A COMPARISON OF TWO METHODS FOR THE SIMULTANEOUS DETERMINATION OF TFM AND BAYER 73 CONCENTRATIONS

ELIMINATION OF ^{14}C-BISAZIR RESIDUES IN ADULT SEA LAMPREY (*PETROMYZON MARINUS*)

Great Lakes Fishery Commission

TECHNICAL REPORT No. 50

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by

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by

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ABSTRACT

A new direct-injection, high-performance liquid Chromatograph (HPLC) method was developed for the simultaneous analysis of 3-trifluoromethyl-4-nitrophenol (TFM) and 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73). A comparison of accuracy and reproducibility was made with an existing HPLC method that requires extraction and concentration steps and the use of an internal standard. Six stream water samples that varied in alkalinity, hardness, and pH were spiked with TFM and Bayer 73 and analyzed by the two procedures. The direct-injection was as accurate as the existing method and resulted in savings of cost and time.

INTRODUCTION

The success of the sea lamprey *(Petromyzon marinus)* control program in the Great Lakes has resulted primarily from the use of the selective toxicant 3-trifluoromethyl-4-nitrophenol (TFM) to kill lamprey larvae in the streams. Applegate et al. (1958) reported that some halogenated nitrophenols were selectively toxic to sea lamprey larvae and TFM was subsequently developed for field use (Applegate et al. 1961). Further research revealed that the addition of small amounts (less than 3% by weight) of 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73) to TFM increased the toxicity without significantly affecting the selectivity (Howell et al. 1964). Since 1963, mixtures of TFM and Bayer 73 have been used to control sea lamprey in the U.S. and Canada (Hamilton 1974), and the use of the two chemicals is expected to continue.

Chemical treatments for sea lamprey control are made with a liquid formulation of TFM, either alone or in combination with a slurry of 70% wettable Bayer 73. There is a range of lampricide concentrations within which sea lamprey larvae are killed without harm to most other forms of aquatic life; however, if the concentration of the lampricide in the stream water rises above the upper limit of that range, it may become toxic to certain other aquatic animals. Consequently, specific concentrations of TFM and Bayer 73 must be maintained during application of the lampricide. Effective monitoring of

treatment concentrations of the lampricide requires an analytical procedure that is simple, fast, accurate, and free of interferences.

TFM has traditionally been quantified in stream water by a calorimetric method based on the yellow color of the TFM molecule under alkaline (buffered) conditions (Smith et al. 1960). The problem of interfering substances was overcome either by preparing standards in stream water or by making corrections for background color.

Dawson et al. (1978) developed a calorimetric technique that was suitable for analyzing Bayer 73 during stream treatments. Bayer 73 was hydrolyzed to 2-chloro-4-nitroaniline (CNA) and then diazotized for calorimetric analyses, but natural color in the water apparently caused some interference. Luhning et al. (1979) developed a method of measurement in which Bayer 73 was quantitatively hydrolyzed with alkali to CNA, partitioned into a solvent, and analyzed by gas liquid chromatography.

A procedure developed by Dawson (1982), here termed the Dawson method, permitted simultaneous determination of TFM and Bayer 73 by high-performance liquid chromatography (HPLC). This method was based on the extraction, liquid chromatographic separation, and quantification of TFM and Bayer 73 through the use of UV detection. Because the analysis was time-consuming, required a number of sample preparation steps, and was costly, a simplified analytical method was considered desirable. In this report, a modification of the Dawson method is described wherein the buffer, the extraction procedure, and the internal standard are eliminated and the volume of the HPLC injection sample is increased. The modified method enabled the injection of a filtered water sample directly into the HPLC.

The objective of this study was to determine if the modified direct-injection method was as accurate and free of naturally occurring interferences as the Dawson method for the simultaneous determination of TFM and Bayer 73 concentrations.

MATERIALS AND METHODS

The simplified direct-injection procedure involves filtering a water sample from the stream and using HPLC with UV detection for analysis. The required equipment, reagents, and stepwise procedure follow:

APPARATUS

1. HPLC, Waters Associates, [1] with Model 440 Detector and optional Data Module. Operating conditions: stationary phase, 3.9 mm x 30 cm, Waters Associates μ Bondapak C-18 reverse phase column; mobile phase, methanol, 9.6 x 10^{-3} mol/L acetate buffer (78:22, v:v); flow rate, 2ml/min; chart speed 0.5 cm/min; wavelength, 313 nm.

1. Reference to trade name does not imply Government endorsement of commercial products

TABLE I Sample data and pH, alkalinity, and hardness for six Lake Huron tributaries.

