SPIRODICLOFEN

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Explanation

Spirodiclofen is the International Organization for Standardization (ISO)–approved name for 3-(2,4-dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl 2,2-dimethylbutyrate (International Union

of Pure and Applied Chemistry [IUPAC]) or 3-(2,4-dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl 2,2-dimethylbutanoate (Chemical Abstracts Service [CAS]), CAS No. 148477-71-8. Spirodiclofen is a selective, non-systemic foliar insecticide and acaricide belonging to the chemical class of ketoenols or tetronic acids, whose pesticidal mode of action is the inhibition of lipid synthesis.

Spirodiclofen is being reviewed for the first time by the present Meeting at the request of the Codex Committee on Pesticide Residues.

All pivotal toxicological studies complied with good laboratory practice (GLP).

Evaluation for acceptable daily intake

1. Biochemical aspects

The toxicokinetics of spirodiclofen have been studied in rats given oral doses of radiolabelled test material. Summaries of the relevant data are presented below.

1.1 Absorption, distribution and excretion

Rats

In a GLP-compliant metabolism and disposition study, [dihydrofuranone-3-^{14}C]spirodiclofen (radiochemical purity >98%; chemical purity 99.2%) was administered to Wistar rats (four males per dose) via oral gavage as a single dose at 2 or 100 mg/kg bw or as a 14-day repeated dose of 2 mg/kg bw of non-labelled material followed by a single dose of labelled material on day 15. An additional single low dose (2 mg/kg bw) group of four female rats was also included, as well as an additional group of four males at the same dose, which was included in order to measure excretion in expired air. A group of six male rats with bile duct cannulae received a single dose of 1 mg/kg bw for assessment of biliary excretion.

Overall recovery of administered radioactivity was 89–107%. Plasma radioactivity data showed that absorption was rapid (time to peak concentration [T_{max}] values of 3–4 h for low dose and 8 h for high dose). Excretion data revealed that expired air was an insignificant route of excretion (<0.05% of the administered dose) and that both urine (57–74% of the administered dose for the low dose and 35% of the administered dose for the high dose) and faeces (23–31% of the administered dose for the low dose and 61% of the administered dose for the high dose) were major routes of elimination. Consistent with plasma kinetics data, excretion was approximately 90% complete at 48 h post-dosing. Tissue burdens were negligible (<0.2% of the administered dose in all tissues examined) and revealed no indication of sequestration or tissue specificity. Approximately 11% of the administered dose appeared in the bile, which contributed up to one third of the radioactivity excreted in the faeces. A decrease in urinary excretion of radioactivity with a commensurate increase in faecal excretion of radioactivity, combined with a comparison of area under the curve (AUC) and maximal concentration (C_{max}) values (neither of which reflected the 50-fold difference in dose), were indicative of decreased absorption at the high dose. Sex-related differences were observed, in that female rats tended to exhibit greater urinary excretion of radioactivity compared with males, whereas males excreted significantly more radioactivity via the faeces compared with females, indicating higher oral absorption in females. In addition, radioactive residues in the organs and tissues were higher in male rats than in female rats. For example, 0.138% and 0.0125% of the dose were detected in the liver and 0.010% and 0.002% in the kidney for males and females, respectively. As a whole, only 0.55% and 0.02% of the administered radioactivity remained in the body of male and female rats, respectively, after sacrifice (Table 1) (Andersch & Köster, 2000a).

Table 1. Radioactive inventory and selected pharmacokinetic parameters in rats following oral administration of [dihydrofuranone-3-^{14}C]spirodiclofen (n = 4)

Parameter	Single dose, 2 mg/kg bw	Single dose, 100 mg/kg bw	Single dose, 2 mg/kg bw	Single dose, 2 mg/kg bw	14-day repeated dose, 2 mg/kg bw per day	Single dose, 1 mg/kg bw
	Male	Male	Male	Female	Male	Male
	48 h	168 h	48 h	48 h	48 h	24 h
Expired air (% of AD)	0.05	ND	ND	ND	ND	ND
Urine (% of AD)	62.1	35.1	57.3	74.2	66.6	24.3
Faeces (% of AD)	30.9	61.3	31.1	23.3	27.6	31.3
Bile (% of AD)	ND	ND	ND	ND	ND	11.9
Carcass/organs[a] (% of AD)	0.378	0.0003	0.551	0.0156	0.150	26.2
Gastrointestinal tract (% of AD)	0.130	ND	0.0818	0.0226	0.0665	13.3
Total (% of AD)	93.6	96.3	89.0	97.5	94.5	107
C_{max} (µg/ml)	ND	51.3	2.1	2.7	2.1	ND
T_{max} (h)	ND	8.0	3.0	3.0	4.0	ND
AUC (µg·h/ml)	ND	772.5	36.7	23.7	21.2	ND

From Andersch & Köster (2000a)
AD, administered dose; ND, not determined
[a] Carcass/organs without gastrointestinal tract.

A second GLP-compliant metabolism and disposition study examined the effect of a 15-week dietary administration ("pretreatment") of unlabelled spirodiclofen to Wistar rats (four of each sex per dose) at doses of 50 or 2500 ppm, followed by a single oral gavage dose of [dihydrofuranone-3-^{14}C]spirodiclofen (radiochemical purity >98%) in carboxymethyl cellulose/acetic acid (99.5/0.5 weight per volume [w/v]) at 2 mg/kg bw. Absorption, excretion, plasma kinetics and carcass burdens were assessed over a 48 h period.

Overall recovery of the administered radioactivity was 87–103%. Analysis of plasma concentration–time course data and plasma kinetics data revealed fairly rapid absorption, as exemplified by T_{max} values of 3–4 h for both sexes in the low dose groups and males in the high dose pretreatment group. The T_{max} value in high dose treatment females was higher (8 h). Urinary excretion data and body burden data indicated that at least 58–75% of the test material was absorbed. The urine was found to be the major route of excretion, accounting for approximately 56–75% of the administered dose over a 48 h period. Elimination via the faeces accounted for approximately 22–31% of the single oral dose over the same period. Total excretion was greater than 90% over 48 h for all groups except the 2500 ppm pretreatment female group, in which total excretion was 85% over 48 h. Tissue distribution was not assessed in this study; however, mass balance data and radioactivity recovered in the skin and residual carcass indicated that little bioaccumulation occurred even after the 15-week dietary treatment. The results of this study indicated that the absorption and elimination of spirodiclofen were not significantly altered by prior dietary exposure to the test substance for 15 weeks (Table 2) (Andersch & Köster, 2000b).

In an additional GLP-compliant study designed to investigate the depletion of radioactive residues from plasma, liver, kidneys and excreta, a single oral gavage dose of [dihydrofuranone-3-^{14}C]-

Table 2. Radioactive inventory and selected pharmacokinetic parameters in rats following oral administration of [dihydrofuranone-3-^{14}C]spirodiclofen[a]

Parameter	Dietary concentration (ppm)			
	50	50	2500	2500
	Male	Female	Male	Female
Urine (% of AD)	72.0	74.7	61.4	56.4
Faeces (% of AD)	30.5	21.6	30.8	27.6
Skin (% of AD)	0.034	0.020	0.048	0.029
Carcass/organs[b] (% of AD)	0.49	0.35	1.2	1.8
Gastrointestinal tract (% of AD)	0.24	0.49	1.4	0.95
Total (% of AD)	103	97.2	94.8	86.8
C_{max} (µg/ml)	2.09	3.24	1.63	1.73
T_{max} (h)	4.0	4.0	3.0	8.0
AUC (µg·h/ml)	28.9	35.1	18.9	20.9

From Andersch & Köster (2000b)
AD, administered dose
[a] One animal from each of the male (#501) and female (#506) 2500 ppm pretreatment groups were considered outliers and were excluded from the means. For male #501: C_{max} = 0.757 µg/ml and T_{max} = 4 h; for female #506: C_{max} = 1.374 µg/ml and T_{max} = 32 h.
[b] Carcass/organs without gastrointestinal tract.

spirodiclofen (radiochemical purity >98%) in 0.5% aqueous carboxymethyl cellulose was administered to three groups of Wistar rats (four of each sex per group) at a dose of 2 mg/kg bw. The groups were sacrificed at 3, 6 and 24 h, respectively, following administration of radiolabelled spirodiclofen, and urine, blood (plasma and erythrocytes), liver, kidney, skin, gastrointestinal tract (including faeces) and carcass were collected from each animal for analysis of residues.

At 3 h post-dosing, 52% and 32% (for males and females, respectively) of the administered dose were detected in the organs and tissues of rats, whereas 44% (male) and 53% (female) were detected in the gastrointestinal tract and 1.8% (male) and 5.1% (female) in the urine. Residue levels in the organs and tissues declined over time, whereas those in the urine increased. Other than the gastrointestinal tract, which also included faeces, the highest residue concentrations were detected in the liver; levels in this organ were highest at the 3 h sampling period, after which they declined. The values in plasma were approximately 10-fold higher than those in the skin and carcass, indicating a delayed equilibrium of the radioactivity between plasma and the tissues in the rat. Residue concentrations in the kidney were comparable with those in the plasma at all time points in both sexes (Table 3) (Köster & Weber, 2002).

In two GLP-compliant rat quantitative whole-body autoradiography studies, [dihydrofuranone-3-^{14}C]spirodiclofen (radiochemical purity >98%) was administered to five male Wistar Hsd/Cpb:WU rats as a single oral (gavage) dose at 3 mg/kg bw in the first study and then to five female rats at the same dose in the second study. In each study, a single animal was sacrificed at 1, 4, 8, 24 and 48 h post-dose and subjected to quantitative whole-body autoradiography to examine the distribution of radioactivity among tissues. In addition, one control animal received unlabelled test substance and was sacrificed at 4 h.

Based on the recovery of radioactivity in the urine, the dosed radioactivity was readily absorbed and excreted, with 53–58% (in males) and approximately 51% (in females) of the dose being recovered in the urine by 24 h post-dose. Autoradiograms of longitudinal sections through the ^{14}C-treated rats showed extensive distribution of radioactivity throughout the entire body within 1 h of dosing.

Table 3. Balance of radioactivity in excreta and tissues of rats sacrificed 3, 6 and 24 h after oral administration of [dihydrofuranone-3-^{14}C]spirodiclofen

Matrix	Radioactivity (% of administered dose)					
	Males			Females		
	3 h	6 h	24 h	3 h	6 h	24 h
Urine	1.8	20.9	57.7	5.1	34.9	74.8
Erythrocytes	0.51	0.47	0.04	0.43	0.25	0
Plasma	4.0	2.6	0.20	2.8	2.1	0.02
Liver	26.0	17.9	1.5	13.5	12.4	0.09
Kidney	1.4	0.88	0.13	1.4	0.2	0.01
Carcass	12.8	10.2	0.93	9.3	7.3	0.13
Skin	7.2	5.7	0.54	4.3	3.7	0.05
Gastrointestinal tract and faeces	43.6	35.6	40.4	52.9	33.1	23.6
Total	97.3	94.3	101.3	89.8	94.5	98.7

From Köster & Weber (2002)

Data from the quantitative whole-body autoradiography indicated that concentrations of radioactivity in males were highest in the small intestines at 1 h post-dose, followed by liver and renal cortex. Although the same trend was reported for females, values were not shown for the small intestine. Based on the data provided, the highest levels at 1 h in females were noted in the liver and renal medulla. Concentrations in all other tissues (except fat and dental root in males and renal cortex and the ovary in females) were below the concentration in blood at 1 h. In females, levels peaked in the ovary at 1 h, in the urinary bladder at 4 h and in most other tissues by 8 h and had declined significantly by 24 h in all tissues. In males, levels peaked in the small intestine at 1 h and then in all other tissues at 4 or 8 h. Concentrations in most male tissues had declined by 6- to 10-fold by 24 h post-dose, with concentrations remaining highest in liver, bladder, dental root and renal cortex. Generally, levels were higher in tissues from males than in those from females. By the 48 h interval, concentrations were below the limit of detection in all tissues for females and all except liver and renal cortex in males. There was no evidence of accumulation in specific organs or tissues in this study (Table 4) (Köster, 2000, 2001).

1.2 Biotransformation

In studies of metabolism, groups of Wistar rats (four males per dose) were given [dihydrofuranone-3-^{14}C]spirodiclofen (radiochemical purity >98%) as a single oral dose at 2 or 100 mg/kg bw on its own or as a single oral dose of 2 mg/kg bw per day following a 14-day administration of unlabelled spirodiclofen at 2 mg/kg bw per day. An additional single low dose (2 mg/kg bw) group of four female rats was also included, as well as an additional group of four males at the same dose in order to measure excretion in expired air. A group of six male rats with bile duct cannulae received a single dose of 1 mg/kg bw for assessment of biliary excretion. Metabolite characterizations were conducted from animal excreta (bile, urine and faeces). Parent compound and metabolites were isolated by semipreparative high-performance liquid chromatography (HPLC) and identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) investigations.

Analyses of excreta revealed that the test article underwent extensive metabolism. The parent compound was detected only in small quantities in the faeces, but not in the urine or bile. Identified

Table 4. Concentration of radioactivity in selected tissues or organs of rats following treatment with [^{14}C]spirodiclofen at 3 mg/kg bw (n = 1)

Tissue/organ	Concentration of radioactivity (µg equivalents/g)							
	Sacrifice interval (h post-dose)							
	1	4	8	24	1	4	8	24
	Males				Females			
Liver	1.83	5.54	5.46	0.631	0.569	1.74	2.83	0.012
Renal cortex	1.34	2.29	1.56	0.262	0.220	0.615	0.886	0.006
Renal medulla	0.240	0.734	0.882	0.066	0.506	1.84	2.93	0.021
Adrenal	0.426	1.02	0.825	0.097	0.186	0.373	0.550	<LOD[a]
Urinary bladder	0.251	3.72	6.46	0.372	1.81	7.43	6.48	0.024
Small intestine	5.69	3.82	3.00	0.092	NR	NR	NR	NR
Blood	0.471	1.52	1.28	0.140	0.189	0.427	0.657	<LOQ[b]
Brown fat	0.797	2.70	1.67	0.190	0.074	0.390	0.429	<LOD
Thyroid	0.166	0.480	0.417	0.052	0.079	0.143	0.158	<LOD
Dental root	0.485	1.40	4.01	0.304	NR	NR	NR	NR
Testis	0.019	0.171	0.139	0.015	NA	NA	NA	NA
Caput epididymis	0.044	0.162	0.204	0.014	NA	NA	NA	NA
Caudate epididymis	0.071	0.199	0.226	0.025	NA	NA	NA	NA
Preputial gland	0.177	0.673	0.878	0.030	NA	NA	NA	NA
Prostate	0.103	0.578	0.404	0.056	NA	NA	NA	NA
Ovary	NA	NA	NA	NA	0.272	0.130	0.206	<LOQ
Uterus	NA	NA	NA	NA	0.046	0.241	0.264	<LOD

From Köster (2000, 2001)

LOD, limit of detection; LOQ, limit of quantification; NA, not applicable; NR, not reported

[a] LOD established as 2 times the background radioactivity multiplied by the calibration factor derived from the microstandards used.

