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Organophosphate Poisoning of Atlantic Salmon in Connection with Treatment against Salmon Lice

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Horsberg, T. E., T. Høy and I. Nafstad: Organophosphate poisoning of Atlantic salmon in connection with treatment against salmon lice. Acta vet. scand. 1989, 30, 385-390. - Three incidents with high mortality in Atlantic salmon after trichlorfon treatment against salmon lice are described. All 3 incidents occurred at water temperatures of 12°C or higher. The mean brain acetylcholinesterase (AChE) activity of dead fish was less than 20% of normal activity, while survivors showed mean activities of 22-61% of normal levels. Dichlorvos residues in muscular and liver tissues ranged from nondetectable levels to 0.2 μg/g tissue. The strongest inhibition of brain AChE was found in association with the highest dichlorvos residues. Substantial AChE-inhibition was, however, also found in samples in which dichlorvos residues could not be detected. AChE-determination was found to be more reliable than residue analysis for the diagnosis of organophosphate poisoning in salmon.

trichlorfon; dichlorvos; residues; acetylcholinesterase.

Introduction

The ectoparasitic copepod *Lepeophtheirus salmonis,* commonly known as the salmon louse, causes severe annual disease problems in Norwegian fish farms. Atlantic salmon (*Salmo salar*), as well as rainbow trout (*Salmo gairdneri*) in marine aquaculture may be infested with this parasite.

Treatment with the organophosphorous compound trichlorfon (metrifonate) was introduced by *Søgnen* in 1974 (pers. comm.). Trichlorfon and other organophosphates act by inhibition of the enzyme acetylcholinesterase (AChE) in the parasite, causing overstimulation, paralysis of parts of the nervous system, and death. Unfortunatly, the nervous system of the fish is also affected by the drug. Until recently, treatment has been carried out according to the regime of *Brandal & Egidius* (1979). Delousing is carried out as a bath treatment by transferring the fish in a net-pen from the pen into a treatment unit, consisting of a net-pen of 50-100 cubic meters capacity surrounded by a tarpaulin bag. Trichlorfon (Neguvon® vet., Bayer) has previously been dissolved in the treatment unit. The fish in a particular pen are transferred and treated in one or more operations depending on the numbers of fish involved. After treatment, the fish are transferred back to the net-pen. The treatment unit is thus floated from net-pen to net-pen, the same trichlorfon solution being used for treatment of several groups of fish. *Brandal & Egidius* (1979) recommended a dosage of 300 mg/l trichlorfon and a treatment time of 15-45 min, depending on water temperature. They recommended that the same trichlorfon solution could be used for up to 10 h.

When trichlorfon is dissolved in seawater, it is spontaneously transformed into the much more toxic organophosphate dichlorvos in a

time-, temperature- and pH-dependent reaction (*Ecobichon* 1979, *Samuelsen* 1987). The greater toxicity of dichlorvos is partly due to it being approx. 100 times more potent as an AChE inhibitior than trichlorfon (*Hofer* 1981), and partly due to the fact that dichlorvos is more fat-soluble, and thus able to penetrate biological membranes at a higher rate.

Since 1985, several incidents with high mortality in fish after trichlorfon treatment have been reported. Extensive formation of dichlorvos from trichlorfon, resulting in organophosphate poisoning of the fish was suspected in all cases.

This study was performed to gain information about the degree to which dichlorvos accumulates in muscular and liver tissues before organophosphate poisoning occurs. Another purpose was to study the possible relationship between dichlorvos levels in liver or muscular tissues and AChE-inhibition in the brain.

Materials and methods

Samples of dead fish and survivors from 3 incidents of high mortality after treatment as well as of untreated control fish of approximately the same size from the same area were frozen and sent as quickly as possible to the Norwegian College of Veterinary Medicine, Department of Pharmacology and Toxicology, for verification of organophosphate poisoning by determination of dichlorvos and AChE. The samples arrived well frozen and were stored at -20°C. Parallel samples were sent to the National Veterinary Institute for routine pathological examination.

