

Toxicological evaluation of certain veterinary drug residues in food

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Diflubenzuron

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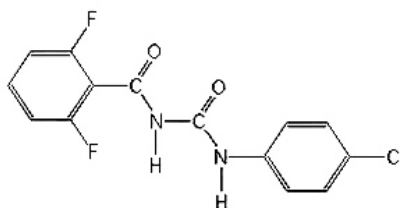
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1. Explanation

Diflubenzuron (International Union of Pure and Applied Chemistry name: 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea, Chemical Abstracts Service no. 35367-38-5; Fig. 1) is an acyl urea derivative (halogenated benzoylphenylurea). Diflubenzuron is approved for use as a veterinary drug in Norway and Chile in the treatment of sea lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*) infestations in Atlantic salmon (*Salmo salar*) as an oral dosage of 3–6 mg/kg body weight (bw) in feed for 14 consecutive days, with a withdrawal period in the range of 105–300 degree-days. It is also used as an insecticide/acaricide in agriculture and forestry against larvae of *Lepidoptera*, *Coleoptera*, *Diptera* and *Hymenoptera* (USEPA, 1997) and as a vector control agent in drinking-water sources and drinking-water storage containers (WHO, 2008).

Fig. 1

Structure of diflubenzuron



The mechanism of action of diflubenzuron is to inhibit the formation of new chitin in the insect cuticle during the moulting process by inducing both chitinase and phenoloxidase.

Diflubenzuron has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated diflubenzuron at the current meeting at the request of the Twenty-second Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO,

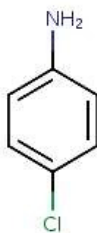
2015). The Committee was asked to establish an acceptable daily intake (ADI) and recommend maximum residue limits (MRLs) for diflubenzuron in salmon muscle and skin in natural proportion.

The Committee noted that the toxicity of diflubenzuron had been previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1981, 1985 and 2001 (FAO/WHO, 1982, 1986, 2002) and by a number of other scientific or regulatory bodies, such as the WHO Task Group on Environmental Health Criteria for Diflubenzuron, which prepared Environmental Health Criteria 184 (IPCS, 1996). In 2001, JMPR established an ADI of 0–0.02 mg/kg bw for diflubenzuron, based on the no-observed-adverse-effect level (NOAEL) of 2 mg/kg bw per day for haematological effects observed in 2-year toxicity studies in rats and a 52-week toxicity study in dogs (FAO/WHO, 2002).

The metabolism of diflubenzuron is known to lead to the formation of 4-chloroaniline (*p*-chloroaniline [PCA]; Fig. 2) in some species, but not the rat, and it is not known whether PCA is formed in salmon or humans. PCA might also occur as an impurity in the product formulation or as a degradation product generated during food processing. PCA is considered by many scientific and regulatory bodies (e.g. the International Agency for Research on Cancer; IARC, 1993) as genotoxic and carcinogenic. The Committee therefore evaluated the toxicity of PCA, focusing particularly on its genotoxicity and carcinogenicity as well as on its possible carcinogenic mode of action, based on studies retrieved from a search of published literature.

Fig. 2

Structure of *p*-chloroaniline (PCA)



Because the sponsor did not provide any toxicological data on diflubenzuron, the Committee relied mainly on the summary evaluation prepared by JMPR in 2001. The Committee considered JMPR's summary evaluation of studies on the short-term and long-term toxicity, reproductive and developmental toxicity, and genotoxicity and carcinogenicity of diflubenzuron.

In addition, the Committee considered information obtained from literature searches on diflubenzuron and PCA. A literature search was conducted in June 2015 on the following search terms using PubMed, Embase and Web

of Sciences: diflubenzuron and toxicity or toxicology, diflubenzuron and toxicokinetics. In total, 215 unique references were retrieved. Of these, five were cited in the current monograph. The Committee also considered information obtained from literature searches conducted in October 2015 using PubMed and the broad search term 4-chloroaniline or *p*-chloroaniline. Four of the 323 references retrieved were cited in the current monograph.

The original studies provided to the 2001 JMPR were performed over a period of approximately 40 years, and all the studies were considered adequate for their intended purpose unless otherwise specified in the JMPR monograph. Some of the critical studies did not comply with good laboratory practice (GLP) regulations, as the data were generated before the implementation of GLP regulations. Overall, however, the present Committee considered that the database was adequate to assess the risk of diflubenzuron.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Oral administration

(i) Mice

When mice (strain not specified) were given a single oral dose of diflubenzuron at 12, 64, 200 or 920 mg/kg bw, excretion was almost complete within 48 hours. The cumulative percentage of the dose excreted in the urine decreased from 15% at the lowest dose to about 2% at the highest dose, showing that the relationship between urinary excretion and dose in mice is similar to that in rats (see below) (de Lange & Post, 1978; GLP status not specified).

(ii) Rats

Radiolabelled diflubenzuron, with the ^{14}C label uniformly distributed in the anilino moiety, was administered by oral gavage to Wistar rats as a single dose of 4, 16, 48, 128 or 1000 mg/kg bw. Urine was collected every 24 hours up to 120 or 144 hours. The intestinal absorption of diflubenzuron in rats decreased with increasing dose. The cumulative excretion in urine as a percentage of the dose decreased from 28% at the lowest dose to only 1% at the highest dose, while the recovery remained constant (90%) (de Lange, Fronik & Post, 1977; Willems et al., 1980; GLP status not specified).

In female Wistar rats with cannulated bile ducts orally administered [^{14}C]diflubenzuron at a dose of 4, 16, 128 or 900 mg/kg bw, bile was collected at

6, 24, 48 and 72 hours, and urine and faeces were collected at 24-hour intervals for 72 hours. The sum of the excretion in urine and bile decreased from 42% of the dose at the lowest dose to about 4% at the highest dose. In Wistar rats that had received radiolabelled diflubenzuron with the ^{14}C in the carbamoyl group of the benzoyl moiety, 1% of the radiolabel was found in expired air. Seventy-two hours after administration of a single oral dose of 5 mg/kg bw of double-labelled diflubenzuron (^{14}C in the anilino moiety and ^3H in the benzoyl moiety), 1.3% of the ^{14}C and 3.5% of the ^3H were retained in the carcass (de Lange, Fronik & Post, 1977; Willems et al., 1980; GLP status not specified).

An extensive GLP-compliant investigation of the absorption, distribution and excretion of diflubenzuron was reported. Groups of Sprague-Dawley rats received [^{14}C]diflubenzuron uniformly labelled in both phenyl rings (specific activity 37 or 722 kBq/mg) by gavage in 1% gum tragacanth. Details of the dosing and sampling are given in Table 1. Samples were retained for subsequent determination of metabolites (see section 2.1.2 below). Radiolabel was determined by liquid scintillation counting (LSC) with correction against an external standard after appropriate processing. The results (Table 2) showed that at a dose of 5 mg/kg bw, about 30% of the administered dose was excreted in urine and bile; absorption was less complete at 100 mg/kg bw. In animals with bile duct cannulae, reduced excretion was seen in urine (about 7%), indicating some degree of enterohepatic recirculation. $^{14}\text{CO}_2$ in exhaled air represented less than 0.1% of the administered dose. The peak concentrations of radiolabel in blood (C_{max}) were achieved at 4 hours (T_{max}) after administration. The highest tissue concentrations at 4 hours were seen in liver and fat. More radiolabel was found in erythrocytes relative to plasma with increasing duration after dosing. Autoradiography showed a wide distribution of radiolabel, the highest concentrations being found in the

Table 1

Design of study of absorption, distribution and excretion of [^{14}C]diflubenzuron in rats

No. of animals	Dosing schedule	Routine samples ^a	Terminal samples
5/sex	1 × 5 mg/kg bw	Urine, faeces, air	Fluids, tissues at 168 h
5/sex	14 × 5 mg/kg bw per day (^{12}C)	—	—
	1 × 5 mg/kg bw (^{14}C)	Urine, faeces, air	Fluids, tissues at 168 h
5/sex	1 × 100 mg/kg bw	Urine, faeces, air	Fluids, tissues at 168 h
3/sex	1 × 5 mg/kg bw	Blood	—
3/sex per time	1 × 5 mg/kg bw	—	Fluids, tissues at 4, 14, 48 and 72 h
3/sex	1 × 5 mg/kg bw	Bile, urine, faeces	Gastrointestinal tract, carcass at 24 h
1/sex per time	1 × 5 mg/kg bw	—	Autoradiography ^b

bw: body weight

^a Urine and faecal samples were taken at 8 and 24 hours, then every 24 hours until 168 hours. Air samples were taken at 8 and 24 hours. Tail vein blood was taken at 0.25, 0.5, 1, 2, 4, 8, 16, 24 and 48 hours. Bile samples were taken hourly for 24 hours.

^b At times corresponding to plasma C_{max} , $C_{\text{max}/2}$ and 48 hours.

Source: Dunsire, Cameron & Spiers (1990)

Table 2
Absorption, distribution and excretion of [¹⁴C]diflubenzuron in rats (mean values)

	1 × 5 mg/kg bw		14 + 1 × 5 mg/kg bw		1 × 100 mg/kg bw	
	Males	Females	Males	Females	Males	Females
Urine (%)						
0–8 h	8	9	4	4	1	1
0–24 h	20	20	14	12	3	2
Faeces (%)						
0–8 h ^a	16	11	<1	11	66	35
0–24 h	73	75	64	72	95	93
Total recovery (%)	97	99	97	99	99	99
Time to blood C _{max} (h)	4	4	ND	ND	ND	ND
Time to blood C _{max/2} (h)	14	14	ND	ND	ND	ND
Plasma concentration (ng eq/mL)						
C _{max}	695	752	ND	ND	ND	ND
C _{max/2}	187	260	ND	ND	ND	ND
48 h	13	20	ND	ND	ND	ND
168 h (7 days)	3	3	4	5	0	10
Liver concentration (ng eq/mL)						
C _{max}	2 090	2 440	ND	ND	ND	ND
C _{max/2}	693	1 170	ND	ND	ND	ND
48 h	336	526	ND	ND	ND	ND
168 h (7 days)	187	151	153	152	330	370
Erythrocyte concentration (µg eq/mL)						
C _{max}	548	449	ND	ND	ND	ND
C _{max/2}	566	318	ND	ND	ND	ND
48 h ^a	360	398	ND	ND	ND	ND
168 h (7 days)	169	251	157	152	590	780
Bile (%)						
0–8 h	3	3	ND	ND	ND	ND
0–24 h	19	15	ND	ND	ND	ND

bw: body weight; C_{max}: maximum concentration; eq: equivalents; ND: not determined

^a Great inter-animal variation.

Source: Dunsire, Cameron & Spiers (1990)

gastrointestinal tract. Excretion was relatively rapid, with more than 90% of the doses of 5 and 100 mg/kg bw being excreted within 24 hours. Animals given repeated doses showed evidence of delayed absorption and excretion, but no change in the pattern of excretion or distribution of radiolabelled residues was seen at 7 days. The highest concentrations of residues of radiolabel 7 days after administration were found in erythrocytes and liver, but the total radiolabelled residues represented less than 1% of the administered dose. There were no notable differences between males and females (Dunsire, Cameron & Spiers, 1990).

(iii) Cats

Diffubenzuron labelled with ^{14}C and ^3H was administered orally to cats (strain not specified) at a dose of about 7 mg/kg bw on day 10 of a 15-day dosing regimen with unlabelled diffubenzuron. Within 72 hours of administration, 9% of the oral dose had been excreted in the urine, and 77% of the ^{14}C and 71% of the ^3H had been excreted in the faeces (Hawkins, Jackson & Roberts, 1980).

(b) **Dermal application**

(i) Rats

In a GLP-compliant study, the dermal absorption of [$\text{U-}^{14}\text{C}$ -anilino]diffubenzuron (purity 97.2%; specific activity 666 kBq/mg) was investigated in groups of four male Sprague-Dawley rats (one control per time point). A suspension of diffubenzuron (100 μL) in 0.25% gum tragacanth was applied to a 10 cm^2 clipped area at a concentration of 0.005 or 0.05 mg/cm^2 and kept under semi-occluded conditions. The rats were killed at 1, 4 or 10 hours after application, and the skin site was washed 5 times with soap solution. Samples of excreta, cage wash, dermal application site and carcass were analysed by LSC after appropriate processing. More than 96% of the applied radiolabel was recovered in all groups, more than 80% being removed in the first wash. The radiolabelled residues at the dermal application site represented 4.5–6% of the applied dose. The mean absorption was less than 0.5% in all groups, with a range of 0.03–1.2%. There was no evidence of increased absorption with longer exposure (Andre, 1996).