[b] LOQ set as either a) 2 times the respective LOD value or b) the lowest value of the calibration microstandards that were included in the calibration curve, whichever was higher.

components in urine and faeces accounted for approximately 59–90% of the administered dose, depending on dose regimen. Ten urinary and 11 faecal components were characterized. With few exceptions, metabolite profiles were generally similar qualitatively but varied quantitatively between dose groups. Greater levels of parent compound were detected in faeces from the high dose group, which was consistent with the observed decreased absorption. The sex-related difference in absorption/excretion processes in the single low dose group was reflected quantitatively and to some extent qualitatively in the metabolite profiles. The predominant metabolite in the excreta of females was the enol metabolite (58% of the administered dose), followed by the 4-hydroxy-enol (16%) and the 3-hydroxy-enol (5%) (equatorial isomers [eq.]). In low-dose males, the major metabolites in excreta were the 3-hydroxy (eq.)-enol (28–33%), the 4-hydroxy (eq.)-enol (18–22%) and the enol (8–9%). The quantitative metabolic profile shifted slightly for high-dose males, with the enol, the 3-hydroxy (eq.)-enol and the 4-hydroxy (eq.)-enol representing approximately 22%, 14% and 9% of the administered dose, respectively. An assessment of the time course of excretion of metabolites via the faeces revealed that most of the metabolites (generally >90%) were excreted during the 0–24 h period following dosing. Qualitatively, the biliary metabolite profile closely paralleled that of the urine, with the exception of a glucuronide conjugate in the bile, which represented approximately one third of the radioactivity in the bile over a 24 h period following dosing. The metabolite profile

in the bile also lacked several components (glyoxylic acid, dichloromandelic acid, dichlorobenzoic acid, mandelic acid cyclohexyl ester) that were detected in the faeces; these were likely the result of biotransformation processes within the gut.

An additional study examined the effect of 15-week dietary pretreatment with spirodiclofen at 50 or 2500 ppm, followed by a single oral gavage dose of [dihydrofuranone-3-^{14}C]spirodiclofen (radiochemical purity >98%) at 2 mg/kg bw to groups of Wistar rats (four of each sex per dose). Metabolites in urine and faeces (at 48 h) were identified based on HPLC investigations. The dietary pretreatment did not appear to significantly affect the metabolism of spirodiclofen in the rat. Similar to the other study, no parent compound was detected in the urine, whereas nine metabolites were characterized. In the faeces, parent compound accounted for 0.38–13.5% of the administered dose, whereas 12 metabolites accounted for 0.17–8.95% (each) of the dose. Sex-related differences were observed that were independent of the pretreatment regimen. The most notable difference was that the enol metabolite in urine accounted for approximately 5% of the administered radioactivity in male rats, compared with 40–52% in female rats. In male rats, the 3-hydroxy-enol was the major metabolite (~23–34% versus 3.5–4.5% in females). Other minor quantitative and qualitative sex-related differences were observed in metabolites that each represented less than 5% of the administered radioactivity.

Major metabolites (i.e. the enol, the 3-hydroxy-enol [equatorial and axial isomers] and the 4-hydroxy-enol [equatorial and axial isomers]) were characterized in urine and plasma, as well as in liver and kidney extracts, in an additional study using three groups of Wistar rats (four of each sex per group) administered a single oral gavage dose of [dihydrofuranone-3-^{14}C]spirodiclofen (radiochemical purity >98%) in 0.5% aqueous carboxymethyl cellulose. The groups were sacrificed at 3, 6 and 24 h, respectively, and the major metabolites were quantified in the various matrices. These investigations were conducted to further elucidate the metabolism of spirodiclofen, as there were sex differences noted in the amounts of these major metabolites in previous studies conducted. These investigations confirmed the previously noted sex differences. In urine, the enol metabolite was dominant in females, whereas the hydroxylated enol metabolites were the major metabolites in males. Metabolite levels increased over time from 3 to 24 h in urine samples. In plasma, liver and kidney samples, the enol was the major metabolite quantified in both sexes. However, the hydroxylated enols were present in higher amounts in males. In females, the hydroxylated enols were not detected at all in plasma and only at very low levels in liver (<1.3% of total radioactive residues) and kidney (<5% of total radioactive residues). In plasma, liver and kidney samples, metabolite levels declined over the 24 h sampling period.

Based on the metabolism studies conducted, it was concluded that the metabolism of [dihydrofuranone-3-^{14}C]spirodiclofen in the rat generally proceeds via formation of an enol that undergoes hydroxylation or is metabolized to a mandelic acid derivative and dichlorobenzoic acid (Figure 1) (Andersch & Köster, 2000a,b; Köster & Weber, 2002).

2. Toxicological studies

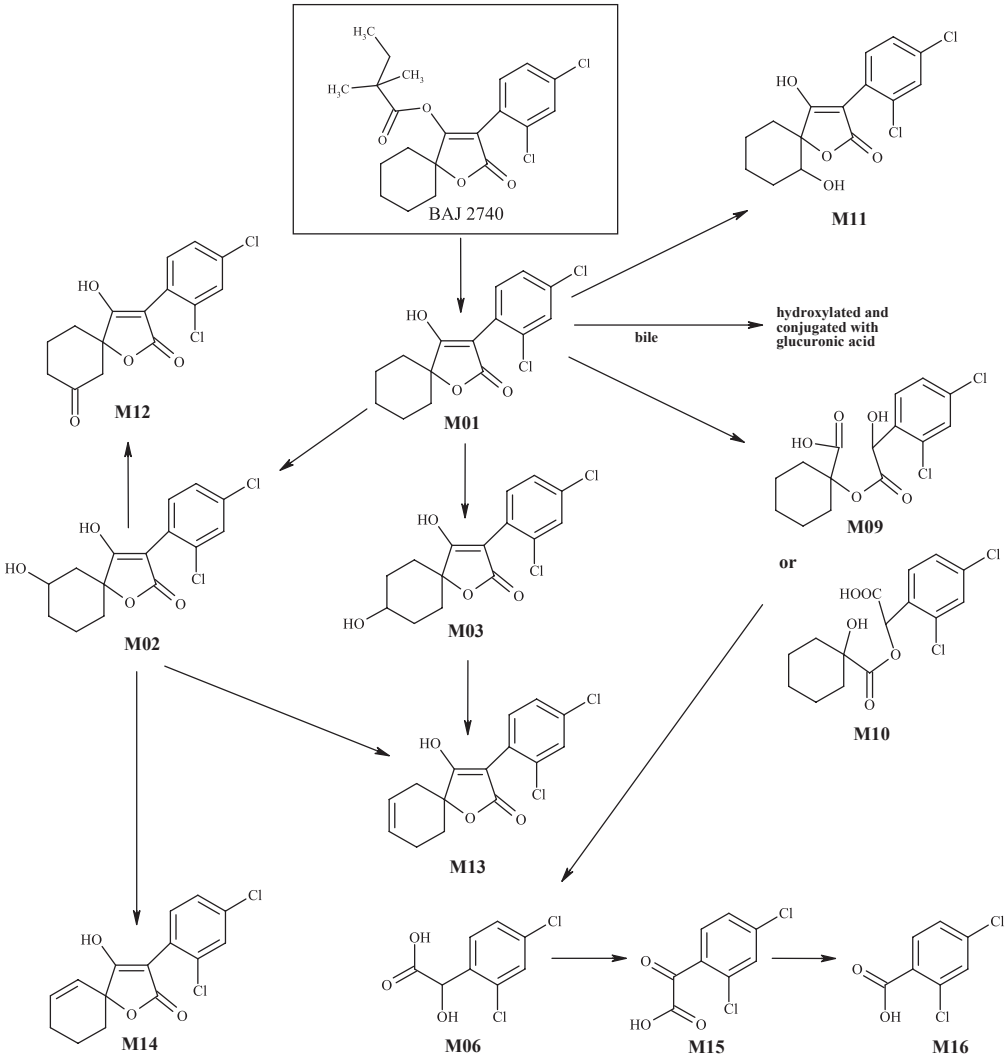
2.1 Acute toxicity

(a) Lethal doses

The results of the acute toxicity studies with spirodiclofen are summarized in Table 5. All studies complied with GLP.

Spirodiclofen is of low acute toxicity via the oral, dermal and inhalation routes. No clinical signs of toxicity or other treatment-related findings were noted in any of the acute toxicity studies.

Figure 1. Proposed metabolic pathway of spirodiclofen (BAJ 2740) in rats



M01: BAJ 2740-enol
M02: 3-hydroxy (eq.)-BAJ 2740-enol / 3-hydroxy (ax.)-BAJ 2740-enol
M03: 4-hydroxy (eq.)-BAJ 2740-enol / 4-hydroxy (ax.)-BAJ 2740-enol
M06: 2,4-dichloromandelic acid
M09: 2,4-dichloromandelic acid cyclohexyl ester (isomer 1)
M10: 2,4-dichloromandelic acid cyclohexyl ester (isomer 2)
eq. equatorial
ax. axial

M11: 2-hydroxy (eq.)-BAJ 2740-enol
M12: 3-keto-BAJ 2740-enol
M13: 3-ene-BAJ 2740-enol
M14: 2-ene-BAJ 2740-enol
M15: 2,4-dichloroglyoxylic acid
M16: 2,4-dichlorobenzoic acid

From Kaune, Temerowski & Krautstrunk (2008)

Table 5. Acute toxicity of spirodiclofen[a]

Species	Strain	Sex	Route	Vehicle	LD_{50} (mg/kg bw)	LC_{50} (mg/l)	Reference
Rat	HsdCpb:WU Wistar	M & F	Oral	Carboxymethyl cellulose sodium salt	>2500[b]	—	Kroetlinger (1996a)
		M & F	Dermal	Water[c]	>2000	—	Kroetlinger (1996b)
		M & F	Inhalation	None[d]	—	>5.03	Pauluhn (1997)

F, female; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; M, male

[a] Spirodiclofen of >99% purity was used in the acute studies.

[b] The reported LD_{50} was based on the Organisation for Economic Co-operation and Development (OECD) draft guideline "Acute Oral Toxicity—Acute Toxic Class Method" from 1995. Based on the revised OECD test guideline 423 (adopted in 2001), the company indicated that the interpretation would be as follows: LD_{50} oral >2000 mg/kg bw, equivalent to category "unclassified" of the Globally Harmonized System of Classification and Labelling of Chemicals, and LD_{50} cut-off >5000 mg/kg bw.

[c] The test substance was mixed with water to form a paste.

[d] The test substance was administered as an aerosolized powder.

(b) Dermal irritation

In a dermal irritation study performed according to Organisation for Economic Co-operation and Development (OECD) test guideline 404, three male Himalayan rabbits were administered 500 mg of spirodiclofen (purity 99.1%; moistened with water) dermally to a 6 cm^2 area of intact skin on the trunk of each animal for 4 h. Animals were observed for 3 days, and dermal reactions were evaluated according to the Draize method at 1, 24, 48 and 72 h following patch removal. Statements of adherence to quality assurance (QA) and GLP were included. No signs of dermal irritation were observed (Leuschner, 1997a).

(c) Ocular irritation

In an eye irritation study performed according to OECD test guideline 405, 100 mg of spirodiclofen (purity 99.1%) was instilled into the conjunctival sac of the right eye of three young adult male Himalayan rabbits for 24 h. The untreated left eye served as the control. Animals were observed for 3 days, and irritation was scored by the method of Draize at 1, 24, 48 and 72 h following administration. Statements of adherence to QA and GLP were included. No signs of irritation were noted at any point during the study (Leuschner, 1997b).

(d) Dermal sensitization

In a GLP-compliant dermal sensitization study conducted according to the Magnusson and Kligman guinea-pig maximization test (OECD test guideline 406), spirodiclofen (purity 99.1%) in physiological saline containing 2% Cremophor EL was tested using young adult female Hsd Poc:DH guinea-pigs. Animals were divided into three groups consisting of an irritation screening group (5 animals), a test group (10 animals) and a control group (5 animals). Based on the results of the irritation screening studies, the test material was administered at 5% for the intradermal injection (0.1 ml) and 50% for the topical induction (0.5 ml) as well as for the challenge applications (0.5 ml). A positive control study using 2-mercaptobenzothiazole (used at 2.5% for intradermal inductions and 40% for the topical induction and challenge treatments), a known skin sensitizer, formulated with physiological sodium chloride containing 2% by volume (v/v) Cremophor EL, was conducted within 6 months of the conduct of this study. Results of the study demonstrated the ability of the performing laboratory to identify a dermal sensitizer using the guinea-pig maximization method.

The first induction phase involved three 0.1 ml paired intradermal injections consisting of complete Freund's adjuvant diluted 1:1 with sterile physiological saline solution, 5% spirodiclofen

in vehicle and 5% spirodiclofen in equal parts vehicle and complete Freund's adjuvant. The animals of the control group were treated in the same manner as the test group, replacing the test substance with vehicle. One week later, a topical induction was performed with 0.5 ml of 50% spirodiclofen in the vehicle for a 48 h exposure period. The challenge was performed 3 weeks after the intradermal induction. A 0.5 ml topical application of 50% spirodiclofen in vehicle was administered for 24 h to test animals; control animals were treated with vehicle only. A patch loaded only with the vehicle was placed on the left flank of control animals. Skin reactions were assessed 48 and 72 h after the start of the application in accordance with the Buehler scoring scale. A second challenge was performed in the same way, with the exception that the applications were made to the opposite side of the animals.

Following the topical induction, open wounds were noted at the treatment site on day 9. These sores were healed by day 17, and no other irritation was noted. Skin reactions were observed in 4 of 10 animals (40%) in the test group and 0 of 10 animals in the control group following both the first and second challenges. Only one animal showed a positive reaction after both challenge treatments. It is concluded that spirodiclofen is a potential dermal sensitizer under the conditions of the guinea-pig maximization test. However, it should be noted that the interpretation of the test result is limited by the fact that only one animal showed a positive reaction following both challenge treatments (Stropp, 1996).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a GLP-compliant short-term feeding study, spirodiclofen (purity 99.1%) was administered to CD-1 mice (10 of each sex per dose) in the diet (which had been premixed with 1% peanut oil) at dose levels of 0, 100, 1000 or 10 000 ppm (equal to 0, 15, 164 or 1640 mg/kg bw per day for males and 0, 30, 234 or 2685 mg/kg bw per day for females) for 13 weeks. Clinical signs were assessed daily, and body weights, food consumption and water intake were recorded weekly. Blood was collected for haematology and clinical chemistry investigations on weeks 12/13 and 13/14, respectively. Ophthalmological examinations and urinalysis were not conducted. At study termination, all animals were subjected to gross necropsy, and the liver, adrenal glands and all necropsy findings were examined microscopically. In addition, the kidneys, testes, epididymides, spleen, thyroid gland and intestines from control and high-dose animals were also examined microscopically. Livers, spleen, kidney, testes, ovary, brain and adrenal glands from all animals were weighed.

