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Residues of Dichlorvos in Atlantic Salmon (*Salmo salar*) after Delousing

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Liver and muscle tissues from Atlantic salmon (*Salmo salar*) were analyzed for residues of dichlorvos after treatment against salmon lice infestations at water temperatures of 4 and 12 °C. Dichlorvos was extracted from tissue samples with cyclohexane and acetone, cleaned by gel permeation chromatography, and determined by gas chromatography (glass capillary column and alkali flame nitrogen–phosphorus specific detector). Though at a water temperature of 4 °C residues were detectable in muscular tissue for up to 3 days after treatment, at 12 °C they could only be detected in samples taken directly after treatment. In liver tissue, residues were detectable for up to 3 days after treatment at 4 °C, while at 12 °C traces of dichlorvos were evident in some samples for up to 6 days after treatment.

The ectoparasitic copepod *Lepeophtheirus salmonis*, commonly known as the salmon louse, causes severe, annually recurring disease problems in farmed salmonids in Norway.

Bath treatment with the organophosphorus compounds trichlorfon [metrifonate, *O,O*-dimethyl (1-hydroxy-2,2,2-trichloroethyl)phosphonate] or dichlorvos [DDVP, *O,O*-dimethyl *O*-(2,2-dichlorovinyl) phosphate], has been employed to control this pest. The structures of these organophosphates are shown in Figure 1. Both substances act by lethal inhibition of cholinesterases in the parasite. Residue studies have shown that trichlorfon is

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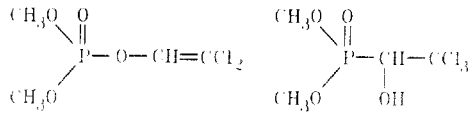

Figure 1. Structural configuration of dichlorvos and trichlorfon.

detectable in muscular tissue of salmon up to 12 days after treatment (Brandal, 1979).

Therapeutic applications of dichlorvos in other animals include control of internal parasites in horses and swine, and the substance is also used to control houseflies in animal housing. In man, trichlorfon is used in the treatment of schistosomiasis, the drug acting by the slow release of the active substance dichlorvos (Nordgren et al., 1981).

Kinetic studies have revealed that trichlorfon and dichlorvos are rapidly metabolized and eliminated in man. Nordgren et al. (1981) were unable to detect either substance in human plasma for more than 8 h after oral administration of trichlorfon. However, the situation is different in poikilothermic species, many xenobiotics being more slowly metabolized and excreted.

As the parasitic infestation is controlled by bath treatment, absorption of drugs occurs mainly via the gills. Absorption and excretion rates depend on a number of factors, such as pesticide application rate, duration of treatment, water temperature and salinity, and fish size.

This study was performed to gain information on which to base the establishment of withdrawal times for dichlorvos in Atlantic salmon.

EXPERIMENTAL PROCEDURES

Fish Experiments. Two experiments were conducted, one at a water temperature of 12 °C and one at 4 °C, the aim being to determine whether variations in water temperature significantly affect excretion of dichlorvos. The temperature remained relatively constant during the sampling period in both trials (±1 °C). In each experiment, the material consisted of 40 Atlantic salmon weighing between 900 and 1500 g, which were treated by bathing in a 2 ppm dichlorvos bath (Nuvan 500 EC, Ciba Geigy, 4 mL/1000 L of water) for 60 min. This dosage is as recommended at 4 °C, but double that recommended at 12 °C (Horsberg et al., 1987). Before and after treatment, the fish were kept in a sea-cage consisting of a net-pen measuring 2 × 2 × 2 m attached to a floating collar. The treatment at 12 °C was performed in a fiber-glass tank containing 1000 L of well oxygenated seawater. The fish were netted, treated, and then returned to the sea-cage. Treatment at 4 °C was performed directly in the sea-cage after this had been enclosed by a tarpaulin. The water salinity was 3.5% and 3.2%, respectively. The fish were starved 1 day prior to treatment. Precautions were taken when handling the pesticide emulsion such as the use of protective clothing and rubber gloves and avoidance of vapor inhalation. The concentrate was first mixed with 10 L of water and then added to the water in the tank (12 °C) or cage (4 °C). During the sampling period, the fish were fed a commercial pelleted fish diet 3 times a day. In both experiments, samples were taken from four fish immediately after treatment and again after 1, 2, 3, 4, 6, 8, 10, 14, and 21 days. The fish were killed by a blow to the head, frozen, and kept at −20 °C until analysis was performed.