(ii) Rabbits

The dermal absorption of [$\text{U-}^{14}\text{C}$ -anilino]diffubenzuron (purity >99%; specific activity 11.2 $\mu\text{g}/\text{g}$) was investigated in a group of eight male New Zealand rabbits. A 10% suspension of diffubenzuron in 1% gum tragacanth was applied at 150 mg/kg bw to shaved areas of the skin and occluded for 6 hours, when the application site was washed. Excretion of radiolabel in urine and faeces was determined over 48 hours. The concentration of radiolabel in faeces was at the limit of quantification (LOQ) (<1% of the applied dose), and about 0.1% of the applied dose was recovered in urine. Diffubenzuron is thus poorly absorbed through rabbit skin (de Lange, 1979; GLP status not specified).

2.1.2 **Biotransformation**

(a) Rats

The basic metabolic pathways of diffubenzuron were established in studies in rats. The main pathway involves hydroxylation of the phenyl moieties of the intact compound. About 80% of the metabolites in rat urine were identified as

2,6-difluoro-3-hydroxybenzuron, 4-chloro-2-hydroxydiflubenzuron and 4-chloro-3-hydroxydiflubenzuron. Approximately 20% underwent scission at the ureido bridge, and most was excreted as 2,6-difluorobenzoic acid (de Lange, Fronk & Post, 1977; Willems et al., 1980).

In the study described above in [section 2.1.1](#), in which Wistar rats were administered radiolabelled diflubenzuron as a single dose of 5 mg/kg bw by oral gavage, there was no indication of the presence of either PCA or 4-chlorophenylurea (CPU), a metabolite that may be reduced to PCA, in the urine or bile of the rats (Willems et al., 1980).

In a GLP-compliant study, the metabolites produced by Sprague-Dawley rats after administration of [^{14}C]diflubenzuron labelled in both phenyl rings were identified and characterized in samples from the study of Dunsire, Cameron & Spiers (1990) (see [section 2.1.1](#) above). Pooled (0–24 hours) samples of urine, faeces or bile were prepared from males and females orally dosed once at 5 or 100 mg/kg bw or 15 times at 5 mg/kg bw per day. After extraction, the metabolites were determined by high-performance liquid chromatographic (HPLC) separation, LSC and comparison with standards. The presence of conjugates was determined by the use of a glucuronidase–arylsulfatase preparation. A specific investigation for the presence of PCA was performed, as routine methods failed to separate this compound from CPU. The metabolic profiles were similar for males and females. Biliary samples were difficult to analyse, as 75–80% of the radiolabel was present in a five-component peak. Enzyme hydrolysis of bile increased the proportion of 2-hydroxydiflubenzuron from about 5% to 19%, at the expense of the major multicomponent peak. Diflubenzuron represented about 7% of the biliary radiolabel. Initial investigations of faecal samples gave variable recovery rates, with diflubenzuron representing more than 93% of the recovered radiolabel. A second method involving enzyme hydrolysis and dilution with control urine was used to investigate samples from animals given the high single dose and repeated doses. Various compounds were identified, the main peaks being diflubenzuron (65–75% of recovered radiolabel), 2-hydroxydiflubenzuron (7–10%), CPU (5–6%) and 2,6-difluorobenzamide (2–4%). Investigations of urine samples gave reliable recoveries (85–105%), and various metabolites were identified, although there was an incomplete separation. The results showed that absorbed diflubenzuron was extensively metabolized. Enzyme treatment of the urine samples indicated conjugation of both 2'-hydroxydiflubenzuron and diflubenzuron. The only notable difference between the groups was the higher concentration of unconjugated 2'-hydroxydiflubenzuron and the presence of peak B in the urine of animals given repeated doses. Specific investigations for PCA did not reveal its presence in bile or urine using a method with an LOQ of 7.5 mg/mL (Cameron, Henderson & McGuire, 1990).

The production of PCA and CPU was investigated in four male Fischer 344 rats given a single dose of [U-¹⁴C-anilino]diflubenzuron (specific activity 164 Bq/mg; radiochemical purity 99.9%) at 104 mg/kg bw in 1% gum tragacanth by oral gavage. Urine and faecal samples were taken after 20, 48, 72 and 96 hours. Pooled samples (0–20 hours) were analysed by HPLC, liquid chromatography with mass spectrometry (LC-MS) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Faecal samples were extracted initially with acetonitrile, then sequentially with hexane, acetone and water. Most of the administered dose was excreted within 20 hours, with 68% in faeces and 2% in urine. The only compound detected in faeces was diflubenzuron. Three metabolites were identified in significant amounts in urine: 4-chloroaniline-2-sulfate (45% of the total urinary radiolabelled residues), *N*-(4-chlorophenyl)-oxamic acid (13%) and 4-chloroacetanilide-2-sulfate (2%). Diflubenzuron, PCA, CPU and their *N*-hydroxy derivatives were not identified in urine. 2-Hydroxydiflubenzuron was present at less than 1% of the total radiolabelled residues (Wang & Gay, 1999; GLP status not specified).

There is no clear reason for the marked differences between the findings of Cameron, Henderson & McGuire (1990) and Wang & Gay (1999). The overall conclusion from these studies is that the primary steps in the metabolism of diflubenzuron are hydrolysis of the anilino ring, cleavage of the ureido bridge and conjugation (mainly to sulfate). Both Willem et al. (1980) and Wang & Gay (1999) indicated that PCA and CPU might not be formed directly in rats. It is possible that hydroxylation of the anilino ring occurs before cleavage of the ureido bridge.

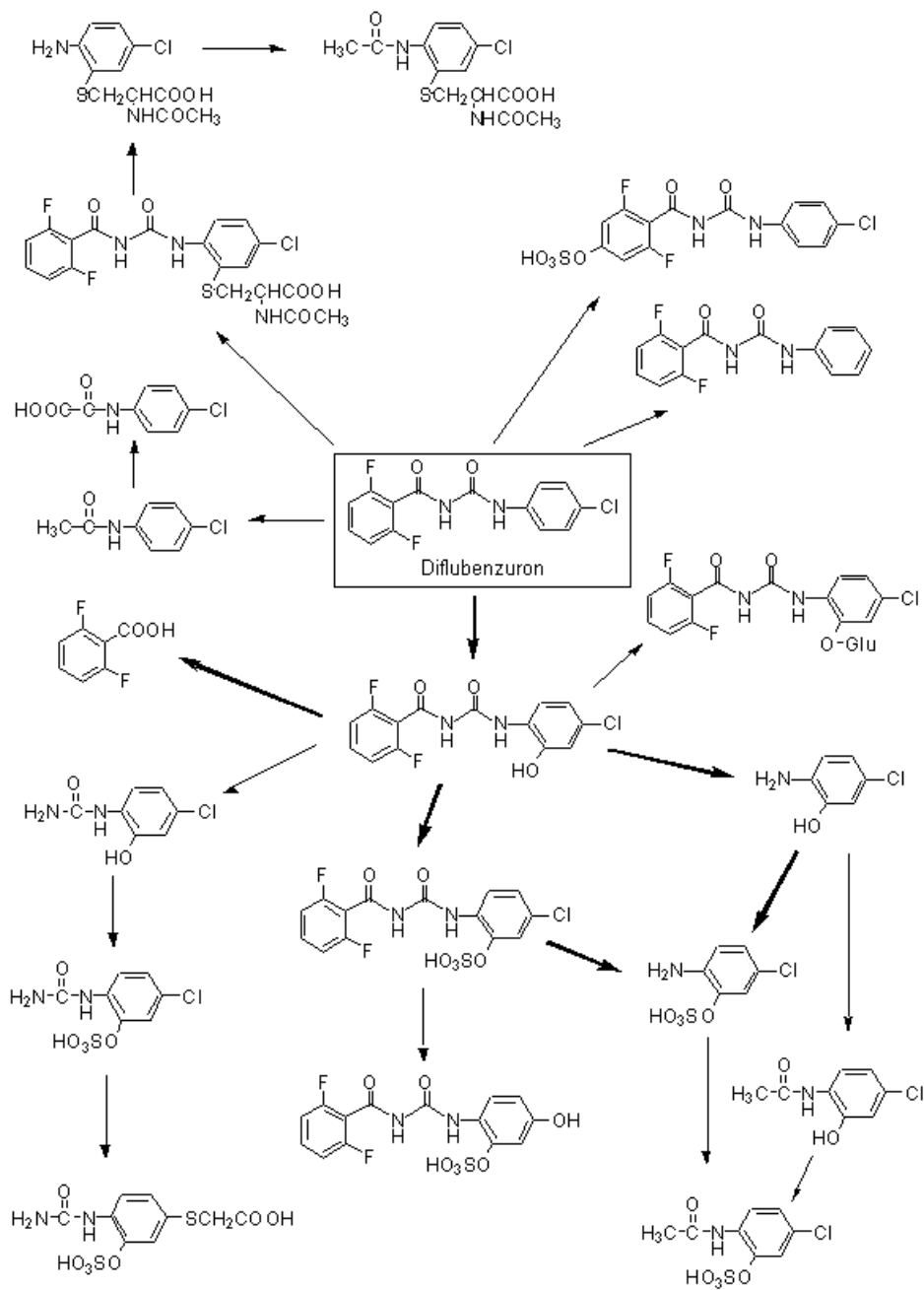
JMPR (FAO/WHO, 2002) proposed a metabolic pathway for diflubenzuron in rats, as presented in Fig. 3.

(b) Other species

PCA was not detected (limits of detection [LODs] not given) in urine or faeces of sheep or cows following administration of a single oral dose of diflubenzuron at 10 mg/kg bw (Ivie, 1978). However, when diflubenzuron was given as a single oral dose of 5 mg/kg bw, PCA was detected in small quantities in the urine of swine (1.03% of the dose; Opdycke, Miller & Menzer, 1982a) and chicken excreta (0.44% of the dose; Opdycke, Miller & Menzer, 1982b).

Following administration of an oral dose of diflubenzuron, CPU was detected in small quantities in the urine of swine (0.82% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982a), in the urine of cows (0.6% of a 10 mg/kg bw dose; Ivie, 1978) and in chicken excreta (3.14% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982b).

Fig. 3
Proposed metabolic pathway for diflubenzuron in rats



Bold arrows: major pathways.
Light arrows: minor pathways.

2.1.3 Effects on enzymes and other biochemical parameters

The effects of diflubenzuron on the concentrations of methaemoglobin and sulphaemoglobin and the incidence of Heinz bodies were investigated in mice and rats dosed once with a 25% weight per weight (w/w) formulation of diflubenzuron. Groups of 15 male Swiss mice (Keet, 1977a) or male Wistar rats (Keet, 1977b) received the formulation at 0 (co-formulants only) or 10 000 mg/kg bw (2500 mg/kg bw as diflubenzuron) in 1% gum tragacanth by oral gavage. Samples of blood were taken 4 and 24 hours after dosing. In mice, there were no effects on body weight, sulphaemoglobin concentration or incidence of Heinz bodies. The methaemoglobin concentrations were increased ($P < 0.003$) at 4 hours, but not at 24 hours. The increase at 4 hours was relatively small (2.2% versus 2.6%), but the ranges (1.3–2.5% versus 1.5–3.3%) indicated a treatment-related effect. A similar pattern was found in rats, with no effects on body weight, sulphaemoglobin concentration or incidence of Heinz bodies. The methaemoglobin concentrations were increased ($P < 0.01$) at 24 hours, but not at 4 hours. The increase at 24 hours was relatively small (1.5% versus 1.9%), but there was a small overlap in the ranges (1.2–1.8% versus 1.6–2.1%), showing a clear treatment-related effect. A single high dose of diflubenzuron (2500 mg/kg bw) can thus increase methaemoglobin concentrations (Keet, 1997a,b).

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies show that diflubenzuron (purity 99.6%) has very low acute toxicity when given by the oral, inhalation or dermal route. The median lethal dose (LD_{50}) in mice and rats given diflubenzuron in 1% tragacanth by oral gavage was greater than 4600 mg/kg bw (van Eldik, 1973). In rats treated dermally for 24 hours, the LD_{50} was greater than 10 000 mg/kg bw (Koopman, 1977). In rats treated by whole-body inhalation of a preparation with a mass median aerodynamic diameter of less than 5 μ m, the median lethal concentration (LC_{50}) was greater than 2.9 mg/L of air (Berczy, Cobb & Cherry, 1973). No clinical signs were seen in these studies, although the level of detail in the study reports was inadequate to permit an independent confirmation. No haematological examinations were performed.

(b) Dermal irritation

In a non-GLP-compliant study, diflubenzuron (purity unknown) was reported not to be irritating to the intact or abraded skin of rabbits (strain and sex not

specified), although the level of detail in the report was inadequate to permit an independent confirmation (Taylor, 1973).

(c) Ocular irritation

In a non-GLP-compliant study, diflubenzuron (purity 99.6%) was slightly irritating to the eyes of New Zealand White rabbits (sex not specified) after instillation of 40 mg (0.1 mL). The findings were similar when the eyes were rinsed 5 minutes or 24 hours after administration (Davies & Liggett, 1973).

