NORFLURAZON

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Explanation

Norflurazon (Fig. 1) is the International Organization for Standardization–approved common name for 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2*H*)-pyridazinone (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 27314-13-2.

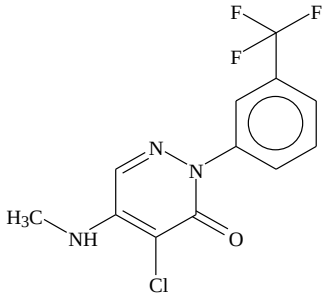
Norflurazon is a fluorinated pyridazinone compound that is applied as a pre-emergence herbicide for selective control of annual grasses and broadleaf weeds in a number of crops, mostly in fruits, but also in cotton, hops, almonds and walnuts. In susceptible plant species, it inhibits carotenoid biosynthesis by inhibition of phytoene desaturase, resulting in chlorophyll photodegradation and eventually chlorosis (Saunders et al., 1985; USEPA, 2001; Lamberth, 2017).

Norflurazon has not previously been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and was reviewed by the present Meeting at the request of the Codex Committee on Pesticide Residues.

Even though all relevant toxicological end-points are covered by submitted studies, it should be noted that most studies are very old, were not performed under good laboratory practice (GLP) and

did not follow current test guidelines, except where otherwise specified. Published literature has been taken into consideration, but is generally very limited.

Fig. 1. Chemical structure of norflurazon



Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution and excretion of ^{14}C-labelled norflurazon (purity 98.8%; radiolabelled in the trifluoromethyl moiety) were investigated in female Sprague Dawley rats (five per dose) following a single oral gavage administration of 2 or 110 mg/kg body weight (bw) in corn oil. A third group of the same size was fed the non-radiolabelled test substance for 14 days at a dietary dose of 2 mg/kg bw per day followed by a radiolabelled dose of 2 mg/kg bw administered 24 hours later by oral gavage. In addition, two females received 2 mg/kg bw of radiolabelled norflurazon as an intravenous injection in physiological saline and ethanol (1:1). Following treatment, rats were caged in all-glass metabolism chambers to collect urine and faeces separately. One of the orally dosed rats in the low-dose group was additionally monitored for expired radiolabelled volatiles. Ninety-six hours after administration of the single or the final repeated dose, the rats were killed, and selected tissues were removed for subsequent analysis for radioactivity.

No analysis of blood for pharmacokinetic parameters was performed.

As no bile duct cannulation group was included, oral absorption could be estimated either on the basis of urinary excretion only or by comparison of faecal elimination following oral and intravenous administration, even though only two animals were in the intravenous group. Based on urinary excretion only, absorption appeared to be as low as 20%. However, faecal excretion accounted for more than 70% of the intravenous dose and was similar to that after oral dosing. Accordingly, oral absorption of norflurazon appeared to be quantitative, at least above 90%. Extensive metabolism (see section 1.2) also suggested high oral absorption. Most of the applied radioactivity was eliminated via the faeces, but urinary excretion was also significant. About 80% of the low dose was eliminated within 24 hours; in contrast, excretion of the high dose was slower, but virtually complete after 96 hours (see Table 1). Previous dietary administration of the non-radiolabelled material had no impact on this pattern.

Exhalation was negligible (0.1% of the applied radioactivity), but investigation was confined to a single animal.

Tissue residues were generally low, with the highest relative radioactivity observed in liver and kidneys (Saunders et al., 1985).

Table 1. Elimination and recovery of ^{14}C-labelled norflurazon in female rats over 96 hours

Matrix (time point)	Mean % of administered radioactivity			
	Oral, low dose	Intravenous, low dose	Oral, high dose	Repeated oral, low dose
Urine (24 hours)	18.4	24.8	8.1	24.7
Urine (96 hours)	21.3	28.4	18.5	27.1
Faeces (24 hours)	58.3	57.0	40.1	54.7
Faeces (96 hours)	77.8	70.7	79.5	65.3
Selected tissues	0.5	0.4	0.2	0.3
Carcass	0.4	0.5	0.4	0.5
Total recovery	100.0	100.0	98.6	93.2

Source: Saunders et al. (1985)

In an older study, excretion of norflurazon labelled with both ^{3}H and ^{14}C was studied in 10 male Wistar rats following daily oral gavage administration of 10 mg of the test substance (a 1% suspension in 0.5% aqueous carboxymethylcellulose) for 15 days. Urine and faecal samples were collected and pooled either for the entire study duration (five animals) or for the individual days (five animals). Twenty-four hours after the final dose, rats were killed, and blood, liver, spleen, kidney, testes, brain, muscle and fat were taken for subsequent analysis for radioactivity.

Total recovery of radioactivity was remarkably variable in the individual rats. Elimination in both urine and faeces accounted for 60% to nearly 100% when the entire treatment period was considered. In the rats for which data from individual days were available, a mean of about 75% of the administered radioactivity was excreted. The fate of the remaining portion is unknown, as only traces were found in the tissues, but no precise measurements were reported. The data obtained with either the ^{3}H or the ^{14}C label demonstrated sufficient parallelism. The predominant excretion route was via the faeces. In the five individual animals observed for the entire study period, faecal excretion accounted for 45–74% of the radiolabels, whereas 13–26% of the radiolabels were found in urine. In the group of five male rats for which daily collections were pooled, an average of 56–59% of the radiolabels were found in faeces and 17–19% in urine, depending on the radiolabel.

This study does not comply with modern standards, and the information provided is limited. However, the study may be considered complementary to the more comprehensive and reliable study by Saunders et al. (1985) reported above and allows the conclusion that excretion in male and female rats is similar (Karapally, 1974).

In the report, reference is made to even older studies that were not available to the current Meeting.

1.2 Biotransformation

In the study described in section 1.1 in which female Sprague Dawley rats were administered ^{14}C-labelled norflurazon by gavage, biotransformation was extensive, as only 2% or less of the administered doses were excreted as unchanged parent. The main metabolic pathways were *N*-demethylation and glutathione conjugation. Replacement of the chlorine in norflurazon by hydrogen was characterized as an additional, but minor, metabolic pathway.

Numerous metabolites, which were analysed by liquid and thin-layer chromatography and mass spectrometry, were found in urine and faeces, of which nine could be identified. Only one of them (metabolite 5, a sulfoxide) accounted for more than 10% of the applied dose (up to 39% in urine,

following intravenous administration), whereas the others represented only 1–2% (Saunders et al., 1985).

The results of the above study were also published in one of the very few articles on the toxicology of norflurazon that were retrieved from the open literature. No new information was given, but the (most abundant in urine) sulfoxide metabolite was designated as metabolite 3 (Quistad et al., 1989).

In the older study by Karapally (1974) in which male Wistar rats were administered norflurazon labelled with both ^{3}H and ^{14}C by gavage (see section 1.1), metabolites were detected and identified by thin-layer and column chromatography and by gas chromatography–mass spectrometry, following amberlite purification and several derivatization steps (methylation, acetylation and trimethylsilylation). Metabolism of norflurazon was extensive, with hydroxylation, demethylation and sulfur conjugation being the main pathways. Only low amounts of the parent compound were found in urine (0.1%) and faeces (5.4%). Metabolites were not fully characterized, and their number cannot be determined from the report.

Consideration of the two studies together allows the conclusion that metabolism in male and female rats is similar (Karapally, 1974; Saunders et al., 1985).

2. Toxicological studies

2.1 Acute toxicity

A summary of acute toxicity, skin and eye irritation, and skin sensitization studies with the active ingredient norflurazon is given in Table 2.

Table 2. Acute toxicity studies (including skin and eye irritation and skin sensitization) with norflurazon (active ingredient)

Study type	Species	Batch and purity	Result	Reference
Oral toxicity	Rat (males only)	Not available	LD_{50} = 9 300 mg/kg bw	Bagdon (1972)
Dermal toxicity	Rabbit (males only)	Not available	LD_{50} > 20 000 mg/kg bw	Bukva (1970)
Inhalation toxicity (4-hour nose-only exposure)	Rat	Lot no. 0920376; purity 98.3%	LC_{50} > 2.4 mg/L air	Hoffman (1996)
Skin irritation	Rabbit	Not available	Not irritating	Bagdon (1972)
Eye irritation	Rabbit	Not available	Not irritating	Bagdon (1972)
Skin sensitization	Guinea-pig	Not available	Inconclusive	Bagdon (1973)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose

In addition, the sponsor submitted a number of (more recent) acute toxicity studies in which formulations containing norflurazon were tested. Even though such studies are normally not taken into consideration by JMPR, it was decided to report those with the plant protection product Norflurazon 80 DF, which contains 80% active ingredient, because of the limited database available for norflurazon. An overview of these studies is given in Table 3. Studies with another formulation containing less than 5% norflurazon were submitted by the sponsor but were excluded from the evaluation.

Table 3. Acute toxicity studies (including skin and eye irritation and skin sensitization) with the formulation Norflurazon 80 DF[a]

Study type	Species	Result	Reference
Oral toxicity	Rat	LD_{50} = 1 210 mg/kg bw (males) and 1 080 mg/kg bw (females)	Gardner (1987a)
Dermal toxicity	Rat	LD_{50} > 2 000 mg/kg bw	Gardner (1987b)
Skin irritation	Rabbit	Very slightly irritating	Liggett & Smith (1988)
Eye irritation	Rabbit	Slightly irritating	Liggett & Smith (1987)
Skin sensitization (Buehler method)	Guinea-pig	Not sensitizing	Kynoch & Parcell (1987)

bw: body weight; LD_{50}: median lethal dose
[a] Batch no. 1130377; 80% norflurazon content.

The studies summarized in the above two tables are described in more detail below.

(a) Lethal doses

Oral administration

In an acute toxicity study in rats, a herbicide with the designation "9789 technical grade" was the test item. The sponsor confirmed that the technical active ingredient was norflurazon. The test compound was suspended in 1% carboxymethylcellulose with 0.2% Tween 80 and administered to 10 male Sprague Dawley rats per group at a dose of 5000, 10 000 or 15 000 mg/kg bw following a 16-hour fast. Following treatment, the survivors were observed for 7 days and then killed. At 5000 mg/kg bw, two deaths occurred within 24 or 72 hours post-dosing, respectively. At 10 000 mg/kg bw, five rats died, all within the first 24 hours. At 15 000 mg/kg bw, nine rats died on the first day, whereas the remaining animal survived. In all groups, toxic signs consisted of decreased locomotor activity, ptosis, sedation, muscle tremors and clonic or tonic convulsions, which became more pronounced with increasing dose. All these signs occurred within 4 hours after application and had resolved in the survivors after 1 or 2 days. Based on the mortality pattern, a median lethal dose (LD_{50}) of 9300 mg/kg bw (standard deviation ± 1358) was calculated (Bagdon, 1972).

In another acute toxicity study, the formulation Norflurazon 80 DF (Zorial Rapid 80 DF; batch no. 1130377; purity 80%) was dissolved in 1% aqueous methylcellulose and administered at a volume of 10 mL/kg bw to male and female CD rats. Subsequent to a preliminary test in which two male and two female rats had been dosed with 1000 or 2500 mg/kg bw, a limit test with a dose of 5000 mg/kg bw was carried out in five rats of each sex. As a next step, further groups of five males and five females each received a dose of 800, 1260 or 2000 mg/kg bw. This last experiment was considered the main study and was reported in the most detail. All surviving animals were killed 15 days post-dosing. All animals (including those that died on test) were necropsied and grossly examined.

The number of presumably treatment-related deaths in all groups is summarized in Table 4.

The mortality data appear somewhat contradictory, in particular when the data from the first and second phases of the study are compared. Apparently based on the main study only, LD_{50} values of 1210 mg/kg bw for males and 1080 mg/kg bw for females were calculated by probit analysis. The combined value for both sexes was 1140 mg/kg bw, with a confidence interval ranging from 900 to 1420 mg/kg bw. Clinical signs were observed in all animals from all groups, occurred within 5 hours after dosing and comprised piloerection, ptosis, hunched posture, lethargy, abnormal gait, decreased respiratory rate, pallor of the extremities and increased salivation. Straub tail and jumping movements were noticed from 1260 mg/kg bw onwards, and collapse or clonic convulsions at 2000 mg/kg bw. In the survivors, recovery was complete by day 6 or 7 at the latest. Body weight development appeared

to be impaired in the survivors during the first week after treatment, but subsequently normalized. Severe body weight losses were noted prior to death, whereas the survivors gained weight. Terminal necropsy did not reveal remarkable gross changes that could be attributed to treatment or could explain the mortalities (Gardner, 1987a).

Table 4. Mortality in an acute oral toxicity study with Norflurazon 80 DF

Dose (mg/kg bw)	Animals on study (M/F)	Males		Females	
		No. of deaths	Days of deaths	No. of deaths	Days of deaths
800	5/5	0	–	2	2
1 000	2/2	1	1	0	–
1 260	5/5	3	2, 4	2	2
2 000	5/5	5	1, 2	5	2
2 500	2/2	0	–	2	1
5 000	5/5	5	1	4	1

bw: body weight; F: female; M: male
Source: Gardner (1987a)

Based on this study, and taking into account the rather high concentration of the active ingredient in this product, it may be concluded that norflurazon is of moderate acute oral toxicity. This result is somewhat contrary to the result of the older study by Bagdon (1972), which suggests either a different impurity profile of the active ingredient or a strong impact of co-formulants in the tested product. As the sponsor denied the possibility that co-formulants might have enhanced the toxicity of norflurazon, the more recent, higher-quality study with the formulation is considered the more reliable study with which to assess the acute oral toxicity of norflurazon.

Dermal application

In an acute dermal toxicity study, SAN 9789 (norflurazon, batch and purity not given) was applied for 24 hours to the shaved backs of male New Zealand white rabbits (three per dose group) at a dose of 5000, 10 000 or 20 000 mg/kg bw. No deaths, clinical signs of toxicity or gross abnormalities at necropsy after 7 days were observed in any dose group. The LD_{50} was in excess of 20 000 mg/kg bw. The occurrence or absence of local effects (skin irritation) was not reported (Bukva, 1970).

In a dermal limit test, the formulation Norflurazon 80 DF (Zorial Rapid 80 DF; batch no. 1130377; purity 80%) was prepared as a 100% (weight/volume) paste in distilled water and administered to the dorsolumbar region of five male and five female CD rats at a volume of 2 mL/kg bw. The applied dose was 2000 mg/kg bw. Exposure time was 24 hours, and the animals were observed for 14 days before being killed. All rats survived, remained healthy and gained weight. No signs of irritation or necropsy findings were noted. The LD_{50} in this study was greater than 2000 mg/kg bw (Gardner, 1987b). This study with a formulation was regarded as better suited for the assessment of the acute dermal toxicity of norflurazon than the outdated study with the active ingredient itself.

Exposure by inhalation

In one of the few studies with norflurazon that have been performed according to current standards, acute inhalation toxicity was examined in five male and five female Crl:CD (SD)BR rats exposed (nose-only) for 4 hours to norflurazon (lot no. 0920376; purity 98.3%) generated as a dust.

The intended concentration was 2 mg/L; the analytically verified mean actual concentration was 2.4 mg/L. A mass median aerodynamic diameter of 2.9 µm was determined, with a geometric standard deviation of 2.2. Animals were monitored for mortality and any signs of toxicity or distress during exposure and for 14 days following exposure. No mortalities were observed. During or immediately after exposure, clinical signs (in 1–5 rats of each sex) were excessive salivation, chromodacryorrhoea, red nasal discharge, dried red or brown material on the facial area, laboured breathing and, occasionally, rales. All animals appeared normal by day 3. No systemic effects were noted, all animals gained weight and no gross lesions were observed at necropsy. The inhalation median lethal concentration (LC_{50}) in rats was greater than 2.4 mg/L (Hoffman, 1996).

(b) Dermal irritation

Application of norflurazon (500 mg) to the shaved and partly abraded skin of three male and three female rabbits for 24 hours under occlusion did not cause skin erythema or oedema (Bagdon, 1972). Furthermore, it was reported in the skin sensitization study (see below) that no signs of irritation were observed in guinea-pigs (Bagdon, 1973).

In a guideline-compliant study with the formulation Norflurazon 80 DF in male New Zealand white rabbits, only marginal evidence of skin irritation was obtained. Following a 4-hour exposure under occlusion, very slight erythema was noted in three out of six animals on day 1 and was still visible in two of them on day 2. No signs of skin irritation were apparent in the remaining rabbits (Liggett & Smith, 1988). This study appears more reliable than the much older study reported above.

(c) Ocular irritation

In a study with norflurazon technical active ingredient in three male and three female rabbits, no signs of eye irritation were observed (Bagdon, 1972).

In a guideline-compliant study, the formulation Norflurazon 80 DF proved slightly irritating to the eyes of New Zealand white rabbits, as conjunctival redness, chemosis and discharge were observed in all animals 1 hour after instillation and afterwards. All these signs had disappeared within 2–3 days. No damage to the cornea or iris was noted in any animal (Liggett & Smith, 1987). This study appears more reliable than the much older study reported above.

(d) Dermal sensitization

The skin sensitization potential of norflurazon was evaluated in groups of six male Hartley strain guinea-pigs following intradermal or topical induction. For intradermal induction, a 0.1% solution of the test compound was prepared in 1% carboxymethylcellulose and 0.2% Tween 80 and injected into the upper dorsal quadrant of the back 3 times per week until 10 injections had been given. For challenge, a further intradermal dose was applied 2 weeks after the final induction. The same treatment regimen was performed in the negative control group receiving only the vehicle and in a positive control group of the same size. The positive control substance was 0.1% phenylenediamine hydrochloride dissolved in 0.9% saline. For topical induction, guinea-pigs were administered either the test compound 10 times (every 2 or 3 days) as an aqueous paste or 2% phenylenediamine hydrochloride in 0.9% saline. Following each treatment, the respective sites were occluded for 6 hours. Topical challenge was performed 2 weeks after the final induction.

