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Bioaccumulation of Cinmethylin in Bluegill Sunfish

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Technical cinmethylin and Cinch 7EC herbicide showed low toxicity against various aquatic organisms. The bioaccumulation potential of [^{14}C]cinmethylin in the bluegill sunfish under a dynamic flow-through system at a constant concentration of 0.1 mg/L was examined. A rapid uptake of radioactivity was observed. Tissue residues plateaued after 3 days, and the mean tissue residues after 28 days of exposure were 35, 12, and 58 μg/g (ppm) for the whole fish, fillet, and viscera, respectively. The corresponding bioconcentration factors for the above tissues were 360, 120, and 600, respectively. Greater than 99% of the tissue residues was eliminated from the exposed fish after the 14-day depuration period. In addition to [^{14}C]cinmethylin, major metabolites isolated from the treated fish were α-carboxycinmethylin and 8-hydroxy-α-carboxycinmethylin. *o*-Toluic acid and α-hydroxycinmethylin were observed as minor components.

Cinmethylin [7-oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-, *exo*-] (1) is the active ingredient of Cinch 7EC herbicide. The exo diastereomer of this compound exhibited broad-spectrum preemergent herbicidal activity against various grassy weeds in soybeans, peanuts, and cotton (Peterson et al., 1983). Technical cinmethylin and Cinch 7EC herbicide showed low toxicity against various aquatic organisms (Table I). In order to assess its potential impact to the environment, the bioaccumulation potential of cinmethylin in the bluegill sunfish was examined. Major objectives of this report are to describe (1) the accumulation and depuration rates of [^{14}C]cinmethylin residues in the fish and (2) characterization and identification of significant tissue residues.

EXPERIMENTAL SECTION

Test Materials and Reference Standards. Radiolabeled [*phenyl*-^{14}C]cinmethylin and appropriate reference standards were synthesized at Shell's Biological Science Research Center, Modesto, CA (Lee et al., 1986). Chemical structures of these compounds are presented in Figure 1. The specific activity and radiochemical purity of the test material were 56.4 μCi/mg and greater than 98%, respectively.

Test System. The in-life portion of the study was conducted by Analytical Bio-Chemistry Laboratories, Inc. The bioaccumulation of [^{14}C]cinmethylin in the bluegill sunfish was conducted in 100-L glass aquaria containing 70 L of water. Test aquaria (one control and one treated) were immersed in a water bath and held at 22 ± 1 °C through the use of submersible heating elements.

A proportional diluter system described by Mount and Brungs (1967), with modifications of McAllister et al. (1972), was used for the intermittent introduction of [^{14}C]cinmethylin in water into the test aquarium. The final specific activity of the ^{14}C test material was 0.57 μCi/mg by the appropriate dilution of the ^{14}C stock solution with unlabeled cinmethylin. Aerated well water (pH 8.2, dissolved oxygen 9.3 ppm) was delivered to the glass aquaria at a rate sufficient for six volume changes in 24 h. The diluter system delivered a nominal concentration of 0.1 mg/L cinmethylin.

Before the uptake portion of this study was initiated, the test solution was allowed to flow through the test aquaria for a 24-h equilibration period. The test concentration was confirmed by radioanalysis before the test fish were introduced. The uptake phase was initiated by transferring groups of 110 fish each to the control and treated aquaria. These fish were observed initially and every 24 h during the exposure period for mortality and adverse behavior. Water and fish were sampled at various time intervals throughout the uptake period.

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Table I. Toxicity of Cinmethylin and Cinch 7EC Herbicide to Aquatic Organisms

	LC_{50}, mg/L					
	technical cinmethylin			Cinch 7EC herbicide		
	24 h	48 h	96 h	24 h	48 h	96 h
freshwater fish						
rainbow trout (*Salmo gairdneri*)			6.6 (4.8–9.2)[a]			
bluegill sunfish (*Lepomis macrochirus*)			6.4 (4.7–8.8)			
freshwater invertebrates						
Daphnia magna		7.2 (6.5–8.0)				
estuarine and marine organisms						
sheepshead minnow (*Cyrinodon variegatus*)	3.8 (2.5–5.0)	2.4 (1.7–3.4)	1.6 (1.1–2.3)	4.0 (2.0–8.0)	3.2 (2.0–8.0)	2.4 (1.7–3.4)
mysid shrimp (*Mysidopsis bahia*)	3.4 (2.7–4.5)	3.0 (2.4–3.9)	2.5 (2.0–3.2)	5.8 (0.6–10)	2.0 (1.5–2.5)	1.3 (0.9–1.6)
fiddler crab (*Uca pugilator*)	>1000		>1000	665 (638–693)		130 (101–211)
eastern oyster (*Crassostrea virginica*)		3.2 (2.3–4.9)[b]			3.7 (2.3–4.9)	

[a] Mean and 95% confidence intervals. [b] The 48-h EC_{50} effect criterion was the reduction of the number of normal larvae (those which developed to the fully shelled, straight-hinged veliger stage within 48 h).

