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Diflubenzuron

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3. Diflubenzuron

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Identity

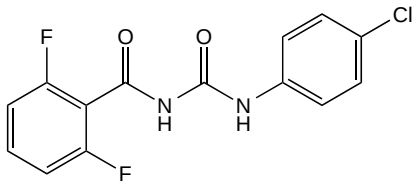
International Non-proprietary Name (INN): Diflubenzuron

Synonyms: Releeze 0.6 g/kg (EWOS AS), EWOS DFB (FAV Recalcine), Dimilin, Micromite, Adept, Du-Dim, Device, DU 112307, PH 60-40, TH 6040, ENT-29054, OMS 1804 (Crompton BV trade names and/or past development codes).

IUPAC Name: 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea

Chemical Abstract Service Number: 35367-38-5

Structural formula:



Molecular formula: $C_{14}H_9ClF_2N_2O_2$

Molecular weight: 310.7 g mol^{-1}

Other information on identity and properties

Pure active ingredient:	Diflubenzuron (purity ≥ 95%)
Appearance:	White crystalline solid
Melting point:	228 °C
Solubility in water:	0.08 mg/L at 25 °C at pH 7
Solubility in acetonitrile:	2.0 g/L
Solubility in acetone:	6.5 g/L
Solubility in dichloromethane:	1.8 g/L
Solubility in n-hexane:	0.063 g/L
Vapor pressure:	≤ 1.2 x 10^{-7} Pa at 25 °C
Log $K_{o/w}$:	3.89 at 22 °C at pH 3
UV_{max}:	257 nm

Background

Diflubenzuron (CAS No. 35367-38-5), besides its use in agriculture, horticulture and forestry against larvae of Lepidoptera, Coleoptera, Diptera, Hymenoptera, and in public health against larvae of mosquitoes, is used as a veterinary drug for the treatment of sea lice (Lepeophtheirus salmonis Krøyer and Caligus rogercresseyi Boxshall and Bravo, 2000) infestations in Atlantic salmon (Salmo salar L.). Diflubenzuron acts by interference with the synthesis of chitin. Demand for chitin synthesis is greatest at the moult between growth stages and hence parasites are killed due to disruption of the moulting process. The fatal effect occurs by the inability of the treated parasites to moult properly due to incomplete development of chitin, with subsequent collapse of the exoskeleton.

The toxicity of diflubenzuron was evaluated by the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) in 1981 (JMPR, 1982), 1984 (JMPR, 1985) and 1985 (JMPR, 1986); an ADI of 0 – 0.02 mg/kg bw, based on NOAELs for methaemoglobin formation in the submitted long-term toxicity/carcinogenicity studies in dogs, rat and mice, was established at the latter Meeting. This ADI was maintained by a 1994 WHO Core Assessment Group that prepared Environmental Health Criteria 184. The USA EPA published a Re-registration Decision for diflubenzuron in August 1997 (EPA, 1997). Diflubenzuron has also been reviewed by the European Commission under Directive 91/414/EC and a MRL of 1000 µg/kg, pursuant to Directive 2377/90, based on an ADI of 0.0124 mg/kg bw/day using the mice studies and applying a safety factor of 100, was published in 1999 (EMEA, 1999).

Under the periodic review program, toxicology data for diflubenzuron were re-evaluated by JMPR in 2001 (WHO, 2002) and residues in 2002 (JMPR, 2002) and 2011 (JMPR, 2012). The JMPR has concluded that the long-term intake of residues of diflubenzuron in food resulting from its uses that have been considered by JMPR is unlikely to present a public health concern.

At the 22nd Session of the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF), concerns were raised about the metabolism of diflubenzuron and formation of the genotoxic metabolite, 4-chloroaniline (p-chloroaniline or PCA). Following discussions, the Committee noted that an ADI of 0-0.02 mg/kg body weight had previously been established by JMPR for diflubenzuron and requested JECFA to recommend MRLs for diflubenzuron in salmon muscle and skin in natural proportion.

Residues in food and their evaluation

Conditions of use

Diflubenzuron is a benzoylurea pesticide used in aquaculture for the treatment of sea lice in Atlantic salmon in the Northern hemisphere and sea lice infestation in salmon in the Southern hemisphere.

Diflubenzuron was first registered as an insecticide in the United States in 1979 (Patterson 2004) and is also used in agriculture, horticulture and forestry against larvae of Lepidoptera, Coleoptera, Diptera, Hymenoptera and in public health against larvae of mosquitoes and other noxious insects.

Dosage

Diflubenzuron is licensed in Norway as a premix (90% pre-concentrate in pelleted diet) at a final concentration of 0.6 g diflubenzuron per kg. The intended oral dose is 3 mg diflubenzuron per kg of fish biomass per day for fourteen consecutive days. The recommended withdrawal period is 105 degree-days. The number of treatment periods per year could be two to three.

In Chile, diflubenzuron is licensed as an oral powder (80% w/w) with an intended oral dose of 6 mg diflubenzuron per kg of fish biomass per day for fourteen consecutive days. The recommended withdrawal period is 300 degree-days.

Pharmacokinetics and metabolism

Pharmacokinetics in laboratory animals

Rats

Single oral doses of 4 to 1000 mg/kg bw, a repeated oral dose of 5 mg/kg bw per day for 14 days, and single dermal doses of 0.05 and 0.5 mg/10 cm^2 of diflubenzuron were administered to rats (EMEA, 1999). Diflubenzuron is absorbed from the gastrointestinal tract and the absorption decreases with increasing dose. Following a dose of 4 mg/kg bw, 42.5% of diflubenzuron was absorbed; however, only 3.7% of a dose of 900 mg/kg bw was absorbed. In rats administered a single oral dose of [^{14}C]-diflubenzuron at 5 mg/kg bw, the highest mean concentrations of radioactivity at 4 h were found in fat (4672 µg eq /kg), ovaries (3737 µg eq /kg), liver (2265 µg eq /kg), heart (1345 µg eq /kg), kidney (1200 µg eq /kg) and brain (984 µg eq /kg). At 48 hours post dose and subsequent times, the highest concentrations were in liver (431 µg eq /kg) and erythrocytes (379 µg eq /kg). No difference was observed in results between males and females. Dermal absorption of diflubenzuron was less than 1%.

The major route of elimination of diflubenzuron is via faeces, urine and bile, as intact diflubenzuron (EMEA, 1999). After administration of a single dose of diflubenzuron, excretion is almost complete within 24 to 48 h, whereas following repeated dosing, the excretion of diflubenzuron and metabolites is slightly slower, being almost complete only after 48 to 96 h. After a single dose of diflubenzuron of 4 mg/kg bw, up to 28%, 30% and 36% of the administered drug could be found in urine, bile and faeces, respectively. Biliary and urinary elimination decreases with increasing dose in a dose dependent manner.

In a study to investigate the intestinal absorption of diflubenzuron in Wistar rats, a mixture labelled with ^{14}C in the amino moiety (31.1 mCi/g) and ^{3}H in the 2,6-difluorobenzoyl moiety (6.3 mCi/g) of diflubenzuron was used (Willems *et al*., 1980). The radiochemical purity, determined by TLC, was >99%. The radiolabelled compound, in suspension (1% tragacanth solution), at doses ranging from 4 mg/kg bw to 1000 mg/kg bw, was administered to female and male rats by gavage. Urine was collected for 6 and 24 h, and also at further 24 h intervals. Faeces were collected at 24 h intervals for 3 days, and then at the conclusion of the experiment. In a second group of female rats, cannulation of the bile duct was performed. Bile was collected at 6, 24, 48 and 72 h, while urine and faeces were collected at 24-h intervals for 72 h. The

cumulative excretion of radioactivity in urine and faeces after oral administration of radiolabelled diflubenzuron is shown in Table 3.1.

Table 3.1. Cumulative excretion (6 days) of radioactivity in urine and faeces after oral administration of [^{3}H, ^{14}C]-diflubenzuron (dose of 5 mg/kg bw) to rats. Results are mean values of 6 animals, with standard deviation in parentheses (Willems *et al*., 1980).

	Percentage of dose	
	[^{3}H]-benzoyl moiety of diflubenzuron	**[^{14}C]-anilino moiety of diflubenzuron**
Urine	24 (3.6)	22 (3.5)
Faeces	69 (3.8)	50 (1.7)
Total	93 (3.6)	72 (3.0

The cumulative excretion of radioactivity in bile and urine over a period of 72 h after oral administration of radiolabelled diflubenzuron to rats with cannulated bile ducts is shown in Table 3.2.

Table 3.2. Cumulative excretion of radioactivity in bile and urine during 72 h after oral administration of [^{3}H, ^{14}C]-diflubenzuron (dose of 5 mg/kg bw) to rats with cannulated bile ducts. Results are given for each of two rats (Willems *et al*., 1980).

	Percentage of dose	
	[^{3}H]-benzoyl moiety of diflubenzuron	**[^{14}C]-anilino moiety of diflubenzuron**
Bile	32 and 23	41 and 27
Urine	19 and 20	22 and 24

In rats with (Table 3.2) and without (Table 3.1) cannulated bile ducts, about 20% of the administered ^{3}H and ^{14}C radiolabelled dose was excreted in the urine. In the bile, an average of 33% of the dose was recovered, with no significant difference between the different labels. The results (sum of the urinary and biliary excretions) indicate that about half of the administered dose was absorbed.

The intestinal absorptions, as a function of dose level, are shown in Tables 3.3 and 3.4. The percentage of the dose excreted in the urine decreased with increasing dosage, while total recoveries remained constant. In bile-cannulated rats, the proportion of biliary to urinary excretion does not change significantly as the dose was increased.