(d) Dermal sensitization

In a GLP-compliant study, the skin sensitizing potential of diflubenzuron (purity 95.6%) was investigated in groups of 10 CRL HA BR guinea-pigs exposed in a maximization test. For induction, 10% diflubenzuron in maize oil with Freund's complete adjuvant was injected, or 30% diflubenzuron in a petroleum jelly product was applied topically. The animals were challenged by application of 10% or 30% diflubenzuron in the petroleum jelly product. Mild responses were seen after induction by either route. After challenge, mild reactions were seen in one control and two test animals. Diflubenzuron was not a skin sensitizer in this study (Prinsen, 1992).

2.2.2 Short-term studies of toxicity

(a) Oral administration

(i) Rats

In a GLP-compliant study, groups of 40 Sprague-Dawley rats of each sex (90 of each sex for controls) received diets containing diflubenzuron (two lots; purity 96% and 97.2%) at a concentration of 0, 160, 400, 2000, 10 000 or 50 000 mg/kg feed (equivalent to 0, 8, 20, 100, 500 and 2500 mg/kg bw per day, respectively) for 13 weeks. About half of the animals were killed at week 7, and the remainder at 13 weeks. Routine clinical examinations were performed. Samples were taken from 10 animals of each sex per group for haematology, clinical chemistry and urine analysis in weeks 7 and 13. All animals were examined grossly, and extensive histological investigations were performed on controls and animals at the highest dose, with more limited examination (but including liver, spleen and bone marrow) of other groups.

There were no treatment-related effects on mortality rate, clinical signs or feed consumption. Body weight gain was reduced by about 20% in females receiving 50 000 mg/kg feed and in males receiving 2000 mg/kg feed and above, but with no clear dose-response relationship. Haematology at week 7 showed a range of dose-related alterations in erythrocyte parameters (erythrocyte counts, haemoglobin, reticulocytes, methaemoglobin and sulfhaemoglobin)

in animals of each sex receiving dietary concentrations of 400 mg/kg feed and above, with minimal effects at 160 mg/kg feed; there were small increases in methaemoglobin concentration in males (statistically insignificant) and females (statistically significant) at 160 mg/kg feed. The haematological findings were reproduced at week 13. Alterations in a number of clinical chemistry parameters were seen in animals at the two highest doses, but the findings were sporadic, generally occurred in only a few animals in a group and were considered to be not biologically significant. The absolute and relative weights of the spleen were increased in males and females at 400 mg/kg feed and above and in males receiving 160 mg/kg feed for 7 weeks. At week 13, the absolute weight of the liver was increased by more than 10% in females at the highest dose, and the relative liver weights were increased by more than 10% in males at the highest dose and in females at dietary concentrations of 400 mg/kg feed and above. Pathological lesions related to treatment were chronic hepatitis, haemosiderosis and congestion of the spleen, and erythroid hyperplasia of the bone marrow in all treated groups and haemosiderosis of the liver at dietary concentrations of 400 mg/kg feed and above. The lesions increased in severity with increasing dose and duration of dosing.

From this study, a NOAEL could not be identified, as there were small but statistically significant increases in methaemoglobin concentration, with associated findings in the spleen (increased spleen weight, spleen haemosiderosis and congestion) and bone marrow (erythroid hyperplasia), at the lowest dose, 160 mg/kg feed (equivalent to 8 mg/kg bw per day) (Burdock et al., 1980; Goodman, 1980).

(ii) Dogs

In a non-GLP-compliant study, purebred Beagle dogs (three of each sex per group), aged 16–23 weeks, received diets (400 g/day) containing diflubenzuron (purity unspecified) at a concentration of 0, 10, 20, 40 or 160 mg/kg feed for 13 weeks. The achieved intakes were 0, 0.4, 0.8, 1.6 and 6.4 mg/kg bw per day, respectively. Blood samples for haematology were taken before dosing and at weeks 2, 4, 6 and 12. Clinical chemistry (limited), urine analysis and ophthalmological investigations were performed before dosing and at weeks 6 and 12. All animals were examined grossly at sacrifice, and an extensive range of tissues was examined microscopically. The results were presented for the two sexes combined.

There were no deaths or adverse clinical signs. Body weights and feed and water consumption were not affected by treatment. The body weights fluctuated considerably during the study, which was expected, given the spread of ages, and there was no indication of an association with treatment. The results of urine analysis, ophthalmology and gross pathological and histopathological

examinations were similar in all groups. Increases in aspartate aminotransferase activity were seen in animals at the highest dose in weeks 6 and 12, but the finding was of dubious significance, as it was not reproduced when the samples were retested. At week 2, haematological end-points were similar in all groups. At week 4, the haemoglobin concentration of animals at 160 mg/kg feed was reduced by about 10%. At week 6, both the haemoglobin concentration and erythrocyte count were reduced and the methaemoglobin and free haemoglobin concentrations were increased in animals at 160 mg/kg feed. At week 12, haematological end-points were similar in all groups, although there was a clear increase in the myeloid:erythroid ratio in bone marrow in dogs at 160 mg/kg feed (1.2 versus 0.7 in controls). There was an indication of an increase in spleen weight (by about 40%) at 160 mg/kg feed, but intergroup variation was high, and the ranges in treated and control groups showed considerable overlap.

The NOAEL was 40 mg/kg feed (equal to 1.6 mg/kg bw per day), on the basis of alterations in haematological end-points and bone marrow at 160 mg/kg bw (equal to 6.4 mg/kg bw per day) (Chesterman et al., 1974).

In a non-GLP-compliant study, groups of six male and six female Beagle dogs (treated groups: six of each sex; controls: 12 of each sex) received gelatine capsules containing diflubenzuron (purity 97.6%) at a dose of 0, 2, 10, 50 or 250 mg/kg bw per day for 52 weeks. The animals underwent routine clinical investigations. Blood samples for haematology and clinical chemistry were taken before dosing and at weeks 4, 7, 13, 26 and 51. Urine was analysed before dosing and at weeks 7, 13, 26 and 51. Ophthalmoscopy was performed before dosing and at weeks 26 and 51. All animals were examined grossly and microscopically.

One female at the highest dose died from liver failure, and one female at 50 mg/kg bw per day died from pneumonia, but neither death was clearly related to treatment. No consistent differences were found between control and test animals with respect to clinical signs, feed consumption, body weight, water consumption or urinary parameters. A range of effects related to impaired erythrocytes was seen at 50 and 250 mg/kg bw per day from week 13 onwards. At doses of 50 mg/kg bw per day and above, signs of haemolytic anaemia, destruction of erythrocytes and compensatory regeneration of erythrocytes were observed. Increases in methaemoglobin and sulfhaemoglobin concentrations were evident at doses of 10 mg/kg bw per day and above. The severity of the haematological alterations appeared to increase with duration of dosing, stabilizing at week 26 and with some evidence of adaptation at week 52. Increased sulfhaemoglobin concentrations seen in animals at the lowest dose in week 26 were considered not to be adverse, as they were not statistically significant and were not seen at week 52. Other findings included increased platelet numbers at doses of 10 mg/kg bw per day and above in females and 50 mg/kg bw per day and above in males; increased lactate

dehydrogenase activity from week 7 in animals of each sex receiving 250 mg/kg bw per day; and increases in liver (10%) and spleen weights (35%) at doses of 50 mg/kg bw per day and above. The only notable histopathological findings were in the liver, comprising increased pigmentation of Kupffer cells and macrophages at 10 mg/kg bw per day and above. The increased incidence of pigmentation of Kupffer cells observed in males at the lowest dose (2 mg/kg bw per day) was considered not to be biologically significant, as it was graded “minimal”, and the frequency was within the normal range of background findings.

The NOAEL was 2 mg/kg bw per day, based on significant effects on methaemoglobin and sulphaemoglobin concentrations and platelet counts and increased incidences of hepatic pigmentation at 10 mg/kg bw per day (Greenough et al., 1985).

(b) Dermal application

In a GLP-compliant study, Crl:CD BR (VAF/Plus) rats (10 of each sex per group) received diflubenzuron (purity 96.7%) in 0.25% aqueous gum tragacanth applied dermally to shaved, unabrased sites under semi-occlusive dressings at a dose of 0, 20, 500 or 1000 mg/kg bw per day, 6 hours/day, for 21 days. The application sites represented about 10% of the body area at the middle and high doses and 1% at the low dose. Routine clinical investigations were performed. Blood samples for haematology and clinical chemistry were taken before sacrifice. All animals were examined grossly, and liver, kidney and skin from controls and animals at the high dose were examined histologically.

One female at the middle dose and one at the high dose died on day 9. As these were isolated findings and there were no subsequent deaths, the relationship to exposure to diflubenzuron was uncertain. Body weight, feed consumption, clinical signs, clinical chemistry parameters and gross and microscopic appearance were unaffected. There were no signs of irritation at the dermal application site. Reductions in erythrocyte parameters were seen in females at 500 mg/kg bw per day and in animals of each sex at 1000 mg/kg bw per day, together with increased incidences and severity of polychromasia, hyperchromasia and anisocytosis in animals of each sex at doses of 500 mg/kg bw per day and above. Statistically significant increases in leukocyte counts were seen in males at 500 mg/kg bw per day and above, and a non-significant increase in leukocyte counts was seen in females at the high dose. The findings were linked to increased numbers of segmented neutrophils and lymphocytes, but the association with diflubenzuron was unclear.

The NOAEL was 20 mg/kg bw per day, based on haematological effects at 500 mg/kg bw per day (Goldenthal, 1996).

(c) Exposure by inhalation

In a GLP-compliant study, Sprague-Dawley rats (10 of each sex per group) were exposed for 6 hours/day, 5 days/week, for 4 weeks, by nose only, to atmospheres of diflubenzuron (purity 96.5%) that were generated from a jet mill and contained particles with a mass median aerodynamic diameter of about 2 µm and a gravimetric concentration of 0.2 (control), 12, 34 or 110 mg/m³. The concentrations varied during the study by ±10%, but homogeneity within the chamber was confirmed. The animals were observed routinely during and after exposure with a basic neurofunctional assessment. Samples for urine analysis, haematology and clinical chemistry parameters were taken before sacrifice. All animals were examined grossly. A wide range of tissues from animals in the control and high-concentration groups as well as kidney, liver and lungs from all groups were examined microscopically.

One animal died during the terminal bleeding. Body weight, feed consumption, clinical signs, urine analysis, and gross and microscopic appearance were unaffected. A reduction in “grid count” was evident in the neurofunctional assessment of male and female rats exposed to 110 mg/m³. Statistically significant ($P < 0.05$) decreases in some erythrocyte parameters were seen at this concentration, with evidence of a concentration-related effect. Slight, but statistically significant, increases in bilirubin concentrations were seen in both male and female rats at the highest level of exposure.

The no-observed-adverse-effect concentration (NOAEC) was 34 mg/m³ (approximately 10 mg/kg bw per day), based on effects on behaviour, haematological end-points and bilirubin concentration at 110 mg/m³ (Newton, 1999).

2.2.3 Long-term studies of toxicity and carcinogenicity**(a) Mice**

In a GLP-compliant study, HC/CFLP mice (controls: 104 mice of each sex; treated: 52 mice of each sex per group) were administered diflubenzuron (purity 97.6%) in the diet at a concentration of 0, 16, 80, 400, 2000 or 10 000 mg/kg feed (equal to 0, 1.2, 6.4, 32, 160 and 840 mg/kg bw per day for males and 0, 1.4, 7.3, 35, 190 and 960 mg/kg bw per day for females, respectively) for 91 weeks. Groups of 24 controls and 12 mice in each treated group were sacrificed at weeks 27, 52 and 78. Detailed observations, feed consumption and body weight determinations were performed weekly. Haematology (weeks 26, 52, 78 and 91), clinical chemistry (weeks 24, 50, 76 and 89), urine analysis (weeks 25, 51, 77 and 90) and water consumption determination were performed periodically. All animals were examined grossly, and a wide range of tissues from all animals was examined microscopically.

The mortality rates were similar in all groups, with survival to week 78 being more than 50% except for females receiving dietary concentrations of 80

or 2000 mg/kg feed. Clinical signs (blue-grey coloration of the extremities) were noted at week 1 in animals receiving dietary concentrations of 2000 mg/kg feed and higher and subsequently in animals in all groups receiving dietary concentrations of 80 mg/kg feed and higher. Body weights and feed consumption were similar in all groups. Water consumption was increased at some time points for females receiving dietary concentrations of 2000 mg/kg feed and higher, with an associated increase in urine volume. Urine analysis did not indicate any impairment of renal function. Statistically significant, dose-related changes were seen in a number of haematological parameters from week 26 onwards in animals receiving dietary concentrations of 80 mg/kg feed and higher. Statistically significant increases in the concentrations of sulphaemoglobin were seen at 16 mg/kg feed in animals of both sexes at week 52 and in females at week 78. A dose-related increase in the incidence of Heinz bodies was seen consistently in mice at dietary concentrations of 400 mg/kg feed and higher and sporadically in males at 80 mg/kg feed. Increased serum alkaline phosphatase and aspartate aminotransferase activities were seen in males and females at the highest dietary concentration at week 26 and in males at this concentration subsequently. Gross pathological examination showed increased absolute liver and spleen weights (Table 3) and cyanotic skin as the only consistent findings. Histopathological examination confirmed that the liver and spleen were the primary target organs for non-neoplastic effects (Table 3). No increase in tumour incidence was associated with administration of diflubenzuron. The increased sulphaemoglobin concentrations at 16 mg/kg feed are of questionable biological significance, as they occurred in only a proportion of animals in the group and the frequency was within the range of fluctuating control values seen during the study.