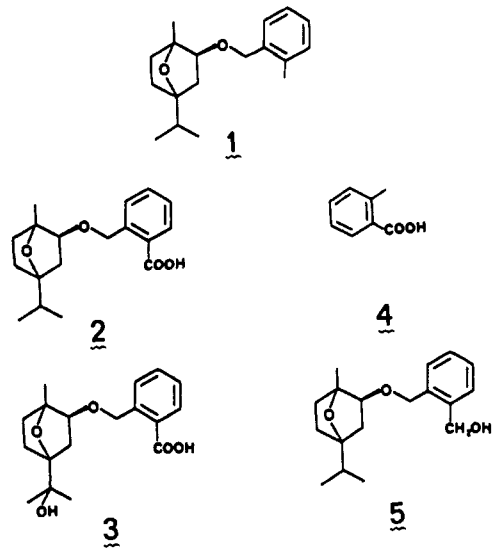

Figure 1. Chemical structures of cinmethylin and related reference metabolites.

The amount of ^{14}C residues in the water, fillet (body, muscle, skin, skeleton), and viscera (fins, head, internal organs) of the control and exposed fish were quantitated by radioassay.

On day 28 of the exposure period, the addition of [^{14}C]cinmethylin to the treated aquarium was terminated. The water in the control and the treated aquaria was removed by siphoning until a depth of approximately 3 in. of water remained in each aquarium. The aquaria were refilled to a volume of approximately 70 L with uncontaminated well water. This water was then removed as described above, and the aquaria were finally filled with approximately 70 L of uncontaminated well water. The fish were then exposed to the uncontaminated environment for 14 days. During the depuration period, water and fish were sampled at various time intervals and the levels of ^{14}C residues were quantitated by radioassay.

Sampling Intervals and Procedures. Water and fish were sampled immediately after exposure (day 0), at days 1, 3, 7, 10, 14, 21, and 28 of the uptake period, and at days 1, 3, 7, 10, and 14 of the depuration period.

On each sampling day, at least 250 mL of water was collected from each aquarium with a 16-oz polyethylene bottle designated for the control and treated aquaria. Duplicate 10-mL aliquots of the water samples were quantitated by LSC in 10 mL of Aquasol-2 scintillation solution.

Three fish from each aquarium were collected and pooled as the control and treated samples. Fish were dissected into fillet and viscera. Two additional fish from each aquarium were pooled and designated for the whole fish analysis. All samples were stored frozen (−20 °C) until radioassay. Individual fish samples were homogenized with dry ice in a grinder and allowed to thaw; subsamples (0.5 g) were combusted in a Packard 306B Tricarb sample oxidizer. Additional fish were collected for tissue residue characterization. On experimental days 3 and 28 of the exposure period and day 3 of the depuration period, 12 fish from each aquarium were sampled and pooled into control and treated groups. They were dissected in the same manner described above.

Fish Tissue Extraction and Residue Characterization. The distribution and chemical nature of the ^{14}C residue in the whole fish, fillet, and viscera samples from the day 3 and day 28 exposure periods and from day 3 of the depuration period were analyzed separately. The analytical methodology designed for this study allowed for the evaluation of the distribution pattern of [^{14}C]cinmethylin and its metabolites in the various subfractions [tissue macromolecules (skin, scale, connective tissues, soluble proteins) and organic extractable and water-soluble conjugates].

Macerated fish tissues (3 g) were homogenized in 15 mL of 0.01 M phosphate buffer, pH 7.4. Proteinaceous materials in the aqueous tissue homogenate were precipitated with 0.75 g of trichloroacetic acid (TCA). This mixture was allowed to stand 16 h at room temperature. Precipitated proteins were separated by centrifugation at 2000 rpm for 10 min. The supernatant, after centrifugation, was transferred, and the resulting protein precipitate was resuspended in 15 mL of phosphate buffer solution (containing 5% TCA). The protein precipitate, after the second homogenization and centrifugation, was dissolved in tissue solubilizer reagent prior to LSC quantitation.