Table 3.3. Excretion of radioactivity in urine and faeces after oral administration of [^{14}C]-diflubenzuron to rats. Duration of the experiment: 120 h. Results are mean values of 6 animals, with standard deviation in parentheses (Willems *et al*., 1980).

Dose (mg/kg)	Sex	Cumulative excretion as % of dose	
		Urine	Urine and faeces
4	female	27.6 (1.4)	88.3 (1.2)
16	female	13.0 (0.7)	86.7 (3.6)
48	male	6.2 (0.9)	92.4 (3.3)
128	female	2.7 (0.3)	91.1 (1.6)
128	male	3.4 (0.5)	91.2 (4.2)
1000	male	1.0 (0.1)	84.5 (9.1)

Table 3.4. Urinary and biliary excretion of radioactivity in female rats with cannulated bile ducts after oral administration of [^{14}C]-diflubenzuron. Duration of the experiment: 72 h. Results are mean values, with standard deviation in parentheses (Willems *et al*., 1980).

Dose (mg/kg)	Number of rats	Cumulative excretion as % of dose		
		Urine	Bile	Total
4	3	12.0 (1.0)	30.4 (5.2)	78.1 (1.3)
16	4	7.7 (1.1)	16.4 (1.6)	78.1 (9.2)
128	4	2.9 (0.4)	6.4 (1.8)	84.0 (3.4)
900	4	2.2 (1.1)	1.5 (0.4)	78.8 (8.7)

The data show that the intestinal absorption, measured as the sum of urinary and biliary excretion, diminished with increasing dose, from about 50% at 4 mg/kg to about 4% at 900 mg/kg.

Bluegill sunfish

Diflubenzuron is accumulated from water into fish tissue at levels up to 80-fold. When bluegill sunfish (*Lepomis macrochirus,* 7 cm length) were exposed to water containing 10 µg/L of diflubenzuron for 24 h, 48 h and 72 h, tissue residues were 158, 306 and 266 µg/kg, respectively (Schaefer *et al*., 1979). After 24 to 48 h exposure, fish degrade and eliminated diflubenzuron and the excretory products were neither the parent compound nor *p*-chlorophenylurea.

The bioconcentration of [^{14}C]-diflubenzuron by bluegill sunfish was also evaluated in a dynamic 42-day study (28 days of treatment with diflubenzuron followed by 14 days

depuration) (IPCS, 1996). Radioanalyses of fillet, whole fish and visceral portions were performed throughout the exposure period. Daily bioconcentration factors ranged from 34 to 200, 78 to 360, and 100 to 550 for fillet, whole fish and viscera, respectively. Tissue concentrations of [^{14}C]-diflubenzuron ranged from 0.25 to 1.7 mg/kg for fillet, 0.58 to 3.3 mg/kg for whole fish, and 0.75 to 4.7 mg/kg for viscera. Radioanalysis throughout the depuration period (test fish were placed in clean water for 14 days) indicated 99% depuration each for fillet, whole fish and viscera. The mean concentrations of [^{14}C]-diflubenzuron in fillet decreased from 1.6 mg/kg on day 28 of exposure to 0.012 mg/kg by day 14 of the depuration period. Residue concentrations in whole fish decreased from 3.3 mg/kg on day 28 of exposure to 0.038 mg/kg by day 14 of the depuration period. Concentrations in viscera depleted from 4.4 mg/kg on day 28 of exposure to 0.056 mg/kg by day 14 of depuration. The maximum bioaccumulation factor (550) found in the bluegill sunfish is much lower than that expected based on lipophilicity (7800), indicating rapid degradation and depuration. In addition to the parent compound (80%), 2,6-difluorobenzamide (10-13%) and three other minor metabolites were identified. 4-Chloroaniline was not detected (limit of detection 0.01 mg/kg).

Pharmacokinetic in food producing animals

Chicken

Pharmacokinetic parameters of [^{14}C]-diflubenzuron in White Leghorn (WL) egg-production chickens and Rhode Island Red/Barred Plymouth Rock (RIR/BPR) meat-production chickens were evaluated (Opdycke and Menzer, 1984). Three chickens of each type were given a single bolus intravenous dose of 1 mg/kg of [^{14}C]-diflubenzuron and 3 chickens of each type were given gelatine capsules containing radiolabelled diflubenzuron at a single dose of 5 mg/kg of [^{14}C]-diflubenzuron. Sequential blood samples were taken by heart puncture at 0.15, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 10, 12 and 22 or 24 hours after injection. Following oral administration, blood was sampled at 2.0, 4.0, 6.0, 8.0, 12, 18, 24, 30 and 36 h and assayed for radioactivity. In addition, excreta were collected continuously during the periods of frequent heart punctures. A two-compartment open model was assumed from the study, following intravenous administration of radiolabelled diflubenzuron. Absorption parameters were estimated using constants determined from the intravenous dose experiment. The half-life of elimination from the central compartment was 14.70 h for WL chickens and 8.45 h for RIR/BPR chickens. Absorption of radiolabelled diflubenzuron after a single oral dose of 5 m/kg bw was both faster and more complete in RIR/BPR chickens. The absorption rate constants were 0.046 h^{-1} and 0.192 h^{-1} for WL and RIR/BPR chickens, respectively. Comparison of the absorption patterns in WL and RIR/BPR chickens indicates both a much faster and greater absorption of diflubenzuron in the RIR/BPR than in the WL chickens. The concentrations of [^{14}C]-diflubenzuron in excreta are shown in Table 3.5.

Table 3.5. Percentage (mean ±SD) of [^{14}C]-diflubenzuron equivalents eliminated following oral and intravenous administration of the radiolabelled compound to chickens {WL: White Leghorn; and RIR/BPR: Rhode Island Red/Barred Plymouth Rock} (Opdycke and Menzer, 1984).

Time post-dose (h)	Percentage of [^{14}C]-diflubenzuron equivalents			
	Intravenous, 1 mg/kg		Oral, 5 mg/kg	
	WL	RIR/BPR	WL	RIR/BPR
0-12	4.2 ± 5	20 ± 4	35 ± 12	33 ± 13
12-24	7.5 ± 5	9 ± 4	10 ± 3[a]	18 ± 5
Total	11.7 ± 5	29 ± 4	45 ± 15	51 ± 18

[a] for 12-36 h; SD = Standard Deviation.

Excretion after a single intravenous dose showed rapid elimination, 11.7% and 29% of the administered dose in 22-24 h for WL and RIR/BPR chickens, respectively.

Salmon

The pharmacokinetic parameters of diflubenzuron in Atlantic salmon smolts (approx. 60 g, 22 fish) were studied after a single dose via gavage of 75 mg /kg bw of [^{14}C]-radiolabelled diflubenzuron at 8 °C (Horsberg and Hoy, 1991). The [^{14}C]-diflubenzuron (18.38 mg) was mixed with non-radiolabelled diflubenzuron (81.67 mg) and suspended in 7 mL peanut oil. After a 21-day acclimatization period, a stomach tube was inserted and 0.3 mL of the suspension was administered to each fish. After 2 h, 12 h, 2 d, 6 d, 10 d, 13 d, 20 d and 27 d, fish were slaughtered and 1 to 2 fish were sampled for autoradiography. Samples were taken from blood, brain, muscle, abdominal fat, kidney, liver, bile cartilage and cutaneous mucus. An estimate of the percentage of the administered 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. Whole-body autoradiography, liquid scintillation counting and TLC were used to evaluate the kinetic properties. The concentration of radioactivity in brain and cartilage was highest 12 h after administration, with concentrations of 13.8 µg/g and 10.9 µg/g, respectively. In bile, the concentration of radioactivity varied between 275 and 1066 µg/g the first 10 days after administration, then dropped to less than 4 µg/g for the rest of the period.

The calculated percentages of the administered dose, which were present in muscle, liver, blood and kidney, are shown in Figure 3.1. The highest amount of radioactivity was detected 12 h after administration of [^{14}C]-diflubenzuron. It was concluded that diflubenzuron is poorly absorbed from the intestine, because only 3.7% of the administered dose was detected in blood, muscle, liver and kidney 12 h after administration. The radioactivity in bile was very high, indicating that the major excretion pathway for the drug is the biliary route.

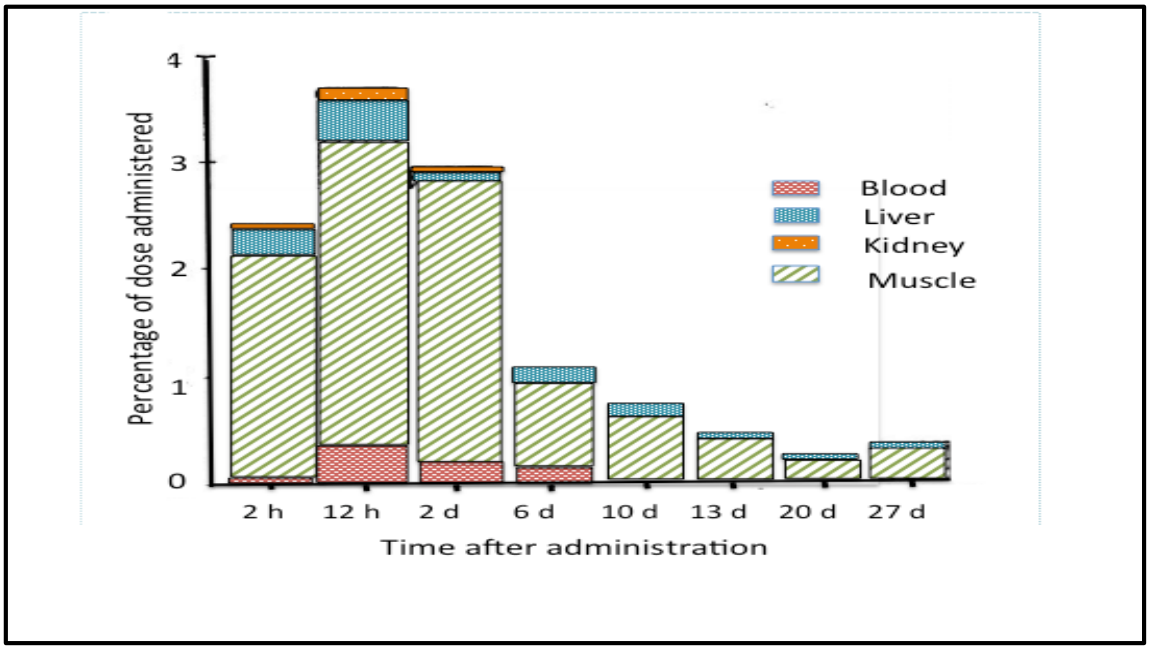


Figure 3.1. Percentage of the administered dose present in muscle, liver, kidney and blood at different intervals after oral administration of 4.3 mg/kg bw [^{14}C]-diflubenzuron to Atlantic salmon (Adapted from Horsberg and& Hoy, 1991).

