



COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

DIFLUBENZURON

SUMMARY REPORT (2)

1. Diflubenzuron, [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea] is an acyl urea derivative for use in the treatment of sea lice (*Lepeophtheirus salmonis*) infestations in Atlantic salmon. Diflubenzuron is admixed as a 90% pre-concentrate in pelleted diet at a final concentration of 0.6 g diflubenzuron/kg. The intended oral dosage is 3 mg diflubenzuron/kg bw/day during 14 consecutive days. The substance is also used as a pesticide in agriculture.

Currently, diflubenzuron is included in Annex III of Council Regulation (EEC) No 2377/90 in accordance with the following table:

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Diflubenzuron	Diflubenzuron	Salmonidae	1000 µg/kg	Muscle and skin in natural proportions	Provisional MRLs expire on 1.7. 2000

2. Diflubenzuron acts by interference with the synthesis of chitin. Demand for chitin is greatest at the moult between growth stages and hence insects are killed due to disruption of the moulting process. No specific studies on pharmacodynamic properties of diflubenzuron were submitted.
3. Pharmacokinetic studies were conducted in rats (single oral doses of 4 to 1000 mg/kg bw, repeated oral dose of 5 mg/kg bw/day for 14 days and single dermal doses of 0.05 and 0.5 mg/10 cm²), mice (single oral doses of 12.5 to 925 mg/kg bw), rabbits (single oral dose of 1 mg/kg bw and single dermal dose of 150 mg/kg bw) and the target species Atlantic salmon (single oral doses of 3 and 75 mg/kg bw, repeated oral dose of 3 mg/kg bw/day for 14 days and single intravenous dose of 3 mg/kg bw) with unlabelled or radiolabelled diflubenzuron.

In rats diflubenzuron is absorbed from the gastrointestinal tract. Following a dose of 4 mg/kg bw 42.5% was absorbed, but following a dose of 900 mg/kg bw only 3.7% of the dose was absorbed. The absorption decreases with increasing dose indicating that the absorption is saturable in rats. In mice a similar pattern was observed. The maximum plasma concentration was 844 ng equivalents/ml in rats 4 hours after a single oral administration of 5 mg/kg bw. Diflubenzuron is rapidly and evenly distributed to all tissues. After administration of a single oral dose of 5 mg [¹⁴C]-diflubenzuron/kg bw to rats the highest mean levels of radioactivity at 4 hours were found in fat (4672 µg equivalents/kg), ovaries (3737 µg equivalents/kg), liver (2265 µg equivalents/kg), heart (1345 µg equivalents/kg), kidney (1200 µg equivalents/kg) and brain (984 µg/kg equivalents/kg). From 48 hours post dose onwards the highest levels were retained in liver (mean level at 48 hours 431 µg equivalents/kg) and erythrocytes (mean level at 48 hours 379 µg equivalents/kg). No difference between males and females was noted. Absorption and distribution patterns were comparable after single and multiple dosing and thus no accumulation of diflubenzuron is expected. Dermal absorption in rats and rabbits was minimal (less than 1%).

In rats and mice the major route of elimination is via faeces as intact diflubenzuron and via bile and urine after absorption. After single dosing the excretion is almost complete after 24 to 48 hours. In rats at doses of 4 mg/kg bw up to 28% of the dose could be found in urine, approximately 30% in bile and 36% in faeces. Biliary and urinary elimination decreases with increasing dose in a dose dependent manner. Following repeated dosing the excretion of diflubenzuron and metabolites was slightly slower than after a single dose in rats, being almost complete after 48 to 96 hours. In rabbits, urinary elimination seems to be the major route. After a single oral dose of 1 mg/kg bw 62% of the dose was excreted in urine after 48 hours. Less than 1% of an oral dose is recovered in exhaled air.