In the intradermal induction group, guinea-pigs exhibited slight erythema and in two cases also slight oedema due to injection of norflurazon. However, following challenge, no erythema was seen; although oedema was noted in one animal only, it disappeared by 48 or 72 hours. After topical induction and challenge, no evidence of irritation or sensitization was noted. These findings cannot be taken as convincing evidence that norflurazon is not sensitizing, as the positive control substance also did not cause a positive response (Bagdon, 1973).

A product study with Norflurazon 80 DF was performed in female guinea-pigs of the Hartley/Dunkin strain by means of a modified Buehler test with nine inductions. For induction and challenge, 60% dilutions of the test item were prepared in distilled water. Both the test group and the negative control group comprised 10 animals. None of the test or control animals exhibited any dermal reaction following induction or challenge (Kynoch & Parcell, 1987). However, no positive control group was included, so the reliability of the test cannot be proven. In addition, the Buehler test is now considered less predictive than the local lymph node or the maximization assay.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

The only available short-term toxicity study in mice was a range-finding experiment performed in preparation for a long-term toxicity and carcinogenicity study (see section 2.3). Groups of five weanling male and female CD-1 (HaM/ICR Swiss) mice were administered the test substance (batch no. CH0004; purity 98.8%) via their diet for 28 days. The nominal dietary concentrations were 0, 70, 210, 420 and 2520 parts per million (ppm) (equivalent to 0, 10.5, 31.5, 63 and 378 mg/kg bw per day, respectively). The animals were monitored for mortality, clinical signs, abnormal behaviour, body weight and feed consumption. At necropsy, the mice were examined for gross changes, and weights of liver and kidneys were determined.

No changes in mortality, clinical signs, behaviour, body weight or feed consumption or remarkable findings were noted that could be attributed to treatment. In males, dose-related increases in absolute and relative liver weights were observed at 210 ppm and above, whereas a similar effect on liver weight in females was confined to the highest-dose group. Absolute kidney weights were not altered, but there was an increase in relative kidney weight in male mice at 210 ppm and above. In females, in contrast, relative kidney weight was lower at the top dose level. The only gross lesions were noted in males and females at the highest dose, as livers in some, but not all, animals had granular or diffuse smooth granular appearance that was interpreted to indicate fatty degeneration. No recommendation for dose selection in the long-term toxicity and carcinogenicity study was given in the report (Tisdel, 1971).

Rats

In a 28-day feeding study, norflurazon (SAN9789; batch and purity not given) was administered to groups of 10 male and 10 female CFE strain rats per group at a dietary concentration of 0, 500, 1000 or 5000 ppm (equal to 0, 52, 105 and 517 mg/kg bw for males and 0, 70, 140 and 717 mg/kg bw for females, respectively). The end-points evaluated in this study included body weight, feed consumption, clinical signs, haematology, clinical chemistry and urine analysis parameters, gross pathology and organ weights (liver, kidneys, adrenals, brain and testes). Histopathological examination comprised a representative range of organs and tissues, but, with the exception of liver and kidney, was confined to the control and high-dose groups.

There were no test substance–related deaths. Clinical signs were confined to excessive shedding of hair, rough fur and mild alopecia in high-dose males and females. Slight to moderate rales were noted in all groups, including the controls, but were a bit more pronounced at the high dose. This sign might indicate infection, which could call the study quality into question.

High-dose males exhibited a lower mean body weight from the beginning of the study, with the difference relative to the control group achieving statistical significance at study termination in week 4. This effect was clearly not due to lower feed intake, as mean feed consumption was not different among the groups. No effects on body weight were noted in low- or mid-dose males or in females in any treatment group.

Haematology, clinical chemistry and urine analysis did not reveal any findings that could be attributed to substance administration. It must be emphasized, however, that the range of examined haematological and clinical chemistry parameters was quite limited and did not comply with current standards.

No treatment-related gross findings were observed at necropsy in any group. No absolute organ weights were reported. Dose-related, statistically significant increases in relative liver weight were seen in the mid- and high-dose groups in males and in all treatment groups in females. Dose-related, statistically significant increases in relative kidney weight were seen in males in all treated groups, whereas relative kidney weight was increased in high-dose females only. Relative testis weight was higher at the high dose, but this finding was presumably due to lower mean body weight. Relative adrenal weights exhibited a very unique pattern that cannot be explained (Table 5). However, it must be noted that adrenal weights generally may be subject to wide variation.

Table 5. Terminal body weight and relative organ weights in the 28-day feeding study in rats

	Relative organ weight (%)							
	Males				**Females**			
Organ	**0 ppm**	**500 ppm**	**1 000 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 000 ppm**	**5 000 ppm**
Terminal body weight (g)	*217.1*	*208.8*	*210.4*	*172.7**	*157.1*	*156.9*	*155.8*	*150.9*
Liver	4.68	5.12	5.77*	7.64*	4.58	5.28*	5.53*	7.77*
Kidneys[a]	0.82	0.90*	0.91*	0.98*	0.87	0.92	1.03	1.12*
Adrenals[a]	0.024	0.032*	0.030	0.024	0.043	0.047	0.053*	0.036**
Testes	1.36	1.52	1.46	1.68*	–	–	–	–

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance)
[a] The left and right kidney weights and the left and right adrenal weights appear to have been combined.
Source: Fogleman (1970)

Histopathology revealed hypertrophy and hyperplasia of liver cells in male and female rats receiving 1000 or 5000 ppm. Cloudy swelling, enlargement of nuclei, karyolysis and karyorrhexis, and necrosis were sometimes described in liver samples taken from these groups. In females, cloudy swelling and vacuolization of the renal tubules and slight tubular calcinosis were observed in a few animals at the intermediate and high doses.

This study does not comply with current standards and is poorly reported. Based on the relatively limited information reported, the no-observed-adverse-effect level (NOAEL) was identified as 500 ppm (equal to 52 mg/kg bw per day), based on relative liver and kidney weight increases, accompanied by histopathological lesions, at 1000 ppm (equal to 105 mg/kg bw per day) (Fogleman, 1970).

In a 90-day feeding study, norflurazon (SAN9789; batch and purity not given) was administered to groups of 20 male and 20 female CFE strain rats per group at a dietary concentration of 0, 250, 500 or 2500 ppm (equal to 0, 24, 45 and 248 mg/kg bw per day for males and 0, 26, 52 and 275 mg/kg bw per day for females, respectively). In addition to mortality and the occurrence of clinical signs, the end-points evaluated in this study included body weight (determined once a week), feed consumption, gross pathology, and weights of liver, kidneys, adrenals, heart, brain, thyroid and testes. Clinical pathological examinations were performed at scheduled termination. Haematological parameters included erythrocyte and leukocyte counts, haematocrit, haemoglobin and differential white blood cell count. The clinical chemistry parameters comprised glucose, urea nitrogen and uric

acid, total serum protein and albumin, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, cholesterol, bilirubin, calcium and phosphate. During urine analysis, pH and urinary sediment were examined, along with tests for sugar, ketone bodies, albumin and occult blood. Histopathological examination at scheduled termination was performed on a representative range of organs and tissues from five males and five females from each group. Five male and five female rats were randomly selected from each group and killed after 4 weeks of feeding. Clinical chemistry parameters were measured, animals were necropsied and organs (except thyroid) were weighed. Histopathology was apparently not performed in these rats.

There were four unscheduled deaths during the study, all of them close to termination. A low-dose female had a liver tumour (diagnosed as reticulum cell sarcoma). Two mid-dose males died of a respiratory infection. (Signs of respiratory disease occurred in all groups, in particular during weeks 4 through 6.) The cause of death of a high-dose female could not be determined as a result of autolysis. In the absence of clinical signs that could be attributed to treatment in any group and without a dose-related pattern, these fatalities were most likely not related to treatment; however, the respiratory infection may raise concerns about the hygienic conditions and quality of this old study.

Effects on body weight and feed consumption were confined to the high-dose group and were relatively minor. In high-dose males, a transient reduction in mean body weight was noted at the beginning of the study. The difference relative to the control group was statistically significant from weeks 2 through 4, but was not greater than approximately 10%. During the second half of the study, the mean body weight approached the control value. It is not clear if this "normalization" might have been partly due to the interim killing of five animals in this group (the mean body weight of the five prematurely killed males in week 4 was 170.2 g, whereas the mean body weight of the 15 males remaining on study at the same time point was 189 g). In high-dose females, a statistically significantly lower body weight relative to the control group was observed after the first week of treatment and again in weeks 8, 10 and 12 (Table 6). These body weight findings were clearly not due to a lower feed intake, as feed consumption, even though extremely variable throughout the individual study weeks, did not differ very much between the groups, and no consistent trend could be observed. The substantial body weight gain from week 0 to week 4 finds its explanation in the fact that treatment of the animals commenced just after weaning.

Table 6. Mean body weights in selected weeks in the 90-day study in rats

	Mean body weight (g)							
	Males				**Females**			
Week (no. per group)	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**
Prior to treatment (*n* = 20)	60.1	60.1	59.8	59.8	61.9	61.0	61.7	61.4
1 (*n* = 20)	97.3	89.7	100.6	91.6*	92.6	89.6	92.2	88.3*
4 (*n* = 20)	206.2	206.2	209.3	184.3**	157.2	156.1	152.2	150.2
8 (*n* = 15)	291.9	297.8	296.5	276.4	203.9	195.3	195.6	191.3*
12 (*n* = 15)	345.0	352.4	346.1	337.3	223.4	218.7	218.9	205.0*

n: number; no.: number; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test)
Source: Fogleman (1971a)

Haematology, clinical chemistry and urine analysis did not reveal statistically significant or dose-related differences among the groups, either at 28 days or at 90 days. No gross abnormalities were noted at necropsy, apart from some lung findings indicative of respiratory infection in all groups, which were apparently not related to treatment. As in the 28-day study described above (Fogleman,

1970), there were changes in relative organ weights. In the animals from the interim groups (killed at week 4), relative liver weights were increased at the high dose in both sexes. In addition, relative kidney weight was higher in high-dose females. These findings were confirmed in the animals that were killed after 90 days. Again, relative liver and kidney weights were elevated in males and females receiving the high dose. In females, a statistically significantly higher relative liver weight was also noted at the intermediate dose. In addition, mean relative thyroid weight was increased in high-dose males (Table 7).

Table 7. Relative organ weights in the 90-day feeding study in rats at termination

	Relative organ weight (%)							
	Males (*n* = 15)				**Females (*n* = 15)**			
Organ	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**
Liver	3.86	3.77	4.33	4.89*	3.96	4.01	4.17*	5.00*
Kidneys	0.76	0.83	0.78	0.94*	0.74	0.75	0.74	0.81
Thyroid	0.005	0.006	0.005	0.01**	0.006	0.006	0.008	0.008

n: number; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test)
Source: Fogleman (1971a)

The only remarkable histopathological findings were moderate follicular cell hypertrophy, moderate depletion of colloid and a slight to moderate increase in interstitial vascularity in the thyroids of high-dose males, suggesting some stimulation of thyroid activity. It is worth noting that the relative liver and kidney weight increases were not accompanied by microscopic lesions, suggesting that they either were due to lower body weight or reflect an adaptive response rather than an adverse effect.

Based on effects on body weight in both sexes and on the relative thyroid weight increase and histopathological findings in the thyroid of males at 2500 ppm (equal to 248 mg/kg bw per day), the NOAEL was identified as 500 ppm (equal to 45 mg/kg bw per day) (Fogleman, 1971a).

It must be noted that this study does not comply with current standards and is poorly reported. There are only a few summary tables, statistical analysis of absolute organ weights was lacking and much of the text and many tables in the submitted electronic version were difficult to read.

In a 9-month feeding study, norflurazon (SAN9789; batch no. 004; purity 98.8%) was administered to 40 male and 40 female Sprague Dawley rats per group at a dietary concentration of 0, 125, 250 or 500 ppm (equivalent to 0, 6.25, 12.5 and 25 mg/kg bw per day, respectively). The control group consisted of 80 rats of each sex receiving basal diet only. Treatment commenced 2 weeks after weaning. Apparently, the animals were mated and produced a litter as part of the study, but no information regarding this reproduction part is given in the report. The animals were monitored for mortality and the occurrence of clinical signs and were weighed weekly. Feed consumption was determined weekly, except during the mating period for males and females and during lactation for females, and feed efficiency was calculated. Haematological and clinical chemistry parameters were measured in five males and five females from the control and high-dose groups at 17 weeks on test and in 10 males and 10 females from the same groups at termination. Haematology included erythrocyte and leukocyte counts, haematocrit, haemoglobin and differential white blood cell count. The clinical chemistry parameters comprised glucose, blood urea nitrogen, total serum protein, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase. During urine analysis in the 10 control and high-dose animals of each sex at termination, pH, glucose, ketone bodies, albumin and occurrence of occult blood were examined. All animals surviving to scheduled termination as

well as those that died on test were subjected to gross necropsy. At termination, weights of liver, kidneys, heart, spleen, thyroid and gonads were determined, and relative organ weights were calculated. Histopathological examination was confined to liver, kidneys and thyroid (even though many more organs and tissues had been collected) and was performed in 40 animals of each sex from the control groups and in all high-dose rats as well as in those that died on test. In addition, neoplasms were examined histologically.

Twelve animals died during the study, five of them in the control group. Nephritis was the most common cause of death. None of these deaths could be attributed to treatment. A low-dose male had a kidney tumour (adenocarcinoma), and a subcutaneous tumour (adenofibroma) was noted in a control female. These findings are considered incidental.

There were no clinical signs that could be attributed to substance administration. Body weight, body weight gain, feed consumption and feed efficiency were not affected by treatment. Haematological, clinical chemistry and urine analysis parameters were also not affected, but the number of animals under examination for these end-points was rather limited. No gross abnormalities were noted at necropsy. In female rats, there was evidence of higher absolute and relative liver weights, but the figures are poorly depicted in the submitted copy of the study, and statistical analysis was not performed. Histopathology did not reveal alterations in the liver or thyroid, but some kidney lesions were more frequent in the animals at the high dose. At 500 ppm, the incidence of tubular degeneration was more than doubled in males (20/40 compared with 8/40 in controls) and was also increased in females (9/40 compared with 1/40 in controls). In addition, in females, kidney pigmentation was increased (18/40 in the high-dose group compared with 4/40 in controls), and hyaline tubules were observed more frequently (22/40 in the high-dose group compared with 8/40 in controls). An increase in hyaline tubules was also noted in high-dose male rats, but the difference relative to the control group (30/40 compared with 19/40) was less pronounced than in females.

Although there were indications of toxicity at least at 500 ppm (equivalent to 25 mg/kg bw per day), with the kidney identified as the main target organ, a NOAEL cannot be identified owing to the deficiencies of this poor quality study (Harris, 1972).

Dogs

The oldest study in beagle dogs (four of each sex per dose) was of limited quality, as the duration of treatment in the different groups varied (18 versus 10 weeks), doses were increased twice during the study in the low-dose (25–150 ppm) group and dietary administration was discontinued at the intermediate and high doses (1000 and 5000 ppm) and changed to daily capsule administration of 25 or 125 mg/kg bw for the duration of the study. The study author reported mainly gastrointestinal signs and poor palatability (Fogleman, 1971b).

Owing to the deficiencies of this study, the results are not reliable. This study may be considered fully replaced by the 6-month study and the modern 1-year study in beagle dogs reported below.

In a 6-month feeding study, norflurazon (batch and purity not given) was administered to beagle dogs (four of each sex per group) at a dietary concentration of 0, 50, 150 or 450 ppm (equal to 0, 1.5, 5.0 and 14.3 mg/kg bw per day for males and 0, 1.6, 4.8 and 17.8 mg/kg bw per day for females, respectively). Parameters under investigation were body weight, body weight gain, feed consumption, clinical signs (including neurological examination), haematology, urine analysis and clinical chemistry parameters (including determination of microsomal liver enzymes at termination), ophthalmology, organ weights, and gross and microscopic pathology. In contrast to other, older studies with norflurazon, histopathology of a wide range of organs was performed in all animals.

There were no unscheduled deaths during the study, and no clinical (including neurological or ophthalmological) signs could be attributed to treatment. Body weight and body weight gain were not affected by treatment, and there were no significant differences in feed consumption. Although the

total feed intake in high-dose females was, on average, higher than in the control group (59.0 kg versus 51.3 kg), even though the mean body weight gain of 0.5 kg was the same, this was mainly due to a single female with an extraordinarily high feed intake of 85.6 kg over the study period. In males, the feed intake in the mid- and high-dose groups was also higher than in controls, but this finding was not dose related and resulted in a slightly higher body weight gain.

Red blood cell count tended to decrease in high-dose females after 3 and 6 months of treatment (Table 8). However, the mean values were still within the reference range of 5.5–8.5 × 10^6/µL for healthy dogs (Moritze, Schwendenwein & Kraft, 2014), suggesting that the effect might be treatment related, but was not necessarily adverse. One of the females in this group had a value of only 5.13 × 10^6/µL after 6 months, the lowest individual value of all females on study, but the pretest value of 5.66 × 10^6/µL in the same animal was also the lowest at this time point. The haematocrit was lower in high-dose females as well, and the difference was even more impressive when compared with the pretest mean in the same group. There was no concomitant impact on haemoglobin. No similar haematological changes were observed in males, in which consistent increases in red blood cell count and haematocrit over the course of the study were apparent in all groups, including the controls.