River sampled	Date collected	PH	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)
Black Mallard	6 Mar 85	7.7	94	104
Black Mallard	6 Feb 85	7.3	92	106
Nagle Creek	18 Dec 84	7.1	66	78
Schmidt Creek	4 Dec 84	8.0	129	138
Mulligan Creek	16 Nov 84	-	61	80
Black Mallard	7 Nov 84	8.1	176	184
Trout River	27 Aug 84	7.9	200	206
Ocqueoc River	20 Aug 84	8.2	136	153
Schmidt Creek	7 Aug 84	-	-	

2. Filter apparatus, General Electric vacuum pump with a stainless steel funnel having a flat, fritted stainless steel base and Reeve Angle 934AH fiberglass filter
3. Eppendorf pipettes
4. Test tubes with screw caps
5. Texas Instruments TI-55 calculator

REAGENTS

1. Methanol, HPLC grade from J. T. Baker, Inc.
2. Water, Type III reagent grade
3. TFM, from Hoechst Aktiengesellschaft Germany, 35.7% active
4. Bayer 73, technical grade from Chemagro Corp., 70% active
5. Acetic acid (glacial), analytical reagent from J. T. Baker, Inc.
6. Sodium acetate, anhydrous analytical reagent from Mallinckrodt
7. Acetate buffer, Stock A (0.11 mol/L): 5-ml glacial acetic acid and 1.552 g sodium acetate diluted to 1 L with reagent water. Stock B (9.6×10^{-3} mol/L) for mobile phase: 100 ml stock A + 1000 ml reagent water.
8. N,N-Dimethylformamide (DMF), HPLC grade from EM Science, a division of EM Industries, Cherry Hill, NJ

PROCEDURE

Water samples that varied in hardness, alkalinity, pH, and color, collected from six tributaries to Lake Huron (Table 1) were used to determine if different water quality characteristics affected the direct-injection method. Standards were prepared by pipetting appropriate aliquots of TFM stock solution (20 g active/L reagent water) and Bayer 73 stock solution (30 mg active/L DMF) into a volumetric flask and diluting to volume with either reagent water or stream water that had been filtered. Linear regressions were calculated to determine the best

TABLE 2. Recovery of TFM and Bayer 73 from spiked stream water samples.

River sampled	Date analyzed	TFM spike conc. (mg/L)	TFM: Direct-inlection method: R. Std.[a] mg/L	%	TFM: Direct-inlection method: Stream water mg/L	%	TFM: Dawson method: R. Std. mg/L	%	TFM: Dawson method: Stream water mg/L	%	Bayer 73 spike conc. (µg/L)	Bayer 73: Direct injection method: R Std. µg/L	%	Bayer 73: Direct injection method: Stream water µg/L	%	Bayer 73: Dawson method: R. Std. µg/L	%	Bayer 73: Dawson method: stream water µg/L	%
Bl. Mallard R.	7 Mar 85	7.5	7.48	99.7	7 58	101.1	7.22	96.3	7.33	97 7	30	*	*	31.7	105.7	33.4	III 3	30.9	103.0
Bl. Mallard R	20 Feb 85	7 .5	7.28	97.1	7.41	98.8	7.50	100.0	-	-	30	30.8	102.7	29.2	97.3	31.5	105.0	-	-
Nagle Cr.	19 Dec 84	7.5	7.28	97.1	7.29	97.2	6.74	89.9	6 35	84.7	30	29.8	99.3	27.5	91.7	36. I	120.3	23.X	79.3
Schmidt Cr.	6 Dec 84	-	-	-	-	-	-	-	-	-	30	34.0	113.3	32.3	107.7	29.4	98.0	26.1	87.0
Mulligan Cr.	19 Nov 84	7.5	7.33	97.7	7.38	98.4	8.19	109.2	7.35	98.0	30	31.5	105.0	31.5	105.0	34.6	115.3	30.8	102.7
Bl. Mallard R.	8 Nov 84	7.5	7.05	94.0	7.50	100.0	7.02	93.6	7.55	100.7	-								
Trout R.	28 Aug X4	8.0	6.82	90.9	8 08	108.7	8.23	109.7	8.01	106.8	36	37.0	102.X	3X.2	106.1	41.2	114.4	3X.6	107.2
Ocqueoc R.	24 Aug 84	4.0	3.24	81.0	4.07	101.7	-	-	3 66	91.5	-	-	-	-	-	-	-	-	-
Schmidt Cr.	Y Aug 84	15.0	13.87	92.5	14.25	95.0	13.92	92.8	14.37	95.8	75	x2 3	109.7	70.3	93.7	74.9	99.9	67.9	90.5
X				93.7		100.1		98.8		96.5			105.5		101.0		109.2		95.0
SE				2.1		1.4		3 0		2.6			2.1		2.5		3.2		4.5

[a]Reagent grade water standard

- Samples not tested

* Contaminated sample

TABLE 3. Recovery of concentrations of TFM (5.0 mg/L) and Bayer 73 (30 μg/L) spiked in reagent and stream water samples as determined by direct-injection method for the HPLC.