4. The major route of metabolism in rats is via hydroxylation of the phenyl moieties of diflubenzuron (approximately 80%). Another pathway is cleavage of the benzoyl-ureido bridge (20%). In urine and bile from rats the major metabolites were determined by HPLC or TLC/MS and identified as 2,6-difluoro-3-hydroxy-diflubenzuron, 2,6-difluorobenzoic acid, 2-hydroxy-diflubenzuron and 4-chloro-2-hydroxy- and 4-chloro-3-hydroxy-diflubenzuron. Other metabolites were 2,6-difluorohippuric acid and 2,6-difluorobenzamide. The cleavage product 4-chlorophenyl urea is also found but to a lesser extent (approximately 3 to 5%). In rats given a very high dose of diflubenzuron (100000 mg/kg feed, equal to 7.8 g/kg bw/day) for 4 days, the metabolite 4-chloroaniline was detected in urine, although in very low concentrations (less than 0.01% of the dose absorbed).
5. Many of the toxicity studies were carried out approximately 20 years ago and do not meet modern standards of design or reporting. Furthermore, the purity of diflubenzuron and data on stability in feed were not reported in many of these studies. However, some of the repeated dose toxicity studies and the carcinogenicity studies were performed according to current requirements.

Diflubenzuron was shown to have a low acute toxicity in all tested species (rats, mice and rabbits). After oral administration of diflubenzuron in rats and mice the LD₅₀ was higher than 4640 mg/kg bw. After dermal exposure of diflubenzuron in rats and rabbits the LD₅₀ was higher than 2000 mg/kg bw. The intraperitoneal LD₅₀ in mice was higher than 2150 mg/kg bw. After inhalation of diflubenzuron the LC₅₀ in rats and rabbits was more than 2.49 mg/l air and 3.75 mg/l air, respectively. The only signs of toxicity seen were lethargy, slight dermal irritation and slight decreases in body weight gain in rabbits.

6. Fourteen repeated dose toxicity studies were performed. Diflubenzuron was evaluated in four strains of rats (SPF, ALA:CFY rats, orally for 4 weeks, Wistar rats orally for 13 weeks, Sprague Dawley rats, orally for 13 weeks and 9 weeks, inhalation for 3 weeks, Charles River CrI:CD rats, dermal for 3 weeks), two strains of mice (CFLP mice, orally for 6 weeks and 14 weeks, B6C3F1 mice, orally for 13 weeks), dogs (Beagle, orally for 6 weeks, 13 weeks and 52 weeks) and rabbits (New Zealand White, dermal for 3 weeks and inhalation for 3 weeks). Oral doses of diflubenzuron were 3.125 to 100 000 mg/kg feed in rats, 16 to 50 000 mg/kg feed in mice and 10 to 160 mg/kg feed or 2 to 250 mg/kg bw in dogs. Dermal doses were 20 to 1000 mg/kg bw/day in rats and 69.6 to 322.5 mg/kg bw/day in rabbits. Doses of diflubenzuron after inhalation were 0.12 to 1.85 mg/l air in rats and 0.15 to 1.99 mg/l air in rabbits. In some of these studies the doses in mg/kg bw were not given.

The primary target organs for the toxicity of diflubenzuron after repeated dosing were erythrocytes, the liver and the spleen. Diflubenzuron causes methaemoglobinaemia and sulfhaemoglobinaemia. Dose dependent methaemoglobinaemia was demonstrated after oral, dermal and inhalatory exposure. This effect is the most sensitive toxicological parameter and can be regarded as the toxicological end-point. The 4-chloroaniline concentration in erythrocytes (up to 392 ng/g) may explain the occurrence of methaemoglobinaemia. Other toxicological effects reported were increased activity of liver enzymes, increased organ weights and histopathological findings, e.g. haemosiderosis and mild cell necrosis. NOELs could only be established in the oral 13-week study in Wistar rats as 12.5 mg/kg feed and in the 6-week oral study in mice as 16 mg/kg feed, equal to 2 mg/kg bw/day. However, a limited range of parameters was investigated in the mouse study. In the dog studies NOELs after oral administration could be established in the 6-week study as 4 mg/kg bw/day and in the 52-week study as 2 mg/kg bw/day. The lowest NOEL established in the dog studies was 2 mg/kg bw/day (52-week oral study) based on methaemoglobin formation.