Table 8. Alterations in selected haematological and clinical chemistry parameters in the 6-month feeding study in beagle dogs (group mean values, with standard deviation if available)

	Males (*n* = 4)				**Females (*n* = 4)**			
Parameter	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**
RBC count (× 10^6/µL)								
Pretest[a]	5.96	5.78	5.88	6.46	6.85	6.95	6.26	6.53
3 months	7.18	7.11	6.86	7.03	7.74	6.71	6.78	6.12
6 months	6.72	7.02	7.02	6.79	6.87	6.79	6.50	5.85
Haematocrit (%)								
Pretest	42.25	40.75	41.25	44.75	49.50	50.25	43.30	45.00
6 months	47.50	49.75	48.25	46.75	48.50	48.00	45.00	40.75
Cholesterol (mg/100 mL)								
Pretest	198 ± 31	209 ± 10	221 ± 22	209 ± 28	189 ± 11	187 ± 7	214 ± 21	201 ± 7
3 months	206 ± 74	198 ± 23	288 ± 14	283 ± 30	190 ± 14	228 ± 48	255 ± 80	283 ± 22
6 months	197 ± 48	196 ± 12	253 ± 38	268 ± 13	247 ± 63	225 ± 40	261 ± 15	254 ± 55
ALP (mU/mL)								
Pretest	60.2 ± 14.5	53.1 ± 4.8	64.8 ± 22.5	53.8 ± 11.2	70.8 ± 29.1	49.6 ± 10.1	78.8 ± 18.5	61.4 ± 20.6
6 months	24.2 ± 9.9	32.7 ± 15.8	57.3 ± 21.6	54.8 ± 16.4	45.7 ± 31.9	37.3 ± 7.9	76.4 ± 44.0	65.8 ± 49.0

ALP: alkaline phosphatase; ppm: parts per million; RBC: red blood cells; U: units

[a] "Pretest": 2 weeks before commencement of study.

Source: Klotzsche & Carpy (1973)

Clinical chemistry revealed an increase in mean cholesterol concentrations in mid- and high-dose males after 3 and 6 months and at least in high-dose females after 3 months (Table 8). With

regard to alkaline phosphatase, a decrease in activity was apparent in both sexes with ongoing duration of the study in the control and low-dose groups. However, such a reduction was not observed in the mid- and high-dose groups, which might point to liver toxicity. For both parameters, large interindividual variability in some groups, including the controls, must be noted. All the other parameters measured in blood or urine did not show any changes that could be attributed to treatment. Even though liver weight was increased (see below), no induction of liver enzymes was observed.

Necropsy did not reveal any gross pathological findings. Absolute and relative liver weights were increased at the middle and high doses in both males and females; although there was not always a clear dose–response relationship and the differences were not always statistically significant (Table 9), this is of limited relevance in a study with such a small number of animals. Extremely high absolute liver weights of 459 or 407 g were noted in two high-dose females, in contrast to rather low liver weights (234 and 249 g) in the two other females in the same group. The higher absolute liver weight in low-dose males was not regarded as potentially adverse, as it was not accompanied by clinical chemistry findings suggesting liver toxicity (see Table 8) or by histopathological lesions (see below).

Table 9. Mean absolute and relative liver weights (with standard deviation) in the 6-month feeding study in beagle dogs

	Males (n = 4)				Females (n = 4)			
Parameter	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**
Absolute liver weight (g)	245.82 ± 24.18	320.52* ± 34.05	340.65** ± 25.88	347.50** ± 27.24	234.50 ± 33.43	235.22 ± 26.99	289.80* ± 14.51	337.20 ± 112.71
Relative liver weight (%)	2.58 ± 0.38	3.02 ± 0.70	3.28 ± 0.46	3.26* ± 0.14	2.82 ± 0.29	2.77 ± 0.32	3.19 ± 0.52	4.20* ± 0.79

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (statistical test not provided)
Source: Klotzsche & Carpy (1973)

There were no changes in other organ weights that might be related to treatment.

Clear treatment-related histopathological findings were confined to the highest dose. The liver and the thyroid were mainly affected, but the effects were rather minor in nature. Liver findings comprised congestion in five out of eight high-dose dogs and hypertrophy in one of them. Regarding the thyroid, the number of animals exhibiting an increase in colloid vacuoles was increased at the high dose. The study authors also mentioned an increase in fatty changes in the myocardium and in epithelial vesiculation in the mammary gland of high-dose dogs, along with some evidence of pneumonia; they interpreted these latter findings as signs of "diminished resistance" and indicative of an unspecific toxic effect of the high dose. It must be noted that the information on histopathological examination is based on a summary table only, as the quality of the copy does not allow meaningful assessment of the raw data.

The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on liver weight increases, histopathological findings in liver and thyroid and alterations in clinical chemistry parameters at 150 ppm (equal to 4.8 mg/kg bw per day) (Klotzsche & Carpy, 1973).

In one of the very few available guideline-compliant toxicological studies performed under GLP conditions, norflurazon (batch no. P.148/86/31; purity 98.2%) was administered to groups of four male and four female beagle dogs at a dietary concentration of 0, 50, 200 or 800 ppm (equal to 0, 1.7, 6.2 and 27 mg/kg bw per day for males and 0, 1.5, 6.3 and 23 mg/kg bw per day for females, respectively) for 1 year. The animals were monitored for clinical signs and weighed weekly. Feed intake was determined on a weekly basis. Comprehensive haematological and clinical chemistry examinations, including urine analysis, were performed at 3-month intervals. At scheduled

termination, the dogs were killed and necropsied. Organ weights were measured, and histopathological examination of a wide range of organs and tissues was conducted in all groups.

There were no unscheduled deaths during the study, but one high-dose female showed subdued behaviour and weight loss. This was apparently due to severe reduction in feed intake during the early part of the study. There is an indication that a palatability problem might have been behind this, as moistening of the diet with water resulted in normalization of feed intake and regaining of body weight and condition. No clinical signs that could be attributed to treatment were noted in other animals.

In general, body weight (gain), feed consumption and feed efficiency were impaired by norflurazon treatment (Table 10). All treated dogs gained less weight than the control animals over the course of the study. This effect was most pronounced in high-dose males and mid- and high-dose females and more equivocal in low- and mid-dose males and low-dose females, as the difference relative to the respective control group was small and, in males, there was no clear dose–response relationship. A marked decrease in feed consumption over the whole study period was observed in both sexes at the high dose, but a weak effect in the low- and mid-dose groups cannot be excluded, suggesting a palatability problem. Based on feed conversion ratios (i.e. the amount of feed consumed divided by body weight gain), feed efficiency appeared to be compromised in high-dose males and mid- and high-dose females over the first 16 weeks of the study. For the subsequent part of the study, this calculation was not performed. This finding might suggest that the lower body weight gain did not result only from lower feed intake, but could also indicate a toxic or metabolic effect. It is not surprising that the mean total body weight was lowest at study termination in the high-dose group in both sexes (see Table 12 below).

Table 10. Mean weekly body weight gain and daily feed consumption in the 1-year study in beagle dogs

	Males				Females			
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**
Body weight gain (kg/week)								
Weeks 0–9	0.21	0.14	0.18	0.08	0.17	0.16	0.10	−0.01
Weeks 0–28	0.10	0.08	0.09	0.04*	0.08	0.08	0.06	0.02
Weeks 0–52	0.06	0.05	0.05	0.03	0.06	0.05	0.04	0.03
Feed consumption (g/day)								
Weeks 0–9	362	359	354	306	338	325	311	252
Weeks 0–52	356	346	325	314	322	293	301	272

ppm: parts per million; *: $P < 0.05$ (analysis of variance, followed by Dunnett's test)
Source: Warren et al. (1990)

Haematology revealed a reduction in red cell parameters (haematocrit, haemoglobin and red blood cell count) in both sexes at the high dose. These findings, suggestive of slight anaemia, were most pronounced at week 12. In high-dose males, platelet count was increased. Clinical chemistry analysis pointed to liver toxicity, with elevated cholesterol levels (confirming the results of the older 6-month study reported above; Klotzsche & Carpy, 1973), at least in mid- and high-dose males, and, occasionally, activities of some enzymes. All these effects were confined to the high-dose or the mid- and high-dose groups (Table 11). The only urine analysis finding that might be related to treatment was a slightly higher specific gravity of urine in males and females receiving 800 ppm, which is normally not considered a pathological effect.

Table 11. Selected haematological, blood clinical chemistry and urine analysis findings from the 1-year study in beagle dogs (group mean values)

	Males				Females			
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**
RBC count (10^6/mm^3)								
Pretest	6.515	6.625	6.688	6.178	6.698	6.635	6.643	6.330
Week 12	7.075	7.038	6.843	6.133*	7.380	7.140	7.198	6.113*
Week 52	7.808	7.873	8.035	7.275	7.718	7.768	7.620	7.055
Platelet count (10^3/mm^3), week 52	286.50	310.25	333.50	399.25	334.75	317.75	337.25	351.0
Cholesterol (mmol/L), week 52	3.44	3.98	5.04	4.78	4.61	3.87	4.82	4.90

ppm: parts per million; RBC: red blood cells; *: $P < 0.05$ (analysis of variance, followed by Dunnett's test)
Source: Warren et al. (1990)

Only a few gross pathological changes could be attributed to treatment. According to the pathology report, these were a discoloured liver with granular or nodular capsule in one high-dose female and gall bladders with black or brown foci in the mucosa in two males and one female from the high-dose group. However, similar gall bladder findings were noted in one low-dose female and one mid-dose male. Dark brown discolouration of the kidney was observed in one high-dose male.

The only treatment-related organ weight change was an increase in (absolute and relative) liver weights in high-dose males and females and in mid-dose males (Table 12), confirming the results from the older 6-month study by Klotzsche & Carpy (1973). No statistical significance was achieved, but such an analysis is of low value in a study with only four animals of each sex per dose group. A substance-related effect is not excluded.

Table 12. Mean absolute and relative liver weights (with standard deviation) in the 1-year feeding study in beagle dogs

	Males (*n* = 4)				Females (*n* = 4)			
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**
Mean body weight (kg)	10.88 ± 2.80	10.55 ± 1.24	10.78 ± 1.40	9.27 ± 0.33	10.98 ± 0.78	10.20 ± 1.15	10.02 ± 1.33	9.77 ± 0.67
Absolute liver weight (g)	281.3 ± 56.0	272.2 ± 40.2	310.0 ± 9.7	321.9 ± 61.0	292.5 ± 32.4	228.5 ± 14.7	247.7 ± 61.2	329.4 ± 44.8
Relative liver weight (%)	2.625 ± 0.360	2.577 ± 0.204	2.909 ± 0.330	3.462 ± 0.573	2.675 ± 0.332	2.262 ± 0.297	2.468 ± 0.506	3.361 ± 0.281

n: number; ppm: parts per million
Source: Warren et al. (1990)

Histopathological examination revealed hepatitis in two high-dose males. Incidences and degree of biliary cholestasis and mucosal hyperplasia were seen only in the high-dose group, even though only one female and one male, respectively, were affected. Male dogs also had an increased incidence of renal cortical tubular pigmentation at 200 and 800 ppm, suggesting that the kidney might also be a target organ. It is noted that this information from the text is not fully supported by the tables.

The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on impairment of nutritional parameters, such as body weight (gain), feed consumption and feed efficiency, in females and alterations in clinical chemistry suggestive of liver toxicity and increased liver weights in both sexes at 200 ppm (equal to 6.2 mg/kg bw per day). Kidney findings of equivocal toxicological significance, including renal tubular pigmentation, were noted in males at 200 ppm and above (Warren et al., 1990).

The outcome of this study is very much in line with the results of an older 6-month study (Klotzsche & Carpy, 1973). Taken together, these studies suggest that the dog might be more sensitive than the rat.

(b) Dermal application

No studies were submitted.

(c) Exposure by inhalation

No studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a combined chronic toxicity and carcinogenicity study, norflurazon (described as "technical formulation of product 9789"; batch no. 004; purity 98.8%) was administered to groups of 125 male and 125 female CD-1 (HaM/ICR Swiss) mice at a dietary concentration of 85, 340 or 1360 ppm (equivalent to 13, 51 and 200 mg/kg bw per day, respectively) for up to 2 years. Two control groups of the same size were included and fed on untreated basal diet. The design of this very old study is unusual and does not comply with current standards or any guideline, as treatment was commenced in weanling mice that had been selected from the F_{1A} litters of a two-generation reproduction study (reported in section 2.5). As their parents had received the test substance (apparently at the same dietary concentrations) over an 18-week premating period and throughout gestation and lactation, preceding prenatal or postnatal exposure of the animals in the treatment groups is likely and could have, at least theoretically, contributed to the findings.

A clear deficiency of this study was the loss of 56 male mice from one of the two control groups and of 56 low-dose males after 20 weeks on study as a result of an accident. To replace these lost animals, a "small group" of new F_0 animals was purchased, placed on either control or low-dose diets and mated to produce new F_{1A} litters, from which 56 males per group were randomly selected and, after weaning, treated for 104 weeks as in the original study. Thus, part of the treatment and investigation took part at a later time. This unusual approach would normally be considered to have severely disturbed the integrity of the study, making it unacceptable according to current guidelines, even though some of the data were reported separately.

The animals were monitored for mortality and clinical signs, body weight, body weight gain and feed consumption. Satellite groups of 10 males and 10 females each were fed either control diet or the high dose of norflurazon for up to 26 weeks. At 10 or 26 weeks, five satellite animals of each sex per group were killed, and blood samples were taken for haematology and clinical chemistry, but the range of clinical chemistry parameters was small and did not comply with current standards.

All surviving animals were killed between weeks 100 and 104 and, together with those animals that died on test, were necropsied and grossly examined. At scheduled termination, the following organs were weighed: liver, heart, spleen, kidneys, pituitary, brain, gonads, thyroid and adrenals. A range of organs and tissues was taken and preserved for histopathology, but microscopic evaluation was in fact confined to the control and high-dose animals, apart from major lesions, which

were examined from all test groups. Because of tumour findings, the livers of all animals were examined histologically.

There were no clinical signs that could be attributed to treatment. In both sexes, survival was sufficient, at greater than 50% overall and greater than 60% in most groups at week 100. A common cause of premature deaths, but apparent in all groups, including the controls, was lymphosarcoma.

A slight reduction in body weight in high-dose males was likely treatment related. During the second year of the study, mean body weight of high-dose males was 37–39 g on average over the individual weeks, compared with 40–42 g in the control, low-dose and mid-dose groups. No statistically significant differences were observed in females, and there were no alterations in feed consumption in either sex. It is questionable whether the rather weak effect on body weight in one sex may be considered sufficient to conclude that the maximum tolerated dose had been reached. However, this conclusion could be drawn because of liver tumours in male mice (see below).

Haematology and clinical chemistry in a small number of mice did not reveal any changes that could be attributed to administration of the test substance for either 10 or 26 weeks.

Gross examination of mice at scheduled termination and of premature decedents suggested an impact at the high dose on the liver and perhaps on the kidneys in both sexes and on the adrenals in males only. In particular, when analysis was made on the basis of "all animals on study", there was a higher number of mice exhibiting macroscopic liver changes and evidence of nephritis, such as pitted surface (Table 13). Even though statistical analysis of these findings was not performed, effects at the high dose and some effects at the intermediate dose appear outside the normal biological variance, as demonstrated by the large differences between the two control groups. With regard to nephritis in males, an impact of the intermediate dose cannot be excluded.

Table 13. Selected gross pathological findings in male and female mice at termination after 2 years and in those animals that died on test (combined incidence)

	Combined incidence of pathological finding				
Parameter	**0 ppm**	**0 ppm**	**85 ppm**	**340 ppm**	**1 360 ppm**
Males					
Number examined	*125*	*124*	*124*	*125*	*125*
Liver: enlarged	2	8	5	7	15
Liver: dark, congested, haemorrhagic	1	2	1	6	15
Kidneys: nephritis	4	8	3	12	14
Adrenals: enlarged[a]	0	2	2	1	9
Females					
Number examined	*125*	*126*[b]	*126*[b]	*125*	*125*
Liver: enlarged	4	9	9	8	15
Liver: dark, congested, haemorrhagic	1	7	7	8	18
Kidneys: nephritis	5	5	6	7	12
Adrenals: enlarged[a]	5	0	11	0	7

ppm: parts per million

[a] Reported only for animals killed at scheduled termination.

[b] Apparently, in both groups, one female had been erroneously taken for a male when the very young animals were allocated to the different groups.

Source: Tisdel (1975a)

Higher absolute and relative liver weights were noted after 24 months in males in all treated groups, revealing a dose-related pattern (Table 14). The slightly lower mean absolute and relative kidney weights in high-dose males, in contrast, reflect the lower terminal body weight. In females, absolute and relative liver weights were also increased at least at the intermediate and high doses, whereas kidney weight was not affected. Occasional changes in other organ weights did not follow a pattern and were considered incidental.