The NOAEL was 16 mg/kg feed (equal to 1.2 mg/kg bw per day), on the basis of increased methaemoglobin and sulphaemoglobin concentrations and incidences of cyanosis at 80 mg/kg feed (equal to 6.4 mg/kg bw per day). The NOAEL for carcinogenicity was 10 000 mg/kg feed (equal to 840 mg/kg bw per day), the highest concentration tested (Colley et al., 1984).

(b) Rats

In a non-GLP-compliant study, groups of CD rats (60 of each sex per group) were fed diets containing diflubenzuron (purity unspecified) at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equivalent to 0, 0.5, 1, 2 and 8 mg/kg bw per day, respectively) for 2 years. The achieved dietary concentrations and homogeneity were not confirmed. All animals were investigated for clinical signs, body weight, feed consumption, and gross and microscopic pathology of a limited range of tissues. Samples from controls and animals at the highest concentration were taken at 3, 6, 12, 18 and 24 months for haematology (10 of each sex), limited

Table 3
Findings in mice given diflubenzuron in the diet for up to 91 weeks

Finding	Sex	0 mg/kg feed	16 mg/kg feed	80 mg/kg feed	400 mg/kg feed	2 000 mg/ kg feed	10 000 mg/ kg feed
Week 26							
Leukocyte count ($10^3/\text{mm}^3$)	Male	6.5	7.7	8.7	8.1	7.2	11*
	Female	6.5	5.3	6.4	6.7	11*	8.3*
Erythrocyte count ($10^6/\text{mm}^3$)	Male	7.5	7.8	7.4	7.6	7.1	7.0
	Female	8.7	9.1	8.4	8.9	8.3	8.1
Haemoglobin (g/dL)	Male	14	15	14	14	14	15
	Female	16	16	16	16	17*	17*
Mean cell haemoglobin (pg)	Male	19	19	19	19	20*	22*
	Female	18	17	18	18	20*	21*
Methaemoglobin (%)	Male	1.6	1.9	2.2*	2.9*	4.9*	5.9*
	Female	1.6	1.8	2.3*	3.4*	5.3*	5.9*
Platelet count ($10^3/\text{mm}^3$)	Male	890	930	900	1 200*	1 200*	1 200*
	Female	1 100	1 100	1 100	1 400*	1 400*	1 400
Absolute liver weight (g)	Male	2.6	2.7	2.5	2.6	3.0*	3.0*
	Female	2.5	2.3	2.5	2.4	2.6	2.8
Absolute spleen weight (g)	Male	0.13	0.16	0.14	0.16	0.22*	0.29*
	Female	0.15	0.14	0.16	0.22*	0.29*	0.41*
Week 78							
Leukocyte count ($10^3/\text{mm}^3$)	Male	10	10	13	14	9.5	13
	Female	7.1	5.7	5.9	5.5	9.4	4.9
Erythrocyte count ($10^6/\text{mm}^3$)	Male	7.3	7.1	6.8	6.9	6.9	6.6
	Female	7.9	8.1	6.9	7.2	7.2	6.3*
Haemoglobin (g/dL)	Male	14	15	12	14	15	16*
	Female	15	15	12	14	14	15
Mean cell haemoglobin (pg)	Male	21	21	18	21	22	24*
	Female	19	18	18	20	20	23*
Methaemoglobin (%)	Male	0.66	0.87	1.4*	2.3*	3.2*	4.6*
	Female	1.3	1.4	1.9*	3.6*	5.2*	6.0*
Sulphaemoglobin (%)	Male	0.1	0.2	0.4*	4.0*	3.8*	6.4*
	Female	0.1	0.6*	0.6*	3.0*	3.5*	3.7*
Platelet count ($10^3/\text{mm}^3$)	Male	1 000	1 000	1 100	1 200	1 500*	1 600*
	Female	590	600	700	930	1 300	1 500
Absolute liver weight (g)	Male	3.4	3.0	3.9	3.5	3.6	3.5
	Female	2.9	2.9	3.4	3.2	3.9	4.3*
Absolute spleen weight (g)	Male	0.22	0.48	0.97	0.52	0.22	0.33
	Female	0.27	0.22	0.38	0.50	0.55	0.46
Week 91							
Total number of tissues/sex per group		104	52	52	52	52	52
Hepatocyte enlargement	Male	36	12	11	23	35*	31*
	Female	15	4	5	11	15*	29*

Finding	Sex	0 mg/kg feed	16 mg/kg feed	80 mg/kg feed	400 mg/kg feed	2 000 mg/ kg feed	10 000 mg/ kg feed
Hepatocyte vacuolation	Male	22	17	9	12	18	26*
	Female	33	17	17	17	12	26
Pigmented Kupffer cells	Male	0	0	1	1	3	15*
	Female	1	0	1	0	±4	11*
Splenic extramedullary haematopoiesis	Male	27	7	15	21	19	22*
	Female	37	18	19	19	25	25
Splenic siderocytes	Male	5	1	2	16*	32*	30*
	Female	15	6	13	30*	31*	43*

*, $P < 0.05$

Source: Colley et al. (1984)

clinical chemistry (five of each sex) and urine analysis (five of each sex). Ophthalmological investigations were performed on controls and animals at the highest concentration at 3, 6, 12, 18 and 24 months. Haematological investigations were performed on animals at 40 mg/kg feed (10 of each sex) at 12, 18 and 24 months.

The survival rate was unaffected by treatment but was low, with less than 30% of animals surviving in all groups at termination. There were no treatment-related effects on urine analysis, clinical chemistry, ophthalmology, feed consumption or body weight gain. Statistically significant ($P < 0.05$) increases in methaemoglobin concentrations were seen at 12 and 18 months in males and females receiving 160 mg/kg feed, but not in those receiving 40 mg/kg feed. Sulphaemoglobin concentration was unaffected by treatment, but the free haemoglobin concentration was reduced in groups at the highest concentration at 3 and 12 months. No increase in the incidence of tumours was seen in treated animals; however, the poor survival and limited range of tissues examined severely limited the power of this study to detect a tumorigenic compound.

The NOAEL was 40 mg/kg feed (equivalent to 2 mg/kg bw per day), on the basis of haematological changes (increases in methaemoglobin concentrations) at 160 mg/kg feed (equivalent to 8 mg/kg bw per day). The NOAEL for carcinogenicity was 160 mg/kg feed (equivalent to 8 mg/kg bw per day), the highest concentration tested (Hunter et al., 1976; Colley & Offer, 1977).

In a GLP-compliant study, Sprague-Dawley rats (controls: 100 of each sex; treated: 50 of each sex per group) were fed diets containing diflubenzuron (purity 97.6%) at a concentration of 0, 160, 620, 2500 or 10 000 mg/kg feed (equal to 0, 7.1, 28, 112 and 472 mg/kg bw per day for males and 0, 9.3, 37, 128 and 612 mg/kg bw per day for females, respectively) for 2 years. All animals were

investigated for clinical signs, body weight, feed consumption, and gross and microscopic pathology of a wide range of tissues. Haematology (10 of each sex per group) was performed at 12 and 24 months. No clinical chemistry, urine analysis or ophthalmological examinations were performed; because these parameters were not affected in rats at 160 mg/kg feed in the previous non-GLP-compliant study, the highest dose tested in the non-GLP-compliant study and the lowest dose tested in this GLP-compliant study, this was considered not to be a significant omission.

The survival rate was similar in all groups and was greater than 50% at termination. Feed consumption was similar in all groups, but body weight gain was reduced by more than 10% in females at 2500 and 10 000 mg/kg feed from week 4, achieving statistical significance at week 26. A range of erythrocyte parameters was altered in both males and females at 620 mg/kg feed and above at 12 and 24 months, although there was no marked progression with duration of dosing (Table 4). The methaemoglobin and sulphaemoglobin concentrations were increased significantly in males at the lowest concentration at 12 months, but there was no clear dose–response relationship, and the biological significance of the finding is debatable. Increased numbers of reticulocytes and the extent of bone marrow hyperplasia indicated that the rats responded to the effects on erythrocytes. The only gross finding was enlarged spleens, and this was confirmed by the data on organ weights. The main treatment-related histopathological findings were pigmented (golden brown) macrophages in the spleen and liver and erythroid hyperplasia of the bone marrow. The background incidence of pigmented macrophages in the spleen was high, however, and there was no clear dose–response relationship. Evidence of irritation in the stomach was seen in males receiving diflubenzuron at dietary concentrations greater than 2500 mg/kg feed that survived to termination. The overall incidence of tumours was low, with no evidence of atypical findings or any dose–response relationship.

No NOAEL for toxicity could be identified, as increased methaemoglobin and sulphaemoglobin concentrations were noted at 160 mg/kg feed (equal to 7.1 mg/kg bw per day), the lowest dose tested, and were consistent with the pattern of toxicity of diflubenzuron seen at other doses. The NOAEL for carcinogenicity was 10 000 mg/kg feed (equal to 472 mg/kg bw per day), the highest dietary concentration tested (Burdock et al., 1984).

2.2.4 Genotoxicity

Table 5 summarizes the eight in vitro and four in vivo studies performed to examine the genotoxic potential of diflubenzuron. JMPR (FAO/WHO, 2002) reviewed all of the genotoxicity studies except for the two recent in vivo studies by de Barros et al. (2013). JMPR indicated that the overall quality of the studies was acceptable, positive controls giving positive results and the concentration ranges

Table 4
Findings in rats given diflubenzuron in the diet for up to 104 weeks

Finding	Sex	0 mg/kg feed	160 mg/kg feed	620 mg/kg feed	2 500 mg/ kg feed	10 000 mg/ kg feed
Week 52						
Erythrocyte count (10 ⁶ /mm ³)	Male	8.7	8.3	7.6*	8.0	7.4*
	Female	8.1	7.8	7.6	7.1*	6.5*
Haemoglobin (g/dL)	Male	17	16	15*	16	15*
	Female	15	15	14	14*	13*
Erythrocyte volume fraction (%)	Male	48	48	44	46	43*
	Female	46	46	45	43	41*
Reticulocyte count (%)	Male	0.9	0.8	1.6	1.7*	2.1*
	Female	1.0	1.1	1.8	3.1*	5.0*
Methaemoglobin (%)	Male	0.2	2.0*	1.5	2.4*	2.5*
	Female	0.6	0.9	1.4	1.9*	2.2*
Sulphaemoglobin (%)	Male	0.1	1.3*	0.9*	0.7	1.1*
	Female	0.1	0.2	1.0*	0.1	0.4
Week 104						
Methaemoglobin (%)	Male	0.8	1.1	1.3	1.8*	1.8*
	Female	0.7	1.3	1.6*	2.1*	2.3*
Sulphaemoglobin (%)	Male	0.2	0.2	0.6	0.8	0.4
	Female	0.2	0.3	0.6	1.1*	1.1*
Myeloid:erythroid cell ratio	Male	2.5	1.6	1.8	1.5*	1.5
	Female	1.9	1.1*	1.3	1.3	1.1*
Absolute spleen weight (g)	Male	1.0	1.0	1.3	1.4*	1.5*
	Female	0.7	0.9	0.9	1.1*	1.2*
Sternum, marrow hyperplasia (%)	Male	16	10	8	60*	72*
	Female	4	6	2	6	2
Sternum, erythroid hyperplasia (%)	Male	4	6	26*	38*	30*
	Female	1	2	12*	22*	26*
Liver, pigmented macrophages (%)	Male	22	20	50*	82*	72*
	Female	26	38	74*	80*	86*
Spleen, pigmented macrophages (%)	Male	66	84	86	88	74
	Female	80	90	100	94	100
Stomach, acanthosis or hyperkeratosis (%)	Male	1	0	3	11*	24*
Stomach, gastritis (%)	Male	1	0	0	7*	21*

*, $P < 0.05$

Source: Burdock et al. (1984)

of diflubenzuron being sufficient to induce signs of toxicity or precipitation. JMPR concluded that diflubenzuron was not genotoxic in vitro (in the presence or absence of metabolic activation) or in vivo (FAO/WHO, 2002).