There were no treatment-related effects noted for mortality, clinical signs, body weights, body weight gains, food or water consumption or haematological parameters. Cholesterol levels were decreased at 10 000 ppm (by 31% for females and by 18% for males); these decreases attained statistical significance for females only. Histopathological examinations revealed effects on the liver and adrenal cortex. Centrilobular hepatocellular hypertrophy was observed in males at 100 ppm and above and in females at 10 000 ppm, and periportal cytoplasmic vacuolation was noted in males and females at 10 000 ppm. Cytoplasmic vacuolation of the adrenal cortex occurred in males at 10 000 ppm and in females at 1000 ppm and above. In 9 out of 10 females at 10 000 ppm, this alteration was increased in severity, frequently leading to a degeneration of cortical cells accompanied by mononuclear infiltrates. Kidney weights were also decreased for males at 10 000 ppm. Originally, no findings were noted in male reproductive tissues. However, due to histopathological lesions that were observed in dogs treated with spirodiclofen, male reproductive tissues (testes, epididymides, prostates, seminal vesicles) from mice in this study were re-examined as an addendum to the main study. This re-examination revealed an increased incidence of hypertrophic Leydig cells in the testes

Table 6. Incidences of selected microscopic findings in the mouse

Histopathological finding	Incidence (no. of animals with finding)			
	Dietary concentration (ppm)			
	0	100	1000	10 000
Males (*n* = 10)				
Liver – centrilobular hepatocellular hypertrophy	0	3	3	6
Liver – periportal cytoplasmic vacuolation	0	0	0	1
Adrenal – cytoplasmic vacuolation[a]	0	0	0	8
Adrenal – degeneration of cortical cells	0	0	0	0
Adrenal – mononuclear infiltrate	0	0	0	0
Testes – Leydig cell hypertrophy	1	1	9	10[b]
Testes – Leydig cell vacuolation	0	0	0	7
Testes – degeneration of germinal epithelium	0	2	0	0
Females (*n* = 10)				
Liver – centrilobular hepatocellular hypertrophy	0	0	0	2
Liver – periportal cytoplasmic vacuolation	0	0	0	3
Adrenal – cytoplasmic vacuolation	0	0	6	10
Adrenal – degeneration of cortical cells	0	0	0	9
Adrenal – mononuclear infiltrate	0	0	0	9

From Leser & Romeike (1997, 1998); Leser & Hartmann (2002)
[a] Only nine examined.
[b] Increased severity compared with other dose levels and controls.

at 1000 ppm and above (incidence of 1/1/9/10), which was characterized by a foamy cytoplasm of the enlarged Leydig cells and was located in the intertubular interstitium and beneath the capsule. At 10 000 ppm, hypertrophy was increased in severity and was accompanied by a minimal to slight vacuolation of the Leydig cells in 7 out of 10 animals. No findings were noted in the prostate or seminal vesicles (Table 6).

The no-observed-adverse-effect level (NOAEL) was 100 ppm (equal to 30 mg/kg bw per day) for females, based on cytoplasmic vacuolation of the adrenal cortex at 1000 ppm. For males, the study authors did not establish a NOAEL owing to the hepatocellular hypertrophy noted at 100 ppm (equal to 15 mg/kg bw per day), and therefore this dose was considered to be the lowest-observed-adverse-effect level (LOAEL) for males. However, the Meeting considered that the findings of centrilobular hepatocellular hypertrophy in male mice at 100 ppm were adaptive and therefore non-adverse. As such, the NOAEL for males was also identified to be 100 ppm (equal to 15 mg/kg bw per day) based on an increased incidence of Leydig cell hypertrophy at 1000 ppm (Leser & Romeike, 1997, 1998; Leser & Hartmann, 2002).

Rats

In a GLP-compliant 4-week feeding study, spirodiclofen (purity 98.2%) was administered to female Wistar rats (five per dose) via diet (containing 1% peanut oil) at dose levels of 0, 100, 500 or 5000 ppm (equal to 0, 10, 49.6 and 569.3 mg/kg bw per day, respectively) for 4 weeks. Clinical observations were performed daily, and body weights and food and water consumption were determined weekly. Clinical laboratory examinations and gross necropsy (with organ weights and tissue sampling) were performed on all surviving animals at termination. Induction of hepatic enzymes was determined, and cell proliferation of hepatic and renal tissues was assessed immunohistochemically

Table 7. Selected clinical chemistry findings and liver enzyme levels in the female rat

Parameter	Dietary concentration (ppm)			
	0	100	500	5000
Plasma levels				
ASAT (U/l)	34.9	36.6	32.0	43.1**
ALAT (U/l)	41.6	45.4	37.7	54.7*
AP (U/l)	403	371	364	1260
Total protein (g/l)	64.5	63.9	62.9	59.2**
Triglyceride (mmol/l)	0.76	1.11*	0.76	0.40*
Cholesterol (mmol/l)	2.07	2.06	1.93	1.08**
Liver tissue levels				
ECOD (nmol/g per minute)	1.5	1.6	1.9*	2.3*

From Kroetlinger & Geiss (2000)
ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; U, units; * $P < 0.05$; ** $P < 0.01$

(using the proliferating cell nuclear antigen technique). Organs and tissues were microscopically examined. Immunotoxicological investigations were also performed, which included measures of cell counts in the spleen, lymph nodes and bone marrow, as well as assessments of subpopulations of spleen and lymph node cells, macrophage activity and responsiveness of the spleen and lymph node cells (by FACScan analyses). Serum antibody titres (immunoglobulins [Ig] IgG, IgM, IgA) were also determined.

There were no treatment-related effects on mortality, clinical signs, body weight, food and water consumption or any of the haematological parameters tested. Treatment-related effects were observed in clinical chemistry of animals receiving 5000 ppm and included increases in aminotransferase and alkaline phosphatase (AP) activities and decreases in total protein, triglyceride and cholesterol levels. While these parameters may be considered incidental on their own, taken together, the study authors suggested that they indicate an effect of treatment on the liver. Biochemical determinations of hepatic tissue showed induction of a phase I, cytochrome P450–dependent mono-oxygenase enzyme (7-ethoxycoumarin deethylase [ECOD]; increased by 53%) at 5000 ppm (Table 7). The study authors indicated that the determination of cell counts in the spleen, mesenteric lymph node and femoral bone marrow and mitogen stimulation did not result in treatment-related effects. They also concluded that FACScan analyses revealed a decrease in splenic T cells after treatment with 5000 ppm and that titres of IgG antibodies were reduced after treatment with 500 and 5000 ppm. The effects on the immune system were considered by the study authors to occur at doses that caused signs of significant toxicity and were so slight that they were not considered to represent adverse immunotoxic effects.

The Meeting concluded that the results of the immune system tests were inconclusive, based on the following considerations: 1) the FACScan analyses were done in pooled (group) samples (in addition, the study report stated that these data were compared with historical control data in order to establish whether the findings were "significant"; however, these historical control data were not provided); 2) only graphs were submitted of antibody (IgG, IgA, IgM) titres; 3) the immunotoxicity report stated that there was an unusually high variation in controls for the IgA titre, size distributions of lymph node cells in the FACScan analyses at 100 ppm as well as all spleen cell counts, which prevented the detection of dose-related effects for these parameters; and 4) no individual data were submitted to validate the results.

Gross pathological and histopathological examinations did not reveal any treatment-related changes. Cell proliferation studies revealed no effect on hepatic cells; however, increases noted in

kidney cell proliferation in both the medulla and the cortex were considered to reflect a toxic effect of treatment.

The NOAEL in this study was 500 ppm (equal to 49.6 mg/kg bw per day), based on clinical chemistry findings (increases in aminotransferase and AP activities and decreases in total protein, triglyceride and cholesterol levels) and liver enzyme induction at 5000 ppm. The Meeting noted that the study was stated to have been conducted in accordance with OECD test guideline 407 (adopted in 1995); however, the study is not acceptable according to this guideline, owing to the use of only one sex (five females per group). According to the guideline, five animals of each sex per group should be used (Kroetlinger & Geiss, 2000).

In a GLP-compliant 14-week feeding study, spirodiclofen (purity 99.1%) was administered to Wistar rats (10 of each sex per dose) via the diet (containing 1% peanut oil) at dose levels of 0, 100, 500, 2500 or 12 500 ppm (equal to 0, 6.6, 32.1, 166.9 and 851.4 mg/kg bw per day for males and 0, 8.1, 47.1, 215.3 and 995.8 mg/kg bw per day for females, respectively) for 14 weeks. Additional groups of 10 males and 10 females were treated with 0 or 12 500 ppm of spirodiclofen (equal to 0 or 902.2 mg/kg bw per day for males and 0 or 1094.7 mg/kg bw per day for females) for 14 weeks and then observed for reversible effects over 4 weeks. A satellite group of rats (five of each sex per dose) was dosed for 4 weeks with the same doses of spirodiclofen as in the main study for immunotoxicological investigations. Clinical observations were performed daily, and body weights and food consumption were determined twice weekly. Ophthalmological examinations were performed on all animals of all groups before the start of the study and at week 11. Clinical chemistry, haematology and urinalysis examinations were conducted on blood or urine samples from weeks 5 and 13 in the main study and from week 17 in the recovery group. Levels of thyroid hormones (triiodothyronine [T_3], thyroxine [T_4] and thyroid stimulating hormone [TSH]) in plasma were also measured at these time points. Gross necropsy (with organ weights and tissue sampling) and histopathological examinations were performed on all animals in both the main study and recovery groups. Four days prior to necropsy, animals in the satellite group were immunized in vivo with sheep red blood cells, and a plaque-forming cell assay (PFCA) was performed. Other immunotoxicological investigations included measures of cell counts in the spleen, lymph nodes and bone marrow (methods not reported) and assessments of subpopulations of spleen and lymph node cells (by FACScan), macrophage activity in spleen and mesenteric lymph nodes following phorbol myristate acetate stimulation as well as serum antibody titres (IgG, IgM, IgA) using enzyme-linked immunosorbent assay.

There were no treatment-related effects on mortality, clinical signs, ophthalmoscopic examinations or water intake. During the treatment period, statistically significant decreases in body weights were observed at 12 500 ppm in both sexes (by 13% in males and by 12% in females). Slight decreases in body weights were also observed in both sexes at 2500 ppm (by 4% in males and by 7% in females). During the recovery period, the decreases in body weights in the 12 500 ppm group became less apparent (decreased by 7% in males and by 5% in females). Food consumption was comparable to that of controls during treatment up to and including 500 ppm in both sexes. At 2500 and 12 500 ppm, it was significantly reduced in the first determination period (possibly due to adaptation) and occasionally during further treatment at 12 500 ppm in both sexes. During the recovery period, food intake in females was increased.

Increased erythrocyte counts and reduced mean corpuscular volume and mean corpuscular haemoglobin values were noted in females at 12 500 ppm during treatment and after the recovery period. These differences were slight and were not regarded as indicative of primary haematotoxicity. Significant decreases in leukocyte counts in the 12 500 ppm group of both sexes, decreases in thrombocyte counts in males at 12 500 ppm and slight but statistically significant increases in thromboplastin times at 2500 and 12 500 ppm for both sexes were also noted. The increases in thromboplastin times were not considered biologically relevant. After the recovery period, the blood coagulation

parameters were comparable to those of controls. Clinical chemistry showed significant increases in AP activity at 2500 and 12 500 ppm (both sexes) and in asparate aminotransferase (ASAT) and alanine aminotransferase (ALAT) activities at 12 500 ppm (both sexes). Decreases in glutamate dehydrogenase (GLDH) activity were also noted in both sexes, but were not always dose dependent. Significantly decreased plasma cholesterol and triglyceride levels were observed at 12 500 ppm in both sexes and at 2500 ppm in males only. Significantly decreased serum phosphate concentrations were noted at 12 500 ppm in both sexes. Differences in ALAT activity, AP activity, cholesterol levels and triglyceride levels were still noted at the end of the recovery period in males. Plasma levels of TSH were slightly increased in females at 2500 and 12 500 ppm. After the recovery period, higher T_3 and T_4 and lower mean TSH concentrations were determined for males. As differences were slight and did not result in histological changes, they were not considered to be toxicologically relevant (Table 8). Increases in absolute and relative adrenal weights were observed at 12 500 ppm (both sexes), which correlated with microscopic findings in the adrenal gland. These consisted of an increase in severity of uniformly small cytoplasmic vacuolation in the cortex of males at doses of 2500 ppm and above and in females at 500 ppm and above. At the high dose, an increase in severity of mixed cytoplasmic vacuolation in the adrenal cortex was also noted. Other histopathological findings included minimal to moderate vacuolation in the small intestine (mostly the jejunum) at 2500 ppm and above (both sexes) and reduced glycogen content of hepatocytes in four high-dose females (Table 9). After the recovery period, there were no toxicologically relevant differences in organ weights or histopathology.

The study authors indicated that the immunotoxicity investigations revealed slight decreases in cell counts (males at 12 500 ppm) and subpopulation changes (males at 2500 ppm and both sexes at 12 500 ppm) in the spleen. In addition, the study authors noted decreases in IgM and IgG antibodies for females at 12 500 ppm in the PFCA, and serum IgA antibody titres were also slightly reduced in females at 2500 ppm and above. Absolute and relative spleen (females) and thymus weights (males) were reduced at 12 500 ppm. The Meeting considered that the results of the immunotoxicity tests were inconclusive based on the following considerations: 1) no individual animal data were submitted, and thus the results could not be validated; 2) the FACScan analysis for lymphocyte subpopulations was performed on pooled samples, and the findings were difficult to interpret; 3) results of the macrophage activity, PFCA and antibody titres were presented in graphs only, and no individual data were submitted; and 4) the report stated that "regarding the PFCA for male rats it is obvious that the animals were not stimulated properly", and therefore the accuracy of the results of the PFCA is questionable. As the immunotoxicity testing is not part of the guideline requirement, elimination of these data does not affect its acceptability for a guideline subchronic study.

The NOAEL for females was determined to be 100 ppm (equal to 8.1 mg/kg bw per day), based on cytoplasmic vacuolation of the adrenal cortex at 500 ppm, whereas the NOAEL for males was 500 ppm (equal to 32.1 mg/kg bw per day), based on clinical chemistry findings (increased AP and decreased cholesterol and triglyceride levels) and vacuolation of the adrenal cortex and the small intestine at 2500 ppm (Wirnitzer & Romeike, 1998; Wirnitzer & Hartmann, 2002a).