Test no.	TFM				Bayer 73			
	Reagent Std.		Stream H_2O		Reagent Std.		Stream H_2O	
	mg/L	percent	mg/L	percent	μg/L	percent	μg/L	percent
1	4.59	91.8	4.14	94.8	27.1	90.3	26.7	89.0
2	4.51	90.2	4.66	93.2	28.2	94.0	27.9	93.0
3	4.94	98.8	5.09	101.8	28.2	94.0	27.9	93.0
4	4.85	97.0	5.00	100.0	28.5	95.0	28.2	94.0
5	4.94	98.8	5.09	101.8	28.6	95.3	28.4	94.7
%	4.17	95.3	4.92	98.3	28.1	93.7	27.8	*92.1*
SE	0.0906	1.81	0.0906	1.81	0.267	0.891	0.296	0.986

fit line for a standard curve of peak area vs. concentration. Typically, the data points on the curves as compared with those determined imperically show correlation coefficients of 0.999 and 0.997 for TFM and Bayer 73, respectively.

Fresh, unfiltered stream water samples were spiked with TFM and Bayer 73, thoroughly mixed, and filtered. Vacuum filtration was used to remove any suspended matter that may have interfered with the HPLC's operation.

The spiked stream water samples were analyzed by HPLC and compared with procedural standards by using 500 μl injections. The data module was programmed to calculate peak areas. Peak areas and standard curves were used to determine the lampricide concentrations in the spiked samples and to calculate percent recovery.

To compare the Dawson method with the direct-injection method, the percent TFM and percent Bayer 73 recovered from each stream water sample were determined; the mean percent recovered and standard error (SE) were then calculated for both TFM and Bayer 73. To determine the precision of the direct-injection method, five samples of water from the Black Mallard River, Michigan, were spiked with TFM (5.0 mg/L) and Bayer 73 (30 kg/L) and the mean percent recovery and SE for the lampricides were determined.

RESULTS

The average recovery of TFM by the direct-injection method was 100% (range, 95-109%) when stream water standards were used, and 94% (range, 81-100%) when reagent water standards were used (Table 2). By the Dawson method, the recovery was 97% (range, 85-107%) when stream water was used, and 99% (range, 90-110%) when reagent water standards were used.

The recovery of Bayer 73 was more difficult than TFM because the initial concentrations were much lower (Table 2). When the direct-injection method was used with stream water standards, the average recovery was 101% (range, 92-108%); the recovery of Bayer 73 was within 2.5 μg/L of the spike for six of

the tests made and was within 5 μg/L of the spike in the seventh test. When reagent water standards were used, Bayer 73 recoveries averaged 106% (range, 99-113%); the recovery was within 2 μg/L of the spike in four tests; and within 4 and 8 μg/L in the two other tests.

When the Dawson method was used with stream water standards, the recoveries averaged 95% (range, 79-107%); the recovery of Bayer 73 ranged from 7 μg/L less to 3μg/L more than the spike. When reagent water standards were used, the recoveries averaged 109% (range, 98-120%); the recoveries ranged from 1 μg/L less to 6 μg/L more than the spike.

Five replicate samples of Black Mallard River water spiked with 5.0 mg/L TFM and 30 μg/L Bayer 73 were analyzed to evaluate the reproducibility of the direct-injection method (Table 3). The respective average percentages of TFM and Bayer 73 recovered were 98% (SE 1.8) and 93% (SE 0.99), when stream water standards were used, and 95% (SE 1.8) and 94% (SE 0.89), when reagent water standards were used.

DISCUSSION

Water quality factors may affect the accuracy of all TFM and Bayer 73 analyses. Natural color has been reported as an interference for calorimetric determinations of TFM (Smith et al. 1960) and Bayer 73 (Dawson 1978). Chromatographic analyses eliminate this interference because the retention times of natural color products differ from those of TFM or Bayer 73. Dawson (1982) also reported poor recoveries of TFM from some unbuffered water samples. He theorized that the TFM was ionized at alkaline pH's and not completely removed from the water sample by the Sep Pak cartridge.

In general, lampricide recovery rates with the direct-injection method, were slightly better with the stream water standards than with the reagent water standards. Stream water standards produced slightly more accurate results than reagent water standards, but the precision was nearly identical.

Typical liquid chromatograms of Lake Huron water samples spiked with TFM (1.0 mg/L) and Bayer 73 (0.1 mg/L) are shown in Fig. 1. The retention times of TFM and Bayer 73 for the Dawson and the direct-injection methods were similar; also the peak shapes were unaffected.