Table 5
Results of genotoxicity assays with diflubenzuron

Test system	Test object	Concentration/dose	Results	References
In vitro				
Reverse mutation (Ames)	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538	8, 40, 200, 1 000 µg/plate	Negative	Koorn (1990)
Reverse mutation (Ames)	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538; <i>Saccharomyces cerevisiae</i> D4	0.1, 1, 10, 500 µg/plate	Negative	Brusick & Weir (1977a)
Reverse mutation (Ames)	<i>S. typhimurium</i> TA98, TA100, TA1537, TA1538	10, 100, 1 000 µg/plate	Negative	MacGregor et al. (1979)
Forward mutation	L5178Y mouse lymphoma	1, 5, 19, 38, 75, 150, 300 µg/mL	Negative	MacGregor et al. (1979)
Cell transformation	BALBc/3T3 cells	20, 40, 80, 160, 310 µg/mL	Negative	Brusick & Weir (1977b)
Unscheduled DNA synthesis	WI-38 human cells	50, 100, 500, 1 000 µg/mL	Negative	Brusick & Weir (1977c)
Unscheduled DNA synthesis	Wistar rat hepatocytes (primary culture)	1, 3, 10, 33, 100, 333 µg/mL	Negative	Enninga (1990)
Chromosomal aberrations	Chinese hamster ovary cells	100, 150, 200, 250 µg/mL	Negative	Taalman & Hoorn (1986)
In vivo				
Dominant lethal mutation	Albino (CR) mice	1 000, 2 000 mg/kg bw (in corn oil, intraperitoneally)	Negative	Arnold, Kennedy & Keplinger (1974) ^a
Micronucleus induction	Bone marrow (Swiss mice)	15, 150, 1 500 mg/kg bw (in corn oil, by oral gavage)	Negative	MacGregor et al. (1979)
Micronucleus induction	Peripheral blood (Swiss mice)	0.3, 1, 3 mg/kg bw	Positive (clastogenic)	de Barros et al. (2013)
Comet formation	Peripheral blood (Swiss mice)	0.3, 1, 3 mg/kg bw	Positive	de Barros et al. (2013)

bw: body weight; DNA: deoxyribonucleic acid

^a Not validated.

The two more recent in vivo genotoxicity studies were identified from a literature search (de Barros et al., 2013; GLP status not specified). The genotoxic potential of diflubenzuron in vivo was examined. Fifty adult male Swiss mice (60 days old, weighing 30–40 g) were divided into five groups (10 per group). Three groups received diflubenzuron (purity 98%) at a single dose of 0.3, 1 or 3 mg/kg bw by oral gavage. The negative control group received the vehicle, corn oil. The positive control group was treated with cyclophosphamide dissolved in saline at a dose of 100 mg/kg bw.

Peripheral blood was collected from each animal for the comet assay and micronucleus test. The comet assay was conducted on blood collected 24 hours after treatment. A total damage score was determined by multiplying the number of cells assigned to each class of damage by the numeric value of the class and

summing all resulting values. The micronucleus test was conducted on blood collected from the tail of animals at 24, 48 and 72 hours after treatment. Two thousand cells per animal (two slides per animal per treatment) were examined. Micronucleated cells were counted using a fluorescence microscope with a 100× magnification, and the frequency of micronuclei was determined.

Diffubenzuron increased the incidence of comet formation at all doses tested. A significant increase in the frequency of micronuclei was observed at the sampled time points at all doses tested when compared with the negative (vehicle) control. The results indicated that diffubenzuron exerts genotoxic effects in a dose-dependent manner. It was noted that the highest dose (3 mg/kg bw) showed a significantly higher frequency of micronuclei than the positive control group at all time points measured.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

In a GLP-compliant two-generation reproductive toxicity study conducted according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 416, groups of 32 male and 32 female CrI:CD(SD)BR (VAF/Plus) rats (28 of each sex in the F₁ generation) received diets containing diffubenzuron (purity 97.1%) at a concentration of 0, 500, 5000 or 50 000 mg/kg feed (equal to 0, 42, 430 and 4300 mg/kg bw per day for males and 0, 36, 360 and 3800 mg/kg bw per day for females, respectively) for 70 days before mating (1:1). The animals were examined routinely for clinical signs and body weight changes. Litters were culled, when possible, to four of each sex on day 4. Blood samples for haematological examination were taken from F₀ and F₁ parents before sacrifice. Haematological end-points and the spleen were not investigated in young animals. All pups and parental animals were examined grossly, whereas histopathological examinations were limited to the reproductive organs, liver and spleen of parental animals. Routine investigations of reproductive performance along with evaluations of developmental end-points, including surface righting reflex, startle reflex, air righting reflex, vaginal opening and preputial separation, were conducted.

Two deaths occurred, a male at the high dose and a female at the low dose, neither of which appeared to be related to treatment. The only clinical sign associated with treatment was pale faeces at the high dose. Animals at this dose had reduced feed consumption at week 1, but not subsequently. Water consumption was increased in all treated groups, but this was considered to be secondary to the high concentrations of diffubenzuron in the diet and not biologically significant. Reproductive parameters and sperm quality and quantity were not impaired by exposure to diffubenzuron. Pup weights were reduced in a dose-related manner

in the F_0 generation, but not in the F_1 generation, and the reduction achieved statistical significance ($P < 0.05$) between day 4 and weaning in animals at 50 000 mg/kg feed. The body weight gain of treated animals after weaning was similar to or greater than that of controls. Attainment of the air righting reflex and startle response was enhanced slightly in diflubenzuron-treated F_0 litters, but unchanged in F_1 animals. Statistically significant ($P < 0.01$) alterations in erythrocyte parameters and increases in lymphocyte counts and platelet numbers were seen in all parental groups. The spleen was the primary target organ, with increases in weight, congestion and haemosiderosis at all doses and an increase in the incidence of congested red pulp in F_0 animals at the middle and high doses. Increased incidences of centrilobular hepatocyte hypertrophy were seen in both generations at concentrations at 5000 mg/kg feed and above, and the prevalence of brown pigmentation of Kupffer cells was increased in all treated groups.

The only effect on reproductive outcome and pup development was a reduction in pup body weight gain during lactation, with statistically significant decreases in F_1 pup weights on days 4, 8 and 21 of lactation. The NOAEL for reproductive effects was 50 000 mg/kg feed (equal to 3800 mg/kg bw per day), the highest dose tested. The NOAEL for toxicity in offspring was 5000 mg/kg feed (equal to 360 mg/kg bw per day), on the basis of reductions in pup body weight at 50 000 mg/kg feed (equal to 3800 mg/kg bw per day) in the F_0 generation. No NOAEL could be identified for toxicity in parental animals, as haematological effects consistent with those seen in other studies with diflubenzuron were observed at all doses tested (Brooker, 1995).

(b) Developmental toxicity

(i) Rats

In a GLP-compliant study, diflubenzuron (purity 97.6%) was administered in 1% tragacanth solution by oral gavage to timed-mated female CrI:CD(SD)BR rats (24 per group) on days 6–15 of gestation at 0 or 1000 mg/kg bw per day (limit dose). The dams were killed on day 20 of gestation and examined grossly, and the uterine contents were removed. About 50% of the fetuses were examined by dissection, followed by staining with alizarin red S for skeletal investigations; the remainder were examined by sectioning and dissection.

There were no deaths or evidence of clinical signs in the dams. Maternal body weights and reproductive parameters were unaffected by treatment. The fetal weights and sex ratio were similar in control and treated groups. Two major malformations were seen in the offspring of treated dams: a conjoined twin and a pup with a short body, malrotated hindlimbs and a horseshoe kidney. Conjoined twins are generally considered to be a preimplantation event and, as such, would have occurred before the first dose of diflubenzuron. The abnormalities in the

other pup had been seen in previous studies in the laboratory, and an isolated incidence was considered not to be related to treatment. The incidences of bilobed thoracic centra and retarded ossification of the third and sixth sternebrae were higher in the treated group than in concurrent controls, but were within the range of incidences in contemporary controls in other studies.

The NOAELs for maternal toxicity and embryo/fetal toxicity were the limit dose of 1000 mg/kg bw per day (Kavanagh, 1988a).

(ii) Rabbits

In a GLP-compliant study, groups of 16 timed-mated female New Zealand White rabbits received diflubenzuron (purity 97.6%) by oral gavage in 1% tragacanth at 0 or a limit dose of 1000 mg/kg bw per day on days 7–19 of gestation. The does were killed on day 28 of gestation and examined grossly, and the uterine contents were removed. Fetuses were examined by dissection, followed by staining with alizarin red S for skeletal investigations.

One control rabbit died, and one treated animal was killed because it developed hindlimb paralysis. The latter effect was considered unlikely to be related to treatment, as it was an isolated finding, and no lesions were found in nerves or muscles in other studies with diflubenzuron. Maternal clinical signs, body weights and reproductive parameters were not affected by treatment. Fetal weights and sex ratios were similar in control and treated groups. The overall incidences of malformations and variations were lower in treated animals than in controls, with no significant increase for any individual finding.

The NOAELs for maternal toxicity and embryo/fetal toxicity were the limit dose of 1000 mg/kg bw per day (Kavanagh, 1988b).

2.2.6 Special studies

(a) Metabolite: 4-chlorophenylurea (CPU)

(i) Metabolism

The metabolism of CPU was studied in four male Fischer 344 rats that received [U-¹⁴C-phenyl]CPU (specific activity 70.3 GBq/mol; radiochemical purity 97%) in 1% gum tragacanth by gavage at 198 mg/kg bw. Urine and faeces were collected at 20, 48, 72, 96, 120 and 144 hours (termination). The total radiolabel in urine, faeces and carcass was determined by LSC after appropriate processing. Pooled faecal and urine samples (0–20 and 20–48 hours) were examined by HPLC, thin-layer chromatography (TLC), LC-MS and gas chromatography with mass spectrometry (GC-MS) and comparison with standards. Metabolites in faeces were identified after sequential extractions with acetonitrile, hexane, acetone and water. Urine samples were also investigated after treatment with glucuronidase or sulfatase.

Over 90% of the administered dose was excreted in urine. Four faecal metabolites were identified, all of which were also found in urine. The major faecal metabolite was 4-hydroxyphenylurea (about 3% of the administered dose), with unchanged CPU present at 0.6%. Thirteen distinct peaks were obtained from urine samples, the major components being 4-chlorophenylurea-2-sulfate (25% of the administered dose), 4-hydroxyphenylurea (18%), 4-chlorophenylurea-2-glucuronide (17%), 4-chlorophenylurea-3-sulfate (8%) and phenylurea-4-sulfate (7%). PCA was not detected in urine at an LOD of 0.02 µg/mL. The metabolic profile of CPU showed that it undergoes extensive ring hydroxylation and conjugation with either sulfate or glucuronide (Gay, Wang & Long, 1999; GLP status not specified).

(ii) Acute toxicity

The acute toxicity of CPU was investigated in male Fischer 344 rats as part of a study of metabolism. The rats were dosed once with CPU (purity 99.3%) at 190, 220 or 230 mg/kg bw in 1% gum tragacanth. No deaths were seen at the middle and high doses, but pronounced clinical signs of central nervous system depression (lethargy, ataxia, loss of righting reflex) were seen within 30 minutes of dosing. The rat given 190 mg/kg bw showed mild signs of central nervous system depression within 90 minutes of dosing. Chromodacryorrhoea lasting 36–48 hours was seen in all animals (Gay, Wang & Long, 1999).

(b) Metabolite: *p*-chloroaniline (PCA)

(i) General toxicity

Repeated exposure to PCA leads to cyanosis and methaemoglobinaemia, followed by effects in blood, liver, spleen and kidneys, as evidenced by changes in haematological parameters, splenomegaly and haemosiderosis (from moderate to heavy) in spleen, liver and kidney, partially accompanied by extramedullary haematopoiesis. The lowest-observed-adverse-effect levels (LOAELs) for a significant increase in methaemoglobin levels in rats and mice were 5 and 7.5 mg/kg bw per day, respectively, for a 13-week oral gavage administration of PCA. The LOAEL for a 103-week oral gavage study in rats (with administration 5 days/week) was 2 mg/kg bw per day, based on a significant increase in methaemoglobin levels and fibrotic changes of the spleen in male rats; hyperplasia of bone marrow was observed in female rats at and above 6 mg/kg bw per day (IPCS, 2003). This information demonstrated that PCA exhibits toxicity end-points similar to those of diflubenzuron, but is more potent than diflubenzuron.

(ii) Carcinogenicity

Mice

Groups of B6C3F1 mice (50 of each sex per group), 6 weeks of age, were fed a diet containing PCA (technical grade, purity unspecified, melting point 68–71 °C) at a concentration of 2500 or 5000 mg/kg feed (equivalent to 375 and 750 mg/kg bw per day, respectively) for 78 weeks, followed by a 13-week observation period. A group of 20 male and 20 female controls received the diet alone. Body weights were recorded once a week for the first 6 weeks, every 2 weeks for the next 12 weeks and monthly thereafter. All animals were inspected twice daily. Feed consumption was measured monthly for 20% of the animals in each group.