AChE-determination

Brain tissue was analyzed for AChE-activity according to the spectrophotometric method of *Ellman et al.* (1961). After thawing, the brain tissue was homogenized in 0.1 mol/l phosphate buffer pH 8.0. The assay contained 0.1 mol/l phosphate buffer pH 8.0, approx. 2.67 mg/ml tissue, 0.33 mmol/l 5,5'-dithiobis 2-nitrobenzoic acid (Sigma) and 0.67 mmol/l acetylthiocholine (Sigma) with a total volume of 3 ml.

Protein was determined by the Bio-Rad method of *Bradford* (1976) using lyophilized bovine serum albumin (1.21 mg/ml) (Sigma) as standard.

Dichlorvos determination

The samples were analyzed according to a method outlined by *Andersson* (1986) with modifications for animal tissues (*Ringstad,* unpubl.). Ten grams of either skeletal muscle or liver tissue were used for analysis. Samples from different fish were pooled when a sufficient amount of tissue could not be taken from 1 fish. After homogenization in a mixture of acetone and hexane, the samples were treated with an ultrasonic disintegrator, centrifuged, and the organic phase was evaporated to approx. 1 ml. After adjustment of the volume to 5 ml with cyclohexane and methylene chloride (1:1), the extract was cleaned by gel permeation chromatography (GPC) on a column packed with Bio-Beads SX-3 gel (Bio-Rad laboratories). The pesticide fraction was concentrated by evaporation and the volume adjusted with cyclohexane, before injection on a Carlo Erba Mega HRGC 5330 gas chromatograph with a capillary column and a nitrogen-phosphorus specific alkali flame detector. The limit of detection was found to be approx. 0.01 µg/g tissue. The recovery of dichlorvos ranged between 61 and 84%.

Results

The anamnestic informations are summarized in Table 1. Incident 1 and 2 have been

Table 1. Anamnestic information in 3 incidents with high mortality after delousing of different salmon groups with the same trichlorfon solution.

Incident	Group	No of fish	Average weight (g)	Water temp. (°C)	Trichlorfon concentration (mg/l)	Approximate age of sol. (h)	Treatment time (min)	Mortality (%)
1	a	11000	32	14.4	280	1	15	
	b	11500	32	"	"	2	11	98
	c	5000	37	"	"	3	8	
2	a	8000	300	12.0	170	1	45	0
	b	38500	200	"	"	2	40	97
	c	40000	200	"	"	3	20	70
3	a	19000	30	12.0	300	1	30	0
	b	11000	60	"	"	2	28	95

previously described in a casuistic report (*Røttereng et al.* 1986).

In incident 1, symptoms were observed about 1 h after the fish had been transferred from the treatment unit back to the net-pen. The fish appeared tetanic, and accumulated on the bottom of the net-pen. Approximately 27000 salmon smolts died during the following hours.

In incident 2, the first treated group (a) appeared to be responding normally to treatment. The second and third groups ((b) and (c)) also seemed to be responding normally, but deaths began occurring very soon after transference back to the net-pen. The treatment of group (c) was aborted when mortality in group (b) was detected. Approximately 55000 salmon smolts in groups (b) and (c) died during the following hours.

In incident 3, the first treated group (a) re-

Table 2. AChE activity in brain and dichlorvos residues in liver and muscular tissue of controls, dead and surviving fish.