Analytical Methods. A gas chromatographic method, based on the method of Andersson (1986) but modified for animal tissue, was used.

Chemicals. Dichlorvos standards were prepared from 99% dichlorvos (Pestanal, Riedel de Haën, Hannover, West Germany). The chemicals used, acetone, cyclohexane, and methylene chloride (Fisons plc, Loughborough, U.K.), were all of high-performance liquid chromatographic quality grade. Bio-Bead SX-3 gel 200–400 mesh was obtained from Bio-Rad, Richmond, CA.

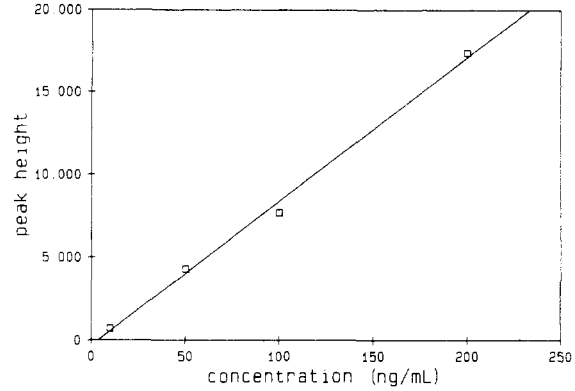

Figure 2. Calibration curve for dichlorvos standards used to determine the dichlorvos concentration in muscular and liver samples.

Procedure. *Extraction.* Ten grams of skeletal muscle or 5–10 g of liver tissue without the gallbladder was homogenized by using an ultraturrax homogenizer for approximately 1 min in a mixture of 20 mL of cyclohexane and 15 mL of acetone. Each sample was treated with an ultrasonic disintegrator for 2 min and centrifuged at 3000 rpm for 10 min, and the organic phase was then transferred to a round-bottom flask. Ten milliliters of cyclohexane and 5 mL of acetone were added to the remaining sample, and sonication and phase separation were repeated. The acetone–water phase with the remaining tissue was discarded. The organic phase was evaporated to approximately 1 mL on a sand bath at 40 °C under a gentle stream of purified air. The volume was adjusted to 2.5 mL with cyclohexane and then to 5 mL with methylene chloride.

Gel Permeation Chromatography (GPC). The extract was cleaned by gel permeation chromatography on a Pharmacia SR 10/50 column (500 × 10 mm) packed with 8.5 g of Bio-Beads SX-3 gel. The column was constantly flushed with cyclohexane/methylene chloride (1:1) by using a high-pressure piston pump (Eldex A-30-S) delivering a constant flow of 1 mL/min through a Supelco LC pressure gauge, 0–6000 psi. All tubings and connections were made of Teflon or stainless steel. Each sample was injected with a Hamilton gastight syringe through a Millipore filter type HV, pore size 0.45 μm, into a 1-mL loop connected to the column, via a Cheminert sample injection valve of the slider type. The loop was then connected with the solvent stream (1.00 mL/min). After approximately 5 min, the loop was disconnected and cleaned with 96% ethyl aclohol. A new sample was injected after 40 min. The same GPC column was used for several hundreds of runs.

The pesticide fraction was evaporated to a volume of less than 1 mL (*not* to dryness) on a sand bath at 40 °C, under a gentle stream of purified air, the volume being afterward adjusted to exactly 1 mL with cyclohexane.

Quantification and Identification. Aliquots of 2 μL of the cyclohexane extracts were analyzed by gas–liquid chromatography using an alkali flame, nitrogen–phosphorus sensitive detector (NPD) in the phosphorus mode. The peak height was compared to a calibration curve prepared by injection of dichlorvos standards from 10 to 200 ng/mL. The standard curve was linear within this range of concentration (Figure 2).

Preparation of Standard Solutions. A stock solution of dichlorvos was prepared in cyclohexane, and working solutions were prepared from the stock solution in concentrations ranging from 10 to 200 ng/mL for quantitative purposes and 20 μg/mL for recovery determination and GPC calibration.