The direct-injection method is much simpler than the Dawson method, requiring only that the samples be filtered before quantification by HPLC. The direct-injection method produced results that were as accurate as those produced by the Dawson method (1982) and was faster, more economical, and free from interferences.

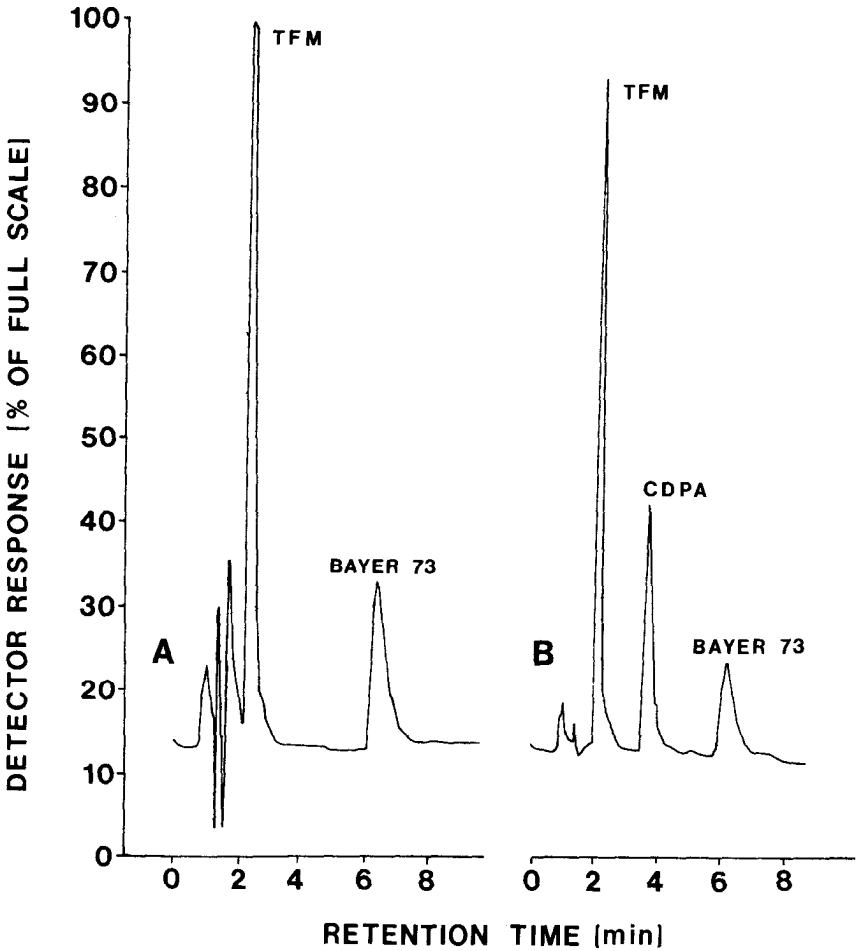

FIG. 1. Liquid chromatography of Lake Huron water sample spiked with TFM (1.0 mg/L) and Bayer 73 (0.1 mg/L). (A) Direct injection method had retention times of 2.47 for TFM and 6.65 for Bayer 73. (B) The Dawson method had retention times of 2.07 for TFM, 3.67 for CDPA-internal standard, and 6.20 for Bayer 73.

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ELIMINATION OF ^{14}C-BISAZIR RESIDUES IN ADULT SEA LAMPREY *(PETROMYZON MARINUS)* [1]

by

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ABSTRACT

Bisazir (P,P-bis[l-aziridinyl]-N-methylphosphinothioic amide), a chemosterilant, was administered to sea lampreys *(Petromyzon marinus)* by intraperitoneal injection of 100 mg/kg or by immersion for 2 h in a 100-mg/L aqueous solution of the chemical. Residues were determined by radiometric analysis of lamprey tissues sampled after 1 to 10 days of withdrawal. Whole body analysis of the injected lampreys showed that total residue concentrations (μg/g as bisazir equivalents) decreased to 4.65 in males and 10.07 in females during the first day after injection, and to 1.46 in males and 3.74 in females after 10 days of withdrawal. Lampreys exposed by bath immersion contained residues of about 25 μg/g of tissue immediately after exposure. The concentration (μg/g) decreased to 1.02 in males and 2.11 in females after 1 day of withdrawal and to 0.51 in males and 0.85 in females after 10 days. Radiometric analysis showed that residues did not concentrate in any of the organs. Concentrations of radioactivity in the blood of immersed lampreys decreased rapidly from more than 20 μg/g immediately after exposure to less than 1 μg/g after 1 day of withdrawal, indicating that residues of the chemical were rapidly cleared from circulation. Residues in the blood decreased more slowly in injected than in immersed lampreys. Unidentified radioactive residues that remained after 48 h withdrawal were tissue-bound and evenly distributed in the subcellular fractions of the liver. Distribution of ^{14}C-bisazir residues was nearly uniform throughout all the subcellular fractions in the liver of a single lamprey treated by bath exposure and withdrawn from the chemical for 48 h.

