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Residues of the Lampricides 3-Trifluoromethyl-4-nitrophenol and Niclosamide in Muscle Tissue of Rainbow Trout

TERRANCE D. HUBERT,* JEFFRY A. BERNARDY, CHUE VUE, VERDEL K. DAWSON, MICHAEL A. BOOGAARD, THERESA M. SCHREIER, AND WILLIAM H. GINGERICH

Biological Resources Division, Upper Midwest Environmental Sciences Center, U.S. Geological Survey, 2630 Fanta Reed Road, La Crosse, Wisconsin 54603

Rainbow trout (*Oncorhyncus mykiss*) were exposed to the ^{14}C-labeled lampricide 3-trifluoromethyl-4-nitrophenol (TFM) (2.1 mg/L) or niclosamide (0.055 mg/L) in an aerated static water bath for 24 h. Fish were sacrificed immediately after exposure. Subsamples of skin-on muscle tissue were analyzed for residues of the lampricides. The primary residues in muscle tissue from fish exposed to TFM were parent TFM (1.08 ± 0.82 nmol/g) and TFM-glucuronide (0.44 ± 0.24 nmol/g). Muscle tissue from fish exposed to niclosamide contained niclosamide (1.42 ± 0.51 nmol/g), niclosamide-glucuronide (0.0644 ± 0.0276 nmol/g), and a metabolite not previously reported, niclosamide sulfate ester (1.12 ± 0.33 nmol/g).

KEYWORDS: TFM; TFM-glucuronide; niclosamide; niclosamide-glucuronide; niclosamide-sulfate; lampricide residues; rainbow trout; muscle

INTRODUCTION

The lampricides 3-trifluoromethyl-4-nitrophenol (TFM; **Figure 1A**) and 2′,5-dichloro-4′-nitrosalicylanilide (niclosamide; **Figure 1B**) are restricted-use pesticides that have been used successfully for more than 40 years to control sea lampreys (*Petromyzon marinus*) in streams tributary to the Great Lakes (*1*). The chemicals are generally applied directly to waterways inhabited by fish. Consequently, there is the potential for lampricide residues to accumulate in fish muscle tissue. Information on the identities and concentrations of these residues in fish muscle tissue will help to ensure human food safety.

The identity of TFM and niclosamide residues in fish muscle tissue has not been well characterized. Studies on the metabolism of TFM in rainbow trout (*Oncorhyncus mykiss*) demonstrated that TFM-glucuronide is the major metabolite of TFM in the bile of rainbow trout (*2*−*5*). However, only one study examined residues in muscle tissue, and no TFM-glucuronide was found (*5*). Schultz et al. (*6*) measured parent TFM in exposed largemouth bass (*Micropterus salmoides*) but did not characterize metabolites. Likewise, a study on the metabolism of niclosamide demonstrated that niclosamide-glucuronide is the major metabolite of niclosamide in the bile of rainbow trout (*7*), but residues of niclosamide in muscle tissue were not characterized. The present work describes the results of experiments conducted to determine the identities and concentrations of TFM and niclosamide residues in rainbow trout muscle tissue. Rainbow trout were chosen as the test organism because they are commonly found in many tributaries of the Great Lakes where lampricide treatments occur.

* Author to whom correspondence should be addressed [telephone (608) 781-6227; fax (608) 783-6066; e-mail thubert@usgs.gov].

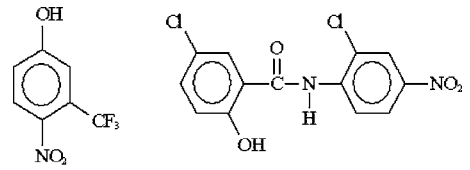

Figure 1. Structures of (**A**) TFM and (**B**) niclosamide.

MATERIALS AND METHODS

Solvents and Reagents. Solvents and reagents were of analytical grade or better. Ready-Safe (Beckman Coulter, Fullerton, CA), Ultima-Flo M, Carbo-Sorb E, Permafluor E, and Combustaid (Perkin-Elmer, Boston, MA) were used for analyses of radioactive compounds. Pronase E, sulfatase, and β-glucuronidase (Sigma Chemical Co., St. Louis, MO) were used for enzymatic processes. Sodium acetate buffer (58 mM, pH 3.8), potassium phosphate buffer (80 mM, pH 7.5), and 0.01 M KCl·NaOH buffer were used for solid-phase extraction (SPE) and high-performance liquid chromatograph (HPLC) analyses.