Analytical Quality Assurance. Each series of samples for analysis consisted of four to six samples of muscular tissue, four to six samples of liver tissue, one blank sample, and one control sample of both muscular and liver tissue spiked with 200–1000 ng of dichlorvos. All samples were subjected to the same extraction and cleanup procedure. Recovery of dichlorvos from the spiked samples was calculated from the chromatographic response by reference to standards of equivalent concentration. Recovery of dichlorvos from the spiked samples seemed not to be influenced by the amount of dichlorvos added. The dichlorvos concentration in the samples was recalculated on the

Table I. Residues of Dichlorvos (Micrograms per Gram) in Muscular and Liver Tissue from Atlantic Salmon According to Water Temperature at Time of Treatment

days after treatment	4 °C				12 °C			
	muscle		liver		muscle		liver	
	av	range	av	range	av	range	av	range
0	0.094	0.087–0.13	<0.01[a]	ND[b]–0.012	0.046	<0.01–0.11	0.041	<0.01–0.075
1	<0.01	ND–<0.01	<0.01	<0.01–0.012	ND		<0.01	ND–0.011
2	ND		<0.01	ND–0.019	ND		<0.01	ND–<0.01
3	<0.01	ND–0.017	0.026	<0.01–0.054	ND		<0.01	ND–<0.01
4	ND		ND		ND		<0.01	ND–<0.01
6	ND		ND		ND		<0.01	ND–<0.01
8	ND		ND		ND		ND	
10	ND		ND		ND		ND	
14	ND		ND		ND		ND	
21	ND		ND		ND		ND	

[a] <0.01, traces detected. [b] ND, not detected.

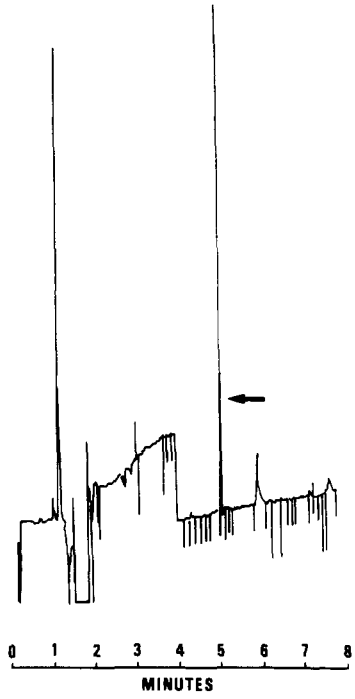

Figure 3. Chromatogram of dichlorvos residues in muscular tissue of an Atlantic salmon killed immediately after exposure to 2 ppm dichlorvos for 60 min at a water temperature of 4 °C. The chromatogram represents injection of the total amount of dichlorvos extracted from 4 mg of tissue. The amount of dichlorvos was 0.13 μg/g, and the recovery was 75%. The arrow indicates the dichlorvos peak.

basis of the recovery percentage obtained in each series of samples. Calibration of the GPC column with a dichlorvos standard (20 μg/mL) and collection of a series of 1 mL/min samples for 40 min showed that the 18–38-mL fraction contained 95% of the organophosphate. The prefraction, 18 mL, contained most of the coextracts and was discarded.

Instrumentation. The samples were injected into a Carlo Erba Mega Series 5330 gas chromatograph, equipped with a DB-1 fused silica capillary column, 25 m × 0.32 mm (i.d.) and an alkali flame nitrogen–phosphorus sensitive ionization detector (NPD).

The operating conditions were as follows: inlet, 260 °C; detector, 270 °C; oven temperature program, 60 °C for 1 min, increase of 40 °C/min to 220 °C, 8 °C/min to 240 °C, and 10 °C/min to 260 °C; carrier gas, He, 2 mL/min; makeup gas, He, 30 mL/min, O_2, 300 mL/min, H_2, 35 mL/min. The splitless injector inlet purge delay time was 30 s. The retention time and peak height were measured by a LCD Milton Roy CI-10 integrator.

RESULTS AND DISCUSSION

The retention time for dichlorvos was about 5.20 min and was very constant for each series of samples (Figure 3). The limit of detection was found to be approximately 0.002 μg/g of tissue. Residues of less than 0.01 μg/g were, however, not quantified due to the uncertainty of the method at such low concentrations. Dichlorvos recovery from different series of samples ranged from 64% to 95%.

The results of the residue analyses are summarized in Table I. In the experiment conducted at a water temperature of 12 °C, dichlorvos residues up to 0.11 μg/g were detected in muscular tissue of fish killed directly after the immersion treatment. Residues could, however, not be detected in muscular tissue in any samples taken later. In liver tissue, residues up to 0.075 μg/g were detected directly after treatment, traces being subsequently evident for up to 6 days.

In the experiment conducted at 4 °C, dichlorvos residues of up to 0.13 μg/g of muscular tissue were detected immediately after treatment. Small amounts of dichlorvos were found in both muscle and liver tissue for up to 3 days after treatment.