INTRODUCTION

The technique of releasing sterilized male screw-worm flies *(Cochliomyia hominivorax)* has been used successfully for the control of this pest in the United States (Knipling 1960). Hanson (1970) proposed use of this technique as an

1. This study was part of a program conducted by the U.S. Fish and Wildlife Service under contract with the Great Lakes Fishery Commission. Mention of a chemosterilant or other products does not constitute recommendation or endorsement by the U.S. Government.

adjunct to lampricide applications for control of the sea lamprey (Petromyzon *marinus)* in the Great Lakes.

The experimental compound bisazir (P,P-bis[l-aziridinyl]-N-methyl-phosphinothioic amide) effectively sterilizes spawning-run sea lampreys without reducing sex drive or mating instinct (Hanson and Manion 1978, 1980). It sterilizes both males and females when administered by intraperitoneal (i.p.) injection. Hanson (1981) showed that spawning-run male sea lampreys can also be effectively sterilized by bath immersion in 100 mg/L of bisazir for 2 h.

Bisazir is highly active and nonspecific in activity; its chemosterilant characteristics have also been demonstrated in several species of insects. The compound is an alkylating agent and is believed to pose potential human health hazards (Borkovec 1972). Therefore, the fate of bisazir in sea lampreys must be determined as part of an evaluation of the potential hazards associated with the release of treated lampreys.

This study was undertaken to determine the rate of elimination of residues of bisazir by adult male and female sea lampreys after bath exposure (Hanson 1981) or i.p. injection (Hanson and Manion 1978, 1980). A holding time of 48 h before release back into the environment will allow the lamprey to recover from the acute effects of bisazir and eliminate most of the residues of the chemical. Therefore, we determined the chemical characteristics of residues of bisazir remaining in lampreys 48 h after treatment with the chemosterilant. The chemical characteristics of residues of bisazir that remained in lampreys 48 h after treatment were described.

MATERIALS AND METHODS

Bisazir (aziridinyl-ring labeled-^{14}C), with a specific activity of 6.52 mCi/mM, was obtained from Pathfinder Laboratories, Inc., St. Louis, Missouri (Fig. 1). Analysis by high performance liquid chromatography showed a radiochemical purity of 98%. Unlabeled bisazir was obtained from the Beltsville Agricultural Research Center, Beltsville, Maryland (U.S. Department of Agriculture code number A 13-6 1585).

Spawning-phase sea lampreys were trapped in the Ocqueoc and Cheboygan rivers (tributaries of Lake Huron), transferred to the National Fisheries Research Center, La Crosse, Wisconsin, and were held in flowing well water for 48 h before treatment. Fifty animals of each sex were exposed to a bath solution of the lowest concentration (100 mg/L bisazir in well water for 2 h) shown to be efficacious by Hanson (1981), and 50 of each sex were injected with the lowest dose (100 mg/kg of bisazir in saline solution) shown to be efficacious by Hanson and Manion (1978, 1980). An isotope dilution of 1 part ^{14}C-bisazir + 9 parts unlabeled bisazir was used in both exposure procedures. Immediately after treatment, the lampreys were transferred to fresh, flowing well water. Temperature was maintained at 12 ± 1°C in all holding units.

Sea lampreys exposed in the bath solution or by injection were sampled immediately and at 1, **2, 3, 4, 7,** and 10 days after treatment. At each sampling interval, five lampreys were taken for whole body analysis, and one was

TABLE I. Mean whole body residues (μg/g) of bisazir equivalents in sea lampreys exposed by bath in 100 mg/L for 2 h or injected i.p. with 100 mg/kg of 'Y-bisazir and placed in fresh, flowing well water for withdrawal."

Withdrawal time (days)	Treatment and sex			
	Bath		Injected	
	Male	Female	Male	Female
0	25.82 (1.31)	25.04 (0.795)	—	95.79 (0.176)
1	1.02 (0.066)	2.11 (0.133)	4.65 (0.713)	10.70 (0.997)
2	0.703 (0.075)	1.20 (0.076)	2.71 (0.175)	6.04 (0.252)
3	0.725 (0.066)	1.11 (0.098)	3.03 (0.516)	4.18 (0.174)
4	0.640 (0.047)	0.957 (0.093)	2.02 (0.138)	3.93 (0.389)
7	0.596 (0.032)	0.909 (0.056)	1.92 (0.265)	3.74 (0.762)
10	0.512 (0.035)	0.853 (0.067)	1.46 (0.077)	3.47 (0.427)

[a]Each entry is the mean of five lampreys (± SE in parentheses).

dissected to determine the distribution of bisazir in the blood, brain, gills, gonads, gut, heart, kidney, liver, and muscle.