Dogs

In a 4-week feeding dose range–finding study, spirodiclofen (purity 99.1%) was administered to groups of Beagle dogs (two of each sex per dose) in the diet (mixed with water to form a paste) at dose levels of 0, 400, 2000 or 10 000 ppm (equal to 0, 11.3, 63.5 and 284.5 mg/kg bw per day for males and females combined) for 4 weeks. Although this study did not comply with GLP, it was performed in a GLP-certified laboratory and followed general GLP procedures. Clinical observations were conducted twice daily, food and water intake were assessed daily and body weights were recorded weekly. Reflexes were assessed, and body temperature and pulse were recorded before the start of the study and at week 4. Blood was collected for haematological and clinical chemistry investigations at pretest and during weeks 2 and 4. Levels of plasma T_3 and T_4 and thyroxine-binding capacity were

Table 8. Selected clinical chemistry findings in the rat

Clinical chemistry parameter	Dietary concentration (ppm)					Historical control range[a]
	0	100	500	2500	12 500	
Males						
Week 5						
Cholesterol (mmol/l)	2.26	2.18	2.02	1.60**	1.15**	
Triglyceride (mmol/l)	1.67	1.75	1.36	0.90**	0.58**	
T_3 (nmol/l)	1.41	1.62	1.61	1.56	1.53	1.35–1.78
T_4 (nmol/l)	59	59	65	64	69*	63–67
TSH (µg/l)	3.75	4.04	6.05	4.04	3.31	
Week 13						
Cholesterol (mmol/l)	2.07	2.04	2.11	1.63**	1.29**	
Triglyceride (mmol/l)	1.79	1.45	2.16	1.22	0.80**	
T_3 (nmol/l)	1.28	1.41	1.44	1.34	1.35	1.28–1.74
T_4 (nmol/l)	56	52	56	54	59	40–65
TSH (µg/l)	2.79	3.42	4.14	3.97	3.71	
Recovery						
Cholesterol (mmol/l)	2.25	—	—	—	1.92*	
Triglyceride (mmol/l)	2.13	—	—	—	1.45**	
T_3 (nmol/l)	1.39	—	—	—	1.63**	1.23–1.85
T_4 (nmol/l)	58	—	—	—	65*	43–59
TSH (µg/l)	4.42	—	—	—	1.95*	
Females						
Week 5						
Cholesterol (mmol/l)	2.01	2.01	1.80	1.71	1.17**	
Triglyceride (mmol/l)	1.40	1.30	0.92*	0.61**	0.46**	
T_3 (nmol/l)	1.65	1.63	1.69	1.52	1.56	1.31–1.72
T_4 (nmol/l)	48	47	50	52	56	48–61
TSH (µg/l)	1.39	1.21	1.29	1.99	2.96**	
Week 13						
Cholesterol (mmol/l)	2.04	2.17	1.88	1.79	1.24**	
Triglyceride (mmol/l)	1.27	1.35	1.19	0.85	0.41**	
T_3 (nmol/l)	1.39	1.48	1.49	1.47	1.46	1.31–1.69
T_4 (nmol/l)	42	44	46	46	45	39–49
TSH (µg/l)	0.84	1.36*	1.25	2.33**	2.87**	
Recovery						
Cholesterol (mmol/l)	1.87	—	—	—	2.02	
Triglyceride (mmol/l)	1.54	—	—	—	1.15	
T_3 (nmol/l)	1.45	—	—	—	1.45	1.42–1.68
T_4 (nmol/l)	44	—	—	—	42	37–50
TSH (µg/l)	1.23	—	—	—	1.44	

From Wirnitzer & Romeike (1998); Wirnitzer & Hartmann (2002a)

* $P < 0.05$; ** $P < 0.01$

[a] Historical control data for T_3 and T_4 collected from five subchronic rat studies ($n = 10$ for each sex) conducted between 1991 and 1994.

Table 9. Incidences and severity of selected microscopic findings in the rat

Histopathological finding	Incidence (no. of animals with finding) / severity[a]				
	Dietary concentration (ppm)				
	0	100	500	2500	12 500
Males					
Main study (n = 10)					
Jejunum – epithelial vacuolation	0	0	0	8 / 1.6	8 / 1.9
Adrenal – small cortical vacuolation	5 / 1.4	9 / 1.7	8 / 1.4	10 / 3.3	10 / 3.3
Adrenal – mixed cortical vacuolation	7 / 2.3	7 / 1.7	6 / 1.5	9 / 1.3	10 / 2.9
Recovery (n = 10)					
Adrenal – small cortical vacuolation	8 / 2.0	—	—	—	8 / 2.3
Adrenal – mixed cortical vacuolation	6 / 2.3	—	—	—	5 / 1.6
Females					
Main study (n = 10)					
Jejunum – epithelial vacuolation	0	0	0	7 / 2.3	7 / 2.4
Adrenal – small cortical vacuolation	4 / 1.5	3 / 1.7	8 / 1.8	8 / 2.0	10 / 3.8
Adrenal – mixed cortical vacuolation	0	0	0	0	0
Liver – reduced glycogen content	0	0	0	0	4
Recovery (n =10)					
Adrenal – small cortical vacuolation	7 / 1.9	—	—	—	7 / 2.0
Adrenal – mixed cortical vacuolation	0	—	—	—	0

From Wirnitzer & Romeike (1998); Wirnitzer & Hartmann (2002a)

[a] The severity scores represent the average score for each group, based on the following scale: Grade 1: Minimal/very few/very small; Grade 2: Slight/few/small; Grade 3: Moderate/moderate number/moderate size; Grade 4: Marked/many/large; Grade 5: Massive/extensive number/extensive size.

included in these investigations. Urinalysis was conducted at the same time points. Gross necropsy (including organ weight assessments) and microscopic examinations were carried out at study termination. In addition, liver triglyceride levels and activities of the following enzymes were determined: microsomal liver enzymes (aminopyrine-*N*-demethylase [N-DEM] and *p*-nitroanisole-*O*-deethylase [O-DEM]), cytochrome P450, cytochrome P450–dependent mono-oxygenases (ECOD, 7-ethoxyresorufin *O*-deethylase [EROD], aldrin epoxidase [ALD]), epoxide hydrolase (EH) and the conjugation enzymes (glutathione *S*-transferase [GST], uridine diphosphate (UDP)–glucuronyltransferase [GLUT]).

There were no compound-related effects on mortality, clinical signs, body weight, food and water consumption, urinalysis or haematological parameters assessed. Increased plasma activities of ASAT, ALAT, AP and lactate dehydrogenase were observed in 10 000 ppm male and female dogs in a time-dependent manner. One female at 2000 ppm also showed an increased ASAT activity at weeks 2 and 4. GLDH levels appeared to be increased for high-dose males, and T_4 levels were slightly decreased for both sexes, beginning at 2000 ppm, in a time-dependent manner. Cholesterol levels were reduced at 10 000 ppm in both sexes at weeks 2 and 4. Serum protein, albumin and iron levels were also decreased at the high dose. A dose-dependent increase in microsomal liver enzymes (O-DEM and N-DEM) was observed in animals at doses of 2000 ppm and above. The cytochrome P450–dependent mono-oxygenases (ECOD and ALD) were also induced by spirodiclofen at doses of 2000 ppm and above, and there was a marginal increase noted in GLUT activity at the high dose. Increased relative adrenal weights were observed at 2000 ppm and above, and increased relative kidney weights

Table 10. Incidences of selected microscopic findings in the dog

Histopathological finding	Incidence (no. of animals with finding)			
	Dietary concentration (ppm)			
	0	400	2000	10 000
Males (*n* = 2)				
Adrenal – cytoplasmic vacuolation	0	0	2	2
Jejunum – vacuolation of epithelium	0	0	1	2
Liver – periportal single-cell necrosis	0	0	0	2
Testes – Leydig cell vacuolation	0	0	2	2
Testes – Leydig cell hypertrophy	0	0	0	1
Testes – immature	0	0	0	1
Prostate – immature	0	0	0	1
Epididymides – oligospermia/aspermia	0	0	0	1
Females (*n* = 2)				
Adrenal – cytoplasmic vacuolation	1	1	2	2
Jejunum – vacuolation of epithelium	0	0	0	1
Liver – periportal single-cell necrosis	0	0	0	2

From Wetzig, Romeike & Sander (2001)

were observed at 10 000 ppm. Although there was a slight, dose-dependent increase in relative liver weight, values from all groups were within historical control ranges. Histopathological examinations revealed that all high-dose animals showed minimal to slight periportal single-cell necrosis in the liver. In the adrenal glands, animals dosed at 2000 ppm and above showed an increased cytoplasmic vacuolation in the cortex. In the intestine (jejunum), a moderate to marked vacuolation of superficial mucosal epithelial cells was found in animals dosed at 2000 ppm and higher. In the testis, vacuolation of Leydig cells was found in males dosed at 2000 ppm and above. In one high-dose animal, this finding was combined with hypertrophy/activation of the Leydig cells. Furthermore, testes and prostate of this animal were considered immature. The epididymides of one male also showed a massive oligospermia/aspermia and slight spermatic debris (Table 10). No correlates to the increased kidney weights were seen in the clinical chemistry, urinary or histopathological assessments.

The NOAEL is 400 ppm (equal to 11.3 mg/kg bw per day, both sexes combined), based on liver enzyme induction, increased adrenal weights and histopathological findings in the adrenal gland and testes at 2000 ppm (Wetzig, Romeike & Sander, 2001).

In a GLP-compliant 14-week dietary study, spirodiclofen (purity 98.6%) was administered to groups of Beagle dogs (four of each sex per dose) in the diet (mixed with water to form a paste) at dose levels of 0, 200, 630 or 2000 ppm (equal to 0, 7.7, 26.6 and 84.7 mg/kg bw per day for males and 0, 8.4, 28.0 and 81.0 mg/kg bw per day for females, respectively) for 14 weeks. Clinical observations were conducted twice daily, food and water intake were assessed daily and body weights were recorded weekly. Ophthalmoscopic and reflex assessments, body temperature and pulse recordings as well as electrocardiogram measurements were conducted before the start of the study (week −2) and at weeks 6 and 13. Blood was collected for haematological and clinical chemistry investigations at pretest (weeks −3 and −1) and during weeks 2, 6 and 13. Levels of plasma T_3 and T_4 and thyroxine-binding capacity were included in these investigations. Urinalysis was conducted at weeks −1, 2, 6 and 13. Gross necropsy (including organ weight assessments) and microscopic examinations were carried out at study termination. In addition, triglyceride levels

Table 11. Selected clinical chemistry findings[a] in the dog

Clinical chemistry parameter	Dietary concentration (ppm)				Historical control range[b]
	0	200	630	2000	
Male (*n* = 4)					
Cholesterol (mmol/l)					
Week −3	3.4 ± 0.5	2.3 ± 0.6	2.9 ± 0.6	3.3 ± 0.7	2.8–4.0
Week 2	3.1 ± 0.5	2.2 ± 0.4	2.9 ± 0.7	2.2 ± 0.5	2.8–3.5
Week 6	3.2 ± 0.4	2.3 ± 0.6	2.4 ± 0.8	1.9 ±0.3	3.2–4.2
Week 13	3.9 ± 0.9	2.8 ± 0.5	2.6 ± 0.5	2.6 ± 0.9	3.2–4.7
T_4 (nmol/l)					
Week −3	29 ± 6.6	24 ± 4.5	30 ± 4.6	28 ± 1.5	19–23
Week 2	29 ± 1.8	25 ± 3.6	26 ± 9.0	24 ± 4.4	19–23
Week 6	32 ± 3.6	28 ± 3.1	25 ± 6.5	19 ± 2.4	19–22
Week 13	27 ± 4.2	26 ± 2.1	24 ± 7.0	16 ± 2.5	19–24
Female (*n* = 4)					
Cholesterol (mmol/l)					
Week −3	2.4 ± 0.4	2.4 ± 0.3	2.7 ± 0.5	3.0 ± 0.8	3.0–4.2
Week 2	2.6 ± 0.4	2.3 ± 0.4	2.4 ± 0.3	2.1 ± 1.0	3.1–3.2
Week 6	2.7 ± 0.3	2.5 ± 0.3	2.4 ± 0.5	1.8 ± 0.5	3.4–4.2
Week 13	2.9 ± 0.9	2.6 ± 0.3	3.1 ± 0.6	1.9 ± 0.5	3.5–4.3
T_4 (nmol/l)					
Week −3	37 ± 5.9	32 ± 3.1	32 ± 9.0	33 ± 10.1	21–25
Week 2	31 ± 1.6	29 ± 2.1	23 ± 5.4	21 ± 4.9	21
Week 6	30 ± 2.8	32 ± 2.2	25 ± 1.5	19 ± 4.0	20–22
Week 13	28 ± 1.4	28 ± 2.1	24 ± 6.2	18 ± 5.1	21–24

From Wetzig & Hartmann (2001b, 2002a)

[a] Mean ± standard deviation.

[b] Historical control data for cholesterol and T_4 collected from three subchronic dog studies (*n* = 4 for each sex) conducted between 1993 and 1995; time periods for blood collection in these studies were prestudy, 2–4 weeks, 5–7 weeks and 13 weeks.

and activities of the following enzymes were determined in liver tissue: microsomal liver enzymes (N-DEM and O-DEM), cytochrome P450, cytochrome P450–dependent mono-oxygenases (ECOD, EROD, ALD), EH and the conjugation enzymes (GST, GLUT).

There were no compound-related effects on mortality, clinical signs, food and water consumption, haematology, urinalysis or ophthalmoscopic examinations. There was a dose-dependent decrease in body weight noted in both sexes, which was considered treatment related at and above the middle dose. Increased plasma aminotransferase (ASAT, ALAT), AP and GLDH activities were observed at doses of 630 ppm and above in females and at 2000 ppm in males. A trend of lower cholesterol and T_4 levels was observed at 630 and 2000 ppm (both sexes), as well as for albumin levels at 2000 ppm. When compared with historical control data (from 1996–1997), all of these parameters were within two standard deviations of the reference ranges (Table 11). There was an induction of phase I enzymes (cytochrome P450–dependent mono-oxygenases [ECOD and ALD]) in response to spirodiclofen administration at all doses in males and at the high dose in females. A slight increase was also measured for cytochrome P450 content in the 630 ppm and 2000 ppm groups. There were no effects on the phase II enzyme activities (GST and GLUT) or on triglyceride levels in the liver. EH was induced at the high dose in females only. Dose-dependent increases in N-DEM and O-DEM levels were seen in both sexes. The changes in liver enzyme activities suggest an induction of hepatic metabolic activity in response to administration of spirodiclofen. These changes were considered adaptive

Table 12. Incidences of selected microscopic findings in the dog

Histopathological finding	Incidence (no. of animals with finding)			
	Dietary concentration (ppm)			
	0	200	630	2000
Males (*n* = 4)				
Adrenal – cytoplasmic vacuolation	0	0	3	4
Adrenal – mononuclear cell infiltrate	0	1	1	4
Kidney – dilatation of proximal tubule	1	0	1	3
Epididymides – aspermia	0	0	1	2
Epididymides – oligospermia	0	0	2	0
Testes – Leydig cell vacuolation	0	0	2	4
Testes – Leydig cell hypertrophy	0	0	2	3
Testes – degeneration, diffuse	0	0	0	2
Prostate – immature	0	0	1	4
Thymus – atrophy	0	0	1	2
Females (*n* = 4)				
Adrenal – cytoplasmic vacuolation	0	2	3	4
Adrenal – mononuclear cell infiltrate	0	2	0	4
Kidney – dilatation of proximal tubule	0	1	0	2
Liver – periportal single-cell necrosis	0	0	0	1
Liver – cytoplasmic change	0	0	0	3
Liver – inflammatory infiltrates	0	0	0	4
Thymus – atrophy	0	0	0	1

From Wetzig & Hartmann (2001b, 2002a)

and thus not regarded as adverse. Dose-dependent increases in relative organ weights were observed in both sexes in the liver, pituitary and adrenal gland at 630 and 2000 ppm and in the kidney at 2000 ppm. Increased adrenal weights were also observed in two of four females at the low dose (200 ppm). Decreased prostate and thymus weights were also observed in males at 630 and 2000 ppm.