It was not possible to make any exact calculations concerning the kinetics of dichlorvos in Atlantic salmon due to the small number of fish sampled on each occasion, the low dichlorvos concentrations found in most samples, and the considerable variation between individual samples. Some facts concerning the disposition of dichlorvos in salmon could, however, be ascertained.

Dichlorvos is absorbed by fish during treatment. Immediately after treatment, concentrations are higher in muscle than in liver. However, levels in muscular tissue decrease rapidly, with the result that low concentrations of dichlorvos are detectable in the liver somewhat longer than in muscular tissue. The decline in dichlorvos levels in muscular tissue seems to be more rapid at a water temperature of 12 °C than at 4 °C. This is consistent with the previously observed temperature-related excretion of other xenobiotics in fish (Borgan et al., 1981). The detection of traces of dichlorvos for up to 6 days in liver of fish treated at 12 °C, but only 3 days at 4 °C, is somewhat more difficult to explain. Traces were, however, only found in one fish on each occasion and might have been due to individual variation. Moderate fatty infiltration of the liver was observed in some of the fish used in the 12 °C experiment, this becoming evident as the samples were prepared. This condition may have resulted in reduced metabolic activity and/or accumulation of the pesticide in liver fat. No macroscopic pathological changes in the liver were seen in the fish used in the 4 °C experiment.

On the basis of simultaneous measurement of dichlorvos and trichlorfon concentrations in plasma from humans given 7.5 mg of trichlorfon/kg of body weight,

Nordgren et al. (1981) estimated that dichlorvos was eliminated much faster than trichlorfon ($t_{1/2}$ trichlorfon = 690 min; $t_{1/2}$ dichlorvos = 0.65 min). Brandal (1979) found residues of trichlorfon in muscular tissue of Atlantic salmon up to 12 days after treatment with 300 ppm for 60 min. A concentration of approximately 0.7 μg/g was detected 1 day after treatment. In the present study, the maximum dichlorvos concentration found in muscular tissue directly after treatment with 2 ppm for 60 min was 0.13 μg/g. The decline in dichlorvos concentrations in muscular tissue to nondetectable levels within 1–3 days after treatment was as expected, assuming that in salmon, as in man, the elimination of dichlorvos is faster than the elimination of trichlorfon.

In conclusion, dichlorvos is rapidly excreted in Atlantic salmon. The withdrawal time of 14 days set by the Norwegian Medicines Control Authority thus has a built-in safety margin which is sufficient to ensure that no residues are present in muscular or liver tissues in fish at slaughter.

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Insecticidal and Antifeedant Bioactivities of Neem Oils and Their Relationship to Azadirachtin Content†

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Neem seed oil, a possible precursor to new botanical insecticides, varies widely with respect to concentrations of the limonoid azadirachtin, the putative active ingredient. Among 12 samples of neem oil analyzed by liquid chromatography, azadirachtin concentrations ranged from <50 (limit of detection) to over 4000 ppm. Oils were bioassayed for larval growth inhibition and antifeedant activity against the variegated cutworm (*Peridroma saucia*) and for molt disrupting activity against the milkweed bug (*Oncopeltus fasciatus*). For each of the three bioassays, bioactivity of the oils, as measured by EC_{50} values, is highly correlated with azadirachtin content of the oils. From 72% to 90% of the variation in bioactivity of the oils can be accounted for by variation in azadirachtin content. As azadirachtin content of neem oil varies widely and is highly correlated to bioactivity against these bioassay species, azadirachtin content may be a useful quality-control criterion for neem oil as a precursor for insecticide production.

INTRODUCTION

Many current neurotoxic insecticides are damaging to the environment and/or pose a threat to public health via food residues, groundwater contamination, or accidental exposure. Although the risks associated with the proper use of these pest control materials should be minimal, the exaggerated perception of these risks among the public is increasing demand for alternative or "organic" produce, which necessitates development of new, environmentally sound pest control materials. A potential source of new pesticidal materials are higher plants; the search for new insecticides or prototypes among plant natural products has heightened in the past decade (Arnason et al., 1989; Balandrin et al., 1985).

One such source of natural insecticides is the neem tree (*Azadirachta indica* A. Juss.; Meliaceae). Native to the Indian subcontinent, this fast-growing shade tree has been widely cultivated in Africa, Australia, the Carribean, and Central and South America. Although the seeds and leaves of this tree have been traditionally used for centuries to control pests (Koul et al., 1990), recent interest in neem

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