Test and Reference Chemicals. Uniformly aromatic-ring labeled [^{14}C]TFM (98% purity, 5.05 mCi/mmol) and [^{14}C]niclosamide (99% purity, 6.55 mCi/mmol) were purchased from Perkin-Elmer Life Sciences, Boston MA (formerly DuPont/New England Nuclear). Niclosamide was uniformly labeled in the chloronitroaniline ring. Non-radiolabeled TFM (99% purity), 2-chloro-4-nitroaniline (97% purity), and 5-chlorosalicylic acid (98% purity) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Non-radiolabeled niclosamide (99% purity) was purchased from Sigma Chemical Co. Reduced TFM, acetylated-reduced TFM, reduced niclosamide, acetylated-reduced niclosamide, and the sulfate ester of niclosamide were synthesized by Derse and Schroeder Associates (Madison, WI) and were 99+% pure. The [^{14}C]glucuronide standards were generated by exposing rainbow trout to water solutions of either [^{14}C]TFM or [^{14}C]niclosamide for 24 h. After the exposure, bile from the fish was collected. Bile containing

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[^{14}C]niclosamide-glucuronide required no purification prior to use. [^{14}C]-TFM-glucuronide was purified from the bile using a C_{18} SPE column. Identities of the glucuronide standards were established by conducting a β-glucuronidase hydrolysis experiment similar to that carried out by Lech (*4*).

Test Organisms. Rainbow trout (~300 g; age, 1.5 years) were reared from eyed-eggs obtained from Ennis National Fish Hatchery (Ennis, MT). Fish were cultured in fiberglass tanks supplied with flowing well water. Fish were acclimated to experimental conditions for at least 1 week before use in the experiments.

Exposure of Test Organisms. Selection of the TFM exposure concentration was based on the pH and alkalinity of the well water used during the experiment. The pH and alkalinity were compared to regression curves developed from historical data relating lethal concentrations to water chemistry (*8*). A concentration of 2.1 mg/L was chosen as a target TFM concentration to maximize residues in tissues while minimizing the potential for mortality. For the niclosamide experiment, a target concentration of 0.055 mg/L was chosen on the basis of toxicity trials that determined the maximum concentration at which the test could be performed without risk of mortality. Ten fish were randomly selected for each exposure and transferred from the acclimation tank into a single test tank. Control systems were prepared which were identical to each test system with the exception that no chemical was added. Water temperature (12 ± 1 °C), pH (7.4−8.3), un-ionized ammonia (<0.01 ppm), and dissolved oxygen (>5 ppm) were monitored throughout the acclimation and exposure periods. Water samples from the control and test tanks were collected throughout the exposure period for liquid scintillation analysis (LSA) to determine the actual exposure concentrations (mean, 2.19 ± 0.03 mg/L [^{14}C]TFM or 0.0525 ± 0.001 mg/L [^{14}C]niclosamide). These concentrations were confirmed by HPLC analysis to determine concentrations of TFM or niclosamide. The concentration of TFM was 2.11 ± 0.090 mg/L, and the concentration of niclosamide was 0.0573 ± 0.001 mg/L. No breakdown products of TFM or niclosamide were observed in the exposure water. Fish were exposed for 24 h. Concentrations of TFM and niclosamide used in the experiments produced no mortality. At the end of each exposure, all fish were euthanized by electrocution and rinsed with fresh water. Skin-on muscle tissues then were collected, frozen, homogenized to a uniform powder with dry ice (*9*), and stored at < −85 °C until analyzed. Fillets from each fish were frozen and stored separately from those of other fish.