Table 14. Organ weight changes in male and female mice at termination after 2 years

Parameter	0 ppm	0 ppm	85 ppm	340 ppm	1 360 ppm
Males					
Number examined	71	81	85	65	74
Mean body weight (g)	37	35	36	36	33
Mean absolute liver weight (g)	1.87	1.92	2.05*	2.18*	2.41*
Mean relative liver weight (% of control)	5.14	5.57	5.69*	6.14*	7.38*
Mean absolute kidney weight (g), left / right	0.393 / 0.394	0.373 / 0.389	0.376 / 0.388	0.385 / 0.401	0.338* / 0.361*
Mean relative kidney weight (% of control), left / right	1.032 / 1.006	1.072 / 1.117	1.047 / 1.081	1.072 / 1.118	1.029 / 1.103
Females					
Number examined	64	70	77	59	71
Mean body weight (g)	31	31	32	32	31
Mean absolute liver weight (g)	1.69	1.30	1.87	1.90*	2.12*
Mean relative liver weight (% of control)	5.43	5.79	5.88	6.02*	6.93*
Mean absolute kidney weight (g), left / right	0.285 / 0.299	0.277 / 0.290	0.277 / 0.294	0.277 / 0.291	0.290 / 0.298
Mean relative kidney weight (% of control), left / right	0.920 / 0.963	0.889 / 0.936	0.872 / 0.926	0.876 0.922	0.955 / 0.978

ppm: parts per million; *: statistically significantly different from at least one control group
Source: Tisdel (1975a)

Histopathology of control and high-dose animals at scheduled termination revealed an increase in liver lesions in male but not in female animals (Table 15). There was a dose-related increase in liver cell adenoma in all male dose groups. This was accompanied by a higher incidence of hepatocellular hypertrophy, at least at the high dose. A similar effect in the low- and mid-dose groups is likely when the low number of animals under examination is taken into account. Hypertrophy often precedes adenoma development. Taken together, these findings are well in line with the increase in liver weight and the gross liver changes and are certainly treatment related. It is surprising that similar gross observations in females did not seem to find a histopathological correlate. Even though carcinoma incidence was increased among mid-dose males at scheduled termination, this finding did not provide convincing evidence of a treatment-related increase in malignant liver tumours in general. There was no dose–response relationship, as the incidence at the high dose was similar to that of the control groups. A few more liver tumours were detected when the premature deaths were taken into consideration (Table 16), but the general pattern did not change. An increase in tumours other than in the liver was not observed in either males or females.

Table 15. Selected histopathological findings in the 2-year feeding study in mice at termination

Pathological finding	0 ppm	0 ppm	85 ppm[a]	340 ppm[a]	1 360 ppm
Males					
Number examined	71	81	37	33	74
Liver: hypertrophy	5	7	6	2	31
Liver: adenoma	3	2	7	10	16
Liver: carcinoma	3	1	–	4	1
Spleen: pigmentation	6	11	–	–	19
Kidneys: pyelonephritis	12	7	3	4	10
Intestines: amyloidosis	5	9	–	–	18
Females					
Number examined	64	70	32	15	71
Liver: hypertrophy	13	4	5	2	13
Liver: adenoma	1	1	2		2
Liver: carcinoma	0	0	1		1
Spleen: pigmentation	13	11	–	–	33
Pancreas: hyperplasia	0	0	–	–	8
Kidneys: pyelonephritis	11	13	2	–	28
Ovaries: cystic	28	35	16	5	48
Bone marrow: pigmentation	0	5	4	–	15

–: organ not examined; ppm: parts per million
[a] Histological examination only of animals with gross lesions.
Source: Tisdel (1975a)

Table 16. Overview of liver cell tumours in the 2-year feeding study in male mice, based on additional examination of the livers of all animals

Group and tumour	0 ppm	0 ppm	85 ppm	340 ppm	1 360 ppm
Males, died on test	54	43	39	60	51
Liver: adenoma	0	1	1	1	0
Liver: carcinoma	1	1	1	1	3
Males, killed at termination	71	81	85	65	74
Liver: adenoma	3	2	7	10	16
Liver: carcinoma	3	1	0	4	1
Males on study	125	124	124	125	125
Liver: adenoma	3	3	8	11	16
Liver: carcinoma	4	2	1	5	4

ppm: parts per million
Source: Tisdel (1975a)

The most prominent and presumably treatment-related non-neoplastic kidney lesion was pyelonephritis, which was much more common in high-dose females than in the controls (Table 15). Gross kidney lesions in mid- and high-dose male mice had no morphological correlate at termination.

However, in premature deaths, nephritis was more often diagnosed in male decedents (17/44 compared with 10/47 and 7/39 in the two control groups). Another adverse effect in female mice was pancreatic hyperplasia, which was noted only at the high dose. Cystic degeneration of the ovaries was more often observed in the high-dose group than in control females. This finding might point to exacerbation of age-related changes and could be hormone mediated. The same assumption with regard to ageing might hold true for the observed increase in intestinal amyloidosis in high-dose males. The toxicological significance of spleen pigmentation in high-dose males and of bone marrow pigmentation in high-dose females is equivocal.

In addition to the original study report, further evaluations of the liver pathology in male mice were performed. In the introductory part of the study report, reference is made to a "second opinion" of an external expert (Ward Richter, A.J. Carlson Animal Research Facility, The University of Chicago), who had concluded, following his review of the histological slides from the terminal males only, that the increase in hypertrophy and adenoma was confined to the high-dose group.[1] In addition, he found no evidence of malignant tumours and considered norflurazon to be non-carcinogenic. A letter to Sandoz Pharmaceutical Research Laboratories dated 9 July 1975 and signed by Professor Richter was submitted by the sponsor in copy, along with some notes, partly handwritten. Different diagnostic criteria and a partly different nomenclature of pathological liver findings were apparently applied for this second evaluation of the slides. The two control groups were combined, and the control and low-dose animals that were recruited late for the study due to the accident described above were also included in Professor Richter's analysis (Richter, 1975). The outcome is summarized in Table 17.

Table 17. Histopathological liver findings in male mice according to Professor Richter

Finding	Control	85 ppm	340 ppm	1 360 ppm
No. ("listed")	152	82	64	74
No. (examined)	144	19	19	74
Hypertrophy, multifocal	5	2	2	11
Hyperplasia, nodular	1	4	2	0
Adenoma	8	8	7	16
Cholangioma	2	0	0	0
Carcinoma	4	1	1	0
Neoplasia, total	14	9	8	16

ppm: parts per million
Source: Richter (1975)

The number of animals "listed" is the same as or at least similar to the number of animals killed at scheduled termination. The evaluation of livers from the low- and mid-dose groups is far from being comprehensive, as only a few animals were included in the review, and no clear justification for the selection was given. Based on what can usually be expected in ageing mice, the incidence of hypertrophy in all groups appears remarkably low. However, the author mentioned that the diagnosis "hypertrophy" was made only when its degree was higher than "normally found". Even then, the difference compared with the previous examination is striking. Statistical analysis of the results was not performed. Based on the results shown in Table 17, the conclusion that norflurazon was not carcinogenic cannot be accepted, as there was a clear increase in adenoma at the high dose compared with the control group (approximately 22% versus 5.6%). The total liver tumour incidence

[1] Even though the pathologist in the first sentence of his letter explained that rat liver tissues had been shipped to him, it can be reasonably assumed that these were, in fact, the mouse liver tissue slides obtained from the long-term study under evaluation.

was also higher (approximately 22% versus 9.7%). The analysis is flawed by ignoring the animals that died on study. This failure results in underestimation of the total tumour incidence, in particular with regard to carcinoma in the mid- and high-dose groups. One might get the wrong impression that the carcinoma incidence even decreased. Meaningful comparison is confined to the control and high-dose groups because of the low number of animals from the low- and mid-dose groups. Therefore, this second histopathological evaluation does not contribute to the identification of a NOAEL for this study.

A third histopathological evaluation of the liver slides from this study was performed by a consultant pathologist (Dr Rust), with its outcome being subject to statistical analysis. This re-evaluation was apparently performed in 1980 on behalf of the company Sandoz in response to questions raised by the United States Environmental Protection Agency (USEPA) in 1979. It was submitted by the sponsor to JMPR as part of the report on the long-term toxicity and carcinogenicity study in mice, even though it is not possible that it was part of the original study when the timelines are taken into consideration. Therefore, it is regarded as a separate document for the current evaluation and is referenced as such (Rust, 1980).

The outcome of this third histopathological evaluation of liver tissues from male mice that either had died on test or were killed at scheduled termination is summarized in Table 18. This time, the incidences of the different histopathological diagnoses were reported separately for the two control groups. The discrepancies with the total number of male mice on study were explained by absent tissues (either from the liver or "any tissue"), which were simply not available for some animals when the re-evaluation was performed, or, more rarely, severe autolysis or incomplete necropsy record. The pathologist also re-evaluated lung tissues, mainly from the control and high-dose groups, but confirmed that there was no evidence of a higher incidence of benign or malignant lung tumours in the high-dose group.

Table 18. Summary of histopathological liver findings in male mice according to Rust (1980)

Finding	0 ppm	0 ppm	85 ppm	340 ppm	1 360 ppm
Tissues provided: males died on test	43	35	32	54	42
Tissues provided: scheduled termination	71	82	82	64	73
Tissues examined	114	117	114	117	115
Nodular hyperplasia/hypertrophy	7	1	6	5	10
Hepatocellular adenoma	0	4 (3[a])	5	1	11
Hepatocellular carcinoma	7	5 (6[a])	3	4	6

ppm: parts per million

[a] According to the pathology report, one animal had both an adenoma and a carcinoma. According to current guidelines, the more severe diagnosis should be used. This would decrease the adenoma incidence and increase the carcinoma incidence in control group 2.

Source: Rust (1980)

Lung metastases of liver cell carcinoma were found in one control group 2 animal and in one mouse of the mid-dose group. The histopathological re-evaluation also revealed amyloidosis, focal necrosis due to acute hepatitis or lymphoid pericholangitis and confirmed the presence of lymphoreticular tumours in some animals. All these findings were not dose related and thus were not attributed to test substance administration.

There are many striking differences between the histopathological evaluation by Rust (1980) and the evaluation in the original study report as well as the reanalysis by Richter (1975). Most likely they are due to the use of different diagnostic criteria, but, after decades, it is difficult to decide which evaluation was most reliable.

The company Sandoz performed a statistical analysis of these results, and this report was also submitted to JMPR as part of the study report. It is based on the additional histopathological evaluation by Rust (1980). For liver cell adenoma, a positive trend was seen, and a pairwise comparison revealed that the increase at the high dose was statistically significant. This statistical analysis can be considered to be replaced by that of Andriano (1981), reported below.

Apparently on the request of the USEPA, Andriano (1981) performed a series of statistical analyses on the liver tumour findings in male mice. For this statistical evaluation, the findings from the re-evaluation by Rust (1980) were apparently used, as the figures were the same. However, the possible impact of the replacement of a large number of animals from one of the control groups and the low-dose group during the study (see above) was taken into consideration by deleting the "accident-involved" treatment groups. Therefore, only data from the second control, mid-dose and high-dose groups were included in the new analysis, making it of little use for identifying the NOAEL. Both tests for trend (Cox's exact test version of Cochran-Armitage's test) and pairwise comparisons (by Fisher's exact test) were applied for analysis of liver lesions and were always one-tailed. Information on the statistical significance of the tumour and hypertrophy findings is taken from this report and summarized here.

For hepatocellular carcinoma, the incidence as determined by Andriano (1981) was as follows: control group: 5/117 (4%); mid-dose group (340 ppm): 4/117 (3%); and high-dose group (1360 ppm): 6/115 (5%). Neither the trend test nor the pairwise comparison revealed a statistically significant difference. This is in line with the previous interpretation of the results. A slightly higher incidence of carcinoma in the control group (6 instead of 5, see Table 18 above) would not alter the outcome.

For hepatocellular adenoma, the incidence was as follows: control group: 4/117 (3%); mid-dose group (340 ppm): 1/117 (1%); and high-dose group (1360 ppm): 11/115 (10%). Statistical analysis gave a positive trend ($P = 0.004$) and a positive result for the pairwise comparison of the high-dose group with the control group ($P = 0.002$). A lower number of adenomas in the control group (3 instead of 4, see Table 18 above) would not change the outcome that much. The adenoma incidence in the mid-dose group was, of course, not statistically significantly different from the control incidence (in fact, it was lower), but, according to the original report, 16 adenomas had been found in 125 animals. In the high-dose group, five adenoma cases were "lost", most likely due to the application of different diagnostic criteria.

For "nodular hypertrophy or hyperplasia", Andriano (1981) came to the following conclusion: control group: 1/117 (1%); mid-dose group (340 ppm): 5/117 (4%); and high-dose group (1360 ppm): 10/115 (9%). Again, there was a positive trend ($P = 0.004$), and the pairwise comparison revealed statistical significance for the increase at the high dose ($P = 0.005$).

For additional analyses, the author compiled all the animals with any hepatic proliferative change or excluded those with tumours. In all cases, the trend was positive, and so was the pairwise comparison for the high-dose group.

The evaluation of liver tumours in this long-term study obviously depends on the histopathological examination that is relied on. The histopathological re-evaluation by Rust (1980) seems the most appropriate, as the number of liver slides was nearly identical for all groups, and potentially preneoplastic liver findings were also considered. However, control group 1 and the low-dose group must be deleted and should not be considered further; these findings are not reliable, as they lack comparability with the other groups and as not all animals were treated in parallel. The same approach was taken by Andriano (1981) in the statistical re-evaluation.

To conclude, the carcinogenic effect on the liver in male mice was confined to the high-dose group (1360 ppm, equivalent to 200 mg/kg bw per day). The NOAEL for carcinogenicity was therefore 340 ppm (equivalent to 51 mg/kg bw per day). No progression to malignancy was observed, and no evidence of a neoplastic effect was seen in females. However, no information on the mode of action for tumour formation is available; accordingly, human relevance cannot be excluded.

For toxicity, a NOAEL could not be identified, as the low-dose group and one of the control groups were excluded, and there were treatment-related and adverse effects at the middle dose (340 ppm): an increase in nodular hyperplasia or hypertrophy in the liver of male mice, an increase in absolute and relative liver weights in both sexes, and a higher frequency of gross kidney changes (nephritis) in males. In addition, no comprehensive histopathological examination for non-neoplastic findings in organs other than the liver was performed at the middle dose. Therefore, no-effect levels for several histopathological findings for which the incidence at the high dose was higher than that in the control group could not be determined. These findings comprised pancreatic hyperplasia, pyelonephritis, splenic and bone marrow pigmentation and cystic ovaries in females as well as pigmentation of the spleen and intestinal amyloidosis in males. Thus, the lowest-observed-adverse-effect level (LOAEL) is 340 ppm (equivalent to 51 mg/kg bw per day), the lowest dose for which reliable data were available (Richter, 1975; Tisdel, 1975a; Rust, 1980; Andriano, 1981).

Rats

In a combined chronic toxicity and carcinogenicity study, norflurazon (described as "technical formulation of product 9789"; batch no. 004; purity 98.8%) was administered to groups of 85 male and 85 female Sprague Dawley rats at a dietary concentration of 125, 375 or 1025 ppm (equivalent to 6.25, 18.9 and 51.3 mg/kg bw per day, respectively). Two control groups of the same size were included and fed on untreated basal diet. Ten animals of each sex from one of the control and each of the treatment groups were randomly selected, killed after 52 weeks and subjected to pathological examinations. A separate report (Tisdel, 1973) on the first year of the study, including haematological, clinical chemistry and organ weight data and results of gross and histopathological examinations of these satellite group rats, was submitted and is referenced here where appropriate. The data obtained from haematological, clinical chemistry and urine analyses have been included and considered in the full report on the 2-year study as well.

The design of this very old study is unusual and does not comply with current standards or any guideline. It can be described as follows: Initially, male and female rats were fed the test substance for 9 months at a dietary concentration of 0, 125, 250 or 500 ppm. This part of the study is reported as a short-term experiment in section 2.2 above (Harris, 1972).

During the treatment period, these animals were mated. The reproduction part of the study is briefly reported in section 2.5 below (Tisdel, 1975c).

For the chronic toxicity and carcinogenicity study, weanling rats (weighing 45–55 g at the commencement of this long-term study) were selected from the F_{1A} litters. It must be kept in mind that their parents had received the test substance via their diet over an 18-week premating period and throughout gestation and lactation at doses that were partly different from those administered in the subsequent chronic toxicity and carcinogenicity study. Preceding prenatal or postnatal exposure of the animals in the treatment groups could have contributed to the findings in the long-term study.

It is not clear how the weanling rats for the chronic toxicity and carcinogenicity study were selected. A further clear deficiency of this study is the fact that the animals were moved, after 43 weeks on test, to a new animal facility.

End-points investigated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, ophthalmology, organ weights, and gross and microscopic pathology. Histopathology of a representative range of organs and tissues in all animals was confined to the control and high-dose groups. At the low and middle dose levels, only major lesions and kidneys were examined microscopically. A severe deficiency was the limited number of animals from which haematological, clinical chemistry and urine analysis data were obtained. Only 10 animals of each sex from one of the control groups and the high-dose group were included in this analysis, which was performed at study weeks 3, 13, 26, 52 and 100. Accordingly, no baseline analysis prior to commencement of treatment via the diet was performed. In addition, the range of blood clinical chemistry parameters was limited (e.g. thrombocytes and other blood coagulation parameters were not

measured). Taking into account that the kidney is a target organ of norflurazon, the absence of creatinine measurements is of particular concern.

The difference in survival between male and female rats was remarkably high. In males, survival was below 50% in all groups from week 96 onwards and at the high dose from week 92. Towards the end of the study, the decline appeared steepest in the high-dose group. Therefore, this group was terminated after week 102, instead of week 103. A lower number of survivors was also observed at the intermediate dose (Table 19). However, if the death rate between, for example, weeks 96 and 100 or from then until termination is compared, there is no evidence of a dose-related effect. It is not clear if the cause of death in the treated groups was treatment related, although the incidence of nephritis appeared higher in high-dose male decedents than in the control groups. In females, there was no impact of treatment on the mortality rate at any dose, and survival at scheduled termination was around or even above 50% in all groups.