Whole lampreys and samples of tissues from individual organs were homogenized by blending them with dry ice in a combination grinder and blender equipped with a stainless steel cup (Benville and Tindle 1970). Subsamples were processed in a Packard model B306 sample oxidizer (Macek et al. 1975) and radioactivity was determined with a scintillation counter. Residues were reported as equivalents of bisazir per gram of wet tissue, since radioactivity in the samples may have resulted from the presence of labeled degradation products, metabolites, or parent bisazir.

To characterize bisazir residues, we extracted homogenates of whole lampreys, collected 2 days after treatment, three times with three solvents-hexane, ethyl ether, and methanol (in that sequence). Sample extracts were concentrated by vacuum rotary evaporation. Radioactivity in the sample extracts was determined by liquid scintillation counting.

Extracts were analyzed by gas chromatography (GC), thin layer chromatography (TLC), and gas chromatography/mass spectroscopy (GUMS). In the analysis by GC, we used a Tracer 220 gas Chromatograph with a 1.8 m x 5 mm glass column packed with 5% OV-7 on 80/100 mesh chromasorb WHP. The column temperature was programmed from 80 to 190°C over 30 min and bisazir residues were identified with a phosphorus selective flame photometric detector.

TABLE 2. Distribution of residues (μg/g) of bisazir equivalents in tissues of sea lampreys exposed by bath to 100 mg/L of ^{14}C-bisazir for 2 h or injected i.p. with 100 mg/kg of ^{14}C-bisazir, and placed in fresh, flowing well water for withdrawal. One lamprey was dissected at each withdrawal time.

Treatment, sex, and withdrawal time (days)	Tissue								
	Blood	Brain	Gills	Gonad	Gut	Heart	Kidney	Liver	Muscle
Bath-exposed									
Male									
0	28.7	87.1	31.0	31.0	35.1	30.6	31.5	33.8	25.4
1	0.631	0.746	1.34	1.01	1.37	0.498	2.29	1.46	0.834
2	0.123	0.908	0.545	1.62	0.509	0.193	2.01	0.983	0.673
3	0.089	0.207	0.495	0.246	0.388	0.183	2.43	2.62	0.484
4	0.092	0.538	0.810	1.25	0.686	0.960	3.17	1.12	0.819
7	0.027	0.233	0.352	0.493	0.431	0.116	0.938	1.03	0.187
Female									
0	22.8	46.6	31.7	10.5	25.1	—[a]	29.6	5.57	21.4
1	0.945	3.26	2.54	4.09	2.63	1.22	5.41	4.42	1.59
2	0.201	1.48	1.59	1.77	1.45	0.418	3.88	2.10	0.823
3	0.136	1.23	1.45	1.49	0.964	0.309	4.27	3.38	0.465
4	0.124	1.31	1.15	1.45	1.35	0.326	3.87	3.41	0.691
7	0.057	0.577	1.46	1.33	0.551	0.290	3.66	2.71	0.317
10	0.084	0.542	0.962	1.41	0.653	0.344	2.19	3.72	0.326
Injected									
Male									
1	5.65	3.02	3.12	9.16	12.3	3.33	12.3	8.74	2.29
2	0.997	3.31	1.56	3.80	6.16	1.24	5.45	4.63	1.20
3	0.397	15.1	1.14	3.27	5.73	1.12	5.30	6.52	0.916
4	0.617	3.82	2.39	5.39	11.6	2.41	10.9	9.00	2.99
7	0.487	1.13	2.83	4.14	8.33	2.17	14.4	12.1	1.39
10	0.464	1.67	1.74	4.67	5.46	1.78	12.5	5.25	1.34
Female									
1	6.79	6.94	6.32	21.3	16.0	5.37	15.9	9.98	4.29
2	1.51	3.81	2.79	9.17	15.8	2.33	10.6	8.95	1.64
3	1.37	3.75	3.51	10.3	11.0	2.83	15.4	22.2	1.31
4	0.676	4.45	2.05	7.52	7.30	1.96	8.51	8.49	1.09
7	0.766	1.44	2.47	5.71	6.04	2.16	13.1	8.34	1.14
10	0.332	2.37	2.26	6.54	7.08	2.54	12.1	13.1	1.10

[a]Sample lost

Preparative TLC was performed on silica gel G plates that were developed with 10% methanol in benzene. We sectioned a vertical section of the plate, 1.5 cm wide over the spot of the extracts into l-cm segments, placed the segments into scintillation vials with Beckman Ready Solv HP, and counted them on the liquid scintillation counter to determine the R_f of the radioactive residues.

