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Tissue Distribution of ^{14}C-Diflubenzuron in Atlantic Salmon (*Salmo salar*)

By *Tor Einar Horsberg* and *Tonje Høy*

Department of Pharmacology and Toxicology,
Norwegian College of Veterinary Medicine, Oslo, Norway.

Horsberg, T. E. and T. Høy: Tissue distribution of ^{14}C-diflubenzuron in Atlantic salmon (*Salmo salar*). Acta vet. scand. 1991, 32, 527–533. – Diflubenzuron is a potent inhibitor of chitin synthesis, with potential use against salmon lice infestations. The absorption, distribution and elimination of the substance in Atlantic salmon was examined after a single, oral dose of 75 mg/kg bodyweight. The kinetic properties were studied by whole-body autoradiography, liquid scintillation counting and thin layer chromatography, using a ^{14}C-labelled isotope of the substance. The drug was poorly absorbed from the intestine, but reached a concentration of more than 4 μg/g in the mucus layer of the skin 2 days after administration. If maintained for several days, this concentration is probably sufficient to control all moulting stages of sea lice in Atlantic salmon. The main route of excretion was via the bile.

salmon lice; kinetics; autoradiography; scintilation counting; metabolism.

Introduction

The ectoparasitic copepods, *Lepeophtheirus salmonis* and *Caligus elongatus,* commonly known as sea lice, cause severe and annually recurring disease problems in salmonids in Norwegian fish farms.

To control these parasites, bath treatment with the organophosphorous compounds trichlorfon (Neguvon®) or dichlorvos (Nuvan®) is performed. This method of treatment has the drawbacks of being labour-intensive, and organophosphorous compounds have a high acute toxicity for mammals and fish. Moreover, discharge of the chemicals may cause undesirable environmental effects (*Egidius & Møster* 1987). Considerable interest has therefore been shown in finding effective drugs which are suitable for oral administration.

Brandal & Egidius (1977) gave trichlorfon orally to Atlantic salmon (*Salmo salar*), and not only observed effects on the degree of parasite infestation, but also toxic reactions to this insecticide in fish. *Palmer et al.* (1987) gave ivermectin orally to Atlantic salmon and also observed effects on the salmon lice infestation, as well as toxic effects in fish. A suitable agent for oral administration must have a high margin of safety, and in respect to this, the insect-growth regulator diflubenzuron seemed to be an interesting candidate. Diflubenzuron interferes with chitin formation (*Post et al.* 1974). It is highly toxic for crustaceans, rendering them incapable of detaching completely from their exuviae during moulting. In the intermoult period, the effect of the pesticide upon crustaceans is negligible. Diflubenzuron has a low acute toxicity in mammals (acute oral LD_{50} for rat and mice: > 4640 mg/kg) and relatively low acute toxicity in

rainbow trout (LC_{50}, 96 h: 140 mg/l, *Worthing & Walker* 1987).

Preliminary trials with oral medication of Atlantic salmon (75 mg diflubenzuron per kilogram bodyweight daily for 14 days) revealed a significant reduction in the number of copepods of all stages (*Høy & Horsberg* 1991).

The aim of this study was to examine the pharmacokinetic properties of ^{14}C-diflubenzuron in Atlantic salmon after a single, oral dose.

Materials and methods

Whole-body autoradiography

Twenty-two salmon smolts adapted to sea water and weighing approx. 60 g were kept in a 500 l fibre glass tank supplied with running water with a salinity of 32 ‰ at 8 ± 1°C. They were handfed a pelleted standard fish diet twice a day throughout the experiment.