Radioactivity associated with the TCA precipitate was considered as protein-bound residues. The protein pellet was further washed twice with 10 mL of methanol followed twice by 10 mL of hexane. Attempts were carried out to characterize the chemical nature of the released radioactivity. Radioactivity remaining with the protein precipitate after solvent washes was considered as unextractable bound residue.

Organic extractable residues in the aqueous tissue homogenate after TCA precipitation were recovered by solvent partitioning (three times with equal volumes of chloroform). The combined chloroform phase represented organic extractable [^{14}C]cinmethylin equivalent residues in the fish tissues.

Radioactivity remaining in the aqueous phase was considered as water-soluble material and was subjected to further acid hydrolysis treatment at pH 1 at 75 °C for 1 h. ^{14}C residues in the aqueous sample, after acid hydrolysis, were extracted three times with equal volumes of chloroform. Radioactivity remaining in the aqueous phase, after acid hydrolysis and solvent partitioning, was considered to be polar, aqueous residues.

For the purpose of generating sufficient amounts of ^{14}C residues for structural characterization, whole fish tissues from the days 1, 3, 7, 10, 14, 21, and 28 exposure period were combined for qualitative analysis. A slightly modified extraction and isolation procedure from that described above was used. Macerated whole fish tissues (60 g) were homogenized in 150 mL of 0.01 M citric acid–phosphate buffer, pH 3.2. The final volume of the aqueous homogenate was adjusted to 500 mL with the same buffer solution and transferred to a Soxhlet liquid extractor. Ether/aqueous extraction was continued for 16 h. The ether extract consisted of organic extractable residues. The aqueous phase (water-soluble conjugates), after the ether extrac-

Table II. TLC R_f Values[a] and Mass Spectral Data of Cinmethylin and Model Metabolites

	R_f value			
	system 1	system 2	system 3	EI-mass spectral data [m/z (% RA)]
cinmethylin (1)	0.69	0.87	0.94	274 (M^+, 8), 216 (5), 169 (20), 154 (18), 133 (10), 123 (47), 107 (42), 105 (100), 93 (24), 83 (20), 71 (35), 55 (15), 43 (95)
α-carboxycinmethylin (2)	0.46	0.64	0.82	TMS derivative 376 (M^+, 12), 286 (42), 241 (25), 223 (4), 207 (37), 169 (15), 133 (30), 123 (25), 107 (30), 93 (20), 73 (80), 55 (20), 43 (100)
8-hydroxy-α-carboxycinmethylin (3)	0.36	0.51	0.38	TMS derivative 374 (M^+ < 1), 359 (<1), 257 (2), 241 (1), 207 (82), 167 (3), 133 (15), 131 (30), 109 (15), 107 (10), 73 (100), 43 (30)
o-toluic acid (4)	0.43	0.67	0.94	TMS derivative 208 (M^+, 35), 194 (100), 149 (40), 119 (85), 91 (43)
α-hydroxycinmethylin (5)	0.43	0.60	0.77	TMS derivative 362 (M^+ < 1), 272 (3), 229 (5), 193 (45), 169 (10), 153 (10), 123 (40), 107 (35), 73 (55), 43 (100)

[a] Key: solvent system 1, toluene–2-propanol–acetic acid (150:20:1.5); solvent system 2, hexane–2-propanol–acetic acid (120:30:1); solvent system 3, ethyl acetate–hexane–chloroform–acetic acid (100:50:5:1.5).

Table III. Concentration of ^{14}C Residues in the Test Water and Fish Tissues during a 42-Day Bioconcentration Study with Bluegill Sunfish

		[^{14}C]cinmethylin equiv residues					
		whole fish		fillet		viscera	
day	water, mg/L	ppm	BCF[a]	ppm	BCF	ppm	BCF
			Uptake Phase				
0	0.082						
1	0.072	10	130	4.6	60	20	260
3	0.080	30	380	9.0	120	47	600
7	0.094	26	320	9.5	120	50	610
10	0.11	30	340	13	150	47	540
14	0.11	31	340	13	140	55	600
21	0.12	41	430	12	130	64	670
28	0.11	35	360	12	120	58	600
			Depuration Phase				
1	0.010	14		7.4		28	
3	0.002	4.8		1.7		7.0	
7	ND[b]	0.59		0.26		0.85	
10	ND	0.46		0.14		0.49	
14	ND	0.30		0.09		0.40	