Additional cleanup for GC/MS analysis was done by gel permeation chromatography (GPC) with an Autoprep Model 1001 having an SX-3 bio bead column and cyclohexane + methylene chloride (1 + 1) mobile phase.

TABLE 3. Subcellular distribution of ^{14}C activity in liver of sea lamprey 48 h after a 2-h treatment with 100 mg/L of bisazir.

Fraction	Centrifugal force X g	Bisazir equivalent (ng/mg) protein
Nuclear	600	15
Mitochondrial	13,300	17
Microsomal	165,000	14
Cytosol	-	20

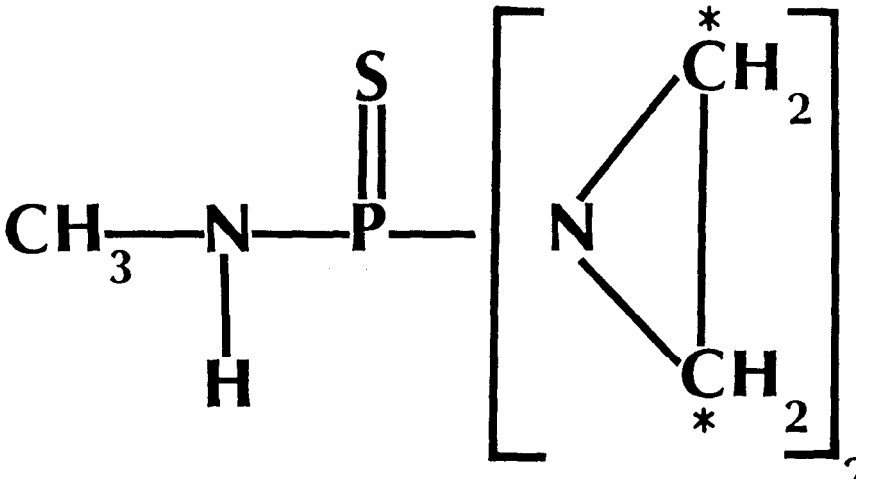

FIG. 1. Chemical structure of (^{14}C)-bisazir (P,P-bis[l-aziridinyl]-N-methyl-phosphinothiotic amide). Asterisk denotes the position of the ^{14}C-label on the aziridinyl rings.

The GC/MS analysis was done at the National Fisheries Contaminant Research Center, Columbia, Missouri, by capillary GC, with positive and negative chemical ionization, and electron impact mass spectroscopy.

The liver of a sea lamprey that had been exposed to 100 mg/L of bisazir for 2 h and then held in flowing water for 48 h was used to study the subcellular distribution of radioactivity in the tissue by the procedures of Gingerich (1986). Subcellular fractionation of the lamprey liver was effected by the method of Statham et al. (1977). Distribution of radioactivity was determined by counting aliquots of each homogenate, and the protein concentration in each fraction was determined by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Whole body radiometric analyses of five female lampreys sacrificed immediately after injection resulted in average recovery of 96% of the injected activity. Whole body residues in males and females exposed in bath solutions of 100 mg/L for 2 h were about 2.5 μg/g immediately after immersion (Table 1). Whole body analyses of lampreys treated with bisazir by either method showed a rapid decrease in bisazir residues within 24 h, but small amounts of radioactive residues remained after 10 days (Table 1). Residues resulting from an injected dose of 100 mg/kg were about four times that in bath-exposed lampreys and remained three to five times higher throughout the withdrawal period. The amount of bisazir taken up during bath immersion was similar in males and females, but the rate of elimination was somewhat slower in females, especially in injected animals.

Nadkarni et al. (1959) noted that the levels of radioactivity after injection of each of three radioactively labeled alkylating drugs into humans followed a common pattern, in that the drug or its radioactive metabolite was rapidly removed from the blood (within 1 h after injection). This rapid decrease in radioactive residues in the blood was followed by a 48-h period of low residue levels. They reported rapid excretion of radioactive residues-but not parent compounds-through the kidneys.

Borkovec et al. (1978) found that boll weevils *(Anthonomus grandis)* exposed to bisazir by fumigation contained from 0.5 to 1.1 μg per weevil. However, no bisazir or its metabolite (P,P-bis[l-aziridinyl]-N-methylphosphinic amide) could be detected in the sterilized weevils 1 day after treatment.

The decrease of ^{14}C in lamprey tissues was similar to that for whole body residues (Table 2). Most residues were eliminated within 1 day, and only a small percentage of the initial radioactivity remained after 10 days (Table 2).

Bisazir was lost most rapidly from the blood. Although residue concentrations were highest in the brain immediately after bath exposure to bisazir, they decreased within 1 day to levels similar to those in the other tissues.