The ^{14}C-diflubenzuron had a specific activity of 4144 MBq/g and was a gift from Duphar, Holland. The radiolabelled diflubenzuron (18.38 mg) was mixed with 81.67 mg of unlabelled diflubenzuron (technical grade), and suspended in 7 ml of peanut oil. After 21 days of adaption, each fish was immobilized, a stomach tube was inserted and 0.3 ml of the suspension was administered. Fish (1 or 2 individuals) were sampled for autoradiography at the following time after administration: 2 h, 12 h, 2 days, 6 days, 10 days, 13 days, 20 days and 27 days. They were killed by an overdose of benzocaine in water and frozen with liquid nitrogen (–196°C). The fish were then embedded in a 1 % solution of sodium carboxymethyl cellulose, frozen in a mixture of n-hexane and solid CO_2 and subjected to whole body autoradiography according to the method of *Ullberg* (1954). Sagittal sections (40 µm) of the whole body were cut on a PMV cryomicrotome (PMV, 450 MP, Stockholm, Sweden), and collected on tape No. 821 (3M Co., St Paul, Minn., USA) at –20°C. The sections were freeze-dried over night, and applied on a Hyperfilm-ßmax (Amersham, U.K.) together with a 2-fold dilution series of a ^{14}C-standard. The films were exposed at –20°C for approx. 3 months before development and production of positive pictures. The films were both visually examined and subjected to densitometric examination.

Liquid scintillation counting

From the remaining material, tissue samples of approx. 10 mg were taken from blood, brain, muscle, abdominal fat, kidney, liver, bile, cartilage and cutaneous mucus. The samples were digested with 1 ml Soluene (Packard) and 200 µl 96 % ethanol at 37°C overnight. The samples were then decoloured by adding 400 µl Perhydrol (Merck) followed by 4 ml of liquid scintillation cocktail (Hionic Fluor, Packard). The number of disintegrations per min (DPM) in the samples were recorded in a Packard Tri-Carb 1900CA liquid scintillation analyzer. The counting efficacy was controlled by a Packard automatic ^{14}C-quenching standard.

An estimate of the percentage of the administered drug dose present in liver, kidney, blood and muscle at different sampling times was calculated using the total content of radioactivity in the organ, the weight of the fish and the total dose of radioactivity administered to each fish. Blood is in fish estimated to constitute approx. 5 % of the body weight (*Ellis et al.* 1985). The other organs represented averagely the following proportions of total body weight (5 fish): Muscle: 68 %, liver: 1.2 %, kidney: 0.9 %.

Thin layer chromatography / phase separation

As whole-body autoradiography and liquid scintillation counting only detect radioactivity, but do not differentiate between the original drug and metabolites, a simple thin-layer chromatographic study of bile at different times after administration was performed to get an estimate of the amount of unchanged drug excreted via this route. Bile from fish sacrificed after 6 h, 1 day and 4 days was collected. The bile was kept frozen at –20°C until analyzed. It was then diluted 1:5 with methanol to achieve precipitation of mucus and proteins, and centrifuged at 10,000 RPM for 10 min. using a Sorvall RC2-B centrifuge equipped with a SS-34 rotor. The supernatant was collected and methanol was removed under a gentle stream of nitrogen. No further purification of the bile was carried out. Aliquots of 10 µl were applied to pre-coated TLC plates (Sil G-25 UV_{254} Machery-Nagel, Düren, W. G.) together with a standard of ^{14}C-diflubenzuron and a non-labelled standard. The TLC-plates were developed in a solvent system of benzene:dioxane:acetic acid 90:30:1 as described by *Ivie et al.* (1980). The plates were first examined under ultraviolet light to determine the Rf-value of the cold standard. They were then exposed to a Kodak XAR-5 film (Eastman Kodak Co., Rochester N.Y., USA) for 3 weeks at –70°C. The films were developed, and the areas on the TLC-plates corresponding to spots on the film were scraped off with a scalpel into liquid scintillation vials. Five ml of liquid scintillation cocktail was added, and the samples were subjected to liquid scintillation counting.

To determine the amount of water soluble metabolites in the bile, the separation of radioactivity in the bile samples between a water phase and an isooctane phase was performed. Ten µl bile was diluted with 40 µl 0.1 M acetate buffer pH 5, after which 450 µl distilled water and 500 µl isooctane (E. Merck, Darmstadt, W.G., analytical quality) were added. The samples were mixed for 10 min. on a whirl-mixer and centrifuged. 300 µl of each phase was collected and 10 ml of liquid scintillation cocktail was added before subsequent analysis in the liquid scintillation counter.