[a] Daily bioconcentration factor (BCF) was obtained by dividing the tissue concentration by the mean measured water concentration up to and including the respective sampling day. [b] Residue less than 0.002 ppm.

tion, was acid-hydrolyzed as described above, and the released radioactivity was recovered by chloroform solvent extraction. The ether and chloroform extracts of the whole fish tissue homogenate were combined, dried over anhydrous sodium sulfate, concentrated to approximately 3 mL, and subjected to further hexane–acetonitrile solvent partitioning prior to TLC analysis. Metabolites were separated by preparative TLC, and individual isolated radioactive components were characterized by TLC and GC–mass spectroscopy.

Chromatography and Radioassay. Radioactivity was quantitated in a Searle Model Delta 300 or a Packard Model 300 liquid scintillation system. Water samples (10-mL aliquot) were counted in 10 mL of the Aquasol-2 scintillation solution. Radioactive residues associated with the fish tissues were analyzed by combusting subsamples (approximately 500 mg) in a Packard Model 306B sample oxidizer. Radioactive areas of the TLC plate, after solvent development and autoradiography, were removed by scraping and analyzed in an Aquasol-2–water (11:4) gel system. All LSC quantitations were corrected for counting efficiency, combustion efficiency, and quenching. Water and fish residues were reported as parts per million (ppm) [^{14}C]cinmethylin equivalent.

Radioactivity recovered in the water and tissue extracts was analyzed by two-dimensional TLC (silica gel F-254, 0.25 mm, E. Merck) and visualized by autoradiography on Kodak SB-5 single-coated X-ray film.

Gas–liquid radiochromatography (RGLC) was carried out on a Varian 1440 gas–liquid chromatograph equipped with a flame ionization detector and a Packard Model 894 gas proportional counter. The column used was a 1 m × 2 mm (i.d.) glass column packed with 3% OV-101 on 80/100-mesh Supelcoport

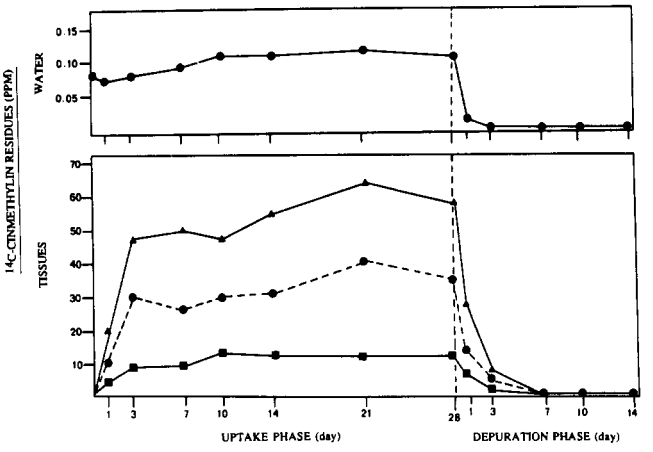

Figure 2. [^{14}C]Cinmethylin equivalent residues in water, whole fish (▲), fillet (■), and viscera (●) during a 42-day bioaccumulation study with bluegill sunfish.

(Supelco Co.). The air, hydrogen, and helium flow rates were 210, 30, and 30 mL/min, respectively. The helium and propane quench gas flow rates for the gas proportional counter were 120 and 15 mL/min, respectively.

Capillary gas–liquid chromatography (GC) was carried out on a 30 m × 0.25 mm (i.d.) fused silica SE-54 WCOT capillary column in a Varian 3700 gas–liquid chromatograph equipped with a flame ionization detector. Temperature-programmed and isothermal analyses were carried out at injector and detector

Table IV. Relative Distribution of ^{14}C Residues in the Various Subfractions in the Fish Tissues at Various Sampling Intervals

	% recovered radioactivity								
	day 3 exposure			day 28 exposure			day 3 depuration		
	WF[a]	F[a]	V[a]	WF	F	V	WF	F	V
aqueous extract	33	27	38	34	30	35	40	46	49
org extr	17	23	18	20	28	18	28	43	27
water sol	16	4	20	14	2	17	12	3	22
acid hydrol	2	b	2	1		2	2		2
polar matl	14		18	13		15	10		20
protein bound	67	73	62	66	70	65	60	54	51
org extr	30	47	29	37	38	36	23	23	20
unextr	37	26	33	29	32	29	37	31	31
total residues, ppm	30	9	47	35	12	58	2	5	7

[a] Key: WF = whole fish, F = fillet, V = viscera. [b] Acid hydrolysis not carried out.

temperatures of 245 and 320 °C, respectively. The helium carrier gas and nitrogen makeup gas flow rates through the detector were 3 and 36 mL/min, respectively. On-column split ratio was controlled at 10:1 ratio.