The numbers of surviving animals at 91 weeks were 18/20 (control), 44/50 (low dose) and 44/50 (high dose) for males; and 20/20 (control), 41/50 (low dose) and 39/50 (high dose) for females. Decreased body weight gain was observed in both treated males and females relative to that of controls. Non-neoplastic proliferative and chronic inflammatory lesions were found in the spleens of treated animals. Haemangiosarcomas occurred in all organs and tissues (e.g. subcutaneous tissue, spleen, liver, kidney) in 2/20 control, 9/50 low-dose and 14/50 high-dose males; one haemangioma was observed in males in the low-dose group. The increased incidence of total vascular tumours was significant ($P < 0.025$, Cochran-Armitage trend test). Among female mice, haemangiosarcomas occurred at all of the sites in 0/18 control, 3/49 low-dose and 7/42 high-dose animals; one haemangioma was observed in females in the high-dose group. The Cochran-Armitage trend test indicated a significant positive association between incidence of combined vascular tumours (haemangiosarcomas or haemangiomas) and doses in females ($P = 0.012$) but not in males ($P > 0.05$); although not significant with the Fisher exact tests for both sexes, the increased incidence of combined vascular tumours in treated animals of both sexes also exceeded the historical control incidence for combined haemangiosarcomas or haemangiomas (about 3% for either sex). It was concluded that there was insufficient evidence to conclude that PCA was carcinogenic in mice under the conditions of this bioassay (USNCI, 1979).

Groups of B6C3F1 mice (50 of each sex per group), 7 weeks old, were administered PCA (purity 99.1%; lot no. 127) by oral gavage in aqueous hydrochloric acid (molar equivalents) at 3, 10 or 30 mg/kg bw per day, 5 days/week, for 103 weeks. Controls received deionized water at a volume of 5 mL/kg bw. All animals were observed twice per day. Clinical signs were recorded once per month. Individual body weights were recorded once every week for the 13 weeks of the study and once every month thereafter. Necropsy was performed on all animals. Histopathological examinations on a standard set of tissues were performed on all vehicle control and high-dose animals, all animals dying before the end of the study

and all grossly visible lesions in all dose groups. Liver and spleen (the target tissues) from the lower-dose groups (3 and 10 mg/kg bw per day) were also examined.

Mean body weights of dosed animals were generally within 5% of those of controls throughout the study. No test article-related clinical signs were observed. Survival after week 103 was 43/50 (controls), 36/50 (low dose), 29/50 (middle dose; $P = 0.005$) and 35/50 (high dose) for males; and 39/50 (controls), 42/50 (low dose), 44/50 (middle dose) and 41/50 (high dose) for females. Incidences of proliferation of haematopoietic cells in the liver were increased in dosed females. Multifocal renal tubular pigmentation (haemosiderin) was observed in high-dose females, but not in any dosed males. Hepatocellular adenomas were observed in male mice with a significant negative trend, and hepatocellular carcinomas occurred with a significantly positive trend. The incidences of hepatocellular carcinomas in mid- and high-dose males and the incidences of hepatocellular adenomas or carcinomas (combined) in low-, mid- and high-dose males were significantly greater than those in vehicle controls; hepatocellular carcinomas metastasized to the lung (see [Table 6](#)). No significant increase in the incidence of such tumours occurred in females. The incidence of haemangiosarcomas in high-dose males was marginally increased relative to that in the controls; nearly all incidences of haemangiosarcomas occurred in the liver or spleen. The incidences of malignant lymphomas in low- and high-dose males and in mid- and high-dose females were significantly lower than those in controls. It was concluded that there was some evidence of carcinogenicity in male mice and no evidence in female mice (USNTP, 1989).

Rats

Groups of Fischer 344 rats (50 of each sex per group), 6 weeks of age, were fed diets containing PCA (technical grade; purity unspecified) at 250 or 500 mg/kg feed (equivalent to 12.5 and 25 mg/kg bw per day, respectively) for 78 weeks, followed by a 24-week observation period. Control groups (20 of each sex) received the diet alone. At the end of the 24-week observation period, the surviving animals were sacrificed. Body weights were recorded once a week for the first 6 weeks, every 2 weeks for the next 12 weeks and monthly thereafter. All animals were inspected twice daily. Feed consumption was measured monthly for 20% of the animals in each group. Gross and comprehensive histological examinations were performed on all animals.

There was no difference in body weight gain in the treated animals compared with the controls. Survival at week 102 was 18/20 (controls), 46/50 (low dose) and 38/50 (high dose) for males; and 18/20 (controls), 49/50 (low dose) and 45/50 (high dose) for females. Non-neoplastic proliferative and chronic inflammatory lesions were found in the spleens of treated rats. Mesenchymal tumours (fibroma, fibrosarcoma, haemangiosarcoma, osteosarcoma and sarcoma

Table 6
Incidences of significant findings in mice treated with PCA hydrochloride

		Incidence of finding ^a			
	Sex	Control	Low dose	Middle dose	High dose
Neoplastic lesions (tumours)					
Hepatocellular adenomas	M	9/50 (18%)*	15/49 (31%)	10/50 (20%)	4/50 (8%)
Hepatocellular carcinoma	M	3/50 (6%)**	7/49 (14%)	11/50 (22%)*	17/50 (34%)**
<i>Metastatic to lung</i>		1/50	1/49	2/50	9/50
Hepatocellular adenomas or carcinomas (combined)	M	11/50 (22%)	21/49 (43%)*	20/50 (40%)*	21/50 (42%)*
Haemangiosarcomas, liver	M	2/50 (4%)	2/50 (4%)	1/50 (2%)	6/50 (12%)
Haemangiosarcomas, spleen	M	3/50 (6%)	2/50 (4%)	0/50 (0%)	5/50 (10%)
Haemangiosarcomas, all sites	M	4/50 (8%)*	4/49 (8%)	1/50 (2%)	10/50 (20%)
Malignant lymphomas	M	10/50 (20%)	3/49 (6%)*	9/50 (18%)	3/50 (6%)*
	F	19/50 (38%)	12/50 (24%)	5/50 (10%)**	10/50 (20%)*
Non-neoplastic lesions					
Proliferation of haematopoietic cells, liver	M	7/50 (14%)	4/49 (8%)	7/50 (14%)	5/50 (10%)
	F	15/50 (30%)	29/50 (58%)	24/50 (48%)	31/50 (62%)
Multifocal renal tubular pigmentation	F	0/50 (0%)	0/7 (0%)	0/4 (0%)	38/49 (78%)

F: female; M: male; *: $P < 0.05$; **: $P < 0.001$ (logistic regression test for neoplastic lesions)

^a Number of animals with the specific lesions/total number of animals examined, or percentage.

Source: USNTP (1989)

not otherwise specified) in the spleen or splenic capsule occurred in 0/20 control, 0/49 low-dose and 10/49 high-dose male rats ($P = 0.001$, Cochran-Armitage trend test) and in 0/18 control, 2/49 low-dose and 5/42 high-dose female rats. The small number of fibromas and sarcomas in the spleens of male rats was considered suggestive of carcinogenicity because of the rarity of these tumours in the spleens of control rats. However, there was insufficient evidence to conclude that PCA was carcinogenic in rats (USNCI, 1979).

In a GLP-compliant study, groups of F344/N rats (49 or 50 of each sex per group), 8 weeks old, were administered PCA (purity 99.1%; lot no. 127) by oral gavage in aqueous hydrochloric acid (molar equivalents) at 2, 6 or 18 mg/kg bw, 5 days/week, for 103 weeks. Controls (50 of each sex) received deionized water at a volume of 5 mL/kg bw. All animals were observed twice per day. Clinical signs were recorded once per month. Individual body weights were recorded once every week for the first 13 weeks of the study and once every month thereafter. Blood samples for haematological parameters and methaemoglobin determination were collected from 15 randomly selected rats from each group at 6, 12, 18 and 24

months. Rats were dosed for 2 consecutive days before blood collection, except at 24 months, when collection was made 11–14 days after administration of the last dose. Animals found moribund or those surviving to the end of the study were humanely sacrificed. Necropsy was performed on all animals. Histopathological examinations were performed on a standard set of tissues from all vehicle control and high-dose animals, all animals dying before the end of the study and all grossly visible lesions in all dose groups. The following tissues in the low- and mid-dose groups were examined: adrenal glands, bone, bone marrow, kidneys, liver, spleen and testes.

The mean body weights of high-dose females were 4–6% lower than those of controls after week 70. The mean body weights of dosed males were generally within 5% of those of controls throughout the study. Mid- and high-dose males and high-dose females had blue extremities, which was indicative of cyanosis. Survival at week 103 in low-dose and mid-dose males was significantly higher than that in the control group: 18/50, 32/50 ($P = 0.007$), 32/50 ($P = 0.005$) and 21/50 for the control, low, middle and high doses, respectively; as was that of low- and high-dose females: 27/50, 39/50 ($P = 0.011$), 36/50 and 37/50 ($P = 0.043$) for the control, low, middle and high doses, respectively. There were changes in haematological parameters at various time points measured, such as decreases in haemoglobin concentration, erythrocyte count, haematocrit value, mean corpuscular volume, nucleated erythrocytes and mean corpuscular haemoglobin. Compound-related non-neoplastic lesions included bone marrow hyperplasia, hepatic haemosiderosis and splenic fibrosis. The incidences of proliferative mesenchymal lesions and fatty metaplasia of the spleen were increased in dosed animals: the incidences of splenic fibrosis were increased in dosed males and females; the incidence of uncommon primary sarcomas of the spleen in high-dose males (Table 7), many of which metastasized to one or more sites, was significantly higher than that in the controls; and cellular infiltration of lipocytes (fatty metaplasia) was observed in high-dose animals (males: 0/49, 0/50, 0/50, 24/50; females: 0/50, 0/50, 0/50, 11/50, for the control, low, middle and high doses). An increased incidence of medullary hyperplasia of the adrenal gland was noted in high-dose females. The incidences of adrenal phaeochromocytomas or malignant phaeochromocytomas combined were significantly higher in the high-dose males. The incidence of mononuclear cell leukaemias was decreased in all treated groups: 21/49, 3/50, 2/50 and 3/50 for males and 10/50, 2/50, 1/50 and 1/50 for females in the control, low-dose, mid-dose and high-dose groups, respectively. Incidences of femoral hyperplasia were increased in high-dose males and mid- and high-dose females (males: 26/49, 36/50, 35/49, 46/50; females: 11/50, 12/48, 21/50, 37/47); and incidences of femoral reticular cell hyperplasia were increased in mid- and high-dose females (males: 0/49, 0/50, 3/49, 0/50; females: 1/50, 2/48, 7/50, 7/47). Incidences of haemosiderin pigmentation were

Table 7
Incidences of findings in the spleen and adrenal gland in rats treated with PCA

Findings	Incidence of finding			
	Control	Low dose	Middle dose	High dose
Spleen, males				
Fibrosis	3/49 (6%)	11/50 (22%)	12/40 (24%)	41/50 (82%)
Fibroma	0/49 (0%)	0/50 (0%)	0/50 (0%)	2/50 (4%)
Fibrosarcoma	0/49 (0%)	1/50 (2%)	2/50 (4%)	17/50 (34%)**
Osteosarcoma	0/49 (0%)	0/50 (0%)	1/50 (2%)	19/50 (38%)**
Haemangiosarcoma	0/49 (0%)	0/50 (0%)	0/50 (0%)	4/50 (8%)
Fibrosarcoma, osteosarcoma or haemangiosarcoma, combined	0/49 (0%)	1/50 (2%)	3/50 (6%)	38/50 (76%)**
Spleen, females				
Fibrosis	1/50 (2%)	2/50 (4%)	3/50 (6%)	42/50 (84%)
Fibrosarcoma	0/50 (0%)	0/50 (0%)	1/50 (2%)	0/50 (0%)
Osteosarcoma	0/50 (0%)	0/50 (0%)	0/50 (0%)	1/50 (2%)
Adrenal gland, males				
Medullary hyperplasia	16/49 (31%)	21/48 (44%)	15/48 (31%)	17/49 (35%)
Phaeochromocytoma	13/49 (27%)	14/48 (29%)	14/48 (29%)	25/49 (51%)*
Malignant phaeochromocytoma	1/49 (2%)	0/48 (0%)	1/48 (2%)	1/49 (2%)
Phaeochromocytoma or malignant phaeochromocytoma, combined	13/49 (27%)	14/48 (29%)	15/48 (31%)	26/49 (53%)*
Adrenal gland, females				
Medullary hyperplasia	4/50 (8%)	4/50 (8%)	7/50 (14%)	24/50 (48%)
Phaeochromocytoma	2/50 (4%)	3/50 (6%)	1/50 (2%)	6/50 (12%)

*: $P < 0.05$; **: $P < 0.001$ (logistic regression test)

Source: USNTP (1989)

increased in high-dose males (males: 1/49, 0/50, 0/49, 26/49; females: 0/50, 0/50, 0/50, 1/50). It was concluded that there was clear evidence of carcinogenicity in male rats and equivocal evidence in female rats (USNTP, 1989).

