control catfish. Recovery of the cyano metabolite was estimated at 92%, as determined by spiking control muscle homogenate with a semi-purified extract of the cyano metabolite from incurred muscle samples.

Channel catfish were orally dosed with NFZ at 10 mg/kg body weight for the depletion study. Semi-logarithmic plots of NFZ and cyano metabolite concentrations versus depletion time in muscle are presented in **Figure 6**. Parent NFZ was depleted very rapidly and undetectable at 96 h after dosing with an elimination half-life of 6.3 h (**Figure 6a**), consistent with a previous study in catfish (*7*). Mean concentrations of the cyano metabolite was highest at 10 h after dosing and could be measured unambiguously above the limit of quantification (signal-to-noise ratio greater than 10) for up to 2 weeks after dosing. In the terminal phase of elimination, the half-life of the cyano metabolite was approximately 81 h.

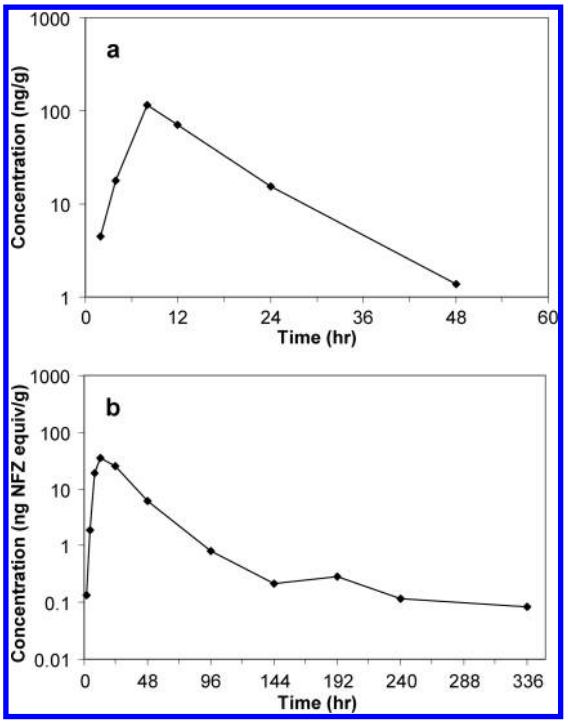

Figure 6. Elimination of (a) parent NFZ and (b) cyano metabolite from the muscle of channel catfish orally dosed with NFZ at 10 mg/kg body weight.

The rapid depletion of NFZ in the tissues of channel catfish is consistent with the disposition of NFs in other animals (*6*, *7*, *15*, *16*, *18*). The intact side chain of covalently bound NF residues has been used as biomarkers for monitoring purposes (*6*, *7*). However, SEM (side chain of NFZ) can contaminate animal products from non-NF sources and lead to misinterpretation of the data when used as a biomarker of NFZ (*8*−*11*). The half-life of the cyano metabolite was longer than that of parent NFZ in catfish, in agreement with previous studies of the cyano metabolite of furazolidone and NFZ in the grouper (*15*) and pig (*16*), respectively.

LC−MS SRM mode can provide a robust tool to characterize the amount of metabolites with a large dynamic linear range. For the cyano metabolite of NFZ, the selection of transition fragments for quantitative purposes was found to provide reliable and reproducible results with the least matrix effects. However, the concentration of cyano metabolite could not be accurately measured because of the unavailability of the commercial standard. The cyano metabolite possesses the side chain (SEM) and the nitroreduced ring portion of the molecule, which together are unique for NFZ. Analysis of the cyano metabolite is more straightforward and time-efficient than the 2-nitrobenzaldehyde derivatization method used for tissue-bound SEM (*6*, *7*). The cyano metabolite can be used as an alternative confirmatory biomarker to monitor the use of NFZ, because of its reasonable stability and relative persistence in muscle compared to the parent drug.

In summary, the cyano derivative was identified as a major metabolite of NFZ in channel catfish. From LC−MS/MS, we could detect and confirm the cyano metabolite in catfish muscle for up to 2 weeks after a single oral dose (10 mg/kg) of NFZ. The cyano metabolite can be used as an alternative confirmatory marker for monitoring the use of NFZ in channel catfish.

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