Determination of Niclosamide Residues in Rainbow Trout (*Oncorhynchus mykiss*) and Channel Catfish (*Ictalurus punctatus*) Fillet Tissue by High-Performance Liquid Chromatography

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Bayluscide [the ethanolamine salt of niclosamide (NIC)] is a registered piscicide used in combination with 3-(trifluoromethyl)-4-nitrophenol (TFM) to control sea lamprey populations in streams tributary to the Great Lakes. A high-performance liquid chromatography (HPLC) method was developed for the determination of NIC residues in muscle fillet tissues of fish exposed to NIC and TFM during sea lamprey control treatments. NIC was extracted from fortified channel catfish and rainbow trout fillet tissue with a series of acetone extractions and cleaned up on C_{18} solid-phase extraction cartridges. NIC concentrations were determined by HPLC with detection at 360 and 335 nm for rainbow trout and catfish, respectively. Recovery of NIC from rainbow trout ($n = 7$) fortified at 0.04 μg/g was 77 ± 6.5% and from channel catfish ($n = 7$) fortified at 0.02 μg/g was 113 ± 11%. NIC detection limit was 0.0107 μg/g for rainbow trout and 0.0063 μg/g for catfish. Percent recovery of incurred radioactive residues by this method from catfish exposed to [^{14}C]NIC was 89.3 ± 4.1%. Percent recoveries of NIC from fortified storage stability tissue samples for rainbow trout ($n = 3$) analyzed at 5 and 7.5 month periods were 78 ± 5.1 and 68 ± 2.4%, respectively. Percent recoveries of NIC from fortified storage stability tissue samples for channel catfish ($n = 3$) analyzed at 5 and 7.5 month periods were 88 ± 13 and 76 ± 21%, respectively.

Keywords: *Niclosamide; fillet residues; channel catfish; rainbow trout; HPLC*

INTRODUCTION

Bayluscide, the ethanolamine salt of niclosamide (NIC, 2′,5-dichloro-4′-nitrosalicylanilide; Figure 1), is a halogenated salicylanilide that has been used successfully for >25 years to control sea lamprey (*Petromyzon marinus*) in the Great Lakes. NIC is sold as the 2-aminoethanol salt (Bayer 73) under the commercial name Bayluscide, a formulation that is ~70% active ingredient by weight. The U.S. Fish and Wildlife Service currently holds the label registration for Bayluscide. Its uses in sea lamprey control operations are limited to combination treatments with 3-(trifluoromethyl)-4-nitrophenol (TFM), to reduce the amount of TFM required for treatment, and simple treatments as a granular bottom-release formulation to survey for lamprey ammocoetes in lentic habitats. The combination treatment with TFM is a cost-saving measure usually used when large quantities of TFM would normally be required, such as the treatment of streams or rivers with high discharge rates. When used in combination, the TFM/NIC ratio ranges from 98:2 to 99.5:0.5 (Associate Committee on Scientific Criteria for Environmental Quality, 1985). Rainbow trout and channel catfish were selected as representative nontarget fish species that are commonly found in the Great Lakes.

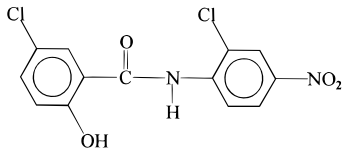

Figure 1. Chemical structure of niclosamide.

Methods for analysis of NIC in water include colorimetry (Dawson et al., 1978) and liquid chromatography (Dawson, 1982). Residues of NIC in fish tissues have been analyzed by gas chromatography (Luhning et al., 1979); however, this procedure requires indirect analysis of the hydrolysis product 2-chloro-4-nitroaniline. A method was needed to analyze for residues of intact NIC that was specific enough to separate NIC from any possible NIC metabolites or TFM. The purpose of this study was to develop an analytical method for the determination of NIC in tissues of fish that were exposed to a typical lampricide treatment. The results will help support reregistration of NIC by the U.S. Environmental Protection Agency (EPA).