(iii) Genotoxicity

The in vitro genotoxicity of PCA was examined in a variety of test systems in the 1989 studies by the United States National Toxicology Program (NTP) (results are summarized in Table 8), which indicated that PCA is clearly genotoxic in vitro. The WHO Concise International Chemical Assessment Document 48 on PCA (IPCS, 2003) provided a table summarizing the results of numerous in vitro genotoxicity studies, including the 1989 NTP studies. Inconsistent and conflicting results have been reported; nonetheless, weak mutagenic activity was repeatedly shown in the presence of metabolic activation (S9).

Table 8
Results of USNTP (1989) genotoxicity assays with PCA

Test system	Test object	Concentration/dose	Results
In vitro			
Reverse mutation (Ames) ^a	<i>Salmonella typhimurium</i> TA97, TA98, TA100, TA1535	33–1 600 µg/plate	Positive (+S9, TA98)
	<i>S. typhimurium</i> TA98, TA100	33–2 000 µg/plate	Positive (+S9, TA98) Weakly positive (+S9, TA100)
	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537	10–3 300 µg/plate	Negative (±S9, all strains)
Forward mutation ^b	L5178Y mouse lymphoma cells	50–600 µg/mL (–S9)	Positive
		9.375–200 µg/mL (+S9)	Positive
		31.3–1 000 µg/mL (–S9)	Positive
		7.8–200 µg/mL (+S9)	Positive
Chromosomal aberrations ^b	Chinese hamster ovary cells	400–600 µg/mL (–S9)	Negative
		800–1 000 µg/mL (+S9)	Positive
		30–600 µg/mL (–S9)	Negative
		1.6–800 µg/mL (+S9)	Negative
		100–550 µg/mL (–S9)	Weakly positive
		100–600 µg/mL (+S9)	Negative
Sister chromatid exchange ^b	Chinese hamster ovary cells	16.7–500 µg/mL (–S9)	Positive
		900–1 200 µg/mL (+S9)	Positive
		0.5–500 µg/mL (–S9)	Equivocal
		50–500 µg/mL (–S9)	Weakly negative
		50–500 µg/mL (–S9)	Negative
		1.6–1 600 µg/mL (+S9)	Negative
		100–600 µg/mL (+S9)	Equivocal

S9: 9000 × g supernatant fraction from rat liver homogenate

^a Parallel tests in three laboratories.

^b Parallel tests in two laboratories.

Source: USNTP (1989)

There are a few in vivo genotoxicity studies that are available in the public literature. In a wing somatic mutation and recombination test, *Drosophila melanogaster* was exposed to PCA at 7.84 mmol/L by 6-hour feeding. PCA was genotoxic in both repair-proficient and repair-defective larvae of the mei-9 cross, which indicates a potential to induce point mutation, chromosome breakages and mitotic recombinations (Graf, Hall & van Schaik, 1990).

In a comet assay, Sprague-Dawley rats (five per group) were treated with PCA orally by gavage at a dose level of 37.5, 75 or 150 mg/kg bw per day at 10 mL/kg bw on three occasions, the second dose being administered approximately 24 hours after the first dose and the third dose being administered approximately 21 hours after the second dose, 3 hours before sampling. A number of clinical

observations, including a blue skin colour, dark eyes, red staining of eyes, underactive behaviour and coat piloerection, were observed at 75 and 150 mg/kg bw per day. Both eyelids partially closed and hunched posture were also observed at 150 mg/kg bw per day. Statistically significant increases in the mean and median per cent tail intensity were observed in the liver and stomach of animals in all treated groups. It was concluded that PCA was clearly positive for genotoxicity in the liver and stomach of rats when administered orally by gavage (Barfield & Burlinson, 2015).

EFSA (2012) reviewed three *in vivo* genotoxicity studies (repeat micronucleus test in mice, rat liver unscheduled DNA synthesis study, rat comet assay) that were submitted by its notifier and concluded that, based on the weight of evidence, PCA is an *in vivo* genotoxic agent.

(c) Microbiological effects

Considering the chemical structure and mode of action of diﬂubenzuron, the Committee did not anticipate any adverse effects of diﬂubenzuron residues on human gastrointestinal microbiota.

2.3 Observations in humans

No reports of adverse effects or poisoning incidents associated with diﬂubenzuron were found, and no adverse effects have been reported during field use (Dykstra, 2001).

Diﬂubenzuron has been produced in a plant in the Netherlands since 1973, where employees involved in its production and formulation underwent regular medical examinations, including haematological analyses. Although no actual exposures have been described, no disturbances in the health status of these employees were observed that could be linked to exposure to diﬂubenzuron (Dykstra, 2001).

3. Comments

3.1 Biochemical data

Diﬂubenzuron is rapidly absorbed to a moderate extent from the gastrointestinal tract. In a single-dose oral study with ¹⁴C-labelled diﬂubenzuron in rats, about 30% of the administered dose was absorbed at 5 mg/kg bw, and less was absorbed at 100 mg/kg bw (Dunsire, Cameron & Spiers, 1990). Once absorbed, diﬂubenzuron is extensively metabolized and rapidly excreted, mostly in the urine, although some enterohepatic circulation occurs. In the radiolabel study, more than 90% of

the administered dose (5 and 100 mg/kg bw) was excreted within 24 hours. When mice were given a single oral dose of diflubenzuron at 12, 64, 200 or 920 mg/kg bw, excretion was almost complete within 48 hours (de Lange & Post, 1978).

The primary metabolic pathways are hydroxylation of the aniline ring, cleavage of the ureido bridge and conjugation, mainly with sulfate. In rats, about 80% of the metabolites were identified as involving hydroxylation of the phenyl moieties of diflubenzuron, and approximately 20% underwent scission at the ureido bridge (de Lange et al., 1975; Willems et al., 1980).

PCA was not detected in bile or urine using a method with an LOQ of 7.5 ng/mL in rats (Cameron, Henderson & McGuire, 1990). A radiolabel study with rats given a single dose of [^{14}C -anilino]diflubenzuron at 104 mg/kg bw also did not identify PCA in urine (Wang & Gay, 1999). PCA was not detected (LODs not given) in urine or faeces of sheep or cow following administration of a single oral dose of diflubenzuron at 10 mg/kg bw (Ivie, 1978) or in rat urine following administration of a single oral dose of diflubenzuron at 5 mg/kg bw (Willems et al., 1980). However, when diflubenzuron was given as a single oral dose of 5 mg/kg bw, PCA was detected in small quantities in swine urine (1.03% of the dose; Opdycke, Miller & Menzer, 1982a) and chicken excreta (0.44% of the dose; Opdycke, Miller & Menzer, 1982b).

When diflubenzuron was given as a single oral dose, CPU, a metabolite that may be reduced to PCA, was detected in small quantities in the urine of swine (0.82% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982a), in the urine of cows (0.6% of a 10 mg/kg bw dose; Ivie, 1978) and in chicken excreta (3.14% of a 5 mg/kg bw dose; Opdycke, Miller & Menzer, 1982b).

3.2 Toxicological data

Critical studies relevant to the risk assessment are summarized in [Table 9](#).

Diflubenzuron was of low acute toxicity when given to mice and rats by the oral, inhalation or dermal route. The oral LD_{50} was greater than 4600 mg/kg bw in mice and rats (van Eldik, 1973), the dermal LD_{50} was greater than 10 000 mg/kg bw in rats (Koopman, 1977) and the inhalation LC_{50} was greater than 2.9 mg/L in rats (Berczy, Cobb & Cherry, 1973).

Diflubenzuron was not irritating to the skin of rabbits (Taylor, 1973) and was slightly irritating to the eyes of rabbits (Davies & Liggett, 1973). Diflubenzuron was not a skin sensitizer in a study in guinea-pigs (Prinsen, 1992).

The primary target for toxicity is the erythrocytes, with secondary effects on liver and spleen. Dose-related methaemoglobinaemia has been consistently demonstrated in both sexes of various species (mice, rats and dogs) after short-term or long-term oral exposure to diflubenzuron.

Table 9
Studies relevant to risk assessment

Species / study type (route of administration)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Diflubenzuron				
Mouse				
Ninety-one-week toxicity and carcinogenicity study (diet)	Males: 0, 1.2, 6.4, 32, 160, 840	Toxicity: Increased methaemoglobin concentration	1.2	6.4
	Females: 0, 1.4, 7.3, 35, 190, 960	Carcinogenicity: None	840 ^a	—
Rat				
Two-year toxicity and carcinogenicity studies ^b (diet)	Study 1: 0, 0.5, 1, 2, 8	Toxicity: Increased methaemoglobin and sulphaemoglobin concentrations	2 ^c	7.1 ^d
	Study 2: Males: 0, 7.1, 28, 112, 472	Carcinogenicity: None	472 ^a	—
	Females: 0, 9.3, 37, 128, 612			
Two-generation reproductive toxicity study (diet)	Males: 0, 42, 430, 4 300	Reproductive toxicity: None	3 800 ^a	—
	Females: 0, 36, 360, 3 800	Parental toxicity: Changes in haematological parameters	—	36 ^e
		Offspring toxicity: Decreased F ₁ pup weights	360	3 800
Developmental toxicity study (gavage)	0, 1 000	Maternal toxicity: None	1 000 ^f	—
		Embryo/fetal toxicity: None	1 000 ^f	—
Rabbit				
Developmental toxicity study (gavage)	0, 1 000	Maternal toxicity: None	1 000 ^f	—
		Embryo/fetal toxicity: None	1 000 ^f	—
Dog				
One-year toxicity study (capsule)	0, 2, 10, 50, 250	Increased methaemoglobin and sulphaemoglobin concentrations, platelet counts and hepatic pigmentation	2	10
4-Chloroaniline (PCA)				
Mouse				
Two-year carcinogenicity study (gavage)	0, 3, 10, 30	Increased incidence of hepatocellular adenomas and carcinomas in male mice	—	3 ^e
Rat				
Two-year carcinogenicity study (gavage)	0, 2, 6, 18	Increased incidence of spleen sarcoma in male rats	6	18

^a Highest dose tested.

^b Two or more studies combined.

^c Overall NOAEL.

^d Overall LOAEL.

^e Lowest dose tested.

^f Limit dose.

In a 13-week study, rats were fed diets containing diflubenzuron at a concentration of 0, 160, 400, 2000, 10 000 or 50 000 mg/kg feed (equivalent to 0, 8, 20, 100, 500 and 2500 mg/kg bw per day, respectively). A range of dose-related changes in erythrocyte parameters (erythrocyte counts, haemoglobin, reticulocytes, methaemoglobin and sulfhaemoglobin) were noted in both sexes at 400 mg/kg feed and above, with minimal effects at 160 mg/kg feed. The absolute and relative weights of the spleen were increased in males at 160 mg/kg feed and above and in females at 400 mg/kg feed and above for 7 weeks. Pathological findings included chronic hepatitis, haemosiderosis and congestion of the spleen, and erythroid hyperplasia of the bone marrow in all treated groups; and haemosiderosis of the liver at 400 mg/kg feed and above. A NOAEL could not be identified, because there were small, but statistically significant, increases in methaemoglobin concentration and associated changes in the spleen (increased spleen weight, spleen haemosiderosis and spleen congestion) and bone marrow (erythroid hyperplasia) at the lowest dose tested (160 mg/kg feed, equivalent to 8 mg/kg bw per day) (Burdock et al., 1980; Goodman, 1980).

In a 13-week non-GLP-compliant study in dogs, animals received diets containing diflubenzuron at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equal to 0, 0.4, 0.8, 1.6 and 6.4 mg/kg bw per day, respectively). At week 6, haemoglobin concentration and erythrocyte count were reduced and methaemoglobin and free haemoglobin concentrations were increased at 160 mg/kg feed. There was an increase in the myeloid:erythroid ratio in bone marrow at 160 mg/kg feed at week 12. A NOAEL of 40 mg/kg feed (equal to 1.6 mg/kg bw per day) was identified, based on changes in haematological end-points and bone marrow at 160 mg/kg feed (equal to 6.4 mg/kg bw per day) (Chesterman et al., 1974).

In a 52-week non-GLP-compliant study, dogs received gelatine capsules containing diflubenzuron at a dose of 0, 2, 10, 50 or 250 mg/kg bw per day. A range of effects related to impaired erythrocytes was seen at the two highest doses from week 13 onwards. Increases in methaemoglobin and sulfhaemoglobin concentrations and in platelet counts were seen at 10 mg/kg bw per day and above. The only histopathological findings were in the liver (increased pigmentation of Kupffer cells and macrophages) at 10 mg/kg bw per day and above. A NOAEL of 2 mg/kg bw per day was identified, based on effects on methaemoglobin and sulfhaemoglobin concentrations, platelet counts and hepatic pigmentation at 10 mg/kg bw per day (Greenough et al., 1985).