Histopathological examination revealed treatment-related findings in the liver, kidney, adrenal gland, prostate, epididymis, testes and thymus. Hepatocellular cytoplasmic changes, inflammatory infiltrates and single-cell necrosis were seen in females at the highest dose. Inflammatory infiltrates and necroses were considered to represent a toxic injury to the liver. Dilatation of the proximal tubules of the renal cortex was seen in both sexes at 2000 ppm. This finding was considered an adaptive effect, as no degenerative changes were noted in the kidney. In the testes, vacuolation and hypertrophy/activation of Leydig cells were observed at 630 and 2000 ppm. In addition, degeneration and/or immaturity of the testicular germinal epithelium, as well as oligospermia and aspermia of the epididymides, were detected at doses of 630 ppm and above. The prostates of all high-dose males and one male of the intermediate dose group were completely undeveloped compared with those of control males of similar age. Mild atrophy of the thymic cortex was observed in one male at 630 ppm and in both sexes at 2000 ppm. This finding was considered more likely due to an indirect toxicity rather than to direct cytotoxicity to thymic lymphocytes. A dose-dependent increase in cytoplasmic vacuolation of the adrenal cortex was observed in females at all doses and in males at 630 ppm and above, which was interpreted as a morphological correlate to effects on lipid and steroid metabolism (Table 12).

The NOAEL for males in this study was 200 ppm (equal to 7.7 mg/kg bw per day), based on decreased body weight gain, decreased prostate and thymus weights, increased relative liver, pituitary and adrenal weights and histopathological findings in the adrenal gland, thymus, epididymides, testes and prostate at 630 ppm. A NOAEL could not be established for females in this study, as adrenal effects were noted in females at all doses tested; the LOAEL for females was therefore 200 ppm, equal to 8.4 mg/kg bw per day (Wetzig & Hartmann, 2001b, 2002a).

In a GLP-compliant 1-year dog study, spirodiclofen (purity 97.8%) was administered to groups of Beagle dogs (four of each sex per group) in the diet (mixed with water to form a paste) at dose levels of 0, 20, 50, 150 or 500/600 (equal to 0, 0.56, 1.38, 4.33 and 16.1 mg/kg bw per day for males and 0, 0.59, 1.52, 4.74 and 17.7 mg/kg bw per day for females, respectively) for 1 year. The highest dose was increased from 500 to 600 ppm in week 4 of the study. Clinical observations were conducted twice daily, food and water intake were assessed daily and body weights were recorded weekly. Ophthalmoscopic and reflex assessments, body temperature recordings, as well as electrocardiogram and blood pressure measurements were conducted before the start of the study (week −3) and at weeks 6, 12, 26/27, 39 and 52. Blood was collected for haematological and clinical chemistry investigations at pretest (weeks −3 and −1) and during weeks 3, 6, 12, 26, 39 and 52. Levels of plasma T_3 and T_4 and thyroxine-binding capacity were included in these investigations. Additional blood samples were taken from high-dose animals during week 20 in order to determine the plasma concentration of spirodiclofen and its metabolite, BAJ 2510, at 0, 2, 4, 7 and 24 h following dosing. Urinalysis was conducted at the same time points as blood collections. Gross necropsy (including organ weight assessments) and microscopic examinations were carried out at study termination. Additional urine samples were collected at week 28 from some high-dose and control animals in order to determine concentrations of the BAJ 2510 metabolite. In addition, triglyceride levels and activities of the following enzymes were determined in liver tissue: microsomal liver enzymes (N-DEM and O-DEM), cytochrome P450, cytochrome P450–dependent mono-oxygenases (ECOD, EROD, ALD), EH and the conjugation enzymes (GST, GLUT).

There were no compound-related effects on mortality, clinical signs, food and water consumption, body weight gain, urinalysis, haematology or ophthalmoscopic examinations. Cholesterol levels were decreased slightly in the 150 and 600 ppm groups; however, values were found to be within historical control ranges (Table 13). No other findings were noted in the clinical chemistry investigations. Increases in N-DEM and O-DEM activities in liver tissue were noted. These increases were dose dependent for N-DEM, beginning at 50 ppm, and noted at the high dose only for O-DEM; they were indicative of an induction of hepatic metabolic activity in response to spirodiclofen administration, which was not considered to be adverse. Dose-related increases in relative weight were observed in adrenal glands of both sexes (more pronounced in females), as well as in testes and prostates in males (Table 14). Histopathological examinations revealed an increased incidence of cortical vacuolation in the zona fasciculata of the adrenal gland in both sexes at and above 150 ppm. In the testes, an increased incidence of Leydig cell vacuolation was noted at the high dose. Slight Leydig cell hypertrophy and tubular degeneration were each observed in one male at 500/600 ppm (Table 15). There were no histopathological findings correlating with the increased prostate weights.

The NOAEL is 50 ppm, equal to 1.38 mg/kg bw per day and 1.52 mg/kg bw per day for males and females, respectively, based on increased relative adrenal weights in both sexes, increased relative testis weight in males and histopathological findings in the adrenal gland of both sexes at 150 ppm (Wetzig & Ruehl-Fehlert, 2001; Wetzig & Hartmann, 2002b).

Table 13. Cholesterol findings in the dog (1-year study)

Week	Cholesterol level[a] (mmol/l)					
	Dietary concentration (ppm)					Historical control data[b]
	0	20	50	150	500/600	
Males (*n* = 4)						
Week −3	3.93 ± 0.78	4.62 ± 0.70	4.84 ± 0.54	4.81 ± 0.44	4.70 ± 0.89	3.93 ± 0.78
Week 3	3.29 ± 0.47	3.75 ± 0.64	3.79 ± 0.76	3.73 ± 0.46	3.52 ± 0.90	3.16 ± 0.27
Week 6	3.16 ± 0.27	3.13 ± 0.49	3.62 ± 0.77	3.06 ± 0.34	2.32 ± 0.59	2.75 ± 0.71
Week 12	2.75 ± 0.71	3.30 ± 0.47	3.90 ± 0.76	3.78 ± 0.27	2.92 ± 0.66	3.26 ± 0.62
Week 26	3.26 ± 0.62	4.26 ± 0.51	4.07 ± 0.91	4.27 ± 0.59	3.50 ± 0.52	3.55 ± 0.73
Week 52	3.28 ± 0.57	4.82 ± 0.99	4.09 ± 1.05	3.88 ± 0.33	3.77 ± 0.31	3.28 ± 0.57
Females (*n* = 4)						
Week −3	3.93 ± 0.66	3.76 ± 0.44	4.09 ± 0.74	4.12 ± 0.80	3.99 ± 0.25	3.93 ± 0.66
Week 3	3.88 ± 0.68	3.18 ± 0.32	3.26 ± 0.59	3.05 ± 0.36	3.07 ± 0.42	3.16 ± 0.77
Week 6	3.16 ± 0.77	2.77 ± 0.18	2.73 ± 0.33	2.82 ± 0.32	2.67 ± 0.64	2.73 ± 0.29
Week 12	2.73 ± 0.29	3.33 ± 0.39	3.08 ± 0.45	2.87 ± 0.31	3.04 ± 0.48	4.39 ± 0.84
Week 26	4.39 ± 0.84	4.81 ± 1.01	3.88 ± 0.75	3.47 ± 0.89	4.21 ± 0.59	5.90 ± 1.56
Week 52	5.89 ± 1.71	4.58 ± 0.69	4.09 ± 0.02	5.54 ± 2.36	4.77 ± 1.36	5.89 ± 1.71

From Wetzig & Ruehl-Fehlert (2001); Wetzig & Hartmann (2002b)

[a] Mean ± standard deviation.

[b] Historical control data collected from four chronic dog studies (n = 4 in each sex) conducted between 1994 and 1996; time periods for blood collection in these studies were prestudy, 4–6 weeks and weeks 13, 26, 39 and 52.

Table 14. Selected relative organ weight findings in the dog (1-year study)[a]

Organ	Dietary concentration (ppm)				
	0	20	50	150	500/600
Males (*n* = 4)					
Body weight (kg)	15.0 ± 2.9	14.8 ± 1.2	15.5 ± 0.67	15.1 ± 1.8	16.28 ± 2.20
Liver (g/kg)	29.0 ± 4.9	34.9 ± 5.8	35.8 ± 14.3	34.8 ± 3.8	34.24 ± 4.11
Adrenal (g/kg)	0.106 ± 0.024	0.121 ± 0.027	0.119 ± 0.028	0.115 ± 0.014	0.123 ± 0.027
Testis (g/kg)	1.38 ± 0.16	1.64 ± 0.20	1.4 ± 0.19	1.77 ± 0.13	1.79 ± 0.086
Prostate (g/kg)	0.70 ± 0.28	0.71 ± 0.17	0.72 ± 0.261	0.73 ± 0.25	0.83 ± 0.20
Females (*n* = 4)					
Body weight (kg)	14.2 ± 1.9	13.6 ± 1.7	13.9 ± 1.2	14.4 ± 1.1	14.7 ± 1.2
Liver (g/kg)	32.4 ± 1.5	30.1 ± 6.1	32.6 ± 8.8	30.5 ± 5.5	32.9 ± 4.0
Adrenal (g/kg)	0.131 ± 0.030	0.139 ± 0.021	0.130 ± 0.014	0.145 ± 0.014	0.154 ± 0.012
Kidney (g/kg)	4.2 ± 0.41	4.6 ± 0.54	4.8 ± 1.02	4.8 ± 0.50	5.0 ± 0.14

From Wetzig & Ruehl-Fehlert (2001); Wetzig & Hartmann (2002b)

[a] Organ weights are relative to body weight; mean ± standard deviation.

Table 15. Incidences of selected microscopic findings in the dog (1-year study)

Histopathological finding	Incidence (no. of animals with finding)				
	Dietary concentration (ppm)				
	0	20	50	150	500/600
Males (*n* = 4)					
Adrenal – cortical vacuolation	1	2	0	4	4
Kidney – increased tubular pigment	2	4	3	0	1
Thyroid – vacuoles	2	2	3	1	1
Testes – Leydig cell vacuolation	0	0	0	0	4
Testes – Leydig cell hypertrophy	0	0	0	0	1
Testes – tubular degeneration	0	0	0	0	1
Females (*n* = 4)					
Adrenal – cortical vacuolation	1	1	0	3	4
Kidney – increased tubular pigment	1	1	1	1	3
Thyroid – vacuoles	1	0	1	0	3

From Wetzig & Ruehl-Fehlert (2001); Wetzig & Hartmann (2002b)

(b) Dermal application

Rat

In a GLP-compliant 4-week dermal toxicity study, spirodiclofen (purity 97.9%) was applied (undiluted; moistened with water immediately prior to application) to the shaved skin of Wistar rats (five of each sex per dose) at dose levels of 0 or 1000 mg/kg bw per day (limit dose), 6 h/day, 5 days/week (total of 22 applications), during a 28-day period. Clinical observations (including examinations for signs of dermal irritation) were performed daily, and body weights and food consumption were determined weekly. Swelling of the skin was evaluated at regular intervals throughout the study by measuring skinfold thickness in the treatment area. Clinical laboratory examinations and gross necropsy (with organ weights and tissue sampling) were performed on all surviving animals at termination. Histopathological examination of the liver, lung, heart, spleen, thymus, kidneys, testes, epididymides, brain, adrenal glands and skin was performed. There were no compound-related effects on mortality, clinical signs, body weight, food consumption, haematology, clinical chemistry, organ weights or gross and histological pathology.

Based on the study results, the NOAEL was 1000 mg/kg bw per day (the limit dose) for males and females (Kroetlinger & Sander, 1999). It was noted that several tissues were not weighed or subjected to histopathological assessment, including the prostate and all female reproductive tissues, among others.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a GLP-compliant carcinogenicity study, spirodiclofen (purity 97.6–98.6%) was administered to groups of CD-1 mice (50 animals of each sex per dose) in the diet (in an acetone/corn oil mixture) at concentrations of 0, 25, 3500 or 7000 ppm (equal to 0, 4.1, 610 and 1216 mg/kg bw per day for

males and 0, 5.1, 722 and 1495 mg/kg bw per day for females, respectively) for 18 months. Body weight and food consumption determinations were conducted weekly for approximately 13 weeks and once a month thereafter; detailed clinical examinations of each animal were conducted weekly throughout the study. Standard haematological and differential leukocyte parameters were evaluated at approximately 12 and 18 months into the study. All animals were subjected to a postmortem examination, which included documenting and saving all gross lesions, weighing designated organs and collecting tissues for histopathological evaluation.

There were no compound-related effects on mortality, clinical signs, body weight, food consumption or haematological examinations. Increased absolute and relative liver weights were observed in males at 3500 ppm and in both sexes at 7000 ppm. Increased absolute and relative adrenal gland weights were noted at 3500 and 7000 ppm in both sexes. Increased testis weights (absolute and relative) were observed in males at 3500 and 7000 ppm. Decreased absolute and relative kidney weights were observed at 3500 and 7000 ppm in both sexes. Gross pathology showed enlarged adrenal glands at 3500 and 7000 ppm in both sexes. Additional findings that were noted included discoloured testis in mid- and high-dose males (0, 0, 3, 7) and increased incidence of focal eye opacity in high-dose males (males: 1, 0, 0, 6; females: 1, 3, 0, 0). Histopathological examination revealed increases in the incidence and severity of vacuolation in the adrenal cortex at 3500 and 7000 ppm in both sexes. The Meeting noted that the incidence of vacuolation of the adrenal cortex was also increased in low-dose females and that the incidence of lymphocytic infiltrate was increased in the adrenals of high-dose females (males: 0, 0, 0, 1; females: 0, 0, 2, 5*; * $P \leq 0.05$). Adrenal gland pigmentation was noted in females at all dose levels and in males at 7000 ppm only. The study authors noted that the incidence in low-dose females was within historical control ranges (reportedly up to 37%, based on two studies) and was not considered to be clearly related to treatment at this dose level. Although the incidence of pigmentation was only slightly above the reported historical control range and the increase in vacuolation was not statistically significant for low-dose females, as the adrenal gland is clearly a target organ, the Meeting concluded that these findings were toxicologically relevant and represent a marginal LOAEL for females at this dose level. In males, dose-dependent increases in the incidence and severity of hepatocytomegaly were observed, and increases in the incidence and severity of hypertrophy and hyperplasia of the interstitial cell in the testes were noted at 3500 and 7000 ppm. The lesion in the testis consisted of greatly increased cell size as well as numbers of cells. Other findings noted in male reproductive tissues of high-dose animals included an increase in testicular degeneration and epididymal aspermia. Mineralization in the brain was significantly increased over control in 7000 ppm females. However, as there was no significant change in males and no dose–response relationship for the lesion in either sex, this finding was considered to be a chance occurrence and not treatment related (Table 16). A dose-related increase in the incidence of amyloid was observed in various organs of both sexes. There were no histopathological findings that correlated with the decreased kidney weights.

Neoplastic lesions were noted in the liver in male and female mice. Increased incidences of hepatocellular adenomas and carcinomas were observed at doses of 3500 ppm and above (Table 17). In comparison with historical control data, the combined frequencies of hepatocellular adenoma and carcinoma at 3500 ppm (16% in males and 10% in females) and 7000 ppm (22% in males and 6% in females) were higher than the ranges seen in either in-house control (4–14% in males and 0–2% in females) or literature historical data presented in the study report (0–9.6% in males and 0–2.7% in females). The Meeting noted that although preneoplastic lesions were not observed at doses lower than those at which the liver tumours were observed, this may be due to the large dose spacing. Additionally, it is noted that these tumours were observed only at high doses (≥3500 ppm), which also produced hepatotoxicity, and are likely to exhibit a threshold in their dose–response. The NOAEL for carcinogenicity was 25 ppm (equal to 4.1 mg/kg bw per day for males and 5.1 mg/kg bw per day for females) based on the increased incidence of hepatocellular adenoma and carcinoma at 3500 ppm.