7. Tolerance studies have been performed in Atlantic salmon. Diflubenzuron was well tolerated in Atlantic salmon at single doses up to 1030 mg/kg bw (333 times the recommended dose) and after repeated doses up to 100 mg/kg bw (33 times the recommended dosage level) for 21 days (50% longer duration than the recommended). No significant adverse effects were observed as for mortality, behaviour, inappetence or histopathological lesions.
8. Reproductive toxicity studies were performed in rats and rabbits. Two 2-generation studies in rats (CD strain) with doses up to 160 mg/kg feed and doses up to 50 000 mg/kg feed, respectively, and a one-generation study in rats (ALA:CFY strain) with the doses 1000 and 100 000 mg/kg feed were conducted. There were no treatment-related effects on mating performance, pregnancy rate, duration of gestation, litter parameters or on the type and distribution of abnormalities in the studies. However, in the one-generation study and in the two-generation study with doses from 500 up to 50 000 mg/kg feed in rats dose related toxic effects were seen both in the parent generation and the offspring from all treatment groups. The toxic effects demonstrated were in the liver and spleen e.g. increased weights and centrilobular hepatocyte enlargement and on haematological parameters, e.g. increased values of methaemoglobin in the parents of both generations. A dose of 50 000 mg/kg feed induced adverse effects on the pre-weaning development of offspring. Thus, a NOEL for maternal toxicity could not be established from these studies but a NOEL for reproductive functions of 50 000 mg/kg feed (corresponding to 2403 to 8020 mg/kg bw) can be established.
9. Two teratogenicity studies have been performed in rats (strain CD) using oral doses of 1 to 4 mg/kg bw and 1000 mg/kg bw, respectively, and two studies in rabbits (New Zealand white) using oral doses of 1 to 4 mg/kg bw and 1000 mg/kg bw, respectively. None of the doses elicited maternal toxicity or any evidence of foetotoxicity or teratogenicity. For these effects the NOEL could be established as more than 1000 mg/kg bw.
10. The mutagenic potential of diflubenzuron was investigated in a battery of properly conducted *in vitro* and *in vivo* studies. Tests for gene mutations in prokaryotic systems (5 *Salmonella*-microsomal assays) and in eukaryotic systems (L5178Y mouse lymphoma cells, unscheduled DNA synthesis in rat hepatocytes and in W1-38 cells) as well as tests for chromosomal aberrations (mammalian cytogenetic test, micronucleus test, dominant lethal test in mouse) have been performed. All studies performed showed negative results for diflubenzuron. In addition, two *in vitro* studies (host-mediated transplacental carcinogen assay and malignant transformation in BALB/3T3 cells) were performed which showed that diflubenzuron did not induce cellular transformation. Thus, it can be concluded that diflubenzuron is devoid of mutagenic potential.
11. Two long-term toxicity/carcinogenicity studies were performed in Sprague-Dawley rats where diflubenzuron was administered in the diet for 104 weeks. In the first one (non-GLP) dose levels of 0, 10, 20, 40 and 160 mg/kg feed, equal to 0, 0.35, 0.70, 1.43 and 5.83 mg/kg bw/day for males and 0, 0.43, 0.88, 1.73 and 7.05 mg/kg bw/day for females were used, and in the second one (GLP-compliant) dose levels of 0, 156, 625, 2500 and 10 000 mg/kg feed, equal to 0, 5.8 to 12.5, 23.7 to 49.5, 91.4 to 194.3 and 238 to 790 mg/kg bw/day for males and 0, 7.4 to 15.5, 28.2 to 63.9, 94.7 to 248 and 493 to 1156 mg/kg bw/day for females were used. The tumour profile of treated rats was similar to that of controls in both studies and there was no evidence to suggest that diflubenzuron is carcinogenic in rats. However, in the non-GLP study, survival was too low. Based on elevated levels of methaemoglobin, the NOEL in this study was 40 mg/kg feed, equal to 1.43 mg/kg bw/day for males and 1.73 mg/kg bw/day for females. In the GLP-study no NOEL could be retained as effects (decreased values for the myeloid:erythroid ratio, elevated methaemoglobin and sulfhaemoglobin values and haemosiderosis) were seen in rats at the lowest dose tested (5.8 to 12.5 mg/kg bw).