MATERIALS AND METHODS

Analytical Standards. NIC (99%) was purchased from Sigma Chemical Co. (St. Louis, MO). The sulfate ester of NIC (99+%) was synthesized by and obtained from Derse and Schroeder Associates, Ltd. (Madison, WI). The glucuronide conjugate of NIC, reference material, was isolated from bile extracted from the gall bladder of a rainbow trout that was exposed to [^{14}C]NIC at a rate of 0.045 mg/L for 24 h. The identification of the major component of the bile as the

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10.1021/jf990695r
Published on Web 05/19/2000

glucuronide conjugate of NIC was achieved using the β-glucuronidase hydrolysis procedure described by Lech (1973). TFM (99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Reagents and Chemicals. ACS grade sodium acetate trihydrate was purchased from J. T. Baker (Phillipsburg, NJ). Dibasic sodium phosphate and potassium chloride were purchased from Sigma Chemical Co. Boric acid, glacial acetic acid, 0.1 M sodium hydroxide, and HPLC grade acetone and methanol were purchased from Fisher Scientific (Pittsburgh, PA). HPLC grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI). HPLC grade water was generated by a Barnstead E-Pure water purifier (Dubuque, IA).

Buffers and Standards. *(1) 58 mM Sodium Acetate Buffer (pH 3.8).* Approximately 0.782 g of sodium acetate trihydrate was added to a 1 L volumetric flask, and 500 mL of HPLC grade water was added. Then, 3 mL of glacial acetic acid was added to the flask, the flask was filled to volume with water, and the contents were mixed.

(2) 0.01 M Potassium Chloride (KCl)−Boric Acid (H_3BO_3) Buffer (pH 8.4). Approximately 0.373 g of KCl and 0.309 g of boric acid were placed in a 100 mL volumetric flask, and 50 mL of HPLC grade water was added. Then, 8.6 mL of 0.1 M NaOH was added to the flask, the flask was filled to volume with water, and the contents were mixed.

(3) NIC Stock Standard (100 μg/mL). A 10 mg sample of NIC was dissolved in methanol and made up to 100 mL. This resulted in a 100 μg/mL stock solution from which appropriate dilution standards were made in methanol for the fortification of fish tissue and for the generation of HPLC calibration curves for NIC quantitation. The stock solution was stable at 4 °C for several months.

Apparatus. HPLC analyses were performed with a Waters model 2010 HPLC system (Milford, MA) coupled with a Waters 996 photodiode array detector set at 200−400 nm. Wavelengths for the analysis of rainbow trout and catfish were 360 and 335 nm, respectively. The HPLC system was controlled by Waters Millennium software. Mobile phase was pumped at a flow rate of 1.0 mL/min. A Sys-tec column heater (Minneapolis, MN) set at 40 °C was used to maintain constant temperature on a Phenomenex Prodigy ODS (3) reverse-phase column (Torrence, CA), 150 mm × 4.6 mm, 5 μm, 100 Å, fitted with a YMC ODS AQ guard column (Wilmington, NC), 4 × 23 mm, S5, 120 Å. A Beckman model 5801 liquid scintillation counter was used (Fullerton, CA). A Parkard model 307 biological sample oxidizer was used (Meriden, CT).

Fish Tissue Preparation. Control fish fillets of rainbow trout (skin on) and channel catfish (skinless) were frozen and then homogenized with dry ice (Benville and Tindle, 1970) and stored in plastic containers with a loose screw cap in a freezer at or below −20 °C. After the dry ice sublimed, sample caps were tightened and samples were replaced in the freezer until method validation. Control rainbow trout were provided by the Upper Midwest Environmental Sciences Center fish culturist, and the catfish were obtained from Osage Catfish Farms (Osage, MO).

Control tissue was partially thawed and weighed (5 g) into Corex 25-mL centrifuge tubes. The rainbow trout samples ($n = 7$ and one control) were fortified with 1 mL of a 200 μg/L NIC standard, and the catfish samples ($n = 7$ and one control) were fortified with 1 mL of a 100 μg/L NIC standard using a volumetric pipet. The fortified tissue was allowed to come in contact with the chemical solution for ∼15 min before extraction. A control sample was run with each set of samples.

Incurred Radiolabeled Residues. Catfish were exposed by immersion in a water bath containing 30 μg/L [^{14}C]NIC for 24 h. Tissue samples were extracted as described under Extraction and Cleanup. The final 5-mL concentrated methanol samples were subsampled in triplicate: 1-mL subsample for the control and 0.1-mL subsample for the incurred radiolabeled tissue extracts. The subsamples were placed into 7-mL scintillation vials with liquid scintillation cocktail and analyzed on a liquid scintillation counter. To determine the actual concentration of ^{14}C residues in the incurred catfish tissue, 0.5 g of the tissue was weighed into an oxidizer cone, oxidized, and analyzed on a liquid scintillation counter.