In a field trial, conducted at a commercial fish farm in Norway, with full scale stocking densities of Atlantic salmon, the clearance time of diflubenzuron was established for liver, skin and muscle (Wallace *et al.,* 1997). The study was conducted at a water temperature of 14.6 to 15.6 °C. Diflubenzuron medicated pellets (0.63 g/kg) were administered to the fish by way of automatic feeding machines, for 14 consecutive days. The daily dose of diflubenzuron ranged from 2.66 to 3.2 mg/kg bw. The clearance times (days after treatment) were calculated using a first order kinetic model and were: 15 days for liver, 18 days for muscle and 14 days for skin.

Metabolism in laboratory animals

Rats

In rats, the major route of metabolism for diflubenzuron is via hydroxylation of the phenyl moieties of diflubenzuron (approximately 80%) and, to a lesser extent, cleavage of the benzoyl-ureido bridge (20%) (EMEA, 1999). The main metabolites identified by HPLC or TLC in urine and bile were 2,6-difluoro-3-hydroxy-diflubenzuron, 2,6-difluorobenzoic acid, 2-hydroxy-diflubenzuron and 4-chloro-2-hydroxydiflubenzuron, 4-chloro-3-hydroxydiflubenzuron, 2,6-difluorohippuric acid and 2,6-difluorobenzamide. The cleavage product 4-chlorophenyl urea also was identified in a concentration of approximately 3 to 5%. The metabolite 4-chloroaniline was detected at very low concentrations (less than 0.01% of the absorbed dose)in urine of rats given a very high dose of diflubenzuron (100 g/kg feed, equal to 7.8 g/kg bw/day) for 4 days.

In an ADME study, diflubenzuron was administered by gavage as [^{14}C]-diflubenzuron to male and female Wistar rats either at single dose of 5 or 100 mg/kg bw or at a dose level of 5 mg/kg bw following 14 days of non-radiolabelled diflubenzuron in the diet at a dose level of 5 mg/kg bw/day (EPA, 1997). An additional group of rats, with cannulated bile ducts, was also treated

with a single oral dose of 5 mg/kg bw of [^{14}C]-diflubenzuron. The rats only partially absorbed diflubenzuron from the gastrointestinal tract. In the bile duct cannulated rats, about 33% of the administered dose was absorbed and about 50% of the 33% (17% of the administered dose) was excreted in the bile. By the seventh day, 19-21% of the administered dose had been recovered from the urine and 77-80% from the faeces of rats receiving the lower doses of 5 mg/kg bw. Also by the seventh day, 3% of the administered dose had been recovered from the urine and 96% from the faeces of rats receiving the higher dose of 100 mg/kg bw. The half-life of radioactivity in blood was about 14 hours. Over 98% of the administered radioactivity had been excreted by the seventh day. Very little bioaccumulation in tissues was observed. The highest concentrations of radioactivity were observed in the erythrocytes and liver at 48 hours. Ten urinary metabolites were identified, including 4-chloroaniline and *p*-chlorophenylurea, which together accounted for about 2% of the administered dose (at 5 mg/kg). In the faeces, only unchanged parent compound was detected.

In another study, the metabolic fate of radiolabelled [^{14}C]- and [^{3}H]-diflubenzuron in Wistar rats was investigated (Willems *et al*., 1980). A mixture labelled with ^{14}C in the amino moiety (31.1 mCi/g) and ^{3}H in the 2,6-difluorobenzoyl moiety (6.3 mCi/g) of diflubenzuron was used. The radiochemical purity, determined by TLC, was greater than 99%. The radiolabelled compound, in suspension (1% tragacanth solution), at a dose of 5 mg/kg bw, was administered by gavage after a 16 h fast period. Urine was collected for 6 and 24 h and faeces at 24 h intervals for 72 h. Excretion was almost complete at 72 h after dosing and about 80% of the metabolites appeared to have the basic diflubenzuron structure. Two major routes of degradation were discernible, hydroxylation of the aromatic rings and scission of the benzoyl-ureido bridge. About 20% underwent cleavage of the ureido bridge but neither 4-chlorophenyl urea nor 4-chloroaniline was not present in urine or bile in appreciable quantities.

Metabolism in food producing animals

The metabolic fate of diflubenzuron has been evaluated in various species, including cattle, sheep, swine, chickens and salmon.

Cattle

In a non-GLP compliant study, a single oral dose of 10 mg/kg bw of [^{14}C]-diflubenzuron (equally labelled in both phenyl moieties, specific activity 17.4 µCi/mol, radiochemical purity > 99.0%) was administered, as a slurry in water, by stomach tube to a catheterized 360 kg lactating Jersey cow (Ivie, 1978). The [^{14}C]-diflubenzuron formulation in water was diluted with non-radiolabelled diflubenzuron such that the final treatment mixture contained 3.6 g of diflubenzuron active ingredient and a total of 0.65 µCi of radiolabelled diflubenzuron. After treatment, urine and faeces were collected at 24-hours intervals, and the cow was milked every 12 hours. Seven days after treatment, the animal was slaughtered and tissues collected for analysis of total radiocarbon residues. Radioactive residues in liquid phases were quantified by direct liquid scintillation counting. Metabolites in milk, urine, bile and faeces were resolved by 2-dimensional TLC. The metabolites determined in urine and faeces of samples are presented in Table 3.6.

Table 3.6. Metabolites in urine and faeces from a lactating cow after oral treatment with [^{14}C]-diflubenzuron (10 mg/kg bw) {Adapted from Ivie, 1978}.

	Percentage of TRR	
Metabolite	**Urine[a]**	**Faeces[b]**
2,6-Difluoro-3-hydroxydiflubenzuron	45.0	17.6
4-Chloro-2-hydroxydiflubenzuron	1.6	0.4
4-Chloro-3-hydroxydiflubenzuron	3.7	0.8
4-Chlorophenylurea	0.6	--
2,6-Difluorobenzoic acid	6.0	--
2,6 Difluorohippuric acid	6.9	--

[a] samples collected after 1 day treatment; [b] samples collected 2 days after treatment; -- = not detected.

It was verified that about 85% of the administered dose was eliminated in the faeces and 15% in the urine during the 7-day post treatment period. Only 0.2% was secreted into the milk. Analysis of tissue samples (brain, liver, kidney, muscle, fat and skin) collected 7 days after treatment revealed that only the liver contained appreciable radiocarbon residues, ranging from 2.3 to 3.6 mg eq/kg. Residues of 0.8 mg eq/kg found in skin were attributed to surface contamination through the faeces. In all other tissues collected, residues lower than 0.1 mg eq/kg were determined. In urine, 4 compounds remained unknown and in faeces another 4 compounds also remained unidentified.

In another metabolism study, dairy cows were dosed orally via capsule for up to 28 days with double ring-labelled [^{14}C]-diflubenzuron at rates equivalent to 0.05, 0.5, and 5 mg/kg in the diet (EPA, 1997). At the 0.05 and 0.5 mg/kg dose levels, no radioactive residues, expressed in diflubenzuron equivalents, were detectable in milk. At the 5 mg/kg dose level, radioactive residues in milk plateaued after 4 days between 6.3 and 13.4 µg/kg. After 28 days of dosing, radioactive residues in muscle, fat, and kidney were non-detectable at the 0.05 mg/kg, 0.5 mg/kg and 5 mg/kg dose levels. Radioactive residues in liver were 7.1 µg/kg at the 0.05 mg/kg level, 70.8 µg/kg at the 0.5 mg/kg level, and 540 µg/kg at the 5 mg/kg level.

Swine

[^{14}C]-Radiolabelled diflubenzuron was administered orally at a dose of 5 mg/kg bw (405 µCi) to a female Duroc-Poland China pig (46 kg) (Opdycke *et al*., 1982a). Urine and faeces were collected at 12-h intervals. After 11 days, the pig was slaughtered and samples of brain, heart, lung, liver, gallbladder, kidney, blood, lymph, fat, ovary and oviduct, stomach wall, pancreas, skin and bone were collected for diflubenzuron quantification. More than 88% of the administered dose was accounted for, with over 82% in the faeces and 5% in the urine. The highest concentrations of [^{14}C]-diflubenzuron equivalents were determined in the gallbladder

(0.43 mg/kg), fat (0.30 mg/kg) and liver (0.23 mg/kg). Metabolites identified by TLC and HPLC coupled to a UV detector (HPLC-UV) in the urine included 4-chlorophenyl urea (0.82% of dose), 2,6-difluorobenzoic acid (0.83% of dose), 4-chloroaniline (1.03% of dose) and 2,6-difluorobenzamide (0.29% of dose).