In a 80-week non-GLP tumourigenicity study in CFLP mice, diflubenzuron was given via the diet at dose levels of 0, 4, 8, 16 and 50 mg/kg feed, equal to 0, 0.34, 0.67, 1.39 and 4.30 mg/kg bw/day for males and 0, 0.42, 0.80, 1.58 and 4.87 mg/kg bw/day for females. A positive trend in the incidence of lymphosarcoma was seen in female mice killed at termination. However, pairwise comparisons resulted in significance only in the 8 mg/kg feed group and the combined findings of lymphosarcoma in the treated female mice dying during the treatment and killed at termination of the study was not significantly different from control. The incidence of all types of lymphoreticular tumours in female mice dying during the study or combined with those killed after 80 weeks was not statistically significant.

The overall conclusion from the four rat and mouse studies, taking into consideration the negative results of the mutagenicity studies, is that there is no evidence for carcinogenicity of diflubenzuron in mice and rats.

12. Diflubenzuron (10% diluted in maize oil, 10% and 30% in vaseline and 1:1 mixture of Freund's adjuvant and maize oil) was tested for delayed hypersensitivity in albino guinea pigs. There was no statistical difference between treated and control animals with respect to the incidence of erythema and oedema. No evidence of delayed contact hypersensitivity in guinea pigs treated with diflubenzuron could be seen.

The potential of diflubenzuron to produce skin or eye irritation was investigated in rabbits. Diflubenzuron was considered to be a marginal or very slight irritant to the eye. The skin irritancy of diflubenzuron technical grade could not be evaluated because of a poorly reported study. However, diflubenzuron VC-90 (containing 92.2% diflubenzuron technical grade) can be considered to be non-irritating to rabbit skin.

13. Studies on microbiological properties of diflubenzuron were not submitted and are not considered to be necessary in view of the nature of the compound.
14. No studies were submitted on observations in humans.
15. The Joint FAO/WHO Meeting on Pesticides Residues (JMPR) evaluated diflubenzuron in 1981, 1984 and 1985 and the International Programme on Chemical Safety (IPCS) in 1996. They both established an ADI of 0.02 mg/kg bw based on NOELs for methaemoglobin formation in the submitted long-term toxicity/carcinogenicity studies in dogs (2 mg/kg bw), rats (40 mg/kg feed, corresponding to approximately 2 mg/kg bw) and mice (16 mg/kg feed, corresponding to approximately 2.4 mg/kg bw) and a safety factor of 100. The values for feed consumption were standard figures and not those measured in the rat and mouse studies.
16. A toxicological ADI of 0.0124 mg/kg bw (equivalent to 744 µg/person) was established for diflubenzuron based on the NO(A)EL of 16 mg/kg feed, equal to 1.24 and 1.44 mg/kg bw/day for males and females, respectively, derived from the long-term toxicity/carcinogenicity study in mice, which were considered as the most sensitive species, and applying a safety factor of 100.
17. The pharmacokinetics of diflubenzuron in the target species Atlantic salmon was studied after a single dose of 75 mg/kg of radioactively (¹⁴C) labelled diflubenzuron at +8°C and after a single intravenous dose and oral dose of 3 mg/kg bw at a water temperature of +6°C.

Diflubenzuron was only partially absorbed from the gastro-intestinal tract. After administration of high doses (75 mg/kg or 25 times the recommended dose) only 3.7% of the dose was absorbed after 12 hours. After administration of the recommended dose, the bioavailability was calculated to be 31% at a water temperature of +6°C. The absorption of diflubenzuron is therefore considered to be dose dependent and saturable in the target species. The kinetics of diflubenzuron after oral treatment at a water temperature of +6°C followed a one-compartment open model with first order input and first order output with a lag time of 3.5 hours. The mean peak plasma level (0.141 µg/ml) was reached after 24 hours. Autoradiography showed distribution of diflubenzuron residues in the liver, kidney, brain, bile, fat and cartilage. The highest recovery, 10% of the administered dose, was found in the fillet 1 day post dose. The highest concentrations were found in the liver although they only accounted for less than 0.3% of the given dose. The radioactivity in bile was very high indicating that the biliary route is the major excretory pathway. The elimination half life at +6°C was calculated to be 71.4 hours.