Incident	Group	Status	No. of samples	AChE brain average and range (nmol/min*mg prot)	Dichlorvos liver average (µg/g)	Dichlorvos muscle average and range (µg/g)
1		controls	4	175.8 (150.9-201.6)	N.D.*	N.D.
		dead	7	31.2 (20.0- 39.8)	N.D.	<0.01**
2		controls	6	130.4 (120.4-137.2)	N.D.	N.D.
	a	survived	2	80.2 (77.7- 82.7)	-***	-
	b	dead	4	7.5 (3.6- 11.0)	0.20**	0.06**
		survived	4	59.7 (55.9- 61.9)	-	-
	c	dead	4	22.1 (5.8- 73.4)	0.03**	0.02**
		survived	4	62.6 (56.2- 73.4)	-	-
3		controls	4	120.5 (104.7-137.5)	N.D.	N.D.
	a	survived	4	26.9 (21.2- 32.7)	0.02**	0.03 (0.01-0.06)
	b	dead	4	21.1 (16.1- 31.4)	0.09**	0.04 (0.02-0.05)

* N.D. = not detected
** = pooled samples
*** - = not determined

sponded normally to treatment, and no fish died. The second group (b) seemed to respond normally, but when the fish were back in the net-pen, deaths began occurring immediatly, and most of the 11000 salmon smolts in this group died during the following hours (*Mortensen,* pers. comm.).

In no case did pathological examination explain the high mortality.

Specific brain AChE activity in controls varied between 104.7 and 201.6, expressed as nmol of acetylthiocholine hydrolyzed per min and milligram of protein (Table 2).

In all incidents, dead fish showed a mean brain AChE activity of less than 20%, in incident 2 (b) less than 10% of activity in corresponding control samples. The lowest extreme AChE activity measured was only 2.8% of the levels in the controls. Survivors from groups with mortality ((2 (b), 2 (c)) showed mean activities of 45-48% of normal value. Fish in groups without mortality ((2a), 3 (a)) also showed marked inhibition of brain AChE activity, with mean levels of 23-62% of normal activity.

In all cases and groups, the reduction in brain AChE activity was significant (Students t-test, $p < 0.001$).

The highest residues of dichlorvos were found in fish with the lowest AChE activities (groups 2 (b), 2 (c) and 3 (b)). In most cases concentrations in liver were higher than in muscular tissues. Dichlorvos could only be detected in very small amounts in incident 1, though inhibition of brain AChE activity was substantial.

In incident 1 and 2, samples of the batches of trichlorfon used were analyzed at Statens legemiddelkontroll (The Norwegian Medicines Control Authority). No deviation from standard requirements was detected.

Discussion

Determination of AChE activity in brain tissue is generally recognized as a valuable method for evaluation of spontaneous occurring organophosphate intoxications.

In this study, dead fish showed a mean AChE activity of less than 20% of the controls. This is in agreement with the results of *Coppage & Matthews* (1975) who described brain AChE activities in pinfish (*Lagodon rhomboides*) exposed to the organophosphorous compound 1,2-dibromo-2,2-dichloroethyl dimetyl phosphate (Naled).

The symptoms and course of the intoxications and the relative reduction of AChE activity described here, are similar to those described by *Salte et al.* (1987). The specific activity of AChE in the controls was, however, 1.4-2.1 times higher in our study. This may have been due to slight differences in the methods used, differences in the size of the fish, and differences in water temperature.

There is very little information available about tissue concentrations of dichlorvos and other organophosphorous pesticides in intoxicated animals. In Atlantic salmon used for toxicity testing of dichlorvos under experimental conditions, we have found dichlorvos levels of 0.08 μg/g muscule tissue and 0.07 μg/g liver tissue when death occurred. The residue levels in liver in groups 2 (b) and 3 (c) were above those found in the toxicity experiment. The residue levels in samples from muscular tissue were, however, in all incidents lower than in the experimentally poisoned fish.

The relationship between AChE activity in brain and dichlorvos residues in muscular and liver tissue was inconstant. In incident 1, only traces of dichlorvos were found in muscular tissue, though inhibition of brain AChE activity was substantial. In incidents 2 and 3, however, the strongest AChE inhibi-

tion was found in fish with the highest levels of residues in both tissues.