Sheep

In a similar non GLP-compliant study as reported for cattle (Ivie, 1978), the fate of diflubenzuron was evaluated in four mixed breed castrated male sheep (28-42 kg). For measurement of the elimination of radiocarbon in the bile, the bile ducts of two sheep were cannulated 7 days before treatment. One cannulated and one uncannulated sheep were treated orally with [^{14}C]-diflubenzuron by the same procedure described for the cattle (Ivie 1978). The other two sheep (one cannulated and one uncannulated) were treated orally with [^{14}C]-diflubenzuron at 500 mg/kg bw, in order to allow isolation of larger quantities of metabolites. Total urine, bile and faeces were collected at 24-h intervals after treatment. After 4 days, the two sheep treated at 10 mg/kg bw were slaughtered, and tissue samples were collected for combustion analysis. Analysis of tissue samples (brain, liver, kidney, muscle and fat) collected 4 days after treatment revealed that only the liver contained appreciable radiocarbon residues (3.6 mg eq/kg in the cannulated sheep and 2.30 mg eq/kg in the uncannulated sheep). Kidney samples from the bile-duct cannulated sheep contained low levels of radiocarbon, whereas the uncannulated sheep did not have detectable residues. In all other collected tissues, residues lower than 0.2 mg eq/kg were determined.

In the 4-day post-treatment period, the uncannulated sheep treated with 10 mg/kg bw eliminated 43% of the administered dose in the faeces and 41% in the urine. The cannulated sheep at the same dose eliminated 36% in the bile, 32% in the faeces and 24% in the urine. In the same period the uncannulated sheep treated with 500 mg/kg bw of radiolabelled diflubenzuron eliminated 79% in the faeces and 10% in the urine. The cannulated sheep at this high dose eliminated 5% in the bile, 74% in the faeces and 7% in the urine. The major radioactive component in all faeces extracts was identified as unmetabolized diflubenzuron (97.7% in the bile-duct cannulated sheep and 40.0% in the uncannulated sheep).

Although sheep had qualitatively similar metabolic profiles to cow, there were quantitative differences in the relative amounts of metabolites. The major metabolite in the cow urine resulted from hydroxylation of the 2,6-difluorobenzoyl ring and comprised almost half of the radiocarbon in the first day's urine sample. In contrast, this metabolite was a minor product in sheep urine, in which the major metabolites resulted from cleavage of the amide group at the benzoyl carbon forming 2,6-difluorbenzoic acid that was subsequently conjugated with glycine to the hippuric acid.

The metabolites determined in urine and faeces samples from sheep by 2D-TLC and identified by comparison with reference compounds, followed by mass spectrometry or NMR, are presented in Table 3.7.

Table 3.7. Metabolites in urine and faeces from sheep after oral treatment with [^{14}C]-diflubenzuron at a dose of 10 mg/kg bw (Adapted from Ivie, 1978)**.**

	Percentage of TRR			
	Bile-duct cannulated		Uncannulated	
Metabolite	**Urine[a]**	**Faeces[b]**	**Urine[a]**	**Faeces[b]**
2,6-Difluoro-3-hydroxydiflubenzuron	1.2	ND	1.4	0.4
4-Chloro-2-hydroxydiflubenzuron	0.8	ND	0.2	0.8
4-Chloro-3-hydroxydiflubenzuron	0.4	ND	ND	0.4
4-Chlorophenylurea	ND	ND	ND	
2,6-Difluorobenzoic acid	15.1	ND	26.7	
2,6 Difluorohippuric acid	30.2	ND	22.3	

[a] samples collected after 1 day treatment; [b] samples collected 2 days after treatment; ND = Not Detected (the LOD was not reported).

Goats

In a subsequent metabolism study, four lactating goats were dosed orally via capsule for 3 consecutive days with double ring-labelled [^{14}C]-diflubenzuron (EPA, 1997). Two goats were dosed at a rate of approximately 10 mg/kg in the diet and two at a rate of approximately 250 mg/kg. Radioactive residues in the faeces and urine accounted for approximately 88% of the administered dose for both low- and high-dose goats. After 3 days of dosing, total radiolabelled residues (TRRs) in the low-dose (10 mg/kg) goats were 7 to 9 µg/kg in milk, 217 to 262 µg/kg in liver, 16 to 19 µg/kg in kidney, 1 µg/kg or less in muscle, and at most 4 µg/kg in fat. TRRs in the high-dose (250 mg/kg) goats were 220 µg/kg in milk, 324 to 606 µg/kg in liver, 360 to 1020 µg/kg in kidney, 20 to 50 µg/kg in muscle, and 120 to 300 µg/kg in fat. The radioactive residues were characterized in milk and liver. Extraction of milk released 85% of the TRR. The principle residues identified consisted of *p*-chlorophenylurea (29-55% TRR) and 2,6 difluorobenzamide (6-8% TRR). 4-chloroaniline was non-detectable (less than 1 µg/kg) in milk from either low- or high-dose goats. Extraction of liver recovered 90% of the TRR. The principle residues identified were diflubenzuron (7% TRR), 2-hydroxydiflubenzuron (7% TRR), *p*-chlorophenylurea (16% TRR), and 2,6-difluorobenzamide (1% TRR). 4-Chloroaniline was not detectable in liver from the low dose goats but accounted for approximately 0.4% of the TRR (11 to 28 µg/kg) in the liver of the high-dose goats.

Chicken

The metabolism and fate of [^{14}C]-diflubenzuron in four White Leghorn (WL, 36 weeks old, about 1500 g) egg-production chickens and four Rhode Island Red/Barred Plymouth Rock

(RIR/RB, 46 weeks old, about 2600 g) meat-production chickens after single oral dose of 5 mg/kg bw (25 µCi to WL and 5 µCi to RIR/RB chickens) were investigated (Opdycke *et al.*, 1982b). Administration of the radiolabelled diflubenzuron was achieved by dissolving the drug into 4 mL of acetone and adding 1 mL to each of four gelatine capsules containing feed. Excreta were collected from individual chicken at 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 120, 192 and 288 or 312 h after treatment. The chickens were sacrificed after 12 and 13 days and samples of fat, liver, kidney, gizzard, ovary with internal eggs, breast, muscle, heart, brain and intestine were collected for analysis. Unextracted residues were combusted for radioassay. Diflubenzuron and metabolites in organic fractions were characterized by TLC co-chromatography and high performance liquid chromatography (HPLC) with the reference compounds. A total of 91% of the administered dose was recovered from the WL and 82% from the RIR/BPR excreta, respectively. Rapid elimination of 65% and 43% of the dose within the first 8 h after administration suggests similar excretion patterns for the WL and RIR/BPR chickens. Residual radioactivity in tissues is shown in Table 3.8.

Table 3.8. Residual radioactivity in tissues following treatment of chickens with a single oral dose of diflubenzuron ; 5 mg/kg oral dose, 25 µCi to WL and 5 µCi to RIR/RB chickens (Opdycke *et al.*, 1982b).

	Concentration of radioactivity (mg eq/kg)	
Tissue	**WL**	**RIR/BPR**
Fat	0.01	0.04
Liver	0.06	0.15
Kidney	0.19	0.14
Gizzard	0.01	0.04
Ovary with internal eggs	0.16	0.09
Breast muscle	0.01	0.03
Egg shells	0.40	ND
Brain	ND	0.25
Heart	0.01	ND
Intestine and contents	0.01	ND

ND = None Detected; Limit of detectability was considered to be the mean of the individual background counts plus twice the standard deviation of the background counts; WL = White Leghorn; RIR/BPR = Rhode Island Red/Barred Plymouth Rock.

Table 3.9 presents the percentage of administered dose for each of the metabolites isolated from the organic phase of the chicken excreta. WL chickens metabolized a greater percentage of the radiolabelled diflubenzuron than RIR/BPR chicken and a larger number of compounds were detected. In WL chickens, 16% of the administered dose was transformed to [^{14}C]-labelled metabolites, while RIR/BPR chickens transformed only 3.4% of the dose. The major

residue was unchanged diflubenzuron in the two breeds of chicken. Up to five metabolites were not identified.

Table 3.9. [^{14}C]-Diflubenzuron and metabolites identified in organic fraction of chicken excreta (Adapted from Opdycke *et al.,* 1982b).

	Percentage (%) of dose	
Metabolite	**(WL)**	**(RIR/BPR)**
Diflubenzuron	49.90	63.39
4-Chloroaniline	0.44	0.58
2,6-Difluorobenzamide	1.98	
4-Chlorophenyl(urea)	3.14	0.38
2,6 Difluorobenzoic acid	1.35	0.22

Residual radioactivity in the eggs was entirely from the parent compound; no metabolites were identified.

Salmon

The metabolic profile of diflubenzuron in Atlantic salmon (*Salmo salar*) has been evaluated according to EEC Regulation No 762/92 in two GLP-compliant experiments (Auger, 1997) after single dosing (gavage) of radiolabelled [^{14}C]-diflubenzuron and multiple dosing (13 days of feeding of non-radiolabelled diflubenzuron followed by a single dose of radiolabelled [^{14}C]-diflubenzuron) at the recommended dose of 3 mg/kg bw (water temperature +15 °C). In both experiments, the fish were treated with radiolabelled diflubenzuron at concentrations of 1.0 g/kg and 0.6 g/kg for the single dose and repeated dose, respectively. The higher concentration of the drug used in the single dose study was chosen to reduce gavage to 0.3% of bw in order to minimize risk of stomach rupture. Analysis of the treated feed before and after dosing confirmed a radiopurity higher than 99%.