Radioanalysis of Total Radioactive Residues. Five 0.5-g samples of each fish were weighed into combustion cones, treated with Combustaid, and oxidized with a Packard Tricarb 307 biological sample oxidizer (Meriden, CT). The $^{14}CO_2$ resulting from the oxidation of tissue was trapped in Carbo-sorb and mixed with Permafluor for LSA using a Beckman LS 5801 liquid scintillation system (Fullerton, CA). The efficiency of the sample combustion process (97.5 ± 2.3% for the TFM experiment; 98.8 ± 1.9% for the niclosamide experiment) was determined after every 25 samples by combusting samples spiked with a standard of known radioactivity. Samples were counted for 20 min or until a 2% 2σ counting error was obtained and corrected for quench by automatic external standardization. The radioassay detection limit for tissue samples (0.023 nmol/g TFM equivalent and 0.0058 nmol/g niclosamide equivalent) was considered to be twice the mean background disintegrations per minute of oxidized control muscle tissues mixed with scintillation cocktail and enumerated by LSA. Background activity was subtracted after the sample counting process. Data on the total radioactive residue in tissue samples were not corrected for oxidizer efficiency.

Characterization of Residues. Each sample was processed in triplicate. The procedures used to extract and analyze TFM or niclosamide residues from muscle tissue were essentially the same (**Figure 2**), with the differences between the TFM and the niclosamide experiments noted in parentheses. In general, methanol was used to extract the majority of both lampricide residues, and the remaining pellets were subjected to a water extraction followed by an enzymatic digestion (samples in the niclosamide experiment were subjected further to a 1% NaOH digestion). Radioactivity from all extracts and the final pellets was quantified. Extracts containing >0.05 ppm of parent-equivalent radioactivity were processed for further characterization.

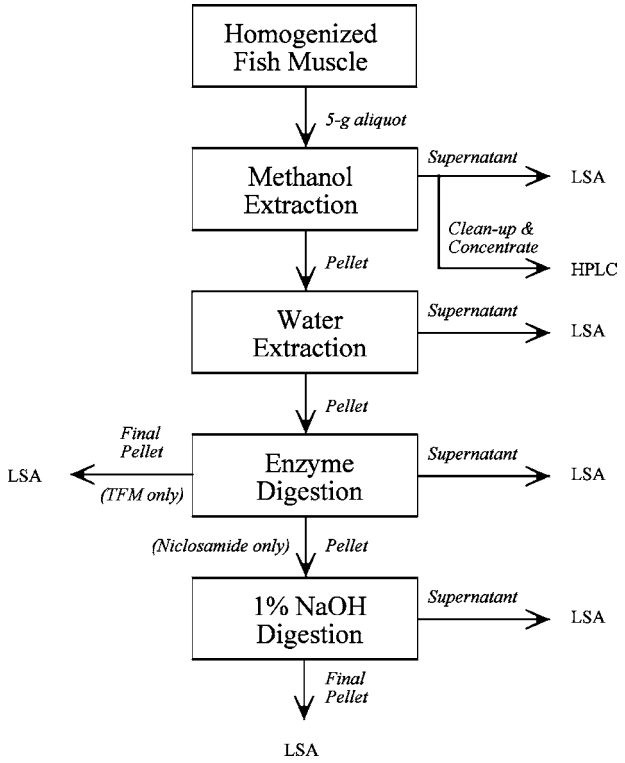

Figure 2. General extraction flow diagram for muscle tissue collected from rainbow trout exposed to [^{14}C]TFM or [^{14}C]niclosamide for 24 h. All extracts and the final pellets were analyzed by LSA. The methanol extracts were further purified, concentrated, and analyzed by HPLC.

Methanol Extraction. Methanol (10 mL) was added to 5-g samples, shaken for 10 min, and centrifuged at 4000*g* for 5 min at 20 °C. The extract then was transferred to a 100-mL flask. The methanol extraction was repeated with the remaining pellet three times (five times for niclosamide). The extracts were combined, concentrated under vacuum, and transferred to a 25-mL volumetric flask. The 25-mL flask was filled to volume with methanol, and subsamples were taken for LSA (3 × 1 mL) and HPLC (2.0 mL) analyses.

Water Extraction. Residues remaining in the pellet after the methanol extraction were extracted further with water. Water (10 mL) was added to the pellet, thoroughly mixed for 10 min, and centrifuged at 4000*g* for 5 min at 20 °C. The procedure was repeated, and the two extracts were combined. Radioactivity in the combined extracts was determined by LSA.