Table 16. Incidences of selected non-neoplastic microscopic findings in the mouse

Histopathological finding	Incidence (no. of animals with finding)			
	Dietary concentration (ppm)			
	0	25	3500	7000
Males (*n* = 50)				
Adrenal pigmentation (corticomedullary junction)	7	5	11	27*
Adrenal vacuolation (cortex)	0	0	31*	37*
Liver hepatocytomegaly	2	6	17*	21*
Heart – atrial thrombus	0	1	1	9*
Testes – tubular degeneration	18	18	22	28
Testes hypertrophy and/or hyperplasia of interstitial cells	6	6	26*	31*
Epididymides aspermia	15	15	15	26*
Brain – mineralization	12	4	12	8
Females (*n* = 50)				
Adrenal pigmentation (corticomedullary junction)	11	20*	45*	42*
Adrenal vacuolation (cortex)	1	6	49*	48*
Brain – mineralization	5	7	4	14*

From Wahle (2000)
* $P < 0.05$

Table 17. Liver tumour incidences in the mouse

Tumour type	Incidence (no. of animals with tumour) / % of total			
	Dietary concentration (ppm)			
	0	25	3500	7000
Males (*n* = 50)				
Hepatocellular adenoma	0	0	5 / 10*	6 / 12*
Hepatocellular carcinoma	1 / 2	1 / 2	3 / 6	5 / 10
Hepatocellular adenoma and carcinoma combined	1 / 2	1 / 2	8 / 16*	10 / 20*[a]
Females (*n* = 50)				
Hepatocellular adenoma	0	0	3 / 6	1 / 2
Hepatocellular carcinoma	0	0	2 / 4	2 / 4
Hepatocellular adenoma and carcinoma combined	0	0	5 / 10*	3 / 6

From Wahle (2000)
* $P < 0.05$
[a] One male had both types of tumours.

The study authors identified 25 ppm (equal to 4.1 mg/kg bw per day for males and 5.1 mg/kg bw per day for females) as the NOAEL for systemic toxicity based on increased liver weights, decreased kidney weights, enlarged adrenal glands and histopathological findings in the adrenal gland in both sexes and the following findings in males: increased liver and testes weights and histopathological findings in the testes and liver at 3500 ppm. However, as the Meeting considered the increased adrenal findings in females at the low dose to be toxicologically relevant, a marginal LOAEL for females

was identified at 25 ppm (equal to 5.1 mg/kg bw per day), and thus a NOAEL for systemic toxicity in females was not identified (Wahle, 2000).

Rats

In a GLP-compliant combined chronic toxicity and carcinogenicity study, spirodiclofen (purity 97.6–98.6%) was administered to groups of Wistar Hsd Cpb:WU rats (50 of each sex per dose) via the diet (mixed with 1% peanut oil) at dose levels of 0, 50, 100, 350 or 2500 ppm (equal to 0, 2.0, 4.1, 14.7 and 110.1 mg/kg bw per day for males and 0, 2.9, 5.9, 19.9 and 152.9 mg/kg bw per day for females, respectively) for 2 years. Additional groups of rats (10 of each sex per dose) were treated likewise with spirodiclofen for interim sacrifice after 1 year. Clinical observations were conducted twice daily, and body weights were recorded weekly for 13 weeks and then every 2 weeks thereafter. Food consumption was recorded twice weekly for 37 weeks and then every 4 weeks thereafter. Water consumption was recorded every 4 weeks throughout the study. Ophthalmological investigations were conducted on all animals before treatment, on control and high-dose animals before interim sacrifice (week 50) and on all animals at the end of treatment (week 102). Samples for clinical chemistry, urinalysis and haematology were collected from animals (10 of each sex per dose) at four time points throughout the study (at approximately weeks 27, 53, 79 and 105). Measurements of thyroid hormones were included in the clinical chemistry investigations. A functional observational battery (FOB) was conducted on 10 rats of each sex per dose at week 77. Gross necropsy and histopathological examinations as well as recording of organ weights were carried out at interim and terminal sacrifices.

There were no treatment-related clinical signs throughout the study. Mortality was increased in 2500 ppm females (incidence of deaths prior to scheduled sacrifice: 21, 18, 19, 15, 24; but with no statistical significance), whereas mortality of 2500 ppm males was slightly decreased (incidence of deaths prior to scheduled sacrifice: 19, 20, 14, 19, 9). Spirodiclofen had no effect on water consumption. At the highest dose, body weight–related food consumption was slightly increased in both sexes. Significant decreases in body weights were observed in both sexes in the 2500 ppm group (decreased by 8–10% for males up to week 101; by 6–7% for females up to week 53). Body weight gains were decreased at 2500 ppm in both sexes up to week 3 and recovered thereafter. The FOB performed in study week 77, the home cage observations and observations during handling did not reveal evidence of a neurotoxic potential. A slightly higher incidence of post-capsular lens opacity in males dosed with 350 and 2500 ppm was noted (22%, 24%, 25%, 39% and 32%, respectively). In females, this finding was distributed evenly across control and treatment groups. Owing to a lack of a clear dose–response relationship and histopathological correlates, combined with the appearance of the effect in one sex only, a clear relationship with treatment was not established for the lens opacity. There were no significant effects on haematological or urinalysis parameters assessed; however, significant increases in AP and decreases in cholesterol and triglyceride levels (not statistically significant) were observed in both sexes at 2500 ppm at all test points (Table 18). Significantly increased T_4 levels were observed in 2500 ppm males at weeks 53 and 105. Increased TSH levels were observed at 2500 ppm in both sexes, but the statistical significance was observed only in females at weeks 79 and 105. There was no clear dose–response relationship or consistency over time for the thyroid hormone findings, and thus a relationship with treatment was not clearly established (Table 19).

At the interim sacrifice, an increased incidence of adrenal cortex vacuolation (microvesicular and/or large vacuoles) in zona fasciculata cells was observed in high-dose males; minimal to slight diffuse adrenocorticocellular hypertrophy was also observed in three high-dose males. In high-dose females, an increased portion of ovarian stroma and slight, possibly spurious, changes in estrous cycle were observed. These changes were characterized as a decrease in the number of females in metestrus/diestrus, with a corresponding increase in the number of those in estrus (based on morphology of the vaginal epithelium). At final necropsy, increased incidences of liver discoloration

Table 18. Selected clinical chemistry findings in the rat

Dietary concentration (ppm)	Week	AP (U/l)	Cholesterol (mmol/l)	Triglyceride (mmol/l)	AP (U/l)	Cholesterol (mmol/l)	Triglyceride (mmol/l)
		Males			Females		
0	27	175	4.44	4.31	122	2.17	2.00
50		196	2.72	1.91	128	2.46	2.13
100		186	2.20	2.18	134	2.04	1.82
350		201	2.57	1.75	150	2.21	1.66
2500		305**	1.90	1.36	226**	2.07	1.16*
0	53	203	3.15	1.73	123	2.32	1.69
50		199	3.90	2.13	112	2.79	2.38
100		180	2.91	2.10	115	2.54	1.89
350		213	3.81	2.09	135	2.11	1.82
2500		279**	2.78	1.81	183	2.06	1.31
0	79	171	3.59	2.70	122	2.25	2.12
50		178	4.25	2.45	100	3.71	6.30
100		163	3.07	2.39	105	2.47	2.73
350		192	4.44	2.42	120	2.24	2.06
2500		277**	2.89	1.84	226**	2.06	1.91
0	105	176	4.11	2.39	117	2.59	2.26
50		167	4.41	2.16	110	2.74	2.66
100		212	3.88	2.42	112	2.77	2.70
350		197	3.77	2.15	135	2.17	2.43
2500		265**	3.29	1.57	188*	2.21	2.06

From Wirnitzer, Bach & Hartmann (2000); Wirnitzer & Hartmann (2002b)
* $P < 0.05$; ** $P < 0.01$

Table 19. Thyroid hormone findings in the rat

Clinical chemistry parameter	Dietary concentration (ppm)				
	0	50	100	350	2500
Males (*n* = 10)					
T_4 (nmol/l)					
- Week 27	45	51	51	53	55
- Week 53	44	46	48	52*	52*
- Week 79	51	50	52	51	57
- Week 105	48	45	48	52	56*
TSH (µg/l)					
- Week 27	3.30	2.26	3.04	3.05	2.88
- Week 53	2.04	1.81	3.03	2.38	3.04
- Week 79	2.67	1.30	3.27	2.98	3.32
- Week 105	1.99	1.00**	2.09	2.15	2.68
Females (*n* = 10)					
T_4 (nmol/l)					
- Week 27	32	31	33	39	35
- Week 53	39	35	34	34	34
- Week 79	40	37	38	44	41

Table 19 (contd)

Clinical chemistry parameter	Dietary concentration (ppm)				
	0	50	100	350	2500
- Week 105	40	42	41	43	44
TSH (µg/l)					
- Week 27	1.93	1.56	1.40	1.80	1.89
- Week 53	0.97	1.30	1.19	0.99	1.48
- Week 79	1.10	1.45	1.41	1.43	2.48*
- Week 105	1.22	1.26	1.47	1.13	2.55**

From Wirnitzer, Bach & Hartmann (2000); Wirnitzer & Hartmann (2002b)
* $P < 0.05$; ** $P < 0.01$

Table 20. Incidences of selected non-neoplastic microscopic and macroscopic findings in the rat at terminal sacrifice

Histopathological finding	Incidence (no. of animals with finding)				
	Dietary concentration (ppm)				
	0	50	100	350	2500
Males (*n* = 50)					
Adrenal vacuolation/hypertrophy	3	1	5	5	25*
Thyroid – colloidal alteration	23	23	28	28	35*
Jejunum – vacuolated enterocytes	3	0	4	3	18**
Nasal cavity – atrophy/degeneration of olfactory epithelium	13	19	15	16	25
Liver – tigroid basophilic foci	12	14	14	10	21*
Females (*n* = 50)					
Jejunum – vacuolated enterocytes	5	0[a]	0	1	17**
Uterus – nodules[b]	8	7	9	9	17

From Wirnitzer, Bach & Hartmann (2000); Wirnitzer & Hartmann (2002b)
* $P < 0.05$; ** $P < 0.01$
[a] $n = 49$.
[b] Macroscopic finding.

(females), dilatation of renal pelvis (females), consistency changes of pancreas (females, only intercurrent deaths) and uterine nodules were seen at the highest dose of 2500 ppm. Terminal histopathological examinations also revealed findings at the high dose. These included an increased incidence of vacuolated enterocytes in the jejunum in both sexes and an increased incidence and severity of vacuolation in zona fasciculata cells of the adrenal cortex, increased adrenocorticocellular hypertrophy, an increased incidence of colloidal alteration in the thyroid, tigroid basophilic foci in the liver and atrophy/degeneration of the olfactory epithelium in the nasal cavity in males (Table 20). The pathology report noted several incidences of age-dependent non-neoplastic findings that exhibited a treatment-related decrease in males and/or females of the high dose group. These findings were as follows: arteritis/periarteritis in the blood vessels of various organs in males, chronic progressive nephropathy in the kidneys of males and females, diffuse transitional cell hyperplasia in the renal pelvis of both sexes, mineralization in the kidneys of females, squamous cell hyperplasia of the uterine cervix in females, peliosis in the adrenal cortex in females, focal hyperplasia in the adrenal medulla in females, pigment deposits in the spleen and mesenteric lymph node in both sexes, and epithelial hyperplasia in the thymus of both sexes.

Table 21. Incidences of neoplastic lesions in the rat

Neoplastic lesion	Incidence (no. of animals with lesions) / % of total					Historical control range[b] (%)
	Dietary concentration (ppm)					
	0	50	100	350	2500	
Males (*n* = 50)						
Testes – Leydig cell adenoma	2 / 4	1 / 2	0 / 0	4 / 8	10 / 20*	2–8
Testes – Leydig cell hyperplasia	4 / 8	4 / 8	4 / 8	7 / 14	19 / 38**	2–10
Thyroid – C-cell adenoma	4 / 8	6 / 12	5 / 10	4 / 8	7 / 14	6–24
Females (*n* = 50)						
Uterus – adenocarcinoma[a]	4 / 8	5 / 10	3 / 6	2 / 4	14 / 28**	2–10
Metastases of uterine origin						
- Ovary	1	0	0	1	5	
- Lung	0	1	1	1	5*	
- Kidney	1	1	2	0	6	
- Pancreas	1	1	1	1	5	
- Liver	0	0	2	1	5*	
- Spleen	0	1	1	0	3	
- Mesenteric lymph node	0	0	0	0	3	
- Bone marrow	0	0	0	0	2	
Thyroid – C-cell adenoma	2 / 4	2 / 4	3 / 6	5 / 10	6 / 12	6–22

From Wirnitzer, Bach & Hartmann (2000); Wirnitzer & Hartmann (2002b)
* $P < 0.05$; ** $P < 0.01$
[a] Incidence includes decedents.
[b] Historical control range from six 24- to 25-month studies conducted in the same laboratory between 1994 and 1996.

An increased incidence of treatment-related neoplastic findings in reproductive organs of males (testes) and females (uterus) was observed (Table 21). In males, a significantly increased frequency of Leydig cell adenomas was observed concurrently with an increased incidence of Leydig cell hyperplasia at the high dose. The average severity of the hyperplasia was also slightly increased at the high dose. The majority of Leydig cell adenomas and focal hyperplasias were found in males at the termination of the study, suggesting a late onset of these alterations. In females, an increased incidence of uterine adenocarcinoma was observed at 2500 ppm. The majority of the uterine adenocarcinomas (11 out of 14) were found in females that died or had to be sacrificed before the termination of the study. The pathology report also indicated that many of the adenocarcinomas had metastasized by invasion and intra-abdominal spread into various organs of the abdominal cavity, such as ovaries, liver, spleen, pancreas, mesenteric lymph node and kidney; metastases were also found in the lung and bone marrow. It was noted that the bone marrow was an uncommon location for metastases of uterine adenocarcinomas. Compared with historical control data (provided in an amendment to the main study), the incidence of Leydig cell adenomas in high-dose males (20%) exceeded the historical control range (2–8%), whereas the incidence of Leydig cell hyperplasia exceeded the historical control range (2–10%) at the middle (14%) and high (38%) doses. The Meeting considered the increase in Leydig cell hyperplasia to be treatment related at both the middle and high doses, based on the exceedance of the historical control range at these doses. The incidence of uterine adenocarcinoma also exceeded the historical control range (2–10%) at the high dose. It was suggested by the study authors that the observed increases in tumours in males and females were not indicative of a primary carcinogenic effect, but rather secondary to interference of spirodiclofen with steroid biosynthesis.