Liver, fillet (muscle and skin), gall bladder (including bile) and residual carcass were collected from 10 fish each at 1 and 7 days (single dose) and 1, 4 and 7 days (repeated dose) post final dose administration. Samples of tissues were collected for TRR determination using liquid scintillation (counting). The limits of detection were 2 µg eq/kg for liver and 0.6 µg eq/kg for fillet and carcass, respectively. Acetonitrile and ethyl acetate tissue sample extracts were also analysed using reversed-phase HPLC-UV at 254 nm. Finally, fish fillet extracts were analysed by liquid chromatography coupled to mass spectrometry (LC-MS).

Diflubenzuron was found as the main TRR both in fillet and in liver, corresponding to 94.8 and 72.2%, respectively, at day 1 after the repeated dosing regimen. For the single dose regime, diflubenzuron represented 88.6% and 69.3% of the TRR for fillet and liver. Diflubenzuron was metabolized 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 was attributed to polar metabolites.

Chromatographic analysis with radio-HPLC of fillet revealed three components. The major component was identified as parent diflubenzuron at concentrations of 389 µg/kg, 99.6 µg/kg and 21.4 µg/kg at 1, 4 and 7 days following repeat administration and 410 µg/kg at 1 day following a single administration. Furthermore, one metabolite was identified as 4-chlorophenyl urea with a maximum concentration of 0.23 µg/kg at 4 days following repeat administration. The third component was not identified (less than 7 µg/kg) but the retention time was in the same range as for 4-chloroaniline. Base hydrolysis of solid residues in liver revealed at least five components at concentrations lower than 9 µg/kg. Three of the 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.

Comparative metabolism in animals

The metabolism studies indicated that diflubenzuron is metabolized in animals via two main routes (Figure 3.2). Reaction pathways A, B and C are hydroxylation reactions of the phenyl groups, which leaves the basic structure of diflubenzuron intact; the metabolites formed are 2,6-difluoro-3-hydroxydiflubenzuron, 4-chloro-3- hydroxydiflubenzuron, 4-chloro-2-hydroxydiflubenzuron and their conjugates. In the other pathway (Fig. 2, D), a cleavage between the carbonyl and amide groups takes place and 2,6-difluorobenzoic acid and 4-chlorophenyl urea are formed. Whereas pathways A, B and C are the major metabolic pathways in rat and cow, pathway D predominates in sheep, swine and chicken (IPCS 1981). Moreover, metabolism of diflubenzuron in laboratory animals was qualitatively similar to that in food-producing animals. In salmon, the second pathway appears to be the main metabolic pathway, with the metabolite 4-chlorophenyl urea identified in both fillet and muscle of salmon administered diflubenzuron.

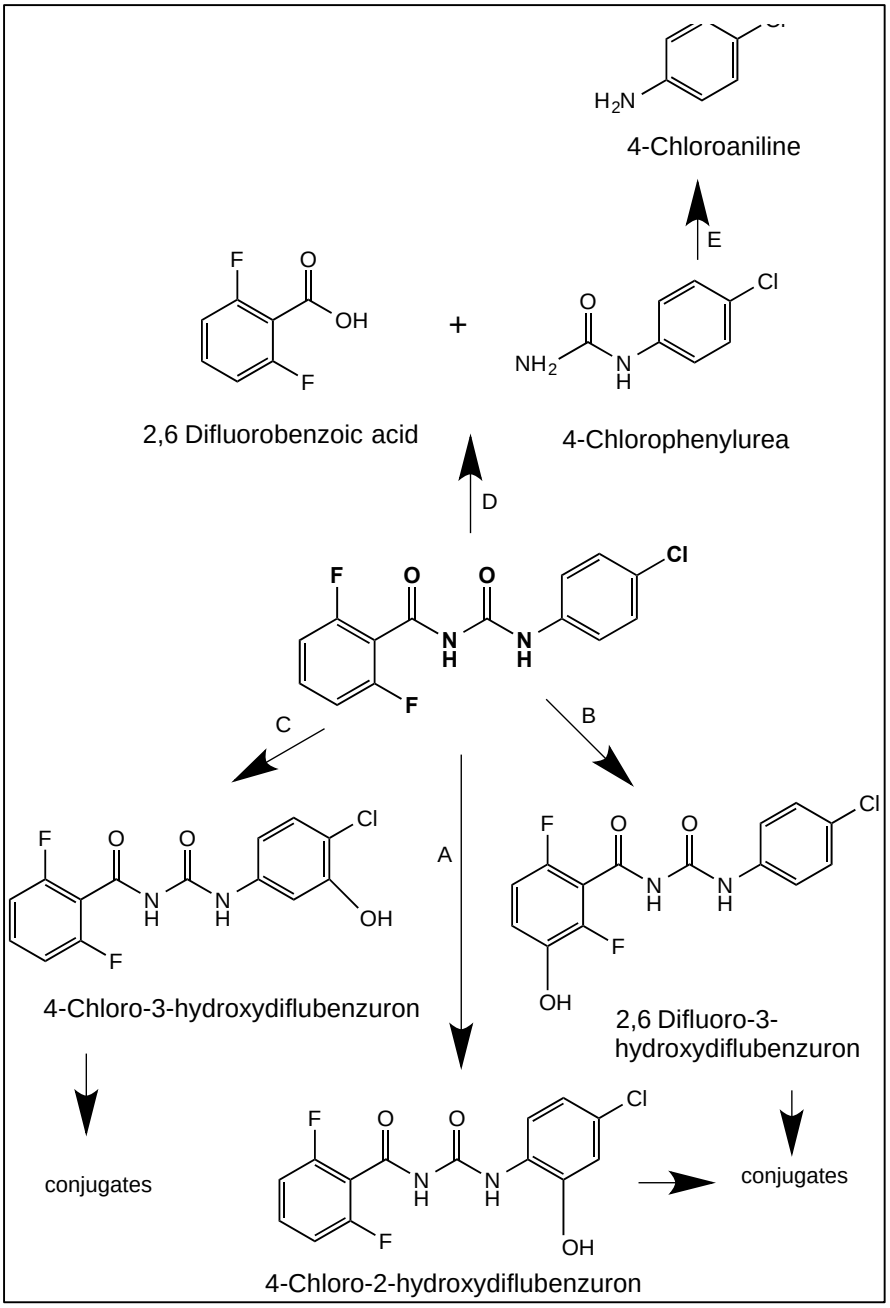


Figure 3.2. Metabolism of diflubenzuron in animals. (Adapted from JMPR, 1982).

Tissue residue depletion studies

Radiolabelled residue depletion studies

Salmon

The total radiolabelled residues were determined in two GLP-compliant studies in which Atlantic salmon (*Salmo salar*), held in sea water of approximately 15 °C, were dosed with [^{14}C]-diflubenzuron at two different regimes: (i) a single dose of 0.3% of bw equal to a dose of 3 mg/kg bw (2 MBq/kg) for one-day by gavage (*Study I*) and (ii) a repeated dose of 0.5% of bw/day equal to 3 mg/kg bw/day for 13 consecutive days using non-radiolabelled diflubenzuron followed by a single dose of radiolabelled [^{14}C]-diflubenzuron by gavage as

performed in the single dose trial (*Study II*) (Auger, 1997). In both studies the fish were treated with radiolabelled diflubenzuron at concentrations of 1.0 g/kg and 0.6 g/kg for the single dose and repeated dose, respectively. The higher concentration of the drug used in the single dose study was to reduce gavage to 0.3% of bw in order to minimize risk of stomach rupture.

Study I – Single oral dose of [^{14}C]-diflubenzuron (Auger, 1997).

Atlantic salmon (*Salmo salar*), weighing 440 to 851 g, were treated with a single dose of 3 mg/kg bw (2 MBq/kg) [^{14}C]-diflubenzuron by gavage. Liver, fillet (muscle and skin), gall bladder (including bile) and residual carcass were collected from 10 fish each at 1 and 7 days post final dose administration. Samples of tissues were collected for TRR determination using liquid scintillation counting. The limits of detection were 2 µg eq/kg for liver and 0.6 µg eq/kg for fillet and carcass. Acetonitrile and ethyl acetate tissue sample extracts were also analysed using reversed-phase HPLC coupled to a UV detector at 254 nm. Finally, fish fillet extracts were analysed by LC-MS.

Diflubenzuron was found as the main TRR both in fillet and in liver corresponding to 88.6% and 69.3% of the TRR for fillet and liver, respectively. The TRR concentrations in tissues are presented in Table 3.10 and the recovery proportions in Table 3.11.

Table 3.10. Change in concentration of radioactivity in tissues of Atlantic salmon (*Salmo salar*) with time following oral administration of a single dose of [^{14}C]-diflubenzuron of 3 mg/kg bw by gavage. Water temperature of 15 ºC (Auger, 1997)**.**

	Concentration of radioactivity (µg eq/kg) ± SD		
Time (days)	**Liver**	**Fillet**	**Carcass**
1	943 ± 106	447 ± 55	1930 ± 973
7	192 ± 51	21 ± 9	42 ± 17

SD = Standard Deviation (n = 10 fish).

Table 3.11. Change in recovery of radioactivity from tissues of Atlantic salmon (*Salmo salar*) with time following oral administration of a single dose of [^{14}C]-diflubenzuron of 3 mg/kg bw by gavage. Water temperature of 15 ºC (Auger, 1997)**.**

	Mean recovery of radioactivity (%) ± SD			
Time (days)	**Liver**	**Fillet**	**Carcass**	**Total recovery***
1	0.29 ± 0.03	9.27 ± 1.14	23.2 ± 12.1	32.8
7	0.06 ± 0.01	0.44 ± 0.19	0.51 ± 0.20	1.0

SD = Standard Deviation (n = 10 fish); * Sum of the average recoveries of liver, fillet and carcass.