Enzyme Digestion. Residues remaining in the pellet after both methanol and water extractions were subjected to digestion with Pronase E. Ten milliliters of 0.0125 g/mL Pronase E solution in phosphate buffer (15 mL of 0.0167 g/mL for niclosamide) was added to the pellet, agitated for at least 16 h at ambient temperature, and stored overnight in a refrigerator at <10 °C. Each sample was centrifuged at 10800*g* for 15 min at 5 °C, and the extract was filtered through Whatman 40 filter paper into a 25-mL volumetric flask. The remaining pellet was rinsed three more times with phosphate buffer, and each rinse (~3 mL) was centrifuged and filtered as before. The flask was adjusted to volume with water and the radioactivity in three 1-mL subsamples determined by LSA.

1% NaOH Digestion (*Niclosamide Experiment Only*). The pellet was resuspended in 1% NaOH/methanol, shaken for 10 min, and centrifuged for 5 min at 10000*g*. The extract was decanted and the rinse repeated twice. The extracts were combined, and the radioactivity in three 1-mL subsamples was determined by LSA. The final pellet was combusted on the Packard Tricarb 307 sample oxidizer and analyzed for radioactivity by LSA.

LSA Analysis. Unextracted tissue samples and the final pellets were analyzed for radioactivity using the procedures outlined under Radioanalysis of Total Radioactive Residues. Extract subsamples were added to 12 mL of Ready Safe scintillation cocktail, stored in the dark for 8

h to minimize chemilumnescence, and counted for radioactivity using the Beckman LS 5801 liquid scintillation system. Samples were counted for 20 min or until a 2% 2σ counting error was obtained and corrected for quench by automatic external standardization.

HPLC Analysis. The water extracts, enzyme extracts (and 1% NaOH extracts for niclosamide), and final pellets contained <0.05 ppm of parent equivalent; therefore, no HPLC analyses were conducted on these extracts. Methanol extracts from each sample were further concentrated and purified using YMC ODS-AQ SPE columns (Wilmington, NC) (J. T. Baker C_{18}, Phillipsburg, NJ, for niclosamide). A 20-mL portion of each methanol extract was concentrated under N_2 to 3−5 mL. Water (2 mL) was added to the concentrate, and the mixture was passed through a preconditioned 500-mg C_{18} SPE column. The column was rinsed with six 1-mL portions of water/methanol (20:80, v/v) (0.01 M KCl/0.01 M NaOH/methanol, 90:10, v/v, for niclosamide). The eluates were combined, concentrated under N_2 to ≤4 mL, and loaded onto a second preconditioned 1000-mg C_{18} SPE column. The column was rinsed three times with 1 mL of acetate buffer. The sample was eluted from the column with six 1-mL volumes of methanol (acetate buffer/methanol, 90:10, v/v, for niclosamide) into a 5-mL volumetric flask, and the content of the flask was concentrated to 5 mL.

Four milliliters of the purified methanol extract was evaporated and reconstituted to 2 mL in acetate buffer/acetonitrile (85:15, v/v; the purified niclosamide extract was not concentrated). Residues of TFM were analyzed on an HP 1090 M HPLC equipped with an ultraviolet (UV) photodiode array detector (Hewlett-Packard, Arlington Heights, IL), a flow-through radioactivity monitor (Packard Instrument, Shelton, CT), and a YMC ODS-AQ reversed-phase column (250 × 4.6 mm, 5 μm, 120 Å). Analytical conditions were a flow rate of 1 mL/min at ambient column temperature (35 °C for niclosamide) with a gradient mobile phase from 85:15 to 30:70 (v/v) acetate buffer/acetonitrile. Niclosamide residues were analyzed on a Waters Millennium HPLC (Milford, MA), a flow-through radioactivity monitor (Packard Instrument), and a YMC ODS-AQ reversed-phase column (250 × 4.6 mm, 5 μm, 120 Å). Analytical conditions were a flow rate of 1 mL/min at a column temperature of 35 °C with a gradient mobile phase from 75:25 to 0:100 (v/v) acetate buffer/acetonitrile. In both methods Ultima-Flo M was introduced into the mobile phase at the flow-through radioactivity monitor to detect radioactive components. Standards of TFM, TFM-glucuronide, reduced TFM, acetylated reduced TFM, niclosamide, niclosamide-glucuronide, reduced niclosamide, acetylated-reduced niclosamide, 2-chlorosalicylic acid, 2-chloro-4-nitroaniline, and niclosamide sulfate ester were processed along with the appropriate sample set to identify and quantify unknowns in the sample. Standards were injected separately but were included in the same run sequence so that standard retention times were recorded under the same conditions.