The NOAEL for oncogenicity is 350 ppm, equal to 14.7 mg/kg bw per day for males and 19.9 mg/kg bw per day for females, based on an increased incidence of Leydig cell adenomas and uterine adenocarcinomas at 2500 ppm. The study authors concluded that the NOAEL for systemic toxicity was 350 ppm, based on decreased body weights, alterations in clinical chemistry parameters (increased AP and decreased cholesterol and triglyceride levels) and histopathological findings in the jejunum, adrenal gland, thyroid, uterus, liver and nasal cavity at 2500 ppm. The Meeting identified the NOAEL for systemic toxicity in males to be 100 ppm, equal to 4.1 mg/kg bw per day, based on the increased incidence of Leydig cell hyperplasia at 350 ppm (Wirnitzer, Bach & Hartmann, 2000; Wirnitzer & Hartmann, 2002b).

2.4 Genotoxicity

A battery of mutagenicity studies with spirodiclofen was conducted to assess the potential for inducing gene mutation or chromosomal aberrations in vitro, as well as its clastogenic potential in vivo. The study results (summarized in Table 22) were negative. Spirodiclofen did not demonstrate any genotoxic potential under the conditions tested.

2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

In a GLP-compliant two-generation reproduction study, spirodiclofen (purity 98.6%) was administered to groups of Crl:WI(WU)BR Wistar rats (25 of each sex per dose; for both F_0 and F_1 generations) for 12–13 weeks prior to mating, as well as during mating, gestation and lactation. Spirodiclofen was administered via the diet at doses of 0, 70, 350 or 1750 ppm (equal to 0, 5.2, 26.2 and 134.8 mg/kg bw per day for F_0 males; 0, 5.5, 27.6 and 139.2 mg/kg bw per day for F_0 females; 0, 6.4, 30.2 and 177.6 mg/kg bw per day for F_1 males; 0, 7.0, 34.4 and 192.7 mg/kg bw per day for F_1 females). Clinical signs, body weights, food intake, mating performance, fertility, duration of pregnancy, estrous cycling and sperm parameters (sperm motility, morphology and counts) were examined in F_0 and F_1 rats. Clinical chemistry parameters were assessed in F_1 adults (10 of each sex per dose) just prior to mating. Litter size, ratio of males to females, clinical signs, pup weight at birth as well as viability, lactation and body weight gain were studied in F_1 and F_2 offspring. Developmental milestones (preputial separation and vaginal opening) were examined in F_1 weanlings. Selected clinical chemistry parameters were examined in F_1 parent animals. Necropsies were done in all rats. Selected organs were weighed (from F_0 and F_1 adults as well as F_1 and F_2 weanlings), and histopathological evaluations were performed on selected organs of F_0 and F_1 rats.

No treatment-related clinical signs or deaths occurred in parental male or female rats of either generation receiving any dose of the test material. Mean body weights were significantly decreased throughout the study in high-dose F_0 males (by 6–8%) and at various time points in mid-dose F_0 males (by 5%). Mean body weights were significantly decreased in high-dose F_1 males (by 17–23%) throughout the study, whereas low- and mid-dose F_1 males had weights similar to those of controls. Body weight gain was also decreased for high-dose F_0 and F_1 males (by 9% and 16%, respectively) over the entire study. In F_0 females, decreases in body weight were observed at the high dose during a few of the premating weeks (by 5–7%; overall gain by 19%) and during gestation and lactation (by 5–10%). Overall body weight gain during premating was also decreased at the middle dose (by 11%) in F_0 females. High-dose F_1 females had significantly decreased body weights during lactation (by 7%), and overall body weight gain during gestation was also decreased (by 12%). Food consumption

Table 22. Results of genotoxicity studies on spirodiclofen

End-point	Test object	Concentration	Purity (%)	Results	GLP compliant	Reference
In vitro						
Reverse mutation[a,b]	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537	0–5000 µg/plate	99.1	Negative	Yes	Herbold (1996a)
Forward mutation[a,c,d,e]	Chinese hamster lung cells (V79), *Hgprt* locus	4–20 µg/ml without S9; 10–80 µg/ml with S9	99.1	Negative	Yes	Brendler-Schwaab (1997)
Chromosomal aberration[a,f,g,h]	Chinese hamster lung cells (V79)	Trial 1: 0–80 µg/ml without S9; 0–160 µg/ml with S9 Trial 2: 0–12 µg/ml without S9	99.1	Negative	Yes	Herbold (1996b)
In vivo						
Micronucleus[i,j]	Mouse (NMRI, 5 of each sex per dose) bone marrow	800 mg/kg bw (intraperitoneal) in 0.5% aqueous Cremophor	99.1	Negative	Yes	Herbold (1996c)

S9, 9000 × *g* rat liver supernatant

[a] Positive and negative controls included; conducted with and without exogenous metabolic activation.

[b] Tested in triplicate, using both the standard plate method and the preincubation method.

[c] Two trials conducted; tested in duplicate in each trial.

[d] Cytotoxicity was observed at ≥15 µg/ml without S9 and at 80 µg/ml with S9; compound insolubility was reported at final concentrations of ≥156 µg/ml.

[e] In Trial 1 with metabolic activation, there was an increase in mutation frequency at all dose levels. The increases were not dose responsive, were not reproduced in Trial 2 and were all within the historical control range; therefore, it was concluded that these increases were not indicative of a mutagenic response.

[f] Two trials were conducted.

[g] Without S9 mix, cytotoxic effects were observed at 0.75 µg/ml and above. With S9 mix, cytotoxic effects were observed at 20 µg/ml and above. Doses were lowered for Trial 2 (without S9 mix) due to the severity of cytotoxicity observed at the concentrations used in Trial 1. Compound insolubility was reported at ≥100 µg/ml.

[h] Statistically significant increases in metaphase aberrations were noted at 3 µg/ml (18 h harvest) without S9 and at 80 µg/ml (30 h harvest) with S9; however, values were within historical control ranges, and therefore it was concluded that this increase did not represent a clastogenic response. Significant cytotoxicity (≥50% decrease in the mitotic index) was observed at these doses.

[i] Analysed five rats of each sex per group. Positive and negative controls were included. Clinical signs included apathy, roughened fur, spasm and eyelids stuck together. One mortality occurred.

[j] A decrease in the ratio of polychromatic to normochromatic erythrocytes was noted in treated groups, which may be indicative of bone marrow toxicity.

was not affected in the F_0 parental generation or in F_1 males. F_1 females at 1750 ppm had slightly increased food consumption. Clinical chemistry parameters were evaluated in a subset of F_1 males and females at the end of the premating period. Dose-responsive decreases in plasma triglyceride, cholesterol and unesterified fatty acid levels were observed in males and females, some of which attained statistical significance at all dose levels (Table 23). AP activity was elevated slightly more than 2-fold in high dose group F_1 male and female rats. Statistically significant decreases in absolute (decreased by 12–15%) and relative (decreased by 8–9%) liver weights were observed in F_0 males at all dose levels and in high-dose F_1 males (decreased by 26% and 13%, absolute and relative, respectively). However, based on the lack of dose dependence or statistically significant findings in the livers of females, these were not considered to be treatment related. Relative adrenal (both sexes) and testes weights were increased at 1750 ppm in F_0 animals. In high-dose F_1 males, a slight increase in absolute adrenal weight and a statistically significant increase in relative adrenal weight were observed. The absolute mean weights for the testes, epididymides, seminal vesicles and prostate were reduced in

Table 23. Selected clinical chemistry findings in F_1 adults

Parameter	Males				Females			
	Dietary concentration (ppm)							
	0	70	350	1750	0	70	350	1750
Cholesterol,[a] mmol/l (% change)	2.55	2.30	2.07** (−19)	1.58** (−38)	2.24	2.33	2.20	1.69 (−25)
Triglyceride,[b] mmol/l (% change)	2.62	1.84**	1.68** (−36)	0.96** (−63)	1.36	1.22	1.02	0.58** (−57)
Non-esterified fatty acid (mmol/l)	0.39	0.38	0.32	0.24**	0.54	0.47	0.44*	0.32**

From Eiben (2000, 2002, 2003, 2004)
* $P < 0.05$; ** $P < 0.01$
[a] Historical control values: male: 2.33 (±2 standard deviations [SD]: 1.52–3.15) mmol/l; female: 2.00 (±2 SD: 1.27–2.73) mmol/l.
[b] Historical control values: male: 1.96 (±5th percentile: 1.02–3.25) mmol/l; female: 1.52 (±5th percentile: 0.71–2.68) mmol/l.

high-dose F_1 males, mainly as a result of four males (Nos 353, 366, 367 and 372) with very low individual values for these organ weights, which also exhibited extremely low body weights.

At 70 ppm, no treatment-related histopathological changes were detected. A slight increase in the severity of the adrenal vacuolation was seen at 350 ppm and above in F_0 and F_1 females and at 1750 ppm in F_0 and F_1 males. In F_0 males, a higher incidence of foamy vacuolation of the jejunal villi epithelium was noted at the high dose. In F_1 males, a higher incidence of testicular and epididymal atrophy was found at the high dose, which was associated with epididymal oligospermia and partly atrophic prostate and seminal vesicles. Testicular and epididymal findings in the other groups were regarded as incidental and within the normal historical range. A slight increase in the severity of vacuolation or degeneration of luteal cells (a morphologically normal luteal regression) was noted in 1750 ppm F_1 females. Although the toxicological relevance was unclear, a relationship with treatment could not be excluded (Table 24).

Evaluation of reproductive performance and function showed no treatment-related effects on the fertility index, gestation index, total number of pups born, number of stillbirths, postimplantation loss or number of live litters produced in either generation, mean gestation interval, estrous cycle length, number of females with prolonged or abnormal cycles, day of vaginal patency in F_1 females, per cent motile sperm or per cent abnormal sperm in both generations, or testicular spermatid and epididymal sperm count in high-dose F_0 males. Decreases in the insemination (mating) index (of 8%), testicular spermatid count (of 33%) and epididymal sperm count (of 18%) were noted in high-dose F_1 males. Both the epididymal sperm and testicular spermatid counts were below the historical control ranges (provided in Amendment III to the study report). It was indicated by the study authors that the mean testicular and epididymal sperm counts were reduced due to the fact that four males in this dose group (Nos 353, 366, 367 and 372), which also had extremely low body weights, had no sperm. In addition, it was noted that there were five high-dose animals that had lower sperm or spermatid levels, which may have also contributed to the decreases in these sperm parameters at the high dose. No alteration in sperm count was found for F_1 males at the middle dose (Table 25).

Evaluation of offspring parameters showed no treatment-related or adverse effects on survival indices (live birth, viability and lactation), sex ratios, live litter size, clinical signs, relative organ weights (brain, thymus and spleen) or gross findings. During the 28-day lactation period, decreases in pup body weight were observed in mid- and high-dose pups of both generations. Decreases were noted throughout lactation in high-dose male F_1 (by 9–23%) and F_2 (by 6–17%) pups, and these pups also gained 24% and 17% less weight, respectively, than controls. Decreases in body weight and body weight gain were noted in high-dose F_1 (body weight: by 5–21%; body weight gain: by 22%)

Table 24. Selected microscopic findings in F_0 and F_1 parental animals

Organ/lesion	Incidence (no. of animals with lesion)[a,b]			
	Dietary concentration (ppm)			
	0	70	350	1750
F_0 generation – males				
Small intestine – epithelial vacuolation	4 (1.3)	0	5 (1.0)	17** (1.4)
Adrenal cortex – vacuolation	24 (2.0)[c]	24 (1.9)	23 (1.8)	25 (2.7)
F_0 generation – females				
Small intestine – epithelial vacuolation	6 (1.3)	7 (1.3)	5 (1.2)	10 (1.4)
Adrenal cortex – vacuolation	22 (1.2)	24 (1.2)	24 (1.6)	25 (2.1)
F_1 generation – males				
Small intestine – epithelial vacuolation	18 (1.3)	15 (1.5)	24 (1.7)	19 (1.6)
Adrenal cortex – vacuolation	22 (1.9)	25 (2.0)	25 (2.1)	24 (2.8)[c]
Testes – diffuse atrophy	0	1 (4.0)	1 (4.0)	4† (3.5)
Prostate – atrophy	0	0	0	3 (2.3)
Epididymides – oligospermia	0	1 (5.0)	1 (5.0)	4† (4.5)
Epididymides – atrophy	0	1 (4.0)	1 (4.0)	4† (3.8)
F_1 generation – females				
Small intestine – epithelial vacuolation	10 (1.5)	12 (1.4)	13 (1.2)	17* (1.6)
Ovaries – vacuolation/degeneration of luteal cells	21 (1.4)	24 (1.3)	25 (1.3)	23 (1.8)
Adrenal cortex – vacuolation	22 (1.2)	20 (1.2)	22 (1.5)	24 (2.1)

From Eiben (2000, 2002, 2003, 2004)

† $P < 0.055$; * $P < 0.05$; ** $P < 0.01$ (calculated by the reviewer)

[a] Number examined = 25 (except where otherwise noted).

[b] Average severity of lesions presented in parentheses, as reported by the study authors: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked, 5 = massive.

[c] Number examined = 24.

Table 25. Spermatid and sperm counts in F_0 and F_1 males

	Dietary concentration (ppm)				Historical control data[a]
	0	70	350	1750	
F_0					
Mean number of spermatids/mg testis	48 760	—	—	46 217	Mean: 49 557 ± 2723 Range: 45 346–53 361
Mean number of sperm/mg epididymis	780 617	—	—	755 865	Mean: 10 268 523 ± 125 304 Range: 887 819–1 243 528
F_1					
Mean number of spermatids/mg testis	47 971	—	47 606	37 087 (77)[b]	Mean: 51 203 ± 601 Range: 50 841–51 897
Mean number of sperm/mg epididymis	889 967	—	982 994	733 837 (82)[b]	Mean: 1 077 723 ± 155 937 Range: 909 309–1 217 101

From Eiben (2000, 2002, 2003, 2004)

[a] Historical data from six studies conducted between 2000 and 2003; for the F_1 generation, there were data from only three of these studies.

[b] Per cent of control.

Table 26. Developmental landmarks in F_1 animals

Dietary concentration (ppm)	Mean age of sexual maturation[a] (days)	
	Preputial separation	Vaginal opening
0	35.4 ± 2.5	37.0 ± 2.7
70	35.0 ± 1.4	37.4 ± 3.4
350	35.4 ± 2.0	37.0 ± 2.9
1750	37.4 ± 1.8*	37.6 ± 2.1

From Eiben (2000, 2002, 2003, 2004)
* $P < 0.05$
[a] Mean ± standard deviation.

and F_2 (body weight: by 12–21%; body weight gain: by 22%) female pups during lactation. Mid-dose F_1 pups had decreased body weights (males: by 2–6%; females: by 3–9%) throughout lactation, whereas mid-dose F_2 pups had decreased body weights (males: by 5%; females: by 7%) only on the day of birth. Sexual maturation as measured by day of preputial separation was delayed by 2 days in high-dose F_1 males (Table 26). The Meeting noted that all observed treatment-related adverse effects on the reproductive system in this study were observed at the highest dose tested (1750 ppm) in second-generation (F_1) males and females, but not in first-generation (F_0) parental animals. This may suggest that early life stage exposure to spirodiclofen may have been a critical factor in eliciting this response, even though a number of the adverse outcomes were not observed until the animals were mature. However, this remains uncertain, considering that F_1 animals began consuming treated diet at an earlier age, experienced a longer duration of dosing and thus were exposed to a higher overall average dose of spirodiclofen compared with the F_0 generation.