Study II – Repeated dose of non-radiolabelled diflubenzuron for 13 consecutive days followed by a single dose of [^{14}C]-diflubenzuron (Auger, 1997).

Atlantic salmon (*Salmo salar*), weighing 514 to 863 g, were treated with diflubenzuron. Medicated feed containing non-radiolabelled diflubenzuron at a dose of 3 mg/kg bw per day was administered for 13 consecutive days. On day14 a single dose of 3 mg/kg bw radiolabelled [^{14}C]-diflubenzuron was administered by gavage. Liver, fillet (muscle and skin), gall bladder (including bile) and residual carcass were collected from 10 fish each at 1, 4 and 7 days post final dose administration. The analyses were carried out as described in *Study I.*

Diflubenzuron was found as the main TRR both in fillet and in liver corresponding to 94.8 and 72.2%, respectively, at day 1 after post-treatment. The TRR concentrations in tissues are presented in Table 3.12 and the recovery proportions in Table 3.13.

Table 3.12. Change in concentration of radioactivity in tissues of Atlantic salmon (*Salmo salar*) with time following repeated dosing. Salmon were administered non-radiolabelled diflubenzuron (3 mg/kg bw) via medicated feed for 13 consecutive days; on day 14 salmon received a single oral dose of [14C]-diflubenzuron (3 mg/kg bw) by gavage. Water temperature of 15 ºC (Auger, 1997)**.**

	Concentration of radioactivity (µg eq/kg) ± SD		
Time (days)	**Liver**	**Fillet**	**Carcass**
1	811 ± 100	466 ± 66	734 ± 118
4	334 ± 60	117 ± 33	181 ± 44
7	181 ± 33	26 ± 11	51 ± 22

SD = Standard Deviation (n = 10 fish).

Table 3.13. Recovery of radioactivity from tissues of Atlantic salmon (*Salmo salar*) with time following repeated dosing. Salmon were administered non-radiolabelled diflubenzuron (3 mg/kg bw) via medicated feed for 13 consecutive days; on day 14 salmon received a single oral dose of [14C]-diflubenzuron (3 mg/kg bw) by gavage. Water temperature of 15 ºC (Auger, 1997)**.**

	Mean recovery of radioactivity (%) ± SD			**Total recovery***
Time (days)	**Liver**	**Fillet**	**Carcass**	
1	0.25 ± 0.04	9.58 ± 1.16	8.78 ± 1.38	18.61
4	0.12 ± 0.02	2.35 ± 0.65	2.43 ± 0.58	4.90
7	0.06 ± 0.01	0.54 ± 0.23	0.62 ± 0.26	1.22

SD: standard deviation (n= 10 fish). * Sum of the average recoveries of liver, fillet and carcass.

The relationship between extractable (in acetonitrile) marker residue (diflubenzuron) and total residue in pooled liver and muscle homogenate samples from Atlantic salmon (*Salmo salar*) held in sea water at 15 ^{0}C is shown in Table 3.14.

Table 3.14. Concentrations (µg/kg) of total radioactive residues (TRR) and diflubenzuron residues (marker residue, MR) in liver and muscle of Atlantic salmon (*Salmo salar*) following repeated dosing. Salmon were administered non-radiolabelled diflubenzuron (3 mg/kg bw) via medicated feed for 13 consecutive days; on day 14 salmon received a single oral dose of [14C]-diflubenzuron (3 mg/kg bw) by gavage. Water temperature of 15 ºC (Auger, 1997).

Administration	Time post-dose (d)	Liver			Muscle		
		TRR (µg/kg)	MR (µg/kg)	MR/TRR ratio (%)	TRR (µg/kg)	MR (µg/kg)	MR/TRR ratio (%)
Single dose	1	922	703	76.3	463	421	91.0
	1	802	617	77.0	410	394	96.0
Repeated dose	4	324	185	57.1	114	100	88.0
	7	177	51.9	30.3	22.9	21.5	93.9

Four other minor metabolites were also detected, including 4-chlorophenylurea at a concentration of 0.23 µg/kg in liver on day 4 in the repeated dose group. The other three metabolites were not identified but were postulated to be mono-hydroxylated products of diflubenzuron. Basic hydrolysis of solid residues in liver revealed at least five components: diflubenzuron, 4-chlorphenyl urea (less than 9 µg/kg), 4-chloroaniline (less than 3 µg/kg) and two unknown substances.

The highest concentrations of [^{14}C]-diflubenzuron-equivalents found in all tissues analysed were at day 1 in both treatment groups (Tables 3.10 and 3.12). Excretion from the Atlantic salmon tissues was rapid with less than 20% of the radiochemical dose remaining in the liver, fillet and carcass 1 day following repeated administration and less than 33% remaining following a single dose administration (Table 3.11). The concentrations decreased to less than 1.5% by 7 days following both dosing regimens (Tables 3.11 and 3.13). The major metabolic pathway is excretion of the parent compound.

Residue depletion studies with non-radiolabelled drug

Salmon

Depletion of diflubenzuron in Atlantic salmon (*Salmo salar*), 600 to 987 g, was evaluated at a water temperature of 6 °C following 14 days of daily medication at a nominal concentration of 0.6 g/kg in feed (actual concentration 0.64 g/kg). This study (Todd, 1997a) was conducted according to EC Council Directive 87/18/EEC and 88/320/EEC and in compliance with GLP. The medicated feed was offered *at libitum* each day at a level of 0.5% of fish biomass per day, equivalent to an intended daily dose of diflubenzuron of 3 mg/kg bw (actual dose of 2.9 mg/kg bw). Livers (without gall bladder) and fillets (muscle and skin) of ten fish were analysed by HPLC-UV on days 1, 7, 14 and 21 post treatment.

The analytical method was validated over the range 0.05 to 5.0 mg/kg for both tissues. Recoveries from fillet ranged from 81 to 108%, with a coefficient of variation of 7.1%. Recoveries from liver ranged from 100 to 108%, with a coefficient of variation of 2.4%. A stability study using fortified tissues (1000 µg/kg) showed that diflubenzuron is stable at -18 °C in both fillet and liver over a storage period of 60 days.

The average concentrations of diflubenzuron in fillet were: 2240 µg/kg, 400 µg/kg, 100 µg/kg and below limit of quantification (LOQ, 50 µg/kg) on days 1, 7, 14 and 21 post-treatment, respectively. The average concentrations of diflubenzuron in liver were 3190 µg/kg, 730 µg/kg, 120 µg/kg and below LOQ on days 1, 7, 14 and 21 post-treatment, respectively. In this study, the withdrawal period was estimated (time where the upper one-side 95% tolerance limit is below the LOQ) to be 22 days for fillet and 21 days for liver. Considering a safety margin, a withdrawal period of 28 days was recommended.

The same protocol was used in a second study at a higher water temperature (15 °C), where Atlantic salmon (*Salmo salar*), 600-987 g, were fed diflubenzuron daily at an intended daily dose of 3 mg/kg bw (actual dose of 3.19 mg/kg bw) for 14 consecutive days (Todd, 1997b). Diflubenzuron was quantified in muscle and liver by a method using HPLC-UV, with a limit of quantification (LOQ) of 50 µg/kg. The average concentrations determined of diflubenzuron in fillet were: 1550 µg/kg and 200 µg/kg on days 1 and 7, respectively, and below LOQ on days 14 and 21 post-treatment. The average concentrations of diflubenzuron in liver were 2170 µg/kg and 260 µg/kg, on days 1 and 7 post-treatment, respectively, and less than 50 µg/kg (LOQ) after 14 days post-treatment. In this study the withdrawal period was estimated to be 18 days for fillet and 17 days for liver. Considering a safety margin, the same withdrawal period of 28 days recommended from the results of the study at 6 °C was recommended for the higher water temperature (15 °C).

In another GLP-compliant depletion study carried out at high temperature (14.6 to 15.6 °C), Atlantic salmon (*Salmo salar*) weighing 4.6 to 5.6 kg were fed *ad libitum* with diflubenzuron (0.63 g/kg) at a level of 0.5% of biomass per day for 14 consecutive days, equivalent to a daily dose of diflubenzuron of 2.66 to 3.2 mg/kg bw (Wallace *et al.*, 1997). Liver, muscle and skin samples collected during the treatment (days 3, 7 and 14) and on days 5, 14, 21 and 28 post-treatment were analysed using a validated HPLC-UV method. During the treatment, the highest average diflubenzuron concentration was found at day 14 in liver (1820 µg/kg) and muscle (2130 µg/kg). For skin, the highest diflubenzuron concentration of 1320 µg/kg was reached on day 7 during the treatment. The maximum diflubenzuron concentration of 3700 µg/kg was in one muscle sample on day 14 during the treatment with the medicated feed. In liver, the average diflubenzuron concentrations (10 fish) were 520 µg/kg (minimum < 50 µg/kg and maximum 890 µg/kg) and 70 µg/kg (minimum <50 µg/kg and maximum 150 µg/kg) on days 5 and 14, respectively. In muscle, the average concentrations were 900 µg/kg (minimum 530 µg/kg and maximum 1900 µg/kg) and 100 µg/kg (minimum <50 µg/kg and maximum 170 µg/kg) on days 5 and 14, respectively. In skin, the average concentrations were 320 (minimum <50 µg/kg and maximum 520 µg/kg) and less than 50 µg/kg (minimum <50 µg/kg and maximum 80 µg/kg), on days 5 and 14, respectively. At 21 days post treatment, all samples analysed had diflubenzuron concentrations lower than 50 µg/kg (LOQ).