RESULTS

The mean terminal radioactive residues (TRR) found in rainbow trout muscle tissue after 24-h exposures were 2.28 ± 1.61 nmol/g TFM-equiv and 3.33 ± 0.55 nmol/g niclosamide-equiv. In the TFM experiment, 104% of the TRR was accounted for by summing the mean percentage of radioactivity of the methanol extract (96.2%), water extract (2.6%), enzymatic digestion extracts (4.2%), and final pellets (0.5%). The HPLC analysis of the concentrated methanol extracts revealed three radioactive compounds (**Figure 3**) averaging 86% of the radioactivity in methanol extracts. Peak 1 (36% of the TRR) exhibited a similar retention time (23 min) and UV spectrum (**Figure 4-1**) as TFM-glucuronide isolated from bile. Hydrolysis of the extract by β-glucuronidase (*4*) confirmed that peak 1 was the TFM-glucuronide. Peak 2 (44% of the TRR, 39 min) cochromatographed with the TFM standard; the UV spectrum also was similar to that of TFM. Peak 3 (5.7% of the TRR, 45 min) cochromatographed with an unidentified impurity in the radiolabeled TFM. The mean concentration of TFM-glucuronide in the muscle tissue averaged 0.44 ± 0.24 nmol/g, and the mean concentration of parent TFM was 1.08 ± 0.82 nmol/g.

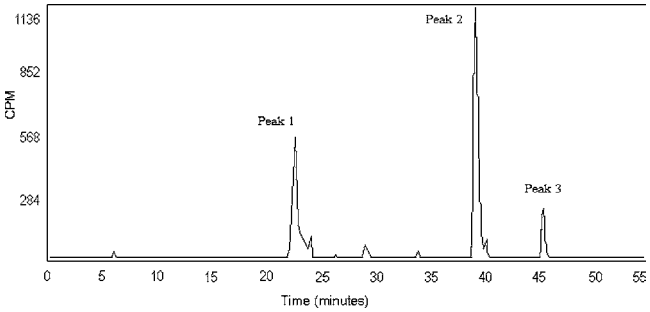

Figure 3. HPLC radiochromatogram of the methanol-soluble fraction of muscle tissue taken from rainbow trout exposed to [^{14}C]TFM. The retention times and UV scans for peaks 1 (TFM-glucuronide), 2 (TFM), and 3 (test material impurity) match the retention times and UV scans of the standards TFM-glucuronide (23 min), TFM (39 min), and the test material impurity (45 min), respectively.