Table 19. Survival in male rats from week 80 until termination

	Number of survivors (out of 85 rats)				
Week	**0 ppm**	**0 ppm[a]**	**125 ppm**	**375 ppm**	**1 025 ppm**
80	59	66	64	58	56
88	45	53	52	43	44
92	42	45	46	41	37
96	33	40	39	32	25
100	27	30	28	22	19
Terminal	23	24	23	16	14

ppm: parts per million
[a] No interim killing after 52 weeks had taken place.
Source: Tisdel (1975b)

No clinical signs could be attributed to treatment in male rats. In females, there was a higher number of rats at the high dose exhibiting spasms at least once during the study. The significance of the higher number of affected animals (15 in the group receiving 1025 ppm compared with 3 or 5 in the two female control groups) is equivocal. Although this finding was not accompanied by any pathological changes, peripheral nerves were not examined histologically. Ophthalmoscopy did not reveal differences between the control and treatment groups.

No significant dose-related or time-consistent effects on body weight or body weight gain were noted in male rats. In high-dose females, in contrast, mean body weight tended to become lower with longer duration of treatment. At termination, the difference (about 297 g compared with 331 or 329 g in the two control groups) was statistically significant. This finding was not due to lower feed consumption or feed efficiency, as both parameters were not affected in either sex or at any dose (although feed efficiency is depicted in tables for the first 13 weeks of the study only). It is also worth noting that body weight gain was not affected consistently in female rats throughout the study. There is no explanation for the adverse effect on body weight in females at the high dose.

Red blood cell parameters, leukocyte number and differential white blood cell count did not exhibit consistent differences between control and high-dose groups, in either the main study or the satellite groups kept for only 52 weeks.

Clinical chemistry parameters sometimes varied between groups, but most of the differences were not consistent over the course of the study. Variations within the same group at different time points probably reflected age-related changes. In the summary tables, no standard deviation is given for any parameter, and significant changes were not indicated, but had to be retrieved from the text. Only two findings might be attributed to treatment and were of potential concern. Blood urea nitrogen

was doubled in high-dose males at termination, compared with the controls. At earlier time points, no difference was noted. This effect could be indicative of the beginning of kidney damage, which was more pronounced in male rats. Also, at termination, 2,3-diphosphoglyceric acid was significantly decreased in high-dose males. The latter is an intermediate in glycolysis that is found mainly in erythrocytes (Juel & Milam, 1979; Rapoport, 1983), but it is an outdated parameter in toxicological studies. It was speculated that a decrease might suggest hypoxia or acidosis and could somehow be related to higher blood urea levels. Charache et al. (1970) reported that increased 2,3-diphosphoglyceric acid concentrations in red blood cells were related to a lower oxygen affinity in humans and that red blood cell–bound 2,3-diphosphoglyceric acid was increased in uraemic patients.

Urine analysis did not reveal meaningful differences between control and high-dose groups.

At interim necropsy after 1 year, higher incidences of nephritis, hydronephrosis and pyelonephritis were seen in treated animals, confirming the kidney as a target organ of norflurazon in rats. In the high-dose satellite male group, 6/10 animals exhibited gross kidney lesions of different stages. In high-dose females, three animals were similarly affected. At the low or intermediate dose, but not in the control groups, diffuse mottled appearance of the kidney surface was occasionally noted in both sexes. Thickened uterine walls and/or endometrial hyperplasia were also noted in the majority of females in all treated groups, whereas no such finding was reported for the controls. In addition, cystic degeneration of the ovaries was observed in three high-dose females. Mean absolute and relative liver weights were increased in high-dose males, and at least absolute liver weight was also higher in the mid-dose group. Kidney weights also tended to be higher in males at the high dose. In females, absolute and relative liver and kidney weights were increased in the high-dose group.

Histopathological examination of the satellite group animals revealed a number of renal findings, such as hyaline casts, tubular and glomerular degeneration and inflammatory changes, but no clear dose–response relationship was apparent, and not all gross lesions were confirmed microscopically. For instance, hydronephrosis was a necropsy finding in five high-dose males, but became a histological diagnosis in only three of them. Nephritis was more common in low-dose males and mid-dose females than in rats at the high dose. Regarding the uterus, histopathology confirmed endometrial hyperplasia in seven females in each of the treated groups, compared with a zero incidence in the control group. There were no other histological findings that could be attributed to treatment. A rather unusual (but clearly not treatment related) finding was the identification of a pancreatic tumour in one male and one female control rat.

At scheduled termination after 2 years, as well as in the decedents that died during the second year of treatment, macroscopic examination revealed some gross alterations, but mostly there were no differences between control and treated groups. At least in high-dose males, the number of animals with rough, pitted kidney surface was increased. In addition, dilated pelvis and hydronephrosis were more frequently observed in high-dose males. In females, incidences of all findings were similar in control and treated groups (Table 20).

Higher absolute and relative liver weights were noted at scheduled termination in high-dose males; a similar increase in low-dose males was not confirmed at the middle dose and, in the absence of a dose–response relationship, most likely occurred by chance. In females, there was a significant increase in relative liver weight at the high dose. This could be due to the lower mean body weight, but might also be indicative of an adaptive response. Kidney weights were markedly increased in male rats receiving the high dose as well as in mid- and high-dose females. In addition, ovary weight was significantly increased in high-dose females. In males, higher mean absolute and relative thyroid weights were noted in the high-dose group (Table 21). Even though the difference relative to the controls was not statistically significant, this effect would be in line with the outcome of the 90-day study in rats by Fogleman (1971a), in which the thyroid was identified as an additional target organ (see section 2.2 above).

Table 20. Selected gross pathological findings in male and female rats at termination after 2 years and in those animals that died on test

Parameter	0 ppm	0 ppm	125 ppm	375 ppm	1 025 ppm
Males					
Number examined	75	85	75	75	75
Kidneys: dilated pelvis	0	0	1	1	6
Kidneys: hydronephrosis	2	1	5	6	7
Kidneys: congestion, haemorrhages, dark	5	10	15	12	12
Kidneys: rough, pitted surface	29	30	25	32	42
Females					
Number examined	75	85	75	76 (?)	75
Adrenals: enlarged	17	14	9	15	22
Uterus: fluid filled	0	6	2	13	8
Uterus: enlarged, distended	15	14	8	13	15
Kidneys: dilated pelvis	0	3	0	2	5

ppm: parts per million
Source: Tisdel (1975b)

Table 21. Organ weight changes in male and female rats at termination after 2 years

Parameter	0 ppm	0 ppm	125 ppm	375 ppm	1 025 ppm
Males					
Number examined	23	24	23	16	14
Mean body weight (g)	484	485	490	493	474
Mean absolute liver weight (g)	21.7	22.5	24.5*	21.9	25.2*
Mean relative liver weight (% of control)	4.48	4.67	5.02*	4.77	5.32*
Mean absolute kidney weight (g), left / right	2.65 / 2.63	2.77 / 2.78	2.81 / 2.80	2.88 / 2.86	3.76** / 3.69**
Mean relative kidney weight (% of control), left / right	0.556 / 0.554	0.584 / 0.586	0.577 / 0.575	0.639 / 0.636	0.798** / 0.784**
Mean absolute thyroid weight (mg)	50.9	51.4	50.3	50.1	65.4
Mean relative thyroid weight (% of control)	0.011	0.010	0.010	0.011	0.014
Females					
Number examined	43	39	42	52	52
Mean body weight (g)	330	322	328	321	302
Mean absolute liver weight (g)	13.9	12.9	14.1	14.4	15.0
Mean relative liver weight (% of control)	4.24	4.05	4.38	4.54	5.02**
Mean absolute kidney weight (g), left / right	1.55 / 1.48	1.45 / 1.43	1.61 / 1.57*	1.67* / 1.66**	1.82** / 1.87**

Parameter	0 ppm	0 ppm	125 ppm	375 ppm	1 025 ppm
Mean relative kidney weight (% of control), left / right	0.484 / 0.461	0.460 / 0.455	0.517 / 0.500	0.534* / 0.533**	0.614** / 0.629**
Mean absolute ovary weight (mg), left / right	61.0 / 61.1	72.3 / 64.5	71.3 / 57.4	69.7 / 74.5	93.5** / 96.4**
Mean relative ovary weight (% of control), left / right	0.019 / 0.019	0.023 / 0.020	0.022 / 0.018	0.022 / 0.024	0.032** / 0.032**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test)
Source: Tisdel (1975b)

Histopathology of control and high-dose animals at scheduled termination revealed increases in renal hyaline casts and nephritis in females, along with higher incidences of endometritis and squamous metaplasia of the uterus. The latter might be a tumour precursor. However, tumour incidences were not increased in females. In contrast to the histological findings in the satellite animals after 52 weeks of treatment, endometrial hyperplasia was visible in the control group females at termination, with an incidence comparable to that in the high-dose group. In male rats, an increase in nephritis was observed at the high dose, with 100% of the animals affected, compared with less than 50% of the controls. In addition, nodular cortical hypertrophy of the adrenals was observed only in treated males, although the total incidence was low. A single benign tumour type (i.e. pituitary adenoma) was seen more frequently in high-dose males. However, four control animals (two from each of the male control groups) that had died on test also had such a tumour, which is common in ageing rats. Thus, it is not likely that this increase can be attributed to treatment (Table 22).

Table 22. Selected histopathological findings in the 2-year feeding study in rats at termination

	Incidence of finding				
Pathological finding	**0 ppm**	**0 ppm**	**125 ppm[a]**	**375 ppm[a]**	**1 025 ppm**
Males					
Number examined	*23*	*24*	*23*	*16*	*14*
Kidneys: nephritis	10	7	10	8	14
Pituitary: (chromophobe) adenoma	4	3	–	–	9
Adrenals: nodular cortical hypertrophy	0	0	–	–	4
Females					
Number examined	*43*	*39*	*42*	*52*	*52*
Kidneys: nephritis	1	1	1	3	4
Kidneys: hyaline casts	22	24	28	43	41
Uterus: endometrial hyperplasia	27	21	–	–	31
Uterus: endometritis	4	2	–	–	9
Uterus: squamous metaplasia	3	0	–	–	9

ppm: parts per million
[a] Histological examination only of kidneys and gross lesions.
Source: Tisdel (1975b)

The NOAEL in this old study of questionable reliability was 125 ppm (equivalent to 6.25 mg/kg bw per day), based on effects on the kidney at 375 ppm (equivalent to 18.9 mg/kg bw per day). No convincing evidence of carcinogenicity was observed (Tisdel, 1973, 1975b).

2.4 Genotoxicity

Norflurazon was evaluated in a few genotoxicity studies, but only in vitro. These studies are summarized in Table 23.

Table 23. Summary of in vitro genotoxicity studies with norflurazon

Type of study	Test system	Concentration range tested	S9	Batch/purity	Result	Reference
Reverse gene mutation test in bacteria (Ames test) and yeast	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and 1538 and *Saccharomyces cerevisiae* D4	0.1–500 µg/plate (or 1 000 µg/plate with TA1537)	±	No information provided	Negative	Brusick (1977)
Reverse gene mutation test in bacteria (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535 and 1537 and *Escherichia coli* (hcr strain)	5–5 000 µg/plate	±	Batch not given; purity 99.8%	Negative	Anonymous (1980)
Rec assay	*Bacillus subtilis* strains H17 and M45	500–10 000 µg/disc	−	Batch not given; purity 99.8%	Negative	Anonymous (1980)
Chromosomal aberration	Chinese hamster ovary cells (12-hour exposure; in addition, 16-hour exposure without activation at highest dose)	32–500 µg/mL (without activation); 63–1 000 µg/mL (with activation)	±	Batch no. 1174/85; purity 97.9%	Negative	Putman (1985)
Unscheduled DNA synthesis and repair	Primary rat hepatocytes	1–1 000 µg/mL	Not needed	Batch no. 1174/85; purity 97.9%	Negative	Curren (1985)

S9: 9000 × *g* supernatant fraction from rat liver homogenate

No evidence of genotoxicity was obtained in the bacterial reverse gene mutation (Ames) assay in five *Salmonella typhimurium* strains and in a yeast system (i.e. *Saccharomyces cerevisiae* strain D4). All the tests, with and without metabolic activation, were negative. In contrast, appropriate positive controls caused a distinct increase in mutation frequency. However, the assay was poorly reported. In particular, it was not actually justified why 500 or (in the case of TA1537) 1000 µg/plate was chosen as the highest tested concentration. Moreover, *Saccharomyces cerevisiae* strain D4 is now considered an outdated test system for this study type. A main deficiency of the study is the absence of any information on batch and purity of the test compound (Brusick, 1977).

In a study from Japan (not signed or dated, but presumably reported in 1980), norflurazon (under designation NP-52; purity 99.8%) was tested in the same five *Salmonella typhimurium* strains as above and also in the *Escherichia coli* hcr strain, with and without metabolic activation. This time, higher concentrations were applied, and the results were again negative. Appropriate positive controls were included and caused a distinct increase in mutation frequency (Anonymous, 1980).

When both Ames tests are considered together, it may be concluded that norflurazon did not induce gene mutations in bacteria.

In the same very brief report, a rec assay in *Bacillus subtilis* was reported that was also negative. It is not clear how many replicates were used, and the description of the test method is very poor. Thus, the scientific validity of this experiment is limited (Anonymous, 1980).

In a valid in vitro chromosomal aberration assay in Chinese hamster ovary cells with norflurazon (purity 97.9%), no evidence of clastogenicity was obtained, either with or without activation. For one of the concentrations (500 µg/mL in the absence of metabolic activation), a cell cycle delay was reported. Positive control substances gave the expected increase in structural aberrations (Putman, 1985).

In an unscheduled DNA synthesis and repair assay, norflurazon (purity 97.9%), in contrast to the positive control compound, did not cause a significant increase in mean net nuclear counts and thus proved negative for direct interaction with DNA. However, it must be taken into account that the relevance of this method is now considered equivocal (Curren, 1985).

In an article from the open literature, induction of primary DNA damage in cells of the planarian *Polycelis felina*, a freshwater aquatic bioindicator organism, was reported. Norflurazon was used as the test substance because of its potential to contaminate groundwater owing to its long half-life. In an in vitro comet assay, tail length, percentage of DNA in the tails and tail moment were significantly increased at norflurazon concentrations of 0.2 and 2 µmol/L from day 1 of treatment onwards. At least with the high concentration, a peak was observed on day 4, whereas some recovery was apparent on day 7. No clear differences with regard to time course were seen when the lower concentration was applied. It must be taken into account that norflurazon also proved directly toxic to the planarians, as demonstrated by damage to the outer mucous layer, epidermis and cell parenchyma. No conclusion regarding effects on intact mammalian organisms may be drawn from this publication (Horvat et al., 2005).

To conclude, norflurazon proved negative for genotoxicity in standard test systems in vitro, but the database is poor. A genotoxicity test in mammalian cells is normally required, but is not available for norflurazon. Furthermore, no in vivo study on genotoxicity was submitted.

2.5 Reproductive and developmental toxicity

(a) Single-generation and multigeneration studies

Mice

A one-generation study was performed in CD-1 (HaM/ICR Swiss) mice. In the F_0 generation, groups of 50 male and 50 female mice received norflurazon at a dietary concentration of 85, 170 or 340 ppm (equivalent to 13, 26 and 200 mg/kg bw per day, respectively). Two control groups of the same size were kept in parallel and fed untreated basal diet. The parental animals were mated after 18 weeks on test. Only one litter was produced. Selected F_{1A} pups (125 males and 125 females from each group) were employed in a subsequent long-term toxicity study (Tisdel, 1975a) that is reported above in section 2.3. However, for this purpose, the 170 ppm dietary concentration was increased to 340 ppm, and the 340 ppm dietary concentration was increased to 1360 ppm.

The reproductive portion of the study did not reveal any indications of adverse effects of the test substance on fertility, reproductive parameters or pup development (Tisdel, 1975d).

Rats

In an old reproductive toxicity study, groups of 40 male and 40 female Sprague Dawley rats in the F_0 generation received the test compound (product 9789; lot no. 004; purity 98.8%) via their diet at a concentration of 125, 250 or 500 ppm (equivalent to 6.25, 12.5 and 25 mg/kg bw per day, respectively). Two control groups of the same size were fed on untreated basal diet. Following an 18-week premating period, females were mated one to one with males from the same group to produce the F_{1A} litter. From these litters, weanling animals were moved to a subsequent combined chronic

toxicity and carcinogenicity study (Tisdel, 1973, 1975b), which is reported in section 2.3 above. The reproduction study was continued 1 week after weaning of the F_{1A} pups, with a second mating to produce the F_{1B} litter. Following weaning of the second litter and a total treatment time of 9 months, the F_0 generation parental animals were killed. This part of the study was considered a separate subchronic study, which is also reported above (see section 2.2) (Harris, 1972). From the F_{1B} litters, male and female pups were randomly selected to become parental animals for the F_2 generation. Group size was the same, but the dietary concentrations were increased from 250 ppm to 375 ppm and from 500 ppm to 1025 ppm. The same concentrations were applied in the chronic toxicity and carcinogenicity study. The F_{2A} litter was employed to produce the F_{3A} and F_{3B} litters, the latter of which was used for teratological examinations of the fetuses after all presumably pregnant F_{2A} dams had been killed on day 20 of gestation.

Toxicity in adult parental rats was not separately reported. Either they were not fully examined for potential adverse effects or these were reported, if occurring, in the short- or long-term toxicity studies. Over all generations and litters, fertility, gestation, pup viability and lactation indices, litter size and pup body weight were not affected by treatment. Time to pregnancy, gestation length and sex ratio in pups also did not differ among the groups. The only remarkable finding was a very low number of pregnancies after the F_{2A} dams had been mated for the second time to produce the F_{3B} litters. In the two control groups, only 16 or 14 dams out of 40 actually produced a litter, and this rate was even lower in the high-dose group (7/40). The study author speculated that the dams might have been disturbed by noise due to construction work in the animal facility building, which would call the study quality into question. With regard to the teratological part of the study, resorption rate and fetal weight were similar across the groups, and there was no evidence of morphological abnormalities (Tisdel, 1975c).