Storage Stability. To simulate the intended application of the method, storage stability was initiated in the field and the samples were maintained and analyzed in the laboratory. Fresh fish samples of both rainbow trout and catfish were fortified with an appropriate amount of NIC standard to achieve a final concentration of 0.5 μg/g. The amount of fortification standard applied depended on the weight of the fish sample and the concentration of the fortification standard. For fortification, fillet samples were cut in half sagittally and the spike was applied to one of the halves. Approximately 10 min was allowed for the solvent to evaporate, and then each fillet half was folded together, wrapped in aluminum foil, bagged in plastic, and frozen. Upon arrival at the laboratory, the fish samples were unwrapped, homogenized, and returned to a freezer set at or below −20 °C. Frozen homogenized tissue for storage stability studies was partially thawed and weighed (5 g) into centrifuge tubes and extracted. The samples were analyzed at time periods of 5 and 7.5 months after initial fortification. These time periods were chosen to bracket the anticipated time of fish collection, sample storage, and tissue analysis projected for a normal lampricide field treatment.

Extraction and Cleanup. Homogenized fish tissue (5 g) was weighed into a centrifuge tube. If necessary, the tissue was fortified as described above. Fifteen milliliters of acetone was added to the tissue and mixed on a wrist-action shaker for 10 min. The tube was centrifuged at 8500 rcf for 5 min at 20 °C, and the supernatant was transferred to a 200-mL pear-shaped rotovap flask using a Pasteur pipet. The acetone extraction procedure described above was repeated three times, and the consecutive acetone extracts were placed into the same flask using the same pipet. The acetone extract was rotary evaporated in a water bath set at 45 ± 5 °C and the vacuum set at 550 ± 50 mbar to ∼5 mL, and then 5 mL of methanol was added to the flask. A C_{18} Bakerbond SPE column (6 mL, 500 mg) was placed on a J. T. Baker model SPE-24G vacuum manifold and conditioned with 2 column volumes of methanol followed by 2 column volumes of 10:90 KCl−H_3BO_3 buffer/methanol. The concentrated tissue extract was transferred with a Pasteur pipet to the conditioned column and processed at a rate of ≤5 mL/min. The rotovap flasks were rinsed with four 2-mL aliquots of 10:90 KCl−H_3BO_3 buffer/methanol and processed through the column, and then the column was rinsed with five 1-mL aliquots of the same solution. The column eluant and rinses were collected in a 25-mL Erlenmeyer flask and then evaporated to ∼3 mL with a nitrogen evaporator. The flask was removed from the evaporator, and 3 mL of water was added to the sample. The concentrated solution containing analyte was transferred to a second preconditioned (2 column volumes of methanol followed by 2 column volumes of 10:90 methanol/acetate buffer) SPE column, processed and rinsed the same as described above for the first SPE cleanup step except the 10:90 methanol/acetate buffer solution was used for rinses. The column was dried for 5 min with air, and the waste from the cleanup was discarded. The analytes were eluted from the column with five 1-mL aliquots of methanol and collected into a 5-mL volumetric flask. The volumetric flask was brought to volume with methanol. The cleaned up extract was transferred with a Pasteur pipet directly into a HPLC sample vial for analysis.

HPLC Analysis. A mobile phase gradient of 58 mM sodium acetate buffer and acetonitrile was generated from initial conditions of 70:30 acetate buffer/acetonitrile to 0:100 acetate buffer/acetonitrile (Table 1). Injection volumes of 100 and 50 μL of the rainbow trout and catfish prepared extracts, respectively, were analyzed on the HPLC system. A three-point standard curve ($r^2 \geq 0.9999$) was used to quantify the NIC concentration. NIC concentrations of samples were calculated on the basis of comparison of peak area responses with those of standards. Tissue concentrations of NIC were reported as micrograms per gram of NIC base.

Method Detection Limit, Accuracy, and Precision. The method detection limit for NIC in fillet tissue was determined using the seven-replicate method (*Federal Register*, 1984), and

Table 1. Mobile Phase Gradient for HPLC Analysis of NIC Residues in Fish Fillet Tissues

time (min)	acetonitrile (%)	58 mM acetate buffer (pH 3.8) (%)
0	30	70
5	35	65
10	50	50
20	60	40
25	80	20
30	100	0
33	30	70
38	30	70

these data were also used to calculate the precision and accuracy of the extraction method. Accuracy was reported as the percent recovery of spiked NIC extracted from the fillet tissue. Precision was reported as the relative standard deviation.