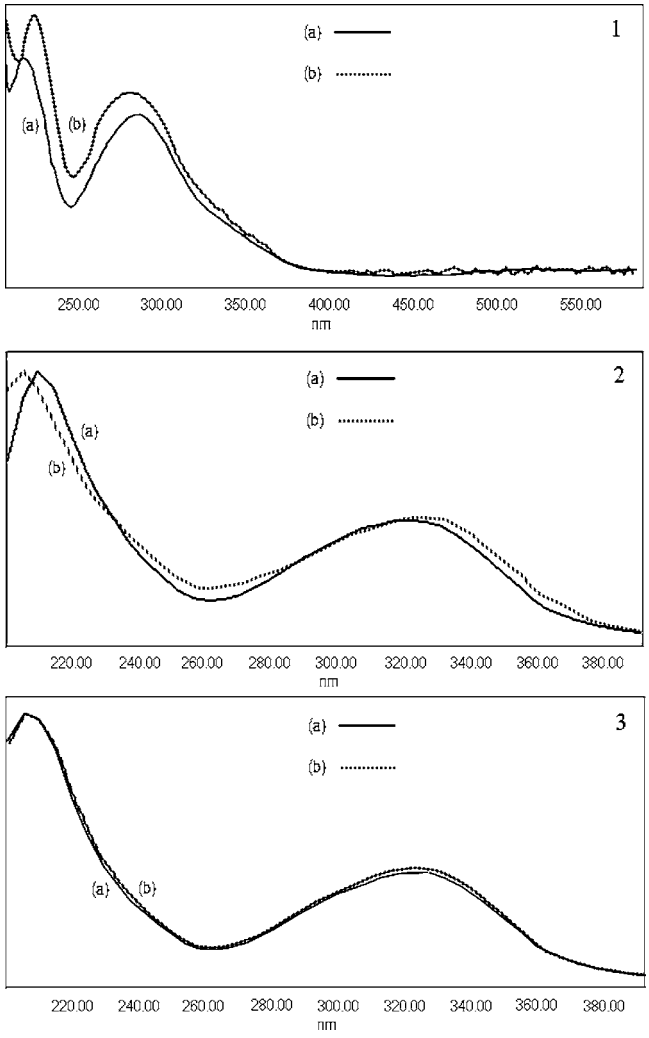

Figure 4. UV spectra of (**1**) (a) TFM-glucuronide from the methanol-soluble fraction of muscle tissue and (b) TFM-glucuronide isolated from the bile of rainbow trout; (**2**) (a) niclosamide-glucuronide from the methanol-soluble fraction of muscle tissue and (b) niclosamide-glucuronide from bile; and (**3**) (a) niclosamide sulfate ester from the methanol-soluble fraction of muscle tissue and (b) the niclosamide sulfate ester standard.

In the niclosamide experiment, 103% of the TRR was accounted for by summing the mean radioactivity of final pellets (<1%) and the extracts of the methanol (95%), water (<1%), enzyme (3%), and 1% NaOH (3%) extractions. The HPLC analysis of the methanol extracts also revealed three radioactive

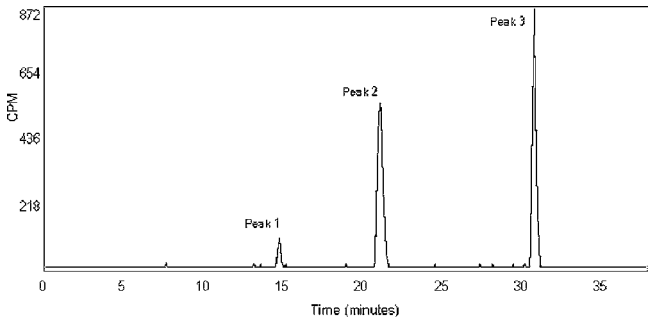

Figure 5. HPLC radiochromatogram of the methanol-soluble fraction of muscle tissue taken from rainbow trout exposed to [^{14}C]niclosamide for 24 h. The retention times and UV scans for peaks 1 (niclosamide-glu), 2 (niclosamide sulfate), and 3 (niclosamide) match the retention times and UV scans of the standards niclosamide-glucuronide (14.7 min), niclosamide-sulfate (21.0 min), and niclosamide (30.8 min), respectively.

peaks (**Figure 5**), with the mean of peak 1 (14.7 min) representing 2.8% of TRR, peak 2 (21.0 min) 38.6%, and peak 3 (30.8 min) 39.6%. Peak 1 showed a retention time and UV spectrum (**Figure 4-2**) similar to those of niclosamide-glucuronide. A hydrolysis experiment with β-glucuronidase (*4*) confirmed its identity as the niclosamide-glucuronide. Peak 2 cochromatographed with the niclosamide sulfate ester standard, and its UV spectrum was similar to that of the sulfate ester standard (**Figure 4-3**). Hydrolysis of this component of the extract with the enzyme sulfatase converted the compound back to parent niclosamide and confirmed the identity as the sulfate ester. Peak 3 cochromatographed with, and had a UV spectrum similar to, the niclosamide standard. The methanol extracts averaged 0.0664 $\pm$ 0.0276 nmol/g niclosamide-glucuronide, 1.12 $\pm$ 0.33 nmol/g niclosamide sulfate ester, and 1.42 $\pm$ 0.51 nmol/g niclosamide in muscle tissue.