In a two-generation reproduction study, norflurazon (batch no. P. 148/86/31; purity 98.1%) was administered in the diet to Wistar Crl:(WI) rats (25 of each sex per dose in both the F_0 and F_1 generations). Dietary concentrations were 0, 150, 750 and 1500 ppm (equal to 0, 10.2, 51.0 and 102.5 mg/kg bw per day, respectively, based on calculated substance intakes in the premating phases in both generations and sexes, with lowest intakes consistently seen in F_0 generation males). The F_0 generation rats were at least 8 weeks old at first dosing and were administered the test substance for 70 days prior to first mating (ratio of one male to one female) to produce the F_{1A} litters. Following weaning of these litters and a minimum premating interval of 10 additional days, the F_0 generation adults were bred again within their respective treatment groups to produce F_{1B} litters. Following weaning on day 21 postpartum, offspring from the F_{1B} litters were selected (one rat of each sex per litter, when possible, avoiding pairing of siblings) to become F_1 adults and, after a premating period of at least 84 days, parents of the F_{2A} and later of the F_{2B} generations. Thus, there were two litters per generation available for toxicological assessment.

All F_0 and F_1 generation parent animals were killed when no longer needed for the assessment of reproductive effects. Pregnant females that failed to deliver until day 24 of presumed gestation were also killed and necropsied. All litters in both generations were culled to four pups of each sex per litter on postnatal day 4. All the pups kept after culling but not needed for further breeding were terminated at weaning. End-points under evaluation included premature deaths, clinical signs, body weight and body weight gain, feed consumption, reproductive parameters (mating, fertility and gestation indices), litter parameters (livebirth, viability and lactation indices) and pup parameters. At scheduled termination, parental adults were necropsied, and liver and kidney weights were determined. Histopathology of the liver and kidneys was examined in all parental animals. Histopathological examinations of other organs were confined to parental animals from the control and high-dose groups in both generations and comprised the following organs: pituitary, ovaries, uterus and cervix, vagina, prostate, seminal vesicles with coagulating gland, and testes with epididymides. The pups were sexed and weighed on days 0, 4 (before and after culling), 7, 14 and 21. At their respective termination, all pups were grossly examined. Liver and kidney weights were determined in randomly selected pups at weaning. Although one would have expected the same

number of male and female pups from the same litter, this does not appear to have been the case (see Tables 24, 26 and 27 below). There was no explanation for this in the study report.

Among parental animals from the F_0 and F_1 generations, the total incidences of premature death were as follows: 1, 0, 2 and 2 in control, low-dose, mid-dose and high-dose males; and 0, 1, 0 and 1 in control, low-dose, mid-dose and high-dose females. As the few parental animals that either were found prematurely dead or had to be killed for humane reasons were nearly equally distributed among the groups and generations, the deaths could not be attributed to treatment. The same was true for the rarely occurring clinical signs, which did not exhibit any dose-related pattern.

There were occasional effects of treatment on body weight and body weight gain. These findings are considered treatment related but were confined to the high dose and were not consistently observed in both sexes or generations. In the F_0 generation, females receiving the high dose exhibited a statistically lower body weight gain ($P < 0.05$; Dunnett's test) in the second half of the initial premating interval (i.e. between weeks 5 and 10). During this period, body weight gain was about 34% lower than in the control group. A much less pronounced decrease of about 14% was also noted for the same period at the intermediate dose, but did not gain statistical significance. Despite these findings, mean body weight was not affected in females, and no effects at all were observed in the F_1 generation. In high-dose males, in contrast, there were no changes in the F_0 generation. However, a decrement in body weight gain by 11% in total was noted in the F_1 generation, achieving statistical significance for weeks 20 through 31, during which body weight gain was decreased by more than one third. The resulting mean body weight in these animals was the lowest among all groups from week 1 onwards, and the difference became statistically significant from week 18 throughout terminal week 31 ($P < 0.05$ or, in weeks 29–31, $P < 0.01$). At termination, the mean body weight was about 9% lower than in the control group.

During the gestation and lactation periods, there were only occasional findings regarding maternal body weights. During the first gestation of F_0 generation dams, mean body weight was significantly lower in the high-dose group on days 0, 7 and 14. Following the second mating, such a difference became apparent only on day 14, following a lower body weight gain in all treated groups from days 7 to 14. During lactation, slightly (but sometimes statistically significantly) lower mean body weight and body weight gain were noted in high-dose F_0 generation females. No such effects were ever seen in the F_1 generation, confirming that body weight parameters in females were affected only in the first generation, and not in the second.

Feed consumption was not affected in any segment of the study, suggesting that the rather weak effects on body weight cannot be attributed to a lower feed intake.

Gross (external and necropsy) pathological examination of the parental animals and the pups did not reveal findings that could be attributed to treatment. However, increases in both mean absolute and relative liver and kidney weights were observed in adult animals in both generations – at the intermediate and high doses in males and at the high dose in females (Table 24) – and also in the pups. Taking into consideration the results of histopathological examination and the lack of clinical chemistry analysis in this study, parallel significant increases in absolute and relative organ weights are considered treatment related, as these findings cannot be assigned to lower body weight. In the low-dose male groups, an effect on liver weight was also noted, but it was weaker and not accompanied by histopathological findings. Statistical significance was gained for either absolute or relative liver weight. On balance, this finding was not considered adverse at this dose.

Liver histopathology in the adult animals revealed hepatocellular hypertrophy, in line with the higher liver weights, at the middle and high doses in both males and females of the F_0 generation. The increase was dose related. Single-cell necrosis was also observed in the same groups, but with a clear dose–response relationship only in females. A nearly identical pattern was seen in the F_1 adults, with the exception that single-cell necrosis was a rare event in this generation, affecting only one male and one female in each of the mid- and high-dose groups. Other liver findings were equally distributed among the groups in both generations. However, it is worth noting that an adenoma was seen in one high-dose F_0 female and in one high-dose F_1 dam, but also in one low-dose F_1 male.

Table 24. Organ weight data in parental rats in the two-generation reproduction study

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Mean liver weight				
F_0 males				
Number examined	25	25	25	24
Absolute weight (g)	21.59	23.52*	25.82*	27.15**
Relative weight (%)	3.66	3.84	4.37**	4.63**
F_1 males				
Number examined	24	25	23	24
Absolute weight (g)	22.77	24.00	24.97*	26.47**
Relative weight (%)	3.61	3.82*	4.00**	4.62**
F_0 females				
Number examined	23	20	18	21
Absolute weight (g)	14.95	14.38	15.24	17.64**
Relative weight (%)	4.52	4.36	4.80	5.86**
F_1 females				
Number examined	20	19	19	21
Absolute weight (g)	15.59	15.67	16.90	18.76**
Relative weight (%)	4.62	4.39	4.77	5.41**
Mean kidney weight				
F_0 males				
Number examined	25	25	25	24
Absolute weight (g)	3.82	3.94	4.54**	5.32**
Relative weight (%)	0.65	0.65	0.77**	0.92**
F_1 males				
Number examined	24	25	23	24
Absolute weight (g)	3.76	3.96	4.39**	6.84*
Relative weight (%)	0.60	0.63	0.70**	1.20**
F_0 females				
Number examined	23	20	18	21
Absolute weight (g)	2.49	2.46	2.48	2.56
Relative weight (%)	0.75	0.75	0.78	0.86**
F_1 females				
Number examined	20	19	19	21
Absolute weight (g)	2.58	2.71	2.96**	3.20**
Relative weight (%)	0.77	0.76	0.83**	0.93**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Eschbach et al. (1994)

Microscopic examination of the kidneys did not reveal nephrotoxicity when the range of findings and the number of affected animals were considered. However, the severity of renal changes tended to be greater at the high dose. In particular, in high-dose F_1 males, there was an extreme variability in absolute kidney weights, as demonstrated by a standard deviation of 4.83 compared with 0.35 in the control group. This was mainly due to three animals with kidney weights of 10.15 (88M), 15.80 (57M) and 25.80 g (55M). A similarly high kidney weight of 13.70 g was noted only in one high-dose F_0 male (97M), whereas 5 g was never exceeded in the controls and only seldom in the other treated groups. Histopathology of these animals revealed that 97M had chronic progressive nephrosis of grade 4. In line with that, both kidneys were markedly enlarged and had a granulated surface. 55M and 57M macroscopically exhibited strongly enlarged kidneys with irregular surface. Microscopic examination revealed chronic progressive nephrosis of grade 4, combined with pelvic dilatation of grade 2, in both animals. In 88M, the enlarged kidneys were accompanied by cortical tubular dilatation and tubular hyperplasia, but also, in other kidney regions, by basophilia and atrophy. In addition, lymphoid cell infiltration and tubular casts were reported, suggesting an inflammatory process. However, all these animals survived until scheduled termination, even though they also suffered from liver changes.

Examination of the reproductive or hormone-producing organs and of gross lesions in the adult rats did not reveal findings that could be attributed to treatment.

There were no effects on fertility or reproductive performance in any generation or mating up to the high dose. Mating behaviour (including time to conception), gestation index and length, parturition, litter size, pup birth weight and sex ratio in pups were not altered. In the second mating of the F_0 generation, there was a statistically significantly higher frequency of stillborn pups at the low and high doses. A nearly identical pattern was observed after the first mating of the F_1 generation (Table 25). Interpretation is difficult, as there was obviously no dose–response relationship. If the increase at the low dose is considered to have occurred by chance, there is little reason for a different view on the high-dose incidences. At the high dose, this finding was not confirmed in the F_{1A} and F_{2B} litters. Furthermore, there was no increase in the number of litters with stillborn pups with dose, and complete litter loss (i.e. litters with all stillborn) was rare and confined to the second mating of the F_0 generation. Overall, an impact of norflurazon on the frequency of stillborn pups is not likely.

Table 25. Number and percentage of stillborn pups, lactation index and terminal pup weight (males and females combined) in the two-generation reproduction study in rats

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
F_{1A} litters				
Number of litters	23	20	20	22
Number of litters with stillborn pups	7	8	8	2
Number of pups in total	319	275	291	305
Number of stillborn pups	14	13	14	14
Percentage stillborn	4.4	4.7	4.8	4.6
Lactation index (%)	95	86	94	87
Mean pup weight on day 21 (g)	51.45	50.84	49.14	47.91
F_{1B} litters				
Number of litters	23	20	18	22
Number of litters with stillborn pups (with all stillborn)	5 (0)	7 (1)	7 (0)	8 (1)
Number of pups in total	334	287	267	331

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Number of stillborn pups	12	28**	11	32**
Percentage stillborn	3.6	9.8	4.1	9.7
Lactation index (%)	90	90	93	95
Mean pup weight on day 21 (g)	49.36	50.31	50.34	47.31
F_{2A} litters				
Number of litters	19	19	23	21
Number of litters with stillborn pups	4	7	4	2
Number of pups in total	257	250	305	277
Number of stillborn pups	5	18**	6	17**
Percentage stillborn	1.9	7.2	2.0	6.1
Lactation index (%)	92	92	76	88
Mean pup weight on day 21 (g)	51.30	50.94	46.03	48.74
F_{2B} litters				
Number of litters	20	19	20	21
Number of litters with stillborn pups	3	9	6	5
Number of pups in total	286	257	285	256
Number of stillborn pups	5	26**	10	7
Percentage stillborn	1.7	10.1	3.5	2.7
Lactation index (%)	87	70	64	59
Mean pup weight on day 21 (g)	50.33	49.05	43.81	42.15*

ppm: parts per million; *: $P < 0.05$ (Dunnett's test); **: $P < 0.01$ (chi-squared and Fisher's exact test)
Source: Eschbach et al. (1994)

Following the second mating of the F_1 generation, the lactation index tended to be lower in treated groups, but the difference relative to the control groups did not gain statistical significance. No effects on pup viability or body weight development were observed in any other generation or litter, but an effect of longer-lasting treatment cannot be excluded, even though it seems rather equivocal. It is worth noting that, in the F_{2B} litters, mean pup weight was statistically significantly lower at the high dose on lactation day 14 and at termination on day 21. In the F_{1A} and F_{1B} litters, mean pup weight at the high dose was the lowest, and it was consistently lower than in the control group. A similar trend was obvious at the intermediate dose, even though no statistical significance was achieved (Table 25). To conclude, a weak effect at least of high dose administration on pup body weight appears likely.

Organ weight changes were observed in male (Table 26) and female pups (Table 27) at the intermediate and high doses. In both sexes, the increases in absolute and relative liver weights were most likely treatment related, whereas a slightly higher relative kidney weight likely reflected a lower pup weight. In F_{2B} male pups, absolute kidney weight was reduced. This was the only organ weight decrease observed in this study. Because of its isolated occurrence and because it seems to be in contrast to the general pattern, this finding is considered incidental and not treatment related.

Table 26. Organ weight data in male rat pups in the two-generation reproduction study

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Mean liver weight				
F_{1A} litters				
Number examined	22	19	19	19
Absolute weight (g)	2.21	2.05	2.22	2.37
Relative weight (%)	4.06	3.92	4.36	4.71**
F_{1B} litters				
Number examined	22	17	15	18
Absolute weight (g)	2.16	2.16	2.30	2.34
Relative weight (%)	4.20	4.15	4.58	4.97**
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	2.26	2.87	2.26	2.52
Relative weight (%)	4.28	4.37	4.71*	4.95**
F_{2B} litters				
Number examined	19	15	14	15
Absolute weight (g)	2.36	2.32	2.31	2.21
Relative weight (%)	4.31	4.48	4.75**	4.87**
Mean kidney weight				
F_{1A} litters				
Number examined	22	19	19	19
Absolute weight (g)	0.59	0.57	0.58	0.58
Relative weight (%)	1.10	1.09	1.15	1.17*
F_{1B} litters				
Number examined	22	17	15	18
Absolute weight (g)	0.58	0.58	0.56	0.55
Relative weight (%)	1.14	1.11	1.13	1.17
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	0.61	0.60	0.57	0.62
Relative weight (%)	1.15	1.15	1.21	1.24**
F_{2B} litters				
Number examined	19	15	14	15
Absolute weight (g)	0.65	0.61	0.59	0.55*
Relative weight (%)	1.20	1.17	1.22	1.22

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Eschbach et al. (1994)

Table 27. Organ weight data in female rat pups in the two-generation reproduction study

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Mean liver weight				
F_{1A} litters				
Number examined	22	19	18	22
Absolute weight (g)	1.98	2.01	2.26	2.34*
Relative weight (%)	3.93	3.99	4.58**	4.91**
F_{1B} litters				
Number examined	21	18	17	20
Absolute weight (g)	2.02	2.05	2.26	2.37
Relative weight (%)	4.03	4.14	4.56**	5.01**
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	2.27	2.36	2.31	2.52
Relative weight (%)	4.40	4.56	5.02**	5.17**
F_{2B} litters				
Number examined	20	15	16	17
Absolute weight (g)	2.39	2.30	2.26	2.30
Relative weight (%)	4.51	4.62	4.93**	5.14**
Mean kidney weight				
F_{1A} litters				
Number examined	22	19	18	22
Absolute weight (g)	0.56	0.59	0.58	0.58
Relative weight (%)	1.12	1.17	1.18*	1.21**
F_{1B} litters				
Number examined	21	18	17	20
Absolute weight (g)	0.57	0.57	0.59	0.59
Relative weight (%)	1.14	1.17	1.19	1.26**
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	0.64	0.63	0.58	0.64
Relative weight (%)	1.24	1.22	1.27	1.33*
F_{2B} litters				
Number examined	20	15	16	17
Absolute weight (g)	0.66	0.62	0.59	0.60
Relative weight (%)	1.25	1.24	1.29	1.35**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Eschbach et al. (1994)

The NOAEL for parental toxicity was 150 ppm (equal to 10.2 mg/kg bw per day), based on decreases in body weight and body weight gain as well as higher liver and kidney weights and histopathological findings in liver and kidney at 750 ppm (equal to 51.0 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 102.5 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 150 ppm (equal to 10.2 mg/kg bw per day), based on impaired body weight development and higher relative liver and kidney weights in pups and on an equivocal impact on viability in the F_{2B} litter at 750 ppm (equal to 51.0 mg/kg bw per day) (Eschbach et al., 1994).

From the parameters investigated, there was no evidence of endocrine disrupting properties. However, in contrast to current data requirements and standards, landmarks of sexual maturation of the pups (i.e. preputial separation or vaginal opening), sperm parameters, estrous cycle and ovarian follicles were not investigated in this rather old study. Thus, no firm conclusion on endocrine disrupting properties may be drawn. However, norflurazon was subject to testing in the Endocrine Disruptor Screening Program of the USEPA. In the Tier I assay battery of this programme, along with other parameters, sexual maturation of male and female pups was investigated in pubertal assays. As they may complement the information obtained from this reproduction study, these studies are reported in section 2.6.

(b) Developmental toxicity

Rats

In a developmental toxicity study, norflurazon (batch no. 0004; purity not provided) was administered by oral gavage to presumably pregnant female Sprague Dawley rats (20 per dose) on gestation days (GDs) 6 through 15. The test substance was applied in 1% carboxymethylcellulose with 0.2% Tween 80 at a dose of 0, 100, 200 or 400 mg/kg bw per day. The doses were selected on the basis of a preliminary range-finding experiment, which had apparently (most likely erroneously) been performed with an 80% wettable powder formulation instead of the technical active ingredient. Dams were euthanized on GD 20. Parameters evaluated in dams were body weight, feed consumption and efficiency, survival, clinical signs, reproductive outcomes and gross pathology. Parameters evaluated in fetuses were fetal weight, sex and incidences of external, visceral and skeletal malformations and variations.