18. The metabolism in salmon was studied after single dosing (single dose of radiolabelled diflubenzuron) or multiple dosing (13 days of feeding of unlabelled diflubenzuron followed by a single dose of radiolabelled diflubenzuron) at the recommended dose of 3 mg/kg bw (water temperature +15°C). Diflubenzuron was metabolised and rapidly excreted, mainly via the bile. Six hours after administration 39% of the radioactivity in bile was identified as diflubenzuron. One and 4 days after administration most of the radioactivity in bile derived from water-soluble metabolites. Chromatographic analysis with radio-HPLC of fillet revealed three components. The major component was identified as diflubenzuron, 98.75%, 99.16% and 99.47% of total residues as determined by chromatography after 1, 4 and 7 days, respectively after repeated dosing (13 days feeding of unlabelled diflubenzuron and 1 day with radiolabelled compound given 3 mg/kg orally) and 97.39% of total residues as determined by chromatography 1 day post treatment after a single dose of radiolabelled diflubenzuron. Furthermore, one metabolite was identified as 4-chlorophenyl urea with maximum concentration of 0.23 µg/kg at 4 days post dose. The third component was not identified (less than 7 µg/kg) but the retention time was in the same range as for 4-chloroaniline. In the liver five components were found. Three components were identified as diflubenzuron, 4-chloroaniline (less than 3 µg/kg) and 4-chlorophenyl urea (less than 9 µg/kg). The two unidentified metabolites were probably mono-hydroxylated products of diflubenzuron.
19. The concentration of marker residue (diflubenzuron) to total residues in salmon (weight 391 to 870 g) was evaluated after single dosing and multiple dosing (13 days feeding of unlabelled diflubenzuron and 1 day with radiolabelled compound) with the recommended dose 3 mg/kg via gavage at a water temperature of +15°C. In fillet (muscle and skin) the total residues after repeated dosing were 466, 117 and 26 µg equivalents diflubenzuron/kg at 1, 4 and 7 days post dose, respectively, and after single dosing the values were 447 and 21 µg equivalents diflubenzuron/kg at 1 and 7 days post dose, respectively. The total residues in liver after repeated dosing were 811, 334 and 181 µg equivalents diflubenzuron/kg at 1, 4 and 7 days post dose, respectively, and after single dosing the values were 943 and 192 µg equivalents diflubenzuron/kg at 1 and 7 days post dose, respectively.

Concentrations of diflubenzuron in fillet, analysed with radio-HPLC, after repeated dosing (13 days feeding of unlabelled diflubenzuron and 1 day with radiolabelled compound given 3 mg/kg orally) were 389, 99.6 and 21.4 µg diflubenzuron/kg at 1, 4 and 7 days post dose, respectively, and after single dosing the value was 410 µg diflubenzuron/kg 1 day post dose. These values result in a ratio of marker residue versus total radioactive residues of 83%, 85% and 82% at 1, 4 and 7 days post dose in fillet after repeated dosing and 92% at 1 day post dose after a single dose, reflecting the low metabolism in Atlantic salmon.

20. Three non-radiometric residue depletion studies that meet the requirements in Volume VI of the Rules Governing Medicinal Products in the European Community were conducted in Atlantic salmon at water temperatures of +15°C and +6°C. The concentrations were measured with an HPLC method with UV detection and a quantification limit for diflubenzuron of 50 µg/kg.

Atlantic salmon (600 to 1346 g) were fed diflubenzuron daily as medicated feed *ad libitum* for 30 minutes each day, for 14 days at a water temperature of +15°C and at a dose of 3.19 mg/kg bw. Liver and fillet (muscle and skin in natural proportions) were analysed on day 1, 7, 14 and 21 post treatment. In the fillet, 1550 (350 to 3080) µg/kg, 200 (70 to 330) µg/kg, less than 50 µg/kg and less than 50 µg/kg of diflubenzuron were measured (mean of ten fish) on day 1, 7, 14 and 21 post treatment, respectively. In the liver, 2170 (720 to 3400) µg/kg, 260 (120 to 350) µg/kg, 40 (less than 50 to 80) µg/kg and less than 50 (less than 50 to 60) µg/kg of diflubenzuron were measured on days 1, 7, 14 and 21 post treatment, respectively. The individual values for each fish are in a broad range probably because of the different weights of the individual fish and of the feeding *ad libitum* which results in different doses in each individual fish.