DISCUSSION

The identification of a sulfate conjugate of niclosamide in rainbow trout is significant. Sulfate metabolites of xenobiotic compounds have been reported previously, and certain xenobiotic substrates that contain hydroxy groups or phenols have been shown to be excreted as a sulfate conjugate by fish (*10*−*13*). However, James et al. reported that trout appear to be relatively deficient in sulfate conjugation, as there was little or no evidence of the presence of sulfate conjugates in vivo or in isolated perfused organ systems or hepatocytes (*13*).

Fish are known to eliminate aromatic compounds as glucuronides, either by conjugation at an existing oxygen on an aromatic ring or by oxidation at an aromatic ring carbon followed by conjugation (*10*−*26*). The conversion of phenolics into glucuronide conjugates in fish is well documented (*10*−*19*). The oxidation−conjugation sequence has been observed with environmental pollutants such as 2,3,7,8-tetrachlorodibenzofuran and benzo[*a*]pyrene and with candidate fishery drugs such as sulfamonomethoxine (*23*−*25*). The liver appears to be the most important site for glucuronidation, although activities have also been noted in kidney, gills, intestines, and heart (*22*). Although not frequent, others have reported the appearance of glucuronides in muscle tissue (*25*, *26*).

The metabolism of TFM was studied by Lech (*3*−*5*) and by Schultz et al. (*6*). Statham and Lech also reported that the glucuronide conjugate of niclosamide was the major metabolite in bile of rainbow trout exposed to [^{14}C]niclosamide (*7*). These studies provided insight into the metabolism of the lampricides, but residues in muscle tissue were not adequately identified. Sills and Allen (*27*) examined muscle tissue from fish exposed to TFM, but did not explore the possibility that residues other than the parent compound might be present. In our experiments, the glucuronides of TFM and niclosamide and the sulfate conjugate of niclosamide were found in measurable concentrations in the muscle tissue. Although muscle tissue of fish exposed to xenobiotic chemicals is expected to contain the parent chemical, none or only small amounts of metabolites are expected, as these are readily converted to more water-soluble forms (*28*).

We recorded observations similar to those of the present work in a study to determine the concentrations of TFM and TFM-glucuronide in the analysis of rainbow trout and channel catfish (*Ictalurus punctatus*) muscle tissue from animals exposed during a sea lamprey control treatment of the Ford River in 1996 (*29*). In contrast, a similar field study on niclosamide showed only the parent compound in the muscle tissue (*30*). However, the exposure concentration and duration in the niclosamide field study were <50% of the exposure concentration and duration used in this study.

During the course of the present study, we conducted a storage stability analysis of subsamples of rainbow trout muscle tissue containing TFM and TFM-glucuronide over a 10-month storage period at ~ -90 °C. In that period, the percentage of TFM-glucuronide was found to increase by ~11% while a corresponding 10% decrease in the concentration of TFM was observed. This would suggest the presence of active glucuronyl transferase enzymes in muscle cells or possibly in the blood. Reports of glucuronyl transferase enzymes isolated from muscle tissue or blood are limited, but the possibility cannot be discounted (*28*).

Analysis of muscle tissue from rainbow trout exposed to TFM or niclosamide has shown that the majority of the residues in the muscle tissue are the parent compound, glucuronide conjugate, or, in the case of niclosamide, sulfate conjugate. No 2-chloro-4-nitroaniline and 2-chlorosalicylic acid were detected in the extracts of muscle tissue of fish exposed to niclosamide, suggesting that the molecule remains intact in the fish. No reduced TFM, acetylated reduced TFM, reduced niclosamide, or acetylated reduced niclosamide was detected in the muscle tissue of rainbow trout. These data expand on the earlier work of Lech and others and suggest that the metabolism of TFM and niclosamide is simple. Absorbed chemical is conjugated with glucuronic acid or sulfate and eliminated.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatograph(y); LSA, liquid scintillation analysis; SPE, solid-phase extraction; TFM, 3-trifluoromethyl-4-nitrophenol; TRR, total radioactive residue; UV, ultraviolet.

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