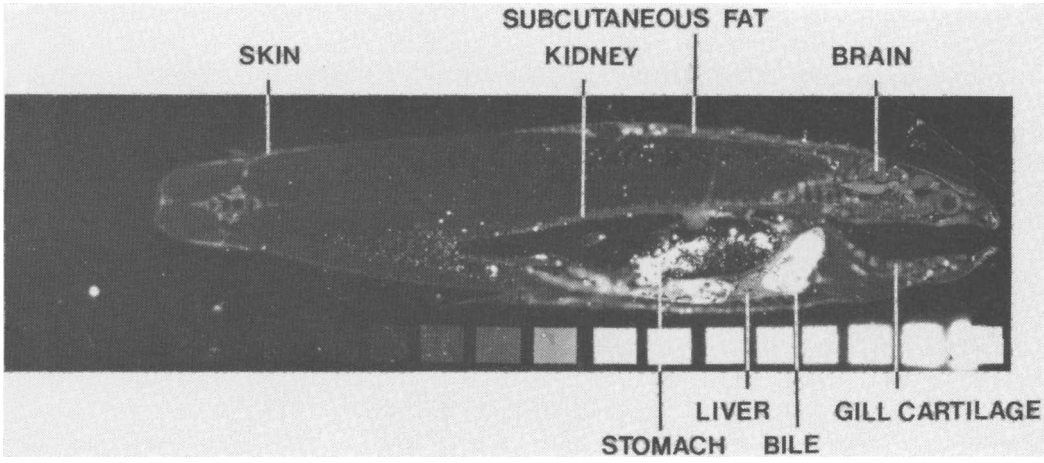

Figure 1. Tissue distribution of ^{14}C-diflubenzuron and metabolites 12 h after oral administration of 4.3 mg diflubenzuron (3.3 Mbq) to an Atlantic salmon smolt.

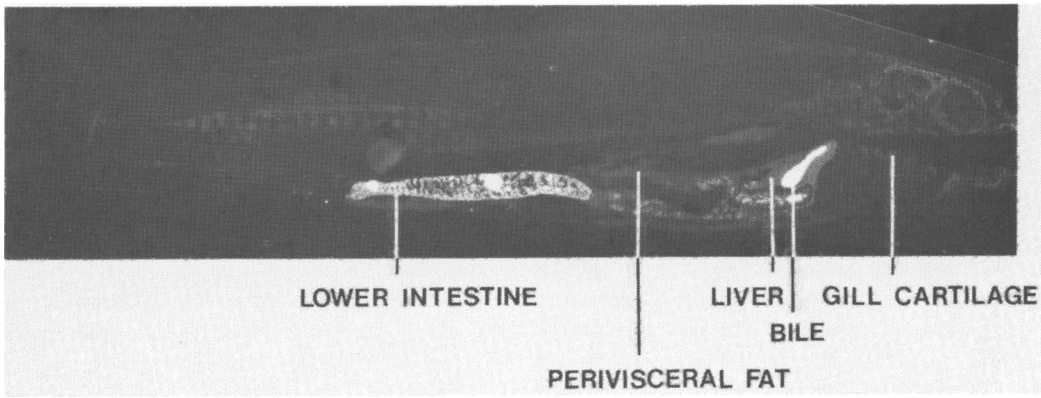

Figure 2. Tissue distribution of ^{14}C-diflubenzuron and metabolites 2 days after oral administration of 4.3 mg diflubenzuron (3.3 Mbq) to an Atlantic salmon smolt.

Results

Whole body autoradiography

Two h after administration, most of the radioactive material was present in the stomach and pyloric caeca. A slight degree of absorption could, however, be observed, with the highest concentrations in cartilage tissue in the head.

After 12 h, most of the administered radioactivity was still present in the intestinal contents. Relatively high concentrations could, however, be observed in the liver, kidney, brain, bile, fat and cartilage. Activity in the cutaneous mucous layer could also be demonstrated. The activity in bile was high, indicating the biliary route to be the major excretion pathway of the drug (Fig. 1).

In all samples taken later, a lower degree of radioactivity was registered in the tissues. The highest activity apart from that in intestinal content and bile, was recorded in the fatty tissues and cartilage, as seen in the autoradiogram from the samples taken 2 days after administration (Fig. 2).