The inconsistency of the relationship between the parameters may be due to several factors. The fish in incident 1 were in a rather poor nutritional condition, while the fish in the other incidents were in a better condition. Dichlorvos is fat-soluble, and the extent to which dichlorvos may build up in the body before an intoxication occurs may vary a great deal. Small fish are generally more susceptible to pesticide poisoning than larger fish of the same species. This is due to larger gill surface area relative to body mass, higher rate of metabolism and smaller lipid pool of the body. Another contributing factor might be the time span between the end of treatment and death. The samples from incident 1 were taken from fish that had survived longer than in the other incidents. Organophosphorous compounds are rapidly metabolized and excreted (*Murty* 1986). In an experiment to determine the excretion rate of dichlorvos from muscular and liver tissues in Atlantic salmon, the residues in muscular tissue dropped to non-detectable levels in 24 h, while residues in liver tissue increased by 100% during the same time-span (*Horsberg & Høy,* unpublished). It therefore seems probable that the dichlorvos levels in the organs might have changed during the periode from the end of treatment until death occurred. The inhibition of AChE by organophosphates is, however, much more persistent.

The trichlorfon preparation used can contain up to 0.3% dichlorvos. Thus, the treatment solution may already contain as much as 0.9 mg/l dichlorvos when it is mixed at a dosage of 300 mg/l. This dichlorvos concentration alone would be sufficient to treat fish against salmon lice with a treatment time of 60 min or less at temperatures above 10°C (*Rae* 1979; *Horsberg et al.* 1987). After 4 h, concentrations of more than 20 mg/l dichlorvos are reached at a temperature of 13.5°C and pH 8.0 (*Samuelsen* 1987). *Horsberg et al.* (1987) found that LC_{50} for the preparation Nuvan® 500 EC, (50% dichlorvos) in salmon smolts with an exposure time of 60 min was 17.4 µl/l water at 12°C. This corresponds to a dichlorvos concentration of 8.7 mg/l. Even though emulsifiable concentrates of pesticides are generally more toxic to fish than the active substance per se (*Murty* 1986), one can assume that highly toxic concentrations of dichlorvos are reached during the 8 to 10 h periode the trichlorfon solution is recommended to be used.

All incidents described occurred at water temperatures of 12°C or higher, under which conditions the turn-over rate for trichlorfon to dichlorvos is rapid. At these temperatures, the metabolic activity and oxygen demand in salmon is high, and the uptake of xenobiotics is increased (*Murty* 1986). There are indications that a low oxygen level in water will increase the toxicity of dichlorvos in salmonids (*Høy & Horsberg,* unpubl.). The treatment unit was in all cases supplied with oxygen from an oxygen tank via perforated rubber tubes, though water oxygen concentrations were in no case recorded during treatment.

Trichlorfon has been widely used against ectoparasites on fish. The recommended doses have varied between 10 000 and 0.25 mg/l, and exposure times between 1 min and 4 days (*Herwig* 1979). In view of the incidents described and several other accidents, the dosage regime of *Brandal & Egidius* (1979) seems hazardous at high temperatures, and has now been abandoned in Norway.

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Sammendrag

Organofosfatforgiftning av atlantisk laks ved behandling mot lakselus.

Tre tilfeller med høy dødelighet hos laks etter behandling med triklorfon mot lakselus er beskrevet. Alle tilfellene oppstod ved vanntemperaturer på 12°C eller høyere. Død fisk hadde gjennomsnittlig under 20% av normal acetylcholinesterase (AChE) aktivitet i hjernen, mens overlevende hadde gjennomsnittlig 22-61% av normal aktivitet. Diklorvosrester i muskel- og levervev varierte mellom ikke påvisbare nivåer og 0.2 µg/g vev. Sterkeste hemning av AChE i hjernen ble funnet i tilfeller med høyeste innhold av diklorvos i vevene. En betydelig AChE-hemming ble imidlertid funnet i prøver hvor diklorvosrester ikke kunne påvises. AChE-bestemelse ble funnet å være mer pålitelig enn restkonsentrasjonsbestemmelse i diagnostikk av organofosfatforgiftning hos laks.

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