One mid-dose dam died on day 10 of gestation. Necropsy revealed no gross lesions. This isolated premature death was not considered treatment related because of dose considerations. There were no clinical signs (with the possible exception of alopecia in one high-dose rat from GD 10 onwards) or abortions among the pregnant dams. Slight maternal toxicity was observed in all treated groups, as mean body weight gain was reduced during the first 3 days of treatment. In high-dose dams, a small body weight loss (a mean of 4 g) was noted. In all these groups, body weight reduction was accompanied by a dose-related decrease in feed efficiency, whereas feed consumption was lower only at the high dose. Because all these parameters normalized during later stages of the study, and taking into account the degree of observed initial changes, these probably treatment-related effects were considered adverse only at the high dose. There were no remarkable gross findings at necropsy.

Resorption rate was slightly higher in the high-dose group (1.1 per litter compared with 0.8 in the control group), but this was mainly due to a single litter with seven resorptions.

No evidence of developmental toxicity was observed in the fetuses. The only malformations were seen in one mid-dose fetus and in one control group fetus. The first one had thoracic spina bifida with haemorrhagic spinal cord, exencephaly and absent tail. The affected control fetus also had spina bifida, but in the lower thoracic and lumbar regions, as well as internal hydrocephalus. Because of the group allocation of these malformations, they cannot be attributed to treatment.

There was no increase in external, visceral or skeletal variations.

The NOAEL for maternal toxicity was 200 mg/kg bw per day, based on initial effects on body weight, feed consumption and feed efficiency at 400 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, the highest dose tested (Hartman & Hrab, 1972).

Rabbits

In a preliminary range-finding study, small groups of five mated New Zealand white rabbit does were administered norflurazon (lot no. 80561; purity 98.4%) via oral gavage at a dose of 0, 10, 40, 80, 100 or 400 mg/kg bw per day from GDs 7 through 19. The test substance was dissolved in 1% carboxymethylcellulose with 0.2% Tween 80. The dosing volume was 1 mL/kg bw. On the last day of treatment, does were killed and necropsied, and live and dead fetuses, resorptions and corpora lutea were counted.

Maternal toxicity was apparent from 40 mg/kg bw per day onwards. Body weight loss was seen at this dose, and it became more pronounced at higher doses. At 80 and 100 mg/kg bw per day, locomotor activity was decreased, the amount of faeces was reduced and resorptions were increased. At the highest dose (400 mg/kg bw per day), does aborted and eventually died or had to be killed on GD 15 or 16.

Based on these results, doses of 10, 30 and 60 mg/kg bw per day were proposed for the main study (Hrab, 1983a).

In the main developmental toxicity study, norflurazon (lot no. 80561; purity 98.4%) was administered to presumably pregnant New Zealand white rabbits (15 per group) on GDs 7–19. The test substance was dissolved in 1% carboxymethylcellulose with 0.2% Tween 80 and administered by oral gavage at a dose of 0, 10, 30 or 60 mg/kg bw per day. The dosing volume was 1 mL/kg bw. Control group animals received the vehicle only. The animals were killed and necropsied on day 30. Parameters evaluated in does were survival, clinical signs, occurrence of abortions, body weight, body weight gain, feed consumption, gravid uterine weights and gross pathology. Live and dead fetuses, early and late resorptions, and corpora lutea were counted. Parameters evaluated in fetuses were fetal weight, fetal sex ratio and incidences of external, visceral and skeletal malformations and variations.

Two high-dose females aborted on GD 20 and GD 24, respectively. Even though both incidents were noted after treatment had been finished, a delayed effect of the test substance cannot be excluded. It was not known whether the abortions were a sign of maternal toxicity or occurred because of severe fetal anomalies. There were no further abortions in any group, premature deaths or clinical signs that could be attributed to treatment. A remarkable finding was delivery of 12 full-term pups by a low-dose animal on day 12. The only explanation was a pre-existing pregnancy of this doe, calling the study quality into question. The doe and her pups were excluded from analysis.

A significant body weight loss was noted in the high-dose group, which was most pronounced during the first week of treatment. This effect was partly reversed by a compensatory increase in body weight gain in the period following termination of treatment. Low- and mid-dose females gained less weight over the course of the treatment period, but the difference relative to the control group was not statistically significant (Table 28). At the intermediate dose, occasional body weight losses (e.g. by 4 g on average from GD 7 to GD 8 or by 14 g from GD 15 to GD 16) were noted, but were much less pronounced than in the group receiving the high dose. On the same days of treatment, the mean losses in the high-dose group were 44 g and 29 g, respectively. The relevance of the lower body weight gain or of sporadic weight losses in the groups receiving 10 and 30 mg/kg bw per day is questionable, as large differences between all the groups were noted in the first week of gestation (i.e. before treatment commenced) (Table 28). The high-dose body weight loss during treatment, in contrast, was clearly adverse when its extent is taken into consideration.

Table 28. Body weight development during gestation

	Mean maternal body weight change (g)			
Days	**0 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**
0–7	11.7	−39.1	−13.9	35.1
7–13	120.3	52.6	41.5	−106.5**
7–19	230.0	157.1	113.7	−129.4**
19–25	42.2	59.8	53.1	193.5*

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance/Dunnett's test)
Source: Hrab (1983b)

Feed consumption was not reported. Therefore, it is not clear whether altered body weight gain was (at least partly) due to changes in feed intake.

There were no differences between the control and treated groups with regard to reproductive parameters such as number of corpora lutea, number of implantations, resorption rate, litter size, number of live or dead fetuses and fetal sex ratio. However, mean fetal weight was reduced at the high dose; even in the absence of statistical significance, this difference is considered indicative of a treatment-related effect (Table 29).

Table 29. Mean fetal weight and skeletal anomalies (fetal/litter incidences)

Parameter	**0 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**
Number of live fetuses per litter	8.5	7.0	7.0	8.8
Mean fetal weight (g)	46.3	46.6	46.5	40.5
Number of fetuses examined	128	98	105	114
Vertebrae absent or malformed	1/1	1/1	1/1	1/1
Fused ribs	0/0	0/0	1/1	1/1
Incomplete ossification, frontal skull bones	10/4	15/6	18*/8	22*/9
Incomplete ossification, caudal vertebrae (16 present in total)	18/11	11/6	28*/10	33*/12
Rudimentary 13th rib	49/14	47/12	38/13	74*/12
Metacarpal bone, not ossified	9/5	6/4	15/7	25*/8
Forelimb digit V, middle phalanx not ossified	5/3	7/5	12*/6	15*/5
Tibia, proximal epiphysis not ossified	39/11	52*/11	38/11	64*/13

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance/Dunnett's test)
Source: Hrab (1983b)

External malformations were confined to two control group fetuses in which acephaly, absence or anomalies of the forelimbs, lordosis, delayed ossification of or malformed vertebrae, and a short tail were described. The only visceral malformation was hydrocephalus in one high-dose fetus (116 fetuses examined). Although it was an isolated finding, historical control data suggest that this type of malformation is rarely seen in this rabbit strain (2/1494 fetuses affected, according to the study report), even though no detailed information on the composition of this historical database is

available. In addition, the incidence of skeletal variations, mainly delayed ossification, was increased at the intermediate and high doses (Table 29). The differences relative to the control group were occasionally statistically significant. However, as the litter (and not the number of affected fetuses) is considered the relevant unit in teratology, there was not much difference between the different doses. The only clearly treatment-related effect was the incomplete ossification of skull bones. It must be emphasized that the total number of fetuses or litters with skeletal or other variations was not affected by treatment. Nonetheless, ossification delay would be well in line with lower mean fetal weight at the high dose. Fused ribs and absent or misaligned vertebrae were considered by the study author as skeletal malformations, but their respective occurrence was rare and not clearly dose related.

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on abortions and body weight loss at the high dose of 60 mg/kg bw per day. The latter finding was strongest during the first week of treatment. Because there are no data for the individual days, it is considered, by default, to be an acute effect.

The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on an increase in skeletal variations, mainly ossification delay, at 30 mg/kg bw per day. By its nature, such an effect is not considered an acute one. A teratogenic potential at the high dose (hydrocephalus) cannot be excluded, and its extent could have been masked by the abortions. Even though such a teratogenic effect should be considered an acute one, it would be confined to the high-dose group (Hrab, 1983b).

2.6 Special studies

(a) Neurotoxicity

No studies were submitted.

(b) Immunotoxicity

No studies were submitted.

(c) Mechanistic studies

No mechanistic studies were submitted. However, there was one article of interest retrieved from the open literature. Because of similar effects in plants, the impact of norflurazon on fatty acids in rat liver cells and liver cell microsomes was investigated in vitro. It was shown that the test compound at concentrations of 0.1 and 1.0 mmol/L in the medium, when incubated for 20 or 60 minutes, reduced the amount of desaturated and chain-elongated fatty acids in rat liver cells. In microsomes, norflurazon at 1.0 mmol/L inhibited desaturation of essential fatty acids, but the mechanism behind it could not be elucidated. The possible toxicological relevance of this finding is unknown, as is its potential to occur in vivo (Hagve, Christophersen & Boger, 1985).

(d) Studies on endocrine properties

A number of studies were performed under the Endocrine Disruptor Screening Program of the USEPA. In total, 10 studies that belong to the Tier I assay battery are accessible on the USEPA website. In all these studies, high-purity norflurazon (99.5 ± 0.5% weight/weight; batch no. 609218), which had been provided by Syngenta Ltd, was applied. This test material may be similar to, but is not the same as, that used in the older toxicological studies reported in this monograph. Only two of these 10 studies are reported here, as they may be used to amend data on the sexual maturation of young animals, an end-point that was apparently not investigated in the study by Eschbach et al. (1994).

Young female Crl:CD(SD) rats were administered norflurazon at a dose of 250 or 500 mg/kg bw per day via oral gavage from postnatal days 22 through 42. A concurrent control group received only the vehicle (i.e. 1% carboxymethylcellulose in deionized water). Each group comprised 15 animals. The rats were monitored for clinical signs of toxicity twice a day. In addition, they were observed for vaginal opening. Once this developmental step had been noted, daily vaginal lavage was performed in each female to determine the stage of estrus. Body weight and feed intake were determined daily. At termination on postnatal day 42, the rats were killed and necropsied. Selected organs (liver, kidneys, adrenals, ovaries, uterus, thyroid, pituitary gland) were taken, weighed and preserved. Clinical chemistry parameters, including levels of thyroxine (T_4) and thyroid stimulating hormone (TSH), were measured.

All animals survived to scheduled termination, and there were no clinical signs of toxicity that could be attributed to treatment. However, when compared with the controls, body weight gain was 7.8% or 9.2% lower in the groups receiving 250 or 500 mg/kg bw per day, respectively. These reductions resulted in a slightly lower mean terminal body weight in these treated groups. These findings cannot be attributed to lower feed intake. Mean age of vaginal opening was 35.5 days in the controls, 36.1 days in the low-dose group and 37.6 days in the high-dose group. The significant delay in vaginal opening at 500 mg/kg bw per day might be related to the impaired body weight development, as two females with the strongest delay gained less weight over the course of the study than the average. At termination, these animals had the lowest absolute body weights among all rats on study and exhibited no or reduced corpora lutea in the ovaries. There was no delay in this developmental landmark in the group receiving norflurazon at 250 mg/kg bw per day. Estrous cycle was not affected at any dose.

Clinical chemistry revealed higher mean bilirubin levels and a decrease in mean bile acid levels at both doses. T_4 levels were significantly reduced at 250 and 500 mg/kg bw per day, by 13% and 23%, respectively. Perhaps in line with that, colloid area in the thyroid was reduced in the high-dose group in 7/15 animals. A similar finding was noted in both the control and the low-dose groups in only two animals each. This alteration was microscopically characterized by smaller follicles and a lower amount of colloid. As there was a concomitant and dose-related increase in absolute and relative liver weights at both doses, increased metabolism of thyroid hormones was suspected. However, TSH levels were not affected. Surprisingly, the lowest mean TSH level was noted at the high dose (5.6 ng/mL compared with 6.3 ng/mL in both the controls and the low-dose group) (Coder, 2012a).

Young male Crl:CD(SD) rats were administered norflurazon at a dose of 185 or 375 mg/kg bw per day via oral gavage from postnatal days 23 through 53. A concurrent control group received only the vehicle (i.e. 1% carboxymethylcellulose in deionized water). Each group comprised 15 animals. The rats were monitored for clinical signs of toxicity twice a day. In addition, beginning from postnatal day 30, the males were observed for preputial separation. Body weight and feed intake were determined daily. At termination on postnatal day 53, the rats were killed and necropsied. Selected organs (liver, kidneys, adrenals, thyroid, pituitary gland and reproductive organs) were taken, weighed and preserved. Clinical chemistry parameters, including levels of testosterone, T_4 and TSH, were determined.

All animals survived to scheduled termination, and there were no clinical signs of toxicity that could be attributed to treatment. Similar to what was observed in the study on young females (Coder, 2012a), body weight gains were 7.4% and 8.2% lower in the groups receiving 185 and 375 mg/kg bw per day, respectively. These reductions resulted in slightly lower mean terminal body weights in these groups (5.7% and 6.6%, respectively). These findings cannot be clearly attributed to lower feed intake, even though lower feed consumption was initially noted in the high-dose group as well as sporadically later in the study. Mean age of complete balanopreputial separation was 45.7 days in the controls, 46.1 days in the low-dose group and 47.5 days in the high-dose group. Mean body weight at attainment of this developmental landmark was similar in all groups. Therefore, the delay was attributed by the study author to the lower body weight gain at the high dose.

Hormone levels were not affected in any group, and the only change in clinical chemistry parameters was a decrease in mean bile acid levels by 54% and 58% at 185 and 375 mg/kg bw per day, respectively.

Absolute and relative liver weights were increased at both doses, whereas mean absolute and relative adrenal weights were reduced in the high-dose group. Decreases in absolute weights of some reproductive organs (prostate, seminal vesicle) were attributed to the lower terminal body weight. Gross and histopathological examination did not reveal findings that could be attributed to treatment (Coder, 2012b).

3. Observations in humans

No data were submitted, and no information on poisoning incidents was retrieved from the public literature.

One case report suggests skin sensitization in a farmer by a plant protection product (Predict®) containing norflurazon at a concentration of 78.6%. A patch test was positive for this formulation in a 53-year-old man who suffered from sunburn-like symptoms that caused almost complete closing of his eyes. Later on, he developed urticaria in the same area (Leow & Maibach, 1996).

In an epidemiological study from the San Joaquin valley of California, USA, a possible association between the occurrence of congenital heart defects and residential proximity of the mothers to commercial agricultural pesticide application during a 3-month periconceptional window was investigated. In this case–control study, including 569 heart defect cases and 785 controls born between 1997 and 2006, norflurazon was specifically included as an example of pyradizinone compounds. Based on 74 cases, the risk for coarctation of the aorta (i.e. narrowing of aorta lumen close to the heart) was increased in infants up to 1 year of age and in fetuses when the mothers had lived within 500 m of pesticide application during the periconceptional period. In fact, six mothers from this group of 74 reported norflurazon exposure, compared with 23 women from the control group giving birth to 785 children. The odds ratio was 2.9, with a confidence interval of 1.1–7.5. Confounding factors were taken into account, but no information on the extent of exposure was available. The San Joaquin valley is an area with heavy pesticide use in general, and therefore co-exposure to other agrochemicals is likely. A similar increase in odds ratio for aortal coarctation was noted with exposure to propiconazole, but also to petroleum oil. For seven other heart defect phenotypes, no association with norflurazon exposure could be detected (Carmichael et al., 2014).

Comments

Biochemical aspects

Following oral administration of a single dose of ^{14}C-labelled norflurazon at 2 mg/kg bw to female rats, more than 90% of the administered dose was absorbed, based on a comparison of excretion following oral and intravenous dosing. Absorption after administration of a single high dose of 110 mg/kg bw was similar. Elimination was nearly complete within 96 hours. The major route of excretion was the faeces, with approximately 20% excreted via the urine. Tissue residues were generally low, with the highest relative radioactivity observed in liver and kidneys (Saunders et al., 1985).

Biotransformation was extensive, with only 2% or less of the administered doses excreted as unchanged parent. The main metabolic pathways were *N*-demethylation and glutathione conjugation. Numerous metabolites were found in urine and faeces, nine of which could be identified. Only one of them (metabolite 5, a sulfoxide) accounted for more than 10% of the applied dose (up to 39% in urine, following intravenous administration), whereas the others represented only 1–2% (Saunders et al., 1985).

An older study provided some evidence that excretion and metabolism are similar in male and female rats (Karapally, 1974).

Toxicological data

Where no reliable information was available on the active ingredient, toxicological data on the 80% formulation were evaluated.

In rats, the acute oral LD_{50} of an 80% formulation was 1080 mg/kg bw (Gardner, 1987a), and the acute dermal LD_{50} was greater than 2000 mg/kg bw (Gardner, 1987b). Although these studies were performed under GLP, there is no reliable information on the acute oral or dermal toxicity of the active ingredient itself.

In a GLP- and guideline-compliant study of the active substance in rats, the acute inhalation LC_{50} was greater than 2.4 mg/L (Hoffman, 1996).

In a study of limited reliability, norflurazon active ingredient did not cause skin or eye irritation in rabbits (Bagdon, 1972). In contrast, the 80% formulation was slightly irritating to the eyes and very slightly irritating to the skin of rabbits (Liggett & Smith, 1987, 1988). For the same formulation, there was no evidence of skin sensitization in a Buehler test in guinea-pigs (Kynoch & Parcell, 1987), whereas an older study with the active ingredient was inconclusive (Bagdon, 1973).