Later on, the activity in all tissues diminished, except in bile, liver and intestinal contents. The activity in the intestinal contents during the later stages was probably due to bile being released into the intestine, as seen on the autoradiograms from 10 days after administration (Fig. 3).

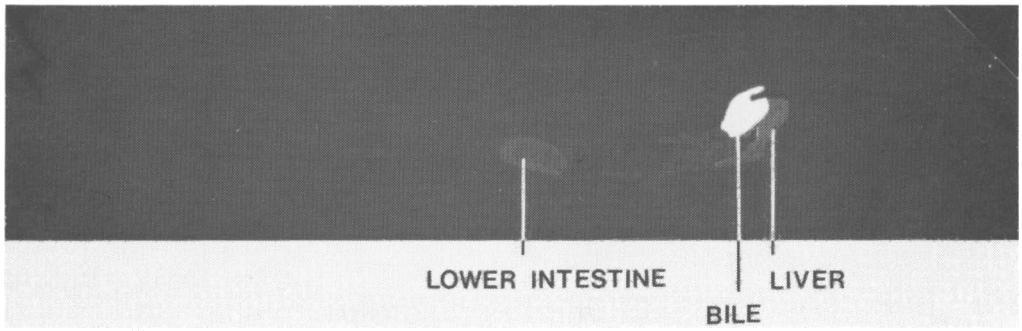

Figure 3. Tissue distribution of ^{14}C-diflubenzuron and metabolites 10 days after oral administration of 4.3 mg diflubenzuron (3.3 Mbq) to an Atlantic salmon smolt.

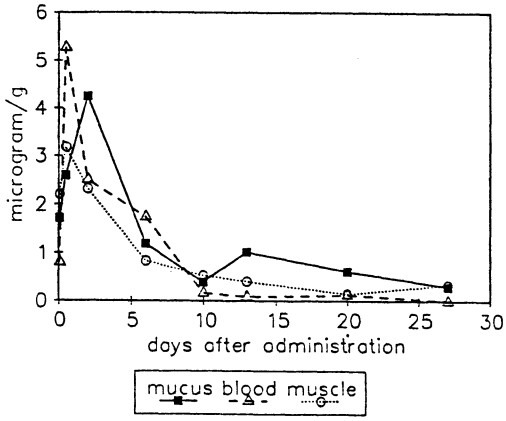

Figure 4. The concentration of diflubenzuron and/or its metabolites in cutaneous mucus, blood and muscle at different intervals after oral administration of 4.3 mg diflubenzuron (3.3 Mbq) to Atlantic salmon smolts.

Liquid scintillation counting

The amount of diflubenzuron or its metabolites (µg/g) in different tissues is presented in Figs. 4 and 5. The results are in agreement with the visual and densitometric evaluation of the autoradiograms.

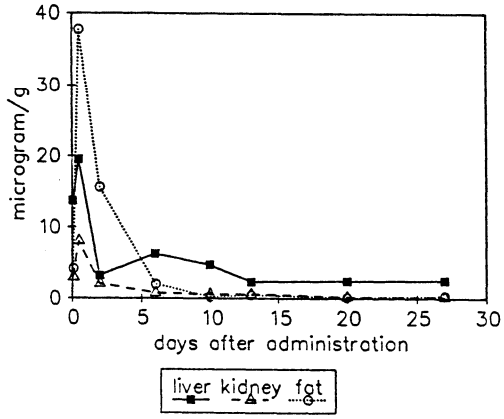

Figure 5. The concentration of diflubenzuron and/or its metabolites in liver, kidney and abdominal fat at different intervals after oral administration of 4.3 mg diflubenzuron (3.3 Mbq) to Atlantic salmon smolts.