After the rapid loss during the first day of withdrawal, residues in the liver and kidney generally persisted at relatively high concentrations. These organs have been shown to be the major organs of biotransformation and elimination of xenobiotics in fish (Cafruny 1971; Mandel 1971).

Residues in the gonad, gut, kidney, and liver remained at higher concentrations in injected than in bath-exposed lampreys. The high residues in these tissues were probably related to the route of administration; higher quantities of bisazir entered the i.p.-injected lampreys in the vicinity of these organs in a very short time.

Parent bisazir was not detected using GC with a minimum detection of <0.01 μg/g in samples from 24- and 48-h withdrawals. These findings are consistent with the results of other analyses of tissues from sea lampreys treated with bisazir in 1978 and are similar to results obtained on insects that had been treated with relatively higher doses of bisazir (Dr. A. B. Borkovec, pers. Comm.).

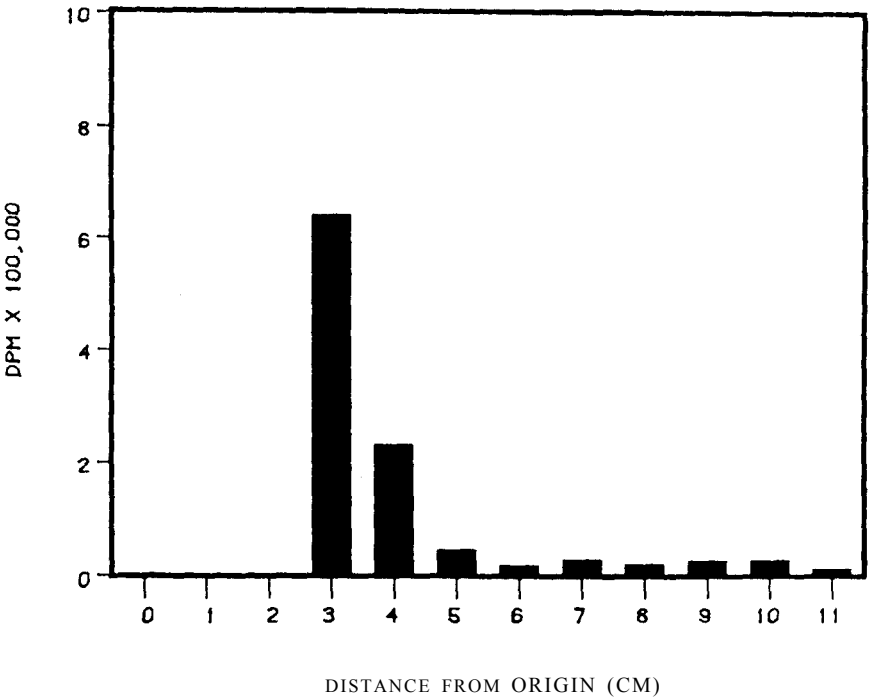

FIG. 2. Radioactivity in disintegrations per minute (DM), in l-cm segments, of a vertical strip of a thin layer chromatogram of an extract of sea lamprey on a silica gel plate developed with 10% methanol in benzene.

Extracts were concentrated under vacuum, taken up in cyclohexane + methylene chloride (1 + 1), and chromatographed by gel permeation chromatography (GPC). A single radioactive band co-eluted with a yellow colored material (apparently lipids). When extracts were cleaned-up by GPC and chromatographed on silica gel thin layer plates, most activity was found at an R_f of approximately 0.3 (Fig. 2). GC/MS analysis of the concentrated extract of this portion of the plate revealed no intact parent compound and any remaining activity was attributed to biogenic material.

Isolation and purification efforts indicated that only a small amount of the radioactivity in lamprey tissues was extractable. Only 15.34% of the total radioactivity was extracted by successive extractions with hexane, ethyl ether, and methanol. Different amounts of radioactive residues. calculated as bisazir equivalents, were extracted by the three solvents during successive extractions: hexane 0.0341 (4.69% of the total), ethyl ether 0.0099 (1.36%), and methanol 0.0651 μg/g (8.96%). Oxidation of tissues that remained after extraction revealed an average of 0.6539 μg/g (89.81% of the total).

The subcellular distribution of radioactive residues in the liver of sea lamprey treated with ^{14}C-bisazir was determined. The liver of a sea lamprey that had been exposed to 100 mg/kg of ^{14}C-bisazir for 2 h by bath immersion and held for 48 h in fresh, flowing water was analyzed. The analysis showed a nearly uniform distribution of the radioactive residues throughout all subcellular fractions (Table 3).

The labile nature of aziridinyl phosphoramides (Stokes et al. 1981) suggests that most of the remaining radioactive residues were probably present in the form of decomposition products, biotransformation products, or both. Since the bisazir used in this study was labeled with carbon 14 in the aziridinyl ring, only the fate of this portion of the molecule could be followed.

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