Atlantic Cod

A non-GLP compliant residue depletion study of diflubenzuron in juvenile Atlantic cod (*Gadus morhua*), a fish species found near Atlantic salmon farms, was conducted at a water temperature of 7.7 ± 0.2 °C (Olsvik *et al.*, 2013). The fish (81 to 122 g) were fed at a nominal dose rate of 3 mg/kg bw (0.6 g/kg in feed), corresponding to a total dose of 42 mg/kg bw after the end of treatment. The highest concentrations of diflubenzuron in liver (181 ± 21 µg/kg) were observed 1 day after the end of the treatment (Day 15). The authors suggest that diflubenzuron is metabolized by phase I enzymes and particularly CYP3A after pregnane X receptor (PXR) activation in cod.

In another study conducted at a water temperature of 7.7 °C, Atlantic cod (65 – 165 g) were fed medicated pellets containing 0.6 g of diflubenzuron per kg for 14 consecutive days (Erdal, 2012). The feed was administered *ad libitum* for a nominal daily dose of 3 mg of diflubenzuron per kg bw. Samples of fillet and skin in natural proportions, liver and terminal colon, were taken during the treatment on days 4, 8, 12 and days 1, 4, 8, 15, 22 and 30 post treatment. At each time point, 10 fish were collected and analysed individually, with the exception of the bile samples, which were accumulated into one or two group samples for each sampling day. After extraction from the sample matrices, diflubenzuron was quantified by LC-MS using teflubenzuron as the internal standard. The LOQ of the validated method was 20 µg/kg. The calculated tissue concentrations in the samples showed high variability, attributed to individual differences in feed consumption and, to a lesser extent, in absorption. The median concentration determined in fillet and skin throughout the treatment period was 36.1 µg/kg, only 1.5% of the mean concentration determined in Atlantic salmon fillet after the same treatment, which indicates that diflubenzuron has a lower gastrointestinal uptake in Atlantic cod compared to Atlantic salmon.

The depletion half-lives for diflubenzuron in fillet and liver ranged from 0.8 to 0.9 days. The concentrations of 4-chloroaniline in all samples analysed by LC-MS/MS were below the detection limit of the method (2 µg/kg). However, these results do not rule out the possibility that 4-chloroaniline could be a metabolite of diflubenzuron in Atlantic cod because the tissue concentrations of the marker residue were so low that the fraction of PCA that might be formed probably would be below the detection limit of the method.

Method of analysis for residues in tissues

Many analytical methods for the determination of diflubenzuron in food, feed and biological matrices have been reported (Table 3.15). The Committee assessed the validation data available for these methods against the analytical requirements as published in CAC/GL71-2009 (FAO/WHO, 2014).Due to the high polarity and low volatility of diflubenzuron, liquid chromatography has been the method of choice. Most protocols use solvent extraction of diflubenzuron from the sample followed by clean-up steps, including solid phase extraction procedures and, more recently, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach (Anastassiades, 2003). Chromatographic separation is commonly performed using reverse-phase chromatography. For the quantification of diflubenzuron, UV and, nowadays, tandem mass spectrometry detectors have been employed. In the latter case,

electrospray ionization is mostly employed in the positive ion mode using acquisition of ions in the selected reaction-monitoring mode (SRM).

Quantitative methods

Liquid chromatography (LC)

Single-residue methods employing HPLC-UV were used in depletion studies carried out in the mid-1990s for the quantification of diflubenzuron in fish tissues. The analytical method (Thus et al., 1995) consisted of extraction of diflubenzuron from the skin, muscle and liver samples (3 to 5 g) by solid-liquid extraction with acetonitrile (2 x 5 mL). The extract was evaporated to dryness at 50 °C, and dissolved in a solution of acetonitrile (1.5 mL), water (0.5 mL) and hexane (4 mL). The solution was vortexed, centrifuged and the hexane layer removed. An additional 4 mL of hexane, 1 mL of water and 4 mL of dichloromethane were added to the test tube. The mixture was vortexed, centrifuged and the dichloromethane layer separated. To the acetonitrile/water layer another 4 mL dichloromethane was added and the separation procedure repeated. The combined dichloromethane layers were mixed with sodium sulphate and the dried dichloromethane layer evaporated to dryness at 50 °C. The residue was dissolved in 4.0 mL of methylethylketone:petroleum ether, 2:25 v/v, with clean-up by solid phase extraction on a Florisil cartridge (500 mg). The chromatographic separation was performed on a C18 column (Zorbax, 250 x 4.6 mm, 7.5 µm particle size) at 35 °C, using acetonitrile:water, 1:1 v/v, as the mobile phase. Quantification was performed using a UV detector at 254 nm. The concentrations of diflubenzuron in the samples were calculated by comparing the peak height of the sample with the peak height of calibration solutions. The method was validated by analysing diflubenzuron fortified tissue samples, ranging from 0 to 3.3 mg/kg, with detection and quantification limits of 20 and 50 µg/kg, respectively. Average recoveries of 88% for liver, 91% for muscle (values corrected for blank) and 103% for skin were determined. Even though matrix effects are not relevant using a UV detector, it is important to consider that interferences could occur at low-concentration measurements in complex food matrices.

Gas chromatography (GC)

Gas chromatography has also been employed, to a lesser extent, for the determination of diflubenzuron in plant and animal products. Due to its thermal instability, high polarity and low volatility, derivatization processes are required. The method is based on hydrolysis of diflubenzuron to 4-chlorophenylurea and 4-chloroaniline followed by derivatization with heptafluorobutyric acid (HFBA) and determination of the N-(4-chlorophenyl)heptafluorobutanamide formed by GC-ECD or NPD (Stan and Klaffenbach, 1991). Mass spectrometry coupled to GC is necessary for confirmation purposes. It is worth emphasizing that any 4-chlorophenylurea or 4-chloroaniline in the sample will be determined as diflubenzuron if not separated before the hydrolysis step.

Confirmatory methods

Liquid chromatography – tandem mass spectrometry (LC- MS/MS)

In recent years, many multi-residue analytical methods for the determination of pesticides and veterinary drugs, including diflubenzuron, in food and biological matrices have been reported.

In general, the methods are based on solvent extraction (acetone, acetonitrile or methanol) with or without hexane liquid-liquid extraction to remove lipids, followed by clean-up over C18 or silica gel solid phase extraction cartridges and determination by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Matrix solid-phase extraction may be used as an alternative technique for the simultaneous extraction and clean-up (fat removal) of lipophilic chemicals. A simple and fast method for the determination and monitoring of eight pesticides, including diflubenzuron, in fish and shellfish by matrix solid-phase dispersion (MSPD) with anhydrous sodium sulphate and C18 as dispersants, silica as an adsorbent and LC-MS/MS quantification, has been reported (Carro *et al.*, 2012).

More recently, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach (Anastassiades *et al.*, 2003), originally developed for the determination of pesticide residues in fruits and vegetables, has been modified and employed for the determination of multi-residues of pesticides, including diflubenzuron, in fish. The original approach is characterized by an extraction step using acetonitrile followed by a subsequent liquid-liquid partitioning of residues through the addition of salts and buffering agents and clean-up steps. Modifications in the original QuEChERS approach include the use of ethyl acetate instead of acetonitrile and incorporating two freezing steps for removal of lipids (Norli *et al.*, 2011). The use of a less polar solvent improves partitioning of analytes from the fatty lipid layer in fish tissues. The pigments that give salmon its trademark pink colour could cause interference with analytes of interests; these pigments could be removed by the use of primary-secondary amine and /or graphitized carbon black (Holmes *et al*., 2015).

Holmes and co-workers presented a single-laboratory ruggedness testing and validation of a modified QuEChERS approach to quantitate residues of 185 pesticides in salmon using UPLC-MS/MS (103 pesticides, including diflubenzuron) and GC-MS/MS (82 pesticides) for analysis (Holmes *et al*., 2015). The pesticides were extracted from the homogenized samples (20 g) with ethyl acetate and two freezing steps and a C18 dispersive solid phase extraction for removal of lipids were carried out. Briefly, 20 g of the homogenized samples were added to 30 mL ethyl acetate and extraction buffer (8 g of $MgSO_4$ and 2 g of NaCl). The samples were shaken and placed into a -20 °C freezer for 30 min, centrifuged and the upper layer decanted. The extracts were concentrated under a stream of nitrogen and diluted with acetonitrile to 15 mL and frozen for a second time at -40 °C for 30 min. After centrifugation, the upper layer was cleaned-up over C18 SPE cartridges prepared by adding 1 g of $MgSO_4$ on the top. The SPE columns were washed with 1% acetic acid in acetonitrile. Eluted extracts were concentrated and re-suspended with acetonitrile. Before LC-MS/MS quantification, an additional clean-up was performed that consisted of the addition of 150 mg of $MgSO_4$, 50 mg of primary-secondary amine (PSA) and 50 mg of C18 to a volume of 1 mL of the extract.

Analytes were separated on a silica-based bonded phase column (Acquity HSS T3 column, 50 x 2.5 mm, 1.8 µm particle size) at 40 °C using a binary mobile phase composed of 0.1% formic acid and 10 mM aqueous ammonium formate. Triphenylphosphate was used as internal standard. For the quantification, a matrix-matched standard of blank salmon extract was prepared at 1, 5 and 10 times the limit of quantification for each pesticide. The method was used in the analysis of 708 salmon samples collected as part of the U.S. Department of

Agriculture's Pesticide Data Program (USDA-PDP). Method validation was accomplished by assessing selectivity and conducting single-laboratory intra- and inter-day precision and accuracy studies and was conducted according criteria set by the USDA-PDP quality assurance program and based on EPA GLP.