In a chronic toxicity and carcinogenicity study, diflubenzuron was given to mice in the diet at a concentration of 0, 16, 80, 400, 2000 or 10 000 mg/kg feed (equal to 0, 1.2, 6.4, 32, 160 and 840 mg/kg bw per day for males and 0, 1.4, 7.3, 35, 190 and 960 mg/kg bw per day for females, respectively) for 91 weeks. Significant, dose-related changes were seen in a number of haematological

parameters from week 26 onwards (methaemoglobin and sulphaemoglobin at 80 mg/kg feed and above; haemoglobin at 2000 mg/kg feed and above; leukocyte and erythrocyte counts at 10 000 mg/kg feed). On week 26, absolute spleen weights were significantly increased at 2000 mg/kg feed and above. Increased incidences of splenic siderocytes at 400 mg/kg feed and above and of pigmented Kupffer cells at 10 000 mg/kg feed were noted. A NOAEL of 16 mg/kg feed (equal to 1.2 mg/kg bw per day) was identified, based on methaemoglobin formation at 80 mg/kg feed (equal to 6.4 mg/kg bw per day). There was no evidence of carcinogenicity in this study (Colley et al., 1984).

In a non-GLP-compliant chronic toxicity and carcinogenicity study in rats, animals received diflubenzuron in the diet at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equivalent to 0, 0.5, 1, 2 and 8 mg/kg bw per day, respectively) for 2 years. The achieved dietary concentrations and homogeneity of diflubenzuron in the feed were not confirmed. The NOAEL was 40 mg/kg feed (equivalent to 2 mg/kg bw per day), based on increases in methaemoglobin concentration and reduced free haemoglobin concentration at 160 mg/kg feed (equivalent to 8 mg/kg bw per day). There was no increase in the incidence of tumours in treated animals. However, the poor survival (<30% in all groups at termination) and limited range of tissues examined limited the power of this study to detect any carcinogenicity of diflubenzuron (Hunter et al., 1976; Colley & Offer, 1977).

In a GLP-compliant combined 2-year toxicity and carcinogenicity study, rats received diflubenzuron in the diet at a concentration of 0, 160, 620, 2500 or 10 000 mg/kg feed (equal to 0, 7.1, 28, 112 and 472 mg/kg bw per day for males and 0, 9.3, 37, 128 and 612 mg/kg bw per day for females, respectively). Erythrocyte parameters (e.g. methaemoglobin and sulphaemoglobin concentrations) were altered, with no marked progression with duration and dosing. The main treatment-related histopathological findings were pigmented macrophages in the spleen and liver and erythroid hyperplasia of the bone marrow at 620 mg/kg feed and above. A NOAEL for toxicity could not be identified, owing to increases in methaemoglobin and sulphaemoglobin concentrations noted at 160 mg/kg feed (equal to 7.1 mg/kg bw per day), the lowest dose tested. The overall incidences of tumours were low, with no treatment- or dose-related findings (Burdock et al., 1984).

The overall NOAEL for toxicity in the 2-year studies in rats was 2 mg/kg bw per day, and the overall LOAEL was 7.1 mg/kg bw per day.

The Committee concluded that diflubenzuron is not carcinogenic in mice or rats.

The genotoxicity of diflubenzuron was evaluated in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found, other than the studies by de Barros et al. (2013), in which positive findings in micronucleus induction and comet formation in the peripheral blood (the target of toxicity) were reported in mice given diflubenzuron at a dose of 0.3, 1 or 3

mg/kg bw. The genotoxicity potency reported in this study was inconsistent with what was reported in other studies and has not been replicated. The Committee concluded that diflubenzuron is not genotoxic based on the weight of evidence of genotoxicity information available.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Committee concluded that diflubenzuron is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study, rats received diets containing diflubenzuron at a concentration of 0, 500, 5000 or 50 000 mg/kg feed (equal to 0, 42, 430 and 4300 mg/kg bw per day for males and 0, 36, 360 and 3800 mg/kg bw per day for females, respectively). Haematological parameters and the spleen were not examined in young animals. Reproductive parameters were not affected. Pup weights were reduced in a dose-related manner in the F_1 generation, but not in the F_2 generation. Alterations in erythrocyte parameters and increases in lymphocyte counts and platelet numbers were seen in all parental groups. The spleen was the primary target organ, showing increases in weight, congestion and haemosiderosis at all doses and an increase in the incidence of congested red pulp in F_0 animals at the middle and high doses. Effects on liver included increased incidences of centrilobular hepatocyte hypertrophy at the middle and high doses and brown pigmentation of Kupffer cells in all treated groups. The NOAEL for reproductive effects was 50 000 mg/kg feed (equal to 3800 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 5000 mg/kg feed (equal to 360 mg/kg bw per day), based on reductions in pup body weight at 50 000 mg/kg feed (equal to 3800 mg/kg bw per day) in the F_1 generation. A NOAEL for parental toxicity could not be identified because of the haematological effects observed at all doses tested (Brooker, 1995).

In a developmental toxicity study, rats were dosed orally by gavage with diflubenzuron at 0 or 1000 mg/kg bw per day (the limit dose) from days 6 to 15 of gestation. The dams were killed on day 20 of gestation. No treatment-related effects on the dams or fetuses were noted. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the only dose tested (Kavanagh, 1988a).

In another developmental toxicity study, rabbits were dosed orally by gavage with diflubenzuron at 0 or 1000 mg/kg bw per day (the limit dose) from days 7 to 19 of gestation. The does were killed on day 28 of gestation. No treatment-related effects on the does or fetuses were noted. There was no evidence of developmental toxicity in rabbits. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the only dose tested (Kavanagh, 1988b).

3.3 Toxicological data on PCA (a metabolite of diflubenzuron)

Repeated exposure to PCA leads to cyanosis and methaemoglobinaemia, followed by effects in blood, liver, spleen and kidneys, as evidenced by changes in haematological parameters, splenomegaly and haemosiderosis (from moderate to heavy) in spleen, liver and kidney, partially accompanied by extramedullary haematopoiesis. The LOAELs for a significant increase in methaemoglobin levels in rats and mice were 5 and 7.5 mg/kg bw per day, respectively, for a 13-week oral gavage administration of PCA. The LOAEL for a 103-week oral gavage study in rats (with administration 5 days/week) was 2 mg/kg bw per day, based on a significant increase in methaemoglobin levels and fibrotic changes of the spleen in male rats; hyperplasia of bone marrow was observed in female rats at and above 6 mg/kg bw per day (IPCS, 2003). This information demonstrated that PCA exhibits toxicity end-points similar to those of diflubenzuron, but is more potent than diflubenzuron.

PCA was tested for carcinogenicity in mice and rats by administration in the diet and by oral gavage.

In a dietary carcinogenicity study in mice, animals received PCA at a concentration of 0, 2500 or 5000 mg/kg feed (equivalent to 0, 375 and 750 mg/kg bw per day, respectively) for 78 weeks, followed by a 13-week observation period. Decreased body weight gain was observed in treated animals. Non-neoplastic proliferative and chronic inflammatory lesions were found in the spleens of treated mice. There was an increased incidence of haemangiosarcomas in the spleen, liver, kidney and subcutaneous tissue (combined) for both sexes. It was concluded that there was insufficient evidence to conclude that PCA was carcinogenic in mice (USNCI, 1979).

In a second carcinogenicity study in mice, animals were administered PCA by oral gavage in aqueous hydrochloric acid at 0, 3, 10 or 30 mg/kg bw per day, 5 days/week, for 103 weeks. Incidences of proliferation of haematopoietic cells in the liver were increased in dosed females. Multifocal renal tubular pigmentation (haemosiderin) was observed in high-dose females. There were increases in the incidences of hepatocellular carcinomas in males dosed at 10 and 30 mg/kg bw per day (3/50, 7/49, 11/50, 17/50), incidences of combined hepatocellular adenomas and carcinomas in all treated males (11/50, 21/49, 20/50, 21/50) and incidences of haemangiosarcomas of the liver and spleen (combined) in males at 30 mg/kg bw per day (4/50, 4/49, 1/50, 10/50). It was concluded that there was some evidence of carcinogenicity in male mice and no evidence in female mice (USNTP, 1989).

In a dietary carcinogenicity study in rats, animals received PCA at a concentration of 0, 250 or 500 mg/kg feed (equivalent to 0, 12.5 and 25 mg/kg bw per day, respectively) for 78 weeks, followed by a 24-week observation period. Mesenchymal tumours (fibroma, fibrosarcoma, haemangiosarcoma,

osteosarcoma and sarcoma not otherwise specified) in the spleen were observed in males at the high dose and in females at both doses; no tumours were found in the controls. It was concluded that there was insufficient evidence to conclude that PCA was carcinogenic in rats (USNCI, 1979).

In a second carcinogenicity study in rats, animals were administered PCA by oral gavage in aqueous hydrochloric acid at 0, 2, 6 or 18 mg/kg bw per day, 5 days/week, for 103 weeks. Changes in haematological parameters (e.g. decreases in haemoglobin concentration, erythrocyte count and haematocrit) were noted at various time points. Non-neoplastic findings included bone marrow hyperplasia, hepatic haemosiderosis and splenic fibrosis. The incidence of uncommon sarcomas of the spleen in high-dose male rats was significantly higher than that in the vehicle controls (fibrosarcomas, osteosarcomas or haemangiosarcomas, combined: 0/49, 1/50, 3/50, 38/50); some of these tumours metastasized to one or more sites. One mid-dose female developed fibrosarcoma, and one high-dose female developed osteosarcoma; the controls showed zero incidence of either of these tumours. The incidence of adrenal phaeochromocytomas or malignant phaeochromocytomas combined was significantly higher in the high-dose males. There was a non-significant increase in the incidence of phaeochromocytomas in high-dose females (2/50, 3/50, 1/50, 6/50). It was concluded that there was clear evidence of carcinogenicity in male rats and equivocal evidence in female rats (USNTP, 1989).

The oral gavage carcinogenicity study is considered to be more appropriate than the dietary admixture feeding study for determining carcinogenicity because (1) PCA is unstable in feed and (2) mice and rats in the feeding studies were dosed for 78 weeks and killed and examined for histopathology following a further 13-week (mice) or 24-week (rats) observation period. Nonetheless, both studies showed some similar effects: splenic toxicity in male and female rats, a treatment-related increase in uncommon splenic sarcomas in male rats and a treatment-related increase in haemangiosarcomas in male mice.

The Committee concluded that PCA is carcinogenic in mice and rats.

PCA has been tested for genotoxicity in various *in vitro* and *in vivo* systems. PCA is genotoxic *in vitro* (USNTP, 1989) and *in vivo* (Barfield & Burlinson, 2015). The Committee is aware of the existence of additional *in vivo* genotoxicity studies (EFSA, 2012); however, these were not available to the Committee.

The Committee concluded that PCA is genotoxic.

There is no established mode of action for PCA carcinogenesis; it is not known whether it is mediated through a genotoxic and/or non-genotoxic mechanism. Several hypotheses regarding the mechanism of splenic carcinogenicity have been proposed. However, because PCA is genotoxic and carcinogenic, the Committee could not exclude the possibility that the carcinogenesis of PCA occurs by a genotoxic mode of action.

3.4 Observations in humans

No reports of adverse effects or poisoning incidences associated with diflubenzuron were found (Dykstra, 2001).

3.5 Microbiological data

Considering the chemical structure and mode of action of diflubenzuron, the Committee did not anticipate any adverse effects of diflubenzuron residues on human gastrointestinal microbiota.

4. Evaluation

In the absence of adequate information on exposure to PCA, a genotoxic and carcinogenic metabolite and/or degradate of diflubenzuron, and on whether diflubenzuron can be metabolized to PCA in humans, the present Committee was unable to establish an ADI for diflubenzuron because it was not possible to assure itself that there would be an adequate margin of safety from its use as a veterinary drug. The Committee also noted that it was not possible to calculate a margin of exposure for PCA in the absence of adequate information on exposure to PCA.

4.1 Additional information that would assist in the further evaluation of the compound

- A comparative metabolism study of diflubenzuron in humans and rats (e.g. in hepatocytes)
- Information on PCA exposure associated with the consumption of treated fish
- Information on the amount of PCA present (if any) as an impurity in the product formulation
- Information on the amount of PCA generated during food processing.

4.2 Recommendation

The Committee recommended that JMPR consider the re-evaluation of diflubenzuron at a future meeting and that the WHO Pesticide Evaluation Scheme (WHOPES) and the WHO *Guidelines for Drinking-water Quality* (GDWQ) Chemical Working Group reconsider their recommendations for the use of diflubenzuron as a vector control agent in drinking-water.

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