Short-term toxicity feeding studies were performed in rats, dogs and, to a lesser extent, mice. In all three species, the liver was a common target organ. Adverse effects on the kidney, thyroid and haematopoietic system (some evidence of anaemia in dogs) were also observed, but did not occur consistently across species and studies.

A poorly reported 28-day study in the mouse was performed as a range-finding experiment. Dietary concentrations were 0, 70, 210, 420 and 2520 ppm (equivalent to 0, 10.5, 31.5, 63 and 378 mg/kg bw per day, respectively). Liver and kidney weights were increased in males from 210 ppm onwards, and a liver weight increase was also observed in females at the highest dose. In males and females at 2520 ppm, gross appearance of the liver suggested some fatty degeneration (Tisdel, 1971).

In a 28-day feeding study in rats, the dietary concentrations were 0, 500, 1000 and 5000 ppm (equal to 0, 52, 105 and 517 mg/kg bw per day for males and 0, 70, 140 and 717 mg/kg bw per day for females, respectively). The NOAEL was 500 ppm (equal to 52 mg/kg bw per day), based on relative liver and kidney weight increases, accompanied by histopathological lesions, at 1000 ppm (equal to 105 mg/kg bw per day) (Fogleman, 1970).

In a 90-day feeding study in rats, the dietary concentrations were 0, 250, 500 and 2500 ppm (equal to 0, 24, 45 and 248 mg/kg bw per day for males and 0, 26, 52 and 275 mg/kg bw per day for females, respectively). Body weight was slightly reduced in both sexes at the high dose. In the same groups, liver and kidney weights were increased, but no concomitant histopathological lesions were noted. In contrast, a higher thyroid weight in high-dose males was accompanied by moderate follicular cell hypertrophy, moderate depletion of colloid and a slight to moderate increase in interstitial vascularity, suggesting some stimulation of thyroid activity. The NOAEL was 500 ppm (equal to 45 mg/kg bw per day) (Fogleman, 1971a).

In a 6-month study, dogs were fed norflurazon at a concentration of 0, 50, 150 or 450 ppm (equal to 0, 1.5, 5.0 and 14.3 mg/kg bw per day for males and 0, 1.6, 4.8 and 17.8 mg/kg bw per day for females, respectively). The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on liver weight increase, minor histopathological findings in liver and thyroid and increases in cholesterol and alkaline phosphatase at 150 ppm (equal to 4.8 mg/kg bw per day) (Klotzsche & Carpy, 1973).

In a guideline- and GLP-compliant toxicological study with norflurazon, dogs were administered a dietary concentration of 0, 50, 200 or 800 ppm (equal to 0, 1.7, 6.2 and 27 mg/kg bw per day for males and 0, 1.5, 6.3 and 23 mg/kg bw per day for females, respectively) for 1 year. The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on impairment of nutritional parameters, such as body weight (gain), feed consumption and feed efficiency, in females and alterations in clinical chemistry suggestive of liver toxicity and increased liver weights in both sexes at 200 ppm (equal to 6.2 mg/kg bw per day). Kidney findings of equivocal toxicological significance, including renal tubular pigmentation, were noted in males at 200 ppm and above (Warren et al., 1990).

The overall NOAEL for the two dog studies was 50 ppm (equal to 1.5 mg/kg bw per day), and the overall LOAEL was 150 ppm (equal to 4.8 mg/kg bw per day).

In a combined long-term toxicity and carcinogenicity study in mice with in utero exposure, norflurazon was administered at a dietary concentration of 85, 340 or 1360 ppm (equivalent to 13, 51 and 200 mg/kg bw per day, respectively) for up to 2 years. Two control groups were included. The study suffered from many deficiencies, and there is good reason to exclude one of the control groups and the low-dose group, as the animals were not treated in parallel with the other groups. The parents of the animals under investigation had already been exposed to norflurazon as part of a reproduction study. Except for a slight reduction in body weight in high-dose males, there were no in-life observations that could be attributed to treatment. Liver weight was increased in all male groups and in mid- and high-dose females, showing a clear dose–response relationship. In females, there was no increase either in neoplastic or in non-neoplastic liver findings in any group (Tisdel, 1975a).

Histopathology of the livers in male mice was evaluated independently by three pathologists (Richter, 1975; Tisdel, 1975a; Rust, 1980) and subjected to an additional statistical reanalysis (Andriano, 1981). Based on the evaluations considered most appropriate by the Meeting (Rust, 1980; Andriano, 1981), there was a clear increase only in liver cell adenoma at 1360 ppm (equivalent to 200 mg/kg bw per day), with a NOAEL for carcinogenicity of 340 ppm (equivalent to 51 mg/kg bw per day). No NOAEL could be identified for non-neoplastic effects, as the lower doses were not sufficiently investigated. The LOAEL was 340 ppm (equivalent to 51 mg/kg bw per day), the lowest dose for which reliable data were available, based on non-neoplastic liver lesions in males, increases in liver weight in both sexes and non-neoplastic microscopic lesions in organs other than the liver (i.e. kidneys, spleen, pancreas, ovaries and bone marrow) mainly in females at the highest dose.

The Meeting concluded that norflurazon at a dose of 200 mg/kg bw per day caused an increase in benign liver tumours in male mice that was preceded by non-neoplastic changes at lower doses. No increase in the incidence of carcinomas was observed in this study. No information on the mode of action is available; thus, human relevance cannot be excluded.

In a combined long-term toxicity and carcinogenicity study in rats, norflurazon was administered at a dietary concentration of 125, 375 or 1025 ppm (equivalent to 6.25, 18.9 and 51.3 mg/kg bw per day, respectively) for 2 years. Two control groups of the same size were included and fed on untreated basal diet. Again, there were a number of deficiencies in this study, including the unusual study design, in which the parents had previously been treated with equal or lower concentrations of the test substance. There was no evidence of carcinogenicity in this study. There were no in-life observations attributable to treatment. The NOAEL was 125 ppm (equivalent to 6.25 mg/kg bw per day), based on increased kidney weights and histopathological changes (increased hyaline casts and nephritis) in the kidneys of females at 375 ppm (equivalent to 18.9 mg/kg bw per day). The uterus was identified as an additional target organ, as there was a higher incidence of squamous metaplasia and endometritis, at least in high-dose females. In low- and mid-dose females, the uteri were not subject to histopathological examination (Tisdel, 1975b).

The Meeting concluded that norflurazon is carcinogenic in male mice, but not in female mice or rats.

Norflurazon was tested for genotoxicity in a limited range of in vitro assays. There was no evidence of genotoxicity in the Ames test (Brusick, 1977; Anonymous, 1980) or the rec assay in bacteria (Anonymous, 1980) or of chromosomal aberrations in mammalian (Chinese hamster ovary) cells (Putman, 1985). The substance gave negative results for unscheduled DNA synthesis in primary rat hepatocytes (Curren, 1985), but was positive in a more recently published comet assay in an aquatic bioindicator organism, but only at cytotoxic concentrations (Horvat et al., 2005).

In spite of the generally poor database, the Meeting concluded, on the weight of evidence, that norflurazon is not genotoxic in vitro and that it is unlikely to be genotoxic in vivo.

In view of the limited evidence of genotoxicity in vitro and taking into account the tumour spectrum of this compound (i.e. adenoma in one sex and one species only at the highest dose), the Meeting concluded that norflurazon is unlikely to pose a carcinogenic risk to humans from the diet.

The reproductive toxicity of norflurazon was investigated in one of the few studies that was performed under GLP and broadly complied with modern test guidelines. In a two-generation study in rats, the test substance was administered at a dietary concentration of 0, 150, 750 or 1500 ppm (equal to 0, 10.2, 51.0 and 102.5 mg/kg bw per day, respectively). No adverse effects on fertility or reproductive performance were observed. Accordingly, the NOAEL for reproductive toxicity was 1500 ppm (equal to 102.5 mg/kg bw per day), the highest dose tested. Parental effects in the adult animals comprised decreases in body weight and body weight gain, but also higher liver and kidney weights and histopathological findings in the liver and kidney, at 750 ppm (equal to 51.0 mg/kg bw per day), with a NOAEL for parental toxicity of 150 ppm (equal to 10.2 mg/kg bw per day). The same NOAEL was identified for offspring toxicity, based on impaired body weight development, higher relative weights of liver and kidney in pups and an equivocal impact on viability (lactation index) in the F_{2B} litters at 750 ppm (equal to 51.0 mg/kg bw per day) (Eschbach et al., 1994).

It was noted that developmental landmarks were not investigated in this study. However, relevant information was available from recent studies from the Endocrine Disruptor Screening Program of the USEPA.

In a pubertal assay in intact young female rats, a delay in vaginal opening was observed at the high dose of 500 mg/kg bw per day, but not at 250 mg/kg bw per day. This effect was attributed to lower body weight gain in this group. Estrous cycle was not affected. An impact on the thyroid was evident, with lower T_4 levels at both doses and histological findings (smaller follicles, lower amount of colloid) in the animals receiving the higher dose (Coder, 2012a).

In a similar study in intact young male rats, balanopreputial separation was slightly delayed at the high dose of 375 mg/kg bw per day. Again, this observation was made in parallel to a decrease in body weight gain. Testosterone, T_4 and TSH levels were not affected (Coder, 2012b).

In a developmental toxicity study in rats, norflurazon was administered by gavage at a dose of 0, 100, 200 or 400 mg/kg bw per day on GDs 6 through 15. The NOAEL for maternal toxicity was 200 mg/kg bw per day, based on initial body weight loss and lower feed consumption and feed efficiency at 400 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, the highest dose tested (Hartman & Hrub, 1972).

In a preliminary range-finding study, pregnant rabbits received norflurazon by oral gavage from GDs 7 through 19 at a dose of 0, 10, 40, 80, 100 or 400 mg/kg bw per day. At 40 mg/kg bw per day, maternal toxicity was apparent as a result of body weight loss, which became more pronounced at higher doses. Resorption rate was increased at 80 mg/kg bw per day and higher. At 400 mg/kg bw per day, does aborted and eventually died or had to be killed on GD 15 or 16 (Hrab, 1983a).

In the main developmental toxicity study, daily doses of 0, 10, 30 or 60 mg/kg bw were administered to pregnant rabbits from GDs 7 through 19. The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on abortions and body weight loss at the high dose of 60 mg/kg bw per day. The latter finding was most marked during the first week of treatment and was considered by the Meeting to be an acute effect. The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on an increase in skeletal variations, mainly ossification delay, at 30 mg/kg bw per day. A teratogenic potential at the high dose cannot be excluded, as hydrocephalus was observed in one high-dose fetus (of 116 fetuses examined). This is a very rare malformation in New Zealand white rabbits (historical background 0.1%), the strain used in this study, and the extent could have been masked by the abortions (Hrab, 1983b).

The Meeting concluded that norflurazon is not teratogenic in rats, but it could not exclude the possibility that norflurazon is teratogenic in rabbits, based on the occurrence of hydrocephalus in a single rabbit fetus in the high-dose group.

No studies on neurotoxicity or immunotoxicity were submitted. There was no evidence for such effects in the routine toxicological studies, but it must be taken into consideration that the range of parameters in these mostly very old and pre-guideline studies was relatively limited. Therefore, the Meeting could not conclude on these end-points.

Toxicological data on metabolites and/or degradates

Desmethyl norflurazon

The desmethyl metabolite of norflurazon (plant metabolite) was found in the urine of male rats (accounting for 2.9%) following oral administration of 10 mg of norflurazon for 15 days (Karapally, 1974). Desmethyl norflurazon was detected in the urine of female rats receiving a single norflurazon dose of 2 mg/kg bw at a maximum of 7% (lower amounts found in other treatment groups). In faeces, the same metabolite accounted for 2.2–5%, depending on the treatment group (Saunders et al., 1985).

This metabolite and its conjugates are considered to be covered by studies of the parent compound.

6-Methyl sulfoxide norflurazon

The metabolite 6-methyl sulfoxide norflurazon appears in rat urine at greater than 10%.

This metabolite is considered to be covered by the studies of the parent compound.

NOA-452075

The metabolite 1-({5-chloro-6-oxo-1-[3-(trifluoromethyl)phenyl]hydropyridazin-4-yl}-ureido)-4-hydroxybutaneurea (i.e. NOA-452075) occurs in the rat only at trace concentrations.

For chronic toxicity, the threshold of toxicological concern (TTC) approach (Cramer class III) could be applied.

Human data

No data were submitted. A case of skin sensitization due to a commercial product containing norflurazon has been reported in the open literature (Leow & Maibach, 1996).

The Meeting concluded that the existing database on norflurazon was adequate to identify the potential hazards to the general population, including fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.005 mg/kg bw for norflurazon, on the basis of an overall NOAEL of 1.5 mg/kg bw per day in the 6-month and 1-year studies in dogs, using a safety factor of 300. This NOAEL was based on impairment of nutritional parameters, such as body weight (gain), feed consumption and feed efficiency, in females and alterations in clinical chemistry suggestive of liver toxicity and increased liver weights in both sexes. An additional factor of 3 was used because of the poor quality of the database. The upper range of the ADI provides a margin of 40 000 relative to the LOAEL for liver adenomas in male mice.

The Meeting established an acute reference dose (ARfD) of 0.3 mg/kg bw from the NOAEL for maternal toxicity of 30 mg/kg bw per day in a developmental toxicity study in rabbits, based on abortions and reductions in maternal body weight at 60 mg/kg bw per day, using a safety factor of 100.

The ADI and ARfD apply to the metabolites desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon.

Levels relevant to risk assessment of norflurazon

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	–	340 ppm, equivalent to 51 mg/kg bw per day[b]
		Carcinogenicity	340 ppm, equivalent to 51 mg/kg bw per day	1 360 ppm, equivalent to 200 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	125 ppm, equivalent to 6.25 mg/kg bw per day	375 ppm, equivalent to 18.9 mg/kg bw per day
		Carcinogenicity	1 025 ppm, equivalent to 51.3 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1 500 ppm, equal to 102.5 mg/kg bw per day[c]	–
		Parental toxicity	150 ppm, equal to 10.2 mg/kg bw per day	750 ppm, equal to 51.0 mg/kg bw per day
		Offspring toxicity	150 ppm, equal to 10.2 mg/kg bw per day	750 ppm, equal to 51.0 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	200 mg/kg bw per day	400 mg/kg bw per day
		Embryo and fetal toxicity	400 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity study[d]	Maternal toxicity	30 mg/kg bw per day	60 mg/kg bw per day
		Embryo and fetal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
Dog	Six-month and 1-year toxicity studies[a,e]	Toxicity	50 ppm, equal to 1.5 mg/kg bw per day	150 ppm, equal to 4.8 mg/kg bw per day

[a] Dietary administration.
[b] Lowest dose tested for which reliable data were available.
[c] Highest dose tested.
[d] Gavage administration.
[e] Two or more studies combined.

Acceptable daily intake (ADI) (applies to norflurazon, desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon, expressed as norflurazon)

0–0.005 mg/kg bw

Acute reference dose (ARfD) (applies to norflurazon, desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon, expressed as norflurazon)

0.3 mg/kg bw

Information that would be useful for the continued evaluation of the compound

Full evaluation of potential for genotoxicity in vivo, neurotoxicity and immunotoxicity; comparative metabolism study in toxicological species and humans; results from epidemiological, occupational health and other observational studies of human exposure

Critical end-points for setting guidance values for exposure to norflurazon

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	>80%
Dermal absorption	No data
Distribution	Highest concentrations in liver and kidney
Potential for accumulation	Low
Rate and extent of excretion	Nearly complete via faeces (65–80%) and urine (19–27%) within 96 hours
Metabolism in animals	Extensive by a variety of pathways (e.g. *N*-demethylation and glutathione conjugation), resulting in numerous metabolites, of which only a sulfoxide accounted for more than 10% of the administered dose
Toxicologically significant compounds in animals and plants	Norflurazon
Acute toxicity	
Rat, LD_{50}, oral	1 080 mg/kg bw (80% formulation)
Rat, LD_{50}, dermal	>2 000 mg/kg bw (80% formulation)
Rat, LC_{50}, inhalation	>2.4 mg/L (active substance)
Rabbit, dermal irritation	Very slightly irritating (80% formulation)
Rabbit, ocular irritation	Slightly irritating (80% formulation)
Guinea-pig, dermal sensitization	Not sensitizing (Buehler, 80% formulation)
Short-term studies of toxicity	
Target/critical effect	Liver changes, alterations in body weight, feed consumption and feed efficiency
Lowest relevant oral NOAEL	1.5 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decrease in body weight; liver weight increase, histological lesions in liver and kidney
Lowest relevant NOAEL	6.25 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in male mice, but not in female mice or rats[a]
Genotoxicity	
	Negative in vitro[a]

Reproductive toxicity	
Target/critical effect	No effects on reproduction
Lowest relevant parental NOAEL	10.2 mg/kg bw per day
Lowest relevant offspring NOAEL	10.2 mg/kg bw per day
Lowest relevant reproductive NOAEL	102.5 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	Abortions and body weight losses in does; skeletal variations and developmental delay in rabbit fetuses
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	10 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
	No studies available on toxicologically relevant metabolites
Human data	
	Evidence of skin sensitization (case report)

[a] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI	0–0.005 mg/kg bw[a]	Short-term toxicity studies in dogs	300
ARfD	0.3 mg/kg bw[a]	Developmental toxicity study in rabbits	100

[a] Applies to norflurazon, desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon, expressed as norflurazon.

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[2] In the sponsor's study directory, these studies were quoted as "Harris". However, Dr Harris approved the studies in his or her responsibility as "Chief pathologist/toxicologist" as indicated in the reports. The study director was apparently Meryl Tisdel, who signed the reports. It is more common to mention the name of the study director when citing a study.