Another simultaneous screening and confirmation procedure for multiple classes of drug residues in fish (salmon, trout, catfish and tilapia), including diflubenzuron, by liquid chromatography-ion trap mass spectrometry, was developed and validated (Smith *et al.*, 2009). Samples (2 g) were added to 2 mL of n-hexane and 10 mL of acetonitrile. After vigorous shaking and centrifugation, the hexane layer was aspirated and discarded. The acetonitrile phase was separated and the remaining tissue pellet was re-extracted with 10 mL acetonitrile and 2 mL n-hexane. The acetonitrile extracts were combined and evaporated just to dryness. Chromatographic separation was achieved using a Phenyl column (YMC, 50 x 4.0 mm, 3 µm particle size) and a mobile phase of 0.1% formic acid with 10 µm NaOH in water and acetonitrile. Analytes were detected on an ion trap mass spectrometer equipped with an ESI source operating at positive and negative mode. With this method, a lower confirmation limit for diflubenzuron of 100 µg/kg was achieved.

A rapid multi-residue screening method for the determination of 128 veterinary antiparasitic drugs and metabolites, including diflubenzuron, in chicken, porcine and bovine meat, was developed and validated according to the European Union Regulation 2002/657/EC for a quantitative screening method (Wei *et al*., 2015). The sample preparation procedure was based on the QuEChERS approach. The drugs were extracted from the chicken, porcine and bovine meat samples (2 g) using 10 mL acetonitrile:ethyl acetate 1:1 v/v and 1 g of $MgSO_4$. After sonication and centrifugation, the upper layer was separated and added to 1 mL of aqueous NH_3. The supernatant was cleaned-up over 200 mg ODS and 1.5 g anhydrous $MgSO_4$. The cleaned extract was evaporated and the residue dissolved in the mobile phase. Quantification was performed by LC-MS/MS, using a C18 chromatographic column (Hypersil, 150 x 2.1 mm, 5 µm particle size) at 40 °C, mobile phase of 12.5 mM ammonium formate at pH 4 in acetonitrile/methanol, 50:50 v/v, and an ESI source was used with both positive and negative ionization mode. The detection capabilities (CCβ) for diflubenzuron in chicken, swine and bovine meat were 2.15, 2.24 and 10.28 µg/kg, respectively.

Matrix solid-phase dispersion and liquid chromatography with UV or atmospheric pressure chemical ionization/mass spectrometry (APCI/MS) detection was reported for the determination of diflubenzuron and 4 other pesticides (hexaflumuron, aflufenuxuron, hexythiazox and benfuracarb) in orange samples from Spain (Valenzuela *et al.*, 2001). In 74.6% of the 150 samples analysed, the pesticide residues were below detection limits, which ranged from 2 to 50 µg/kg. Diflubenzuron residues exceeded 1000 µg/kg in 3 samples.

Several analytical methods used in supervised residue trials and in studies on storage stability in plant products, animal feeding or direct animal treatment were reported and considered by the JMPR in 2011. Most are single-residue methods for either diflubenzuron, 2.6-difluorobenzoic acid, *p*-chlorophenylurea, 4-chloroaniline or 4-chloroacetanilide in only a few substrates. HPLC methods for the determination of diflubenzuron consist of extraction, clean-up and determination with UV, MS or MS/MS detection. In addition, a great number of multi-

residue methods have been reported for residues of diflubenzuron in agricultural products and water (Martinez *et al.*, 2007).

Stability of residues

Residues of diflubenzuron are stable in frozen beef tissue, milk, poultry muscle and eggs at temperatures of at least -20 °C for up to 12 months (EPA, 1997). In Atlantic salmon fillet and liver matrices stored at approximately -18 °C, diflubenzuron was stable for a period at least 60 days (Todd, 1997b).

Table 3.15. Analytical methods for the determination of diflubenzuron in food and biological matrices.

Sample	Extraction	Clean-up	Analytical technique	Linear range	Recovery	LOQ	LOD	Reference
Fish	MSPD	-	LC-MS/MS	5-500 µg/kg	84.9-118% (salmon)	4.7 µg/kg	1.5 µg/kg	(Carro *et al.*, 2012)
Fish (salmon)	SLE1 (ethyl acetate + 2 freezing steps)	QuEChERS2	LC-MS/MS (IS3 triphenyl phosphate)	1, 5 and 10 xLOQ	70-120%	2 µg/kg	not stated	(Holmes *et al.*, 2015)
Fish (salmon, trout, catfish and tilapia)	SLE1 (hexane and acetonitrile)	-	LC-MS/MS	not stated	not stated	100 µg/kg	not stated	(Smith *et al.*, 2009)
Atlantic cod (muscle, fillet and liver)	SLE1 (acetone)	LLE4 (heptane); SPE5 (silica)	LC-MS/MS (IS3 teflubenzuron)	20-75 µg/kg	-	20 µg/kg	10 µg/kg	(Erdal, 2012)
Tilapia fillet	SLE1 (methanol)	SPE5 (C18)	HPLC-DAD	0.1 – 15 mg/L	not stated	110 µg/kg	32 µg/kg	(Luvizotto-Santos *et al.*, 2009)
Fish feed	SLE1 (acetone and THF)	-	HPLC-UV	0.3 – 2.0 g/kg	91.4-93%	0.25 g/kg	not stated	(Hormazabal and Yndestad, 1997)
Milk and cattle tissues	SLE1 (ACN)	QuEChERS2 and SPE5 (C18)	HPLC-DAD	0-25 mg/L	71.8-105.1%	50 µg/kg (kidney, liver,	15 µg/kg (kidney), 16 µg/kg (liver), 14	(Tfouni *et al.*, 2013)

						muscle, fat) 10 µg/kg (milk)	µg/kg (muscle), 6 µg/kg (fat and milk)	
Mushroom	SLE1 (ACN)	QuEChERS2	UPLC-MS/MS	5-5000 µg/L	78.1-107.6%	< 5 µg/kg	< 1.5 µg/kg	(Carro *et al.*, 2012, Du *et al.*, 2013)

[1] SLE = Solid-Liquid Extraction; [2] QuEChERS = Quick, Easy, Cheap, Rugged and Safe; [3] IS = Internal Standard; [4] LLE = Liquid-Liquid Extraction; [5] SPE = Solid Phase Extraction.

Appraisal

Diflubenzuron is a benzoylurea pesticide used in aquaculture for the treatment of sea lice in Atlantic salmon (*Salmo salar*) at a dose of 3-6 mg diflubenzuron per kg of fish biomass per day for fourteen consecutive days, with a withdrawal period in the range of 105–300 degree-days. It is also used in agriculture, horticulture and forestry to control a wide range of insect pests.

Diflubenzuron has not been previously reviewed by the Committee; however, it was evaluated as pesticide by JMPR in 1981, 1984 and 1985. An ADI of 0 – 0.02 mg/kg bw was established by JMPR in 1985.

Metabolism data are available for a variety of animal species, including rats, cattle, swine, sheep, goats, chicken and salmon. Diflubenzuron is predominantly unmetabolized and biliary excretion is the main path for elimination. The metabolic profiles indicated that diflubenzuron is metabolized in animals via two main routes: (i) hydroxylation of the phenyl groups and (ii) cleavage of the carbonyl and amide groups. In the second pathway 4-chloroaniline could be formed. In salmon, the second pathway seems to be the main route.

Metabolic profiling in salmon was available; two studies were carried out following single or repeated dose administration of radiolabelled diflubenzuron to salmon. Diflubenzuron was metabolized and rapidly excreted, mainly via the bile. Two compounds were identified in fillet, the parent drug and 4-chlorophenyl urea. A third compound was not identified, but it could not be confirmed that this compound was not 4-chloroaniline. In liver, diflubenzuron, 4-chlorophenyl urea and 4-chloroaniline were identified. Some metabolites remained unknown.

Radiolabelled residue depletion data are available for salmon at a water temperature of 15 ºC following single or repeated dose. Diflubenzuron was identified as marker residue in salmon muscle and liver.

The highest concentration (less than 500 µg/kg) of diflubenzuron in salmon muscle occurs 1 day after administration of the drug.

The Committee was informed that 4-chloroaniline is a potential hydrolysis product of 4-chlorophenyl isocyanate, which is one of the starting materials for the synthesis of diflubenzuron. Also, 4-chloroaniline could be formed through degradation of diflubenzuron at temperature higher than 100 ºC. Even if these two processes are controlled, it cannot be excluded that 4-chloroaniline is present in the drug used to formulate the medicated pellets. No data were available regarding contaminants and/or degradations products in formulated products. There were also no data available about the stability of diflubenzuron during feed processing, in particular regarding the presence or absence of 4-chloroaniline.

The residue depletion studies in salmon were conducted in the mid 90's using HPLC-UV methods, which required complex sample preparation procedures for extraction and clean-up. The quantification limit was 50 µg/kg in salmon tissues. The state-of-the-art methods (LC-MS/MS) use simpler sample preparation procedures, based on the QuEChERS approach, and have a LOQ of 2 µg/kg. However, an analytical method (LC-MS/MS) for the determination of

diflubenzuron in salmon tissues (muscle and skin), validated according to the criteria described in CAC/GL 71-2009, is not available (FAO/WHO, 2014).

The Committee concluded that the HPLC-UV method provided by the Sponsor lacks in selectivity because of possible interferences from other components in the tissue extracts at the selected wavelength and cannot be recommended for regulatory monitoring of salmon tissues for diflubenzuron.

Maximum Residue Limits

The Committee noted that PCA is a potential hydrolysis product of 4-chlorophenyl isocyanate, which is one of the starting materials for the synthesis of diflubenzuron, and that PCA could be formed through degradation of diflubenzuron at high temperatures during processing of feed or food. The data available to the Committee at the time of the assessment were inadequate regarding the formation or presence of PCA in fish, as well as in processed food.

MRLs for diflubenzuron could not be recommended by the Committee, as the Committee was unable to establish an ADI for diflubenzuron.

The Committee also noted that there is no analytical method suitable for regulatory monitoring purposes.

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