GC–mass spectroscopy was carried out on Finnigan Models 1012 and 4500 mass spectrometers. The TLC R_f values of cinmethylin and reference standards in several solvent systems are presented in Table II.

RESULTS AND DISCUSSION

Bioaccumulation and Depuration Rate Determination. The bioaccumulation potential of [^{14}C]cinmethylin in the bluegill sunfish under a dynamic flow-through system was examined. Fish were maintained at a constant water concentration of 0.097 (±0.018) mg/L [^{14}C]cinmethylin for a 28-day exposure period. Water quality parameters such as the temperature, dissolved oxygen, pH, and ammonia were measured in the treated and control aquarium throughout the test. The dissolved oxygen concentration, which ranged between 6.7 and 8.7 mg/L at 22 °C, and the ammonia concentrations were below the toxic level. The pH values of the treated aquarium were consistent with the control.

Behavioral observations during the study indicated no adverse behavior in the treated or control aquarium. No mortality was observed during the uptake and the depuration phase of this study.

The amounts of [^{14}C]cinmethylin equivalent residues in water, whole fish, fillet, and viscera during the uptake and depuration phase are summarized in Table III. Uptake and depuration curves in the various tissue types are shown in Figure 2. A rapid uptake of radioactivity was observed. Tissues residues appear to plateau after 3 days. The mean tissue residues after 28 days of exposure were 35, 12, and 58 ppm for the whole fish, fillet, and viscera, respectively. The corresponding bioconcentration factors for the above tissues are 360, 120, and 600, respectively. The daily bioconcentration factors for the uptake phase ranged from 130 to 430 for the whole fish, 60 to 150 for fillet, and 260 to 670 for viscera.

Greater than 99% of the tissue residues was eliminated from the exposed fishes after the 14-day depuration period.

Quantitative Distribution of Tissue Residues. The analytical method designed for this study allowed for the evaluation of the relative distribution pattern of [^{14}C]cinmethylin and its degradation products in the various subfractions of the whole fish, fillet, and viscera. Only samples obtained from days 3 and 28 of the exposure periods and from day 3 of the depuration period were examined.

Residue distribution data (Table IV), reported as the percent of recovered radioactivity, showed the majority of the residues were associated with the protein-bound fraction at an approximate 2:1 ratio as compared to the aqueous tissue homogenate. Approximately 50% of radioactivity found in the proteinaceous materials was recovered by successive washing of the tissue precipitates with methanol and hexane and was associated mainly with oily materials. Unextractable tissue-bound residues accounted for 25–40% of the recovered radioactivity. A majority of the water-soluble residues in the whole fish and viscera samples was not recovered by organic solvent extraction after acid hydrolysis.

Table V. Relative Distribution of ^{14}C Residues in the Various Subfractions of the Combined Whole Fish Tissue Homogenate

	% recovered radioactivity
ether extract[a]	44
1	11
2	13
3	14
4	3
5	1
others[b]	2
water-soluble materials[c]	31
organic extractable	11
1	4
3	3
others[b]	4
unextractable	20
solid residues	25

[a] Continuous ether extraction and fractionation procedures recovered an estimated 92% of the total radioactivity in the combined fish tissued homogenate. [b] Including radioactivity associated with the origin of the TLC plate, minor products, and the hexane phase during the initial hexane–acetonitrile partitioning. [c] Aqueous phase analyzed after acid hydrolysis.

Qualitative Characterization of Tissue. Whole fish tissues from the days 1, 3, 7, 10, 14, 21, and 28 exposure period were combined for qualitative analysis. ^{14}C residue in the tissue (43%) was recovered by continuous ether extraction.