Statistical Analysis. Statistical analyses include descriptive statistics involving simple expressions of mean and standard deviation as well as comparisons using ANOVA of storage stability recoveries of NIC in fish tissue (SAS Institute, 1987).

RESULTS AND DISCUSSION

Analytical Method. In previous laboratory exposures of rainbow trout to higher concentrations of NIC (0.055 mg/L), glucuronide conjugate and sulfate ester metabolites of NIC were observed (Dawson et al., 1999). Due to the possible presence of these metabolites and TFM, the method needed to be refined to separate all of these potential analytes and unambiguously identify NIC. The elution times for NIC, potential metabolites of NIC, and TFM with the presented method are NIC = 25.9 min, glucuronide conjugate of NIC = 17.4 min, sulfate ester of NIC = 18.6 min, and TFM = 15.0 min. The method was established to identify and quantitate for all possible analytes in the fish fillet tissue; however, in previous radiolabeled studies, NIC, the parent compound, was found to be the most prominent chemical residue in the fillet tissue (unpublished data).

There was a difference in the interferences in chromatograms of extracts from rainbow trout and channel catfish tissue. The rainbow trout samples contained extraneous peaks of endogenous materials that were extracted from the tissue. Despite a variety of cleanup attempts, we were unable to resolve the chromatogram to baseline in the specific areas of interest. We therefore changed the wavelength from the optimum of 335 nm to 360 nm. The unknown tissue peaks in the rainbow trout extracts were possibly attributed to samples being analyzed with their skin on. Fish were analyzed with skin on or skinless depending on the normal consumption pattern of these fish by humans. Another possible explanation for the unknown peaks is that rainbow trout fillet tissue contains more fat (Nichols et al., 1990) than the catfish tissue (Nichols et al., 1993) and more lipids were extracted during sample preparation accounting for the presence of the extra peaks.

Recovery of NIC from Rainbow Trout and Catfish Tissue. Using the method described above, analysis of rainbow trout tissue fortified at 0.04 μg/g and channel catfish tissue at 0.02 μg/g resulted in recoveries of 77 ± 6.5 and 113 ± 11%, respectively (Table 2; Figures 2 and 3). The precision of the two sets of samples (n = 7) ranged from 8 to 10%. The detection limit for NIC in rainbow trout was 0.0107 μg/g, and that in catfish was 0.0063 μg/g.

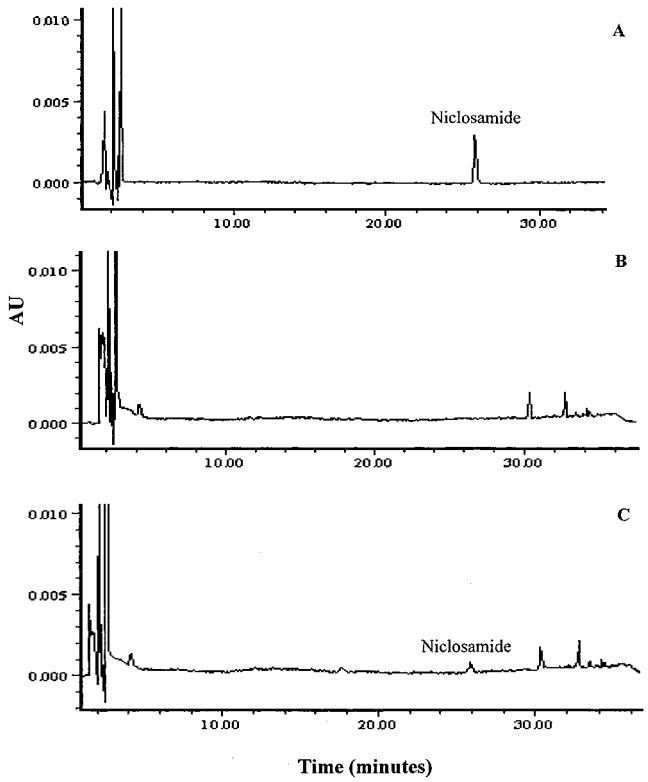

Figure 2. HPLC chromatograms (360 nm) of (A) 200 μg/L NIC standard, (B) extract of rainbow trout control tissue, and (C) extract of rainbow trout control tissue fortified at 0.04 μg/g NIC.