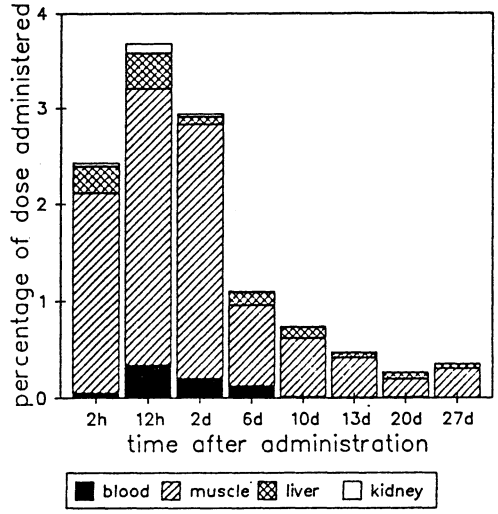

Figure 6. The percentage of the administered dose present in muscle, liver, kidney and blood at different intervals after oral administration of 4.3 mg diflubenzuron (3.3 Mbq) to Atlantic salmon smolts.

Of particular interest is the achieved concentration of more than 4 µg diflubenzuron or metabolites per gram in the cutaneous mucus layer 2 days after administration.

The concentration of radioactivity in brain and cartilage was highest 12 h after administration. The levels corresponded to 13.8 µg/g in brain, and 10.9 µg/g in cartilage. The levels later subsequently dropped.

In bile, high and fluctuating concentrations of diflubenzuron and/or metabolites was detected. The concentration fluctuated between 275 and 1066 µg/g the first 10 days after administration, then suddenly dropped to less than 4 µg/g for the rest of the period.

The calculated percentage of the administered dose which was present in muscle, liver, kidney and blood is presented in Fig. 6. The results show that the highest amount of radioactivity in blood, muscle, liver and kidney could be detected 12 h after administration. Approx. 3.7 % of the administered dose

Table 1. The percentage of radioactivity in spots with the same Rf-value as the standards (Rf 0.88) as determined by thin layer chromatography of bile. The percentage radioactivity in bile derived from water soluble and isooctane soluble metabolites.

	% activity Rf 0.88	% activity water phase	% activity isooctane phase
6 h	39	37	63
1 day	2	95	5
4 days	2	98	2

was present in these organs and tissues at this time point, indicating that diflubenzuron is poorly obsorbed from the intestine.

Thin layer chromatography / phase separation

The results are presented in Table 1. The Rf-value of both the unlabelled and labelled diflubenzuron standards was 0.88 in the chromatographic system described. Six h after administration, 39 % of the radioactivity present in the bile had the same Rf-value as the standards, this dropping to only 2 % in samples taken 1 and 4 days after administration. In these latter samples, most of the radioactivity was present at the origin.

The phase separation study showed that a substantial fraction of the radioactivity in bile of fish taken 6 h after administration was soluble in the organic phase, while in the later samples, most of the radioactivity consisted of more polar compounds which were found in the water phase.

Discussion

The absorption of diflubenzuron from the intestine seemed to be rather poor. Only 3.7 % of the administered dose was detected in blood, muscle, liver and kidney 12 h after administration. This was the sampling time when the highest total level of radioactivity could be detected in these organs/tissues. The total level of radioactivity might, however, have been higher at another time point, as the next samples for autoradiography and scintillation counting were taken as much as 36 h later.

Though the present results point to a rather poor intestinal absorption, up to 4 µg/g diflubenzuron or metabolites were detected in the mucus layer of salmon after the single, oral dose of 75 mg/kg. Although the actual LC_{50} towards different stages of sea lice has not been determined, it is likely that the concentrations achieved are sufficient to interfere with chitin formation in these species. This assumption is based on the conclusion of *Cunningham* (1986), who reviewed a number of publications dealing with the toxicity of diflubenzuron towards freshwater and estuarine crustaceans. The author concluded that diflubenzuron was toxic to larvae of some species subjected to chronic exposure tests in concentrations between 0.1 and 0.5 ng/ml.

High and fluctuating concentrations of xenobiotics and their metabolites in bile, as observed in this study, is commonly seen in fish (*Lech et al.* 1973). Bile seems to be an important excretory pathway for many substances. The fluctuations may be explained by different rates of biliary excretion between individual fish. Bile is released into the intestine when feed passes through the gut. The feeding intensity of individual fsh might therefore influence on the concentration of the xenobiotic or its metabolites in the bile.