The two-dimensional TLC autoradiogram (Figure 3A) of the concentrated ether extract, after removal of the oily materials by hexane–acetonitrile partitioning, showed cinmethylin (11% of the recovered radioactivity), α-carboxycinmethylin (2; 13%), and 8-hydroxy-α-carboxycinmethylin (3; 14%) as the major components. Several minor products were also detected.

For the purpose of isolating sufficient amount of metabolites for structural confirmation, radioactive components in the fish extract were separated by preparative TLC using toluene–2-propanol–acetic acid as the development solvent (Figure 3B). The area of the preparative TLC plate corresponding to the radioactive band was

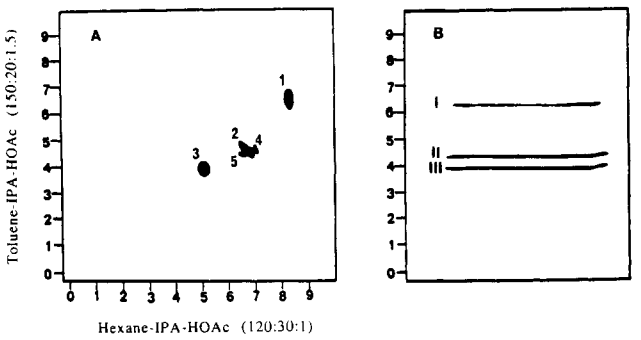

Figure 3. TLC autoradiograms: (A) organic extractable metabolites from the whole fish tissue homogenate; (B) preparative TLC separation of the organic extractable metabolites for structural characterization.

removed, and the radiolabeled materials were extracted from the silica gel with ethyl acetate as the eluting solvent. The concentrated ethyl acetate was subjected to chromatographic and spectroscopic analyses.

The chemical nature of radioactive band I was confirmed as cinmethylin (1) by TLC cochromatography. The retention times and mass spectra of the isolated product and the authentic standard are consistent.

A two-dimensional TLC autoradiogram of the isolated radioactive band II indicated the presence of at least one major and two minor components. The major component had a similar TLC chromatographic property as α-carboxycinmethylin (2). o-Toluic acid (4) and α-hydroxycinmethylin (5) were tentatively identified as minor components based on their relative TLC properties. This sample (containing the above three products) was subjected to further GC–mass spectral analysis following derivatization with a trimethylsilylating reagent (BSTFA). The retention times and mass spectra of the TMS derivatives of the isolated products and the authentic standards were consistent.

Two-dimensional TLC cochromatography showed 8-hydroxy-α-carboxycinmethylin (3) is the major component in radioactive band III. The retention times and mass spectra of the TMS derivative of the isolated product and the authentic standard were consistent.

Characterization of the Water-Soluble Residues. Quantitative distribution data presented in the earlier discussion had indicated a significant amount of the water-soluble ^{14}C residues remained with the aqueous fraction even after acid hydrolysis. The aqueous phase from the whole fish tissue homogenate after the continuous ether extraction was subjected to further acid hydrolysis in an attempt to recover a sufficient amount of ^{14}C residues for structural characterization. Approximately 10% of the total fish residue was recovered after the above treatment. The TLC autoradiogram showed the presence of two major components. Since only a small amount of ^{14}C residue was available for analysis, the structural assignments of the radiolabeled products were tentative and were based only on TLC properties. There were the undegraded parent (4% of the total residue) and 8-hydroxy-α-carboxycinmethylin (3; 6%). A majority of the ^{14}C residues in the water-soluble fraction were polar materials not readily recovered by acid hydrolysis.

CONCLUSION

The physical–chemical properties of cinmethylin such as its water solubility (60 ppm) and octanol–water partition coefficient (6200) suggest a relatively high bioconcentration potential of this product. The maximum bioconcentration factor of cinmethylin was determined to be approximately 600 in the viscera (360 in the whole fish) under the test condition reported in this study. The residue uptake was rapid and plateaued within 3 days of exposure. Cinmethylin metabolized in the fish tissue via hydroxylation and oxidation reactions to yield α-carboxycinmethylin and 8-hydroxy-α-carboxycinmethylin. The metabolic pathways in fish are consistent with those observed in the rat (Lee et al., 1986) and goat (Woodward et al., 1989). The depuration of these tissue residues was rapid. Greater than 90 and 99% of the radioactivity was eliminated within 3 and 14 days after the exposed fish were placed in the uncontaminated water. In view of the low toxicity and the rapid and extensive degradation of this compound, significant impact of cinmethylin to the aquatic environment is not anticipated.

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