Table 2. Validation of Extraction Method for Analysis of NIC in Fortified Rainbow Trout Tissue (0.04 μg/g) and Catfish Tissue (0.02 μg/g)

fish species	actual NIC concn (μg/g)	% recovery	mean % recovery	standard deviation
rainbow trout	0.0308	77	77	6.5
	0.0355	89		
	0.0327	82		
	0.0295	74		
	0.0275	69		
	0.0306	77		
	0.0297	74		
catfish	0.0255	128	113	11
	0.0208	104		
	0.0253	127		
	0.0200	100		
	0.0230	115		
	0.0215	108		
	0.0216	108		

Luhning et al. (1979) reported that their gas chromatograph method was sensitive to 0.01 μg of Bayer 73/g of fish. The sensitivities of the two methods are comparable. The proposed HPLC method is much less labor intensive and allows for direct analysis of NIC. The previous GC method has been shown to be effective on many matrices such as fish tissue, aquatic invertebrates, mud, and water, whereas the proposed HPLC method has been proven to be effective only on rainbow trout and catfish fillet tissue.

Radiovalidation of NIC in Incurred Catfish Tissue. The method was also validated using incurred radiolabeled residues in catfish tissue. Mean percent recovery was 89.3 ± 4.1% for catfish tissue incurred with 30 μg/L of NIC. The radiovalidation of this method provided evidence that the procedure produced acceptable recoveries and was reproducible. Radiovalidation

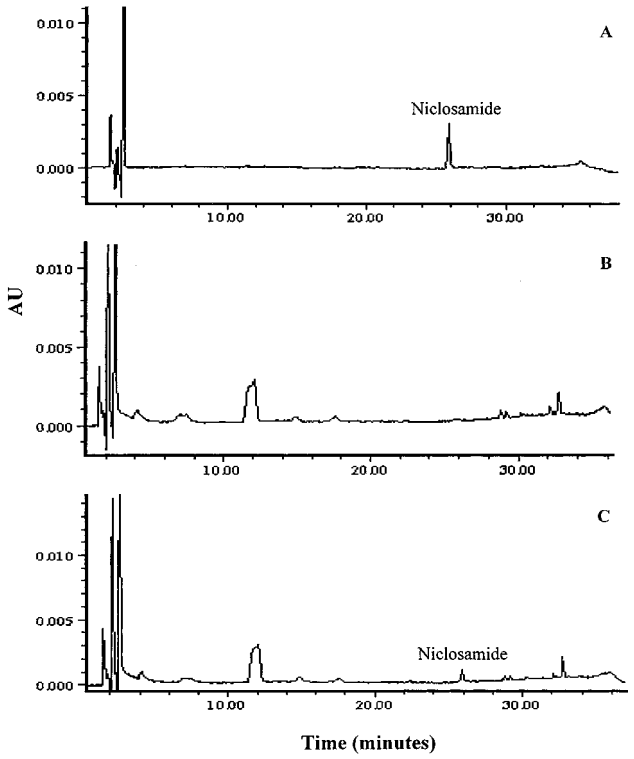

Figure 3. HPLC chromatograms (335 nm) of (A) 200 μg/L NIC standard, (B) extract of channel catfish control tissue, and (C) extract of channel catfish control tissue fortified at 0.02 μg/g NIC.

was not performed in the rainbow trout fillet tissue because incurred radiolabeled tissue was not available.

Storage Stability of NIC in Fish Tissue. One subsample of each homogenate of fortified fish tissue ($n = 3$) was processed and analyzed twice for NIC concentration at 5 months and 7.5 months after fortification. Recoveries were 78 ± 5.1% at 5 months and 68 ± 2.4% at 7.5 months for rainbow trout tissue fortified with 0.5 μg/g of NIC. Recoveries of NIC from channel catfish fillet tissue fortified with 0.5 μg/g were 88 ± 13% at 5 months and 76 ± 21% at 7.5 months. Although recoveries tended to be lower among the fortified samples processed at the end of the study compared with those processed at the beginning, the differences were not statistically significant ($p > 0.05$).

Conclusion. The method was developed for the analysis of NIC in fish fillet tissue. The method generated reproducible results. Acceptable percent recoveries were achieved with distinct separation between integrated peaks. This method may be used for monitoring NIC residues in rainbow trout and channel catfish tissues that have been exposed to NIC lampricide treatments or to obtain additional residue chemistry data required for reregistration of NIC by the EPA.

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Received for review June 25, 1999. Revised manuscript received January 20, 2000. Accepted February 7, 2000. We express our gratitude to the Great Lakes Fisheries Commission for providing financial support for this research project.

JF990695R