The thin-layer chromatographic study indicated that diflubenzuron was rapidly metabolized to more polar compounds. However, 39 % of the radioactivity present in the bile 6 h after administration had the same Rf-value as the diflubenzuron standards, and most probably represented the unchanged drug. This indicates that a substantial part

of the radioactivity present in the body during the first hours after administration was due to the parent substance, and not its metabolites.

The phase separation study confirmed that after 6 h, most of the radioactivity was present in the organic phase, while at 1 and 4 days after administration, most of the radioactivity derived from water soluble metabolites.

Due to the high toxicity of diflubenzuron towards crustaceans, environmental effects must be considered in further studies on this substance or other inhibitors of chitin formation.

Acknowledgements

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References

Brandal PO, Egidius E: Preliminary report on oral treatment against salmon lice, *Lepeophtheirus salmonis* with Neguvon. Aquaculture 1977, *10*, 177–178.

Cunningham PA: A review of toxicity testing and degradation studies used to predict the effects of diflubenzuron (Dimilin®) on estuarine crustaceans. Environ. Pollut. (Ser. A) 1986, *40*, 63–86.

Egidius E, Møster B: Effect of Neguvon® and Nuvan® treatment on crabs (*Cancer pagurus, C. maenas*), lobster (*Homarus gammarus*) and blue mussels (*Mytilius edulis*). Aquaculture 1987, *60*, 165–168.

Ellis AE, Roberts RJ, Tytler P: Die Anatomie und Physiologie der Knochenfische. (Anatomy and physiology of fish). In: R. J. Roberts & H. J. Schlotfeldt (Eds.): Grundlagen der Fischpathologie. Verlag Paul Parey, Berlin und Hamburg 1985, p. 23–63.

Høy T, Horsberg TE: Chemotherapy of sea lice infestations in salmonids. Thesis, Norwegian College of Veterinary Medicine, Oslo 1991.

Ivie GW, Bull DL, Veech JA: Fate of diflubenzuron in water. J. agric. Food Chem. 1980, *28*, 330–337.

Lech JJ, Pepple SK, Statham CN: Fish bile analysis: A possible aid in monitoring water quality. Toxicol. appl. Pharmacol. 1973, *25*, 430–434.

Palmer R, Rodger H, Drinan E, Dwyer C, Smith PR: Preliminary trials on the efficacy of ivermectin against parasitic copecods of Atlantic salmon. Bull. Eur. Ass. Fish Patol. 1987, *7*, 47–54.

Post LC, DeJong BJ, Vincent WR: 1-(2,6-Disubstituted benzoyl)-3-phenylurea insecticides: Inhibitors of chitin synthesis. Pestic. Biochem. Physiol. 1974, *4*, 473–483.

Ullberg S: Studies on the distribution and fate of ^{35}S-labelled benzylpenicillin in the body. Acta radiol. (Stockh.), 1954, *118*, 1–110.

Worthing CR, Walker SB: The Pesticide Manual. 8th ed. The British Crop Protection Council, Thornton Heath, U.K. 1987.

Sammendrag

Fordeling av ^{14}C-diflubenzuron i vev hos atlantisk laks (Salmo salar).

Diflubenzuron er en potent hemmer av kitinsyntese med potensiell anvendelse mot lakselusinfestasjoner. Oppsuging, vevsfordeling og utskillelse av substansen ble undersøkt i atlantisk laks etter en enkelt, oral dose på 75 mg/kg kroppsvekt. De farmakokinetiske egenskaper ble undersøkt ved hjelp av helkroppsautoradiografi, væskescintillasjonstelling og tynnskiktkromatografi. En ^{14}C-merket isotop av substansen ble benyttet i undersøkelsen. Oppsugingen fra tarmen var dårlig, men det ble oppnådd et nivå på mer enn 4 µg/g i hudens slimlag 2 dager etter dosering. Dersom denne konsentrasjonen opprettholdes over flere dager, er den sannsynligvis tilstrekkelig til å gi terapeutisk effekt på de av lakselusas utviklingsstadier som gjennomgår skallskifte. Utskillelsen var hovedsakelig via gallen.

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Reprints may be requested from: T. E. Horsberg, Department of Pharmacology and Toxicology, The Norwegian College of Veterinary Medicine, P. O. Box 8146 Dep, N-0033 Oslo 1, Norway.