In another study Atlantic salmon (619 to 1344 g) were fed diflubenzuron daily as medicated feed *ad libitum* for 30 minutes each day, for 14 days at a water temperature of $+6^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and at a dose of 2.9 mg/kg bw. Liver and fillet (muscle and skin in natural proportions) were analysed on days 1, 7, 14 and 21 post treatment. In the fillet, 2240 (980 to 3670) $\mu\text{g}/\text{kg}$, 400 (120 to 680) $\mu\text{g}/\text{kg}$, 100 (30 to 270) $\mu\text{g}/\text{kg}$ and 40 (30 to 80) $\mu\text{g}/\text{kg}$ of diflubenzuron were measured (mean of 10 fish) on day 1, 7, 14 and 21 post treatment, respectively. In the liver, 3190 (1790 to 4860) $\mu\text{g}/\text{kg}$, 730 (530 to 990) $\mu\text{g}/\text{kg}$, 120 (60 to 280) $\mu\text{g}/\text{kg}$ and less than 50 $\mu\text{g}/\text{kg}$ of diflubenzuron were measured on day 1, 7, 14 and 21 post treatment, respectively. The individual values for each fish are in a broad range probably because of the different weights of the individual fish and of the feeding *ad libitum* which results in different doses in individual fish.

One more study was performed in Atlantic salmon (5000 g) given approximately 2.66 mg diflubenzuron/kg bw as medicated pellets *ad libitum* 6 hours a day, at a water temperature of $+14.6^{\circ}\text{C}$ to $+15.5^{\circ}\text{C}$ for 14 days. Liver, muscle and skin samples were collected and analysed on day 5, 14, 21 and 28 post treatment. In the muscle, 900 (530 to 1900) $\mu\text{g}/\text{kg}$, 100 (less than 50 to 170) $\mu\text{g}/\text{kg}$, less than 50 (less than 50 to 500) $\mu\text{g}/\text{kg}$ and less than 50 $\mu\text{g}/\text{kg}$ of diflubenzuron were measured on day 5, 14, 21 and 28 post treatment, respectively. In the skin, 320 (less than 50 to 520) $\mu\text{g}/\text{kg}$ of diflubenzuron was measured day 5 post treatment and less than 50 $\mu\text{g}/\text{kg}$ the other days. In liver, 520 (less than 50 to 890) $\mu\text{g}/\text{kg}$, 70 (less than 50 to 150) $\mu\text{g}/\text{kg}$, less than 50 $\mu\text{g}/\text{kg}$ and less than 50 $\mu\text{g}/\text{kg}$ of diflubenzuron were measured day 5, 14, 21 and 28 post treatment. The individual values for each fish are in a broad range probably because of the feeding *ad libitum*, which results in different doses in each individual fish.

21. It was concluded from the presented studies that the parent compound diflubenzuron can be considered as the marker residue and that the ratio of marker residue to total residues of 92% at 1 day after single dosing can be used for calculating an MRL for diflubenzuron.
22. The routine analytical method for the determination of residues in muscle and skin of Atlantic salmon is based on HPLC with UV detection. The method was validated in accordance with Volume VI and presented in the ISO 78/2 format. The limit of quantification is 0.05 mg/kg for muscle and skin in natural proportions and the limit of detection is 0.02 mg/kg for muscle and skin in natural proportions

Conclusions and recommendation

Having considered that:

- an ADI of 0.0124 mg/kg bw (i.e. 744 $\mu\text{g}/\text{person}$) is set for diflubenzuron,
- allowing for the partitioning of food intake from vegetable and animal origin approximately 55% of the ADI should be allowed for pesticidal use of diflubenzuron,
- diflubenzuron is the marker residue and a ratio of marker residue to total residues of 0.92 can be assumed from the studies performed,
- a validated routine analytical method is available to quantify residues in muscle and skin in natural proportions;

the Committee recommends the inclusion of diflubenzuron in Annex I of Council Regulation (EEC) No 2377/90 in accordance with the following table:

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Diflubenzuron	Diflubenzuron	Salmonidae	1000 $\mu\text{g}/\text{kg}$	Muscle and skin in natural proportions	

Based on this MRL value, the daily intake of diflubenzuron from veterinary use in fish will represent about 43% of the ADI.