The NOAEL for parental toxicity was 70 ppm, equal to 5.2 mg/kg bw per day for males and 5.5 mg/kg bw per day for females, based on the following findings at 350 ppm: decreased body weight in F_0 males, decreased body weight gain during premating in F_0 females and increased severity of adrenal cortical vacuolation in F_0 and F_1 females. The NOAEL for reproductive toxicity was 350 ppm, equal to 26.2 mg/kg bw per day for males and 27.6 mg/kg bw per day for females, based on delayed sexual maturation, decreased testicular spermatid and epididymal sperm counts (oligospermia), atrophy of the testes, epididymides, prostate and seminal vesicles in F_1 males and increased severity of ovarian luteal cell vacuolation/degeneration in F_1 females at 1750 ppm. The NOAEL for offspring toxicity was 70 ppm, equal to 5.2 mg/kg bw per day for males and 5.5 mg/kg bw per day for females, based on decreased body weight and body weight gain in F_1 male and female pups at 350 ppm (Eiben, 2000, 2002, 2003, 2004).

(b) Developmental toxicity

Rats

In a GLP-compliant developmental toxicity study, spirodiclofen (purity 97.9%) was administered to pregnant Wistar (Hsd Cpb:WU) rats (28 per dose) by gavage (in 0.5% aqueous carboxymethyl cellulose) at dose levels of 0, 100, 300 or 1000 mg/kg bw per day from days 6 through 19 of gestation. On gestation day (GD) 20, all surviving dams were sacrificed and examined grossly, and fetuses were delivered by caesarean section. The number of corpora lutea, implantations, early resorptions, late resorptions, and live and dead fetuses, as well as uterine and placental weight and appearance of the placenta, were assessed at caesarean section. Each fetus was weighed and examined externally for abnormalities, including the palate, and for sex determination. Approximately one half of the fetuses in each litter were examined viscerally by sectioning according to a modified Wilson technique. The remaining one half of the fetuses in each litter were eviscerated and processed for skeletal (bone and cartilage) examination.

No treatment-related deaths or clinical signs of toxicity were observed. There were no treatment-related effects on maternal body weight, body weight gains, food consumption or water intake. Maternal necropsy observations were unremarkable.

No treatment-related differences were noted between the treated and control groups for numbers of corpora lutea and implantations, placental and gravid uterine weights, live fetuses per dam, resorptions, fetal sex ratios, and preimplantation or postimplantation losses. Fetal body weights were similar between the treated and control groups. There were no treatment-related increases in any malformations or in the total number of fetuses or affected litters with malformations. A single case of microphthalmia was noted in the high dose group, which is considered a common spontaneous malformation in the Wistar rat and was thus considered incidental. A slight increase in the fetal and litter incidence of renal pelvis dilatation was observed at 1000 mg/kg bw per day. The study authors concluded that it was within the provided historical control range and therefore was not related to treatment. The Meeting requested additional information regarding the terminology used to describe the renal pelvis dilatation. The manufacturer indicated that "slight renal pelvis dilatation" refers to "renal papilla present though shorter giving the impression of enlarged renal pelvis", whereas "renal pelvis dilatation" (which the Meeting has referred to as "full" renal pelvis dilatation) was stated to refer to "lack of renal papilla, and/or reduced renal parenchyma with large renal pelvis". It had been noted that in the 2 years immediately preceding the study, there had been an apparent decrease in the background incidence of renal pelvis dilatation (Table 27). However, additional historical control data (from 1999 to 2001; Table 27) were also provided by the manufacturer, which showed that in the 2 years following the study with spirodiclofen, there was a rise in the background incidence of slight renal pelvis dilatation. Data were also provided for a range-finding study (Table 28) in which it was noted that although there was an increase in "full" renal pelvis dilatation on a fetal basis, there was no increase on a litter basis (five of six fetuses with this finding were from a single litter). Consequently, the study author concluded that there was no treatment-related effect on this finding in the range-finding study. A reference was provided (Woo & Hoar, 1972) that suggested that renal pelvis dilatation is a "transitory condition" representing "a normal aspect of renal development", although Klaus (2009) noted that an increased incidence of this finding "may be related to delayed fetal development". Klaus (2009) also noted that kidneys will generally be normal at weaning. The Meeting noted that the tendency for this finding to be transient was also supported more recently when a review of the background incidence of this finding indicated that renal pelvis dilatation is more often observed in fetuses than in pups, indicating that this finding should be considered a variation, rather than a malformation (Solecki et al., 2003). The Meeting noted, however, that histopathological examinations of these lesions were not conducted to confirm their nature and considered the increase at the high dose to be related to treatment. However, this finding was considered to be marginal, given that the finding occurred at a high dose (1000 mg/kg bw per day) and that the incidence was within the historical control range for studies conducted before and after the current study.

Variations in ossification rates of numerous bones were common findings in treated and control groups (Table 29); the general trend was towards slightly greater ossification in the treated groups compared with the controls, particularly in some cranial bones (e.g. interparietal, supraoccipital and temporal). Such findings were thought to be incidental and likely related to an incidentally lower body weight of the control fetuses. There appeared to be a dose-dependent increase in asymmetrical sternebrae (fourth) that reached statistical significance at the high dose; however, the fetal (high dose: 10.3%) and litter (high dose: 46.2%) incidence of asymmetrical fourth sternebrae from the current study were within the range of the historical control data provided for skeletal findings (fetal incidence: 5.3–12.5%; litter incidence: 19.2–53.8%) and were therefore not considered to be related to treatment by the study authors. However, the Meeting concluded that while marginal, the increase at the high dose could not be discounted as being related to treatment. The Meeting noted that due to the nature of this finding (Makris et al., 2009), it would be considered unlikely to be caused by a single dose.

Table 27. Incidence of renal pelvis dilatation in rats

Obervations	Dose (mg/kg bw per day)			
	0	100	300	1000
Fetuses				
Number of fetuses examined	133	137	129	144
Renal pelvis dilatation – slight (*N*)	8	5	4	14
Renal pelvis dilatation – full (*N*)	0	0	1	1
Renal pelvis dilatation – total (slight plus full) (*N*)	8 (6.0)[a]	5 (3.6)[a]	5 (3.9)[a]	15 (10.4)[a]
Historical control mean (1995–1996)[b] (%)	*6.0 (1.6–11.0)[a]*			
Historical control mean (1997)[c] (%)	*2.4 (2.2–2.8)[a]*			
Historical control mean (1999–2001)[d] (%)	*7.7 (2.1–11.0)[a]*			
Historical control mean from all studies (1995–2001)[e] (%)	*6.0 (2.1–11.0)[a]*			
Litters				
Number of litters examined	24	25	25	26
Renal pelvis dilatation – slight (*N*)	4	4	2	8
Renal pelvis dilatation – full (*N*)	0	0	1	1
Renal pelvis dilatation – total (slight plus full) (*N*)	4 (17)[f]	4 (16)[f]	3 (12)[f]	8 (31)[f]
Historical control mean (1995–1996)[b]	*41.4 (18.2–69.2)[f]*			
Historical control mean (1997)[c]	*21.3 (20.0–22.2)[f]*			
Historical control mean (1999–2001)[d]	*44.8 (14.0–77.0)[f]*			
Historical control mean from all studies (1995–2001)[e]	*38.6 (14.0–77.0)[f]*			

From Klaus (2000)

[a] Percentage of fetuses affected in parentheses.

[b] Mean incidence of unilateral/bilateral (slight) renal pelvis dilatation from seven studies in Wistar rats conducted between 1995 and 1996.

[c] Mean incidence of unilateral/bilateral (slight) renal pelvis dilatation from four studies in Wistar rats conducted in 1997. Although an additional four studies conducted in 1998 showed fetal and litter incidences similar to those from the studies conducted in 1997, the overall numbers of fetuses and litters examined were not provided, and therefore percentages of litters and fetuses affected could not be calculated.

[d] Mean incidence of unilateral/bilateral (slight) renal pelvis dilatation from eight studies in Wistar rats conducted between 1999 and 2001.

[e] Mean incidence of unilateral/bilateral (slight) renal pelvis dilatation from all provided studies in Wistar rats conducted between 1995 and 2001.

[f] Percentage of litters affected in parentheses.

The NOAEL for maternal toxicity was 1000 mg/kg bw per day. The study authors considered the NOAEL for developmental toxicity to be 1000 mg/kg bw per day. However, the Meeting identified the NOAEL for developmental toxicity as 300 mg/kg bw per day, based on the marginal increases in renal pelvis dilatation and asymmetrical fourth sternebrae at 1000 mg/kg bw per day (Klaus, 2000).

Rabbits

In a GLP-compliant developmental toxicity study, spirodiclofen (purity 97.9–98.5%) was administered to pregnant Himalayan CHBB:HM rabbits (22 per dose) by gavage (in a 0.5% carboxymethyl cellulose sodium salt suspension) at dose levels of 0, 100, 300 or 1000 mg/kg bw per day from days 6 through 28 of gestation. On GD 29, all surviving does were sacrificed and examined grossly, and fetuses were delivered by caesarean section. The number of corpora lutea, implantations, early resorptions, late resorptions, and live and dead fetuses, as well as uterine and placental weight and appearance of the placenta, were assessed at caesarean section. Each fetus was weighed and examined for external abnormalities and for sex determination. Fetuses were examined viscerally by a modified Staples technique, including a transverse section through the brain in about 50% of the

Table 28. Incidence of renal pelvis dilatation from a rat range-finding study with spirodiclofen

Observations	Incidence (no. of fetuses/litters affected)			
	Dose (mg/kg bw per day)			
	0	100	300	1000
Number of fetuses/litters examined	22/6	21/4	34/6	31/6
Renal pelvis dilatation – slight	6/3	4/3	5/2	6/3
Renal pelvis dilatation – "full"	2/2	2/2	2/2	6/2
Renal pelvis dilatation – total (slight plus "full")	8/5	6/3	7/3	12/4

From Klaus (2000)

Table 29. Incidence of selected skeletal findings in rats

Observations	Incidence (no. of fetuses/litters with finding)			
	Dose (mg/kg bw per day)			
	0	100	300	1000
Number of fetuses/litters examined	144/24	154/25	143/25	156/26
Incomplete ossification				
- Parietal bone (bilateral)	10/5	8/4	0**/0	3/3
- Interparietal bone	31/14	16*/10	8**/4**	10**/7
- Supraoccipital bone	13/6	11/8	5/5	1**/1
- Temporal bone (bilateral)	8/5	4/2	1/1	0**/0
Asymmetrical fourth sternebrae[a,b]	3 (2) / 3 (12.5)	10 (6.5) / 10 (40)	12 (8.4) / 10 (40)	16* (10) / 12 (46)

From Klaus (2000)
* $P < 0.05$; ** $P < 0.01$
[a] Mean historical control incidence of asymmetrical fourth sternebrae on a fetal (litter) basis: 8.1% ± 2.3% (39.0% ± 11.5%) from nine studies conducted between 1996 and 1997.
[b] Per cent of total given in parentheses.

fetuses. The eviscerated carcasses were processed for skeletal examination, including cartilage staining. For approximately half of the fetuses, the head was examined via a transverse section through the brain and left intact for skeletal processing and examination; for the remainder of the fetuses, the heads were sectioned by a modified Wilson technique for an evaluation of internal cranial structures.

There were no treatment-related mortalities in maternal animals. Transient decreases in food consumption and faeces were observed in middle and high dose groups. In addition, light-coloured faeces were seen in 14 high-dose animals compared with none in the control or other treatment groups. Increased incidences of alopecia and discoloured urination, accompanied by a decrease in water consumption, were also noted in high-dose does. No statistical differences in absolute body weights were found between the treated and control groups at any time during the study. However, weight loss during GDs 6–9 was significantly greater in the middle (decreased by 55.8 g) and high dose groups (decreased by 72.7 g) compared with the control group (decreased by 23.7 g). Body weight loss and reduced faeces correlated with decreased food consumption values at the middle and high doses (which were 72% and 58%, respectively, of the control levels for GDs 6–9). Body weight changes and food consumption by the low dose group were similar to those of the control group throughout the study. One female in the high dose group aborted on GD 20 after showing distinct signs of systemic toxicity (cold ears, severe body weight loss and nearly no feed intake, as well as

distinct liver lobulation at necropsy). This one abortion resulted in a marginal decrease in gestation rate at the high dose (95.2% versus 100% in controls).

No statistically significant differences were noted between the treated and control groups for numbers of corpora lutea, implantations, live fetuses, resorptions, fetal sex ratios, placental weight and appearance, and preimplantation or postimplantation losses. Fetal body weights were similar between the treated and control groups. No treatment-related external, visceral or skeletal malformations or deviations were observed in fetuses. Although there was an increase in the number of fetuses with distinct liver lobulation in the high dose group (14 versus 3 in controls; 10.1% versus 2.2%; Table 30), there was no increase on a litter basis (three high-dose versus two control litters affected), and the incidence was reportedly within the historical control range. It was noted that the fetal incidence for liver lobulation at the high dose (10.1%) was above the historical control range provided in the study report (0–7.1%) from 18 studies conducted between 1991 and 1996; however, the litter incidence (15%) was within the historical control range if all pilot studies were included (as high as 33% if pilot studies with only three litters are included), but not if these pilot studies were excluded (range without the pilot studies is 0–6.7%). It appeared that the inclusion of the pilot studies artificially inflated the litter incidence of this finding, as only three litters were examined. The manufacturer provided additional historical control data from 1997 to 1998 (Klaus, 2009; added to Table 30). The range reported from these studies was 0–11.6% on a fetal basis and 0–19.0% on a litter basis from nine studies conducted during 1997 and 1998 (note: there were no incidences of this finding noted in the four studies conducted in 1998). When the additional historical control data from 1997 and 1998 are taken into consideration, the incidence of liver lobulation (on both a fetal and litter basis) is within the reported historical control range. Considering this and the fact that the incidence of liver lobulation is significantly increased only on a fetal basis (but not on a litter basis) in the study under review, the Meeting concurred with the study authors that this increase is not likely to be related to treatment with spirodiclofen.

Based on the study results, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on increased body weight loss and decreased food consumption at 300 mg/kg bw per day. The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the limit dose (Holzum, 1998).

2.6 Special studies

(a) Neurotoxicity studies

Rats

In a GLP-compliant acute neurotoxicity study, a single dose of spirodiclofen (purity 97.7–97.9%; mixed batch) was administered to groups of fasted 9-week-old Wistar (Crl:WI(HAN)BR) rats (12 of each sex per group) by gavage in 0.5% methyl cellulose/0.4% Tween 80 in deionized water at doses of 0, 200, 500 or 2000 mg/kg bw. Rats were observed for 14 days, during which time clinical observations were assessed daily and body weights were recorded weekly. Neurobehavioural assessment, which included a FOB and motor/locomotor activity testing in a figure-eight maze, was performed 1 week prior to treatment, approximately 4 h after administration of the dose and 7 and 14 days following treatment. At study termination, all animals were subjected to gross necropsy, and six animals of each sex per group were euthanized and perfused (in situ) for neuropathological examination; brain weights were also recorded for these animals. Of the perfused animals, only the control and high dose groups were subjected to histological evaluation of brain and peripheral nervous system tissues. There were no treatment-related effects on mortality, clinical signs, body weight, brain weight or gross and histological pathology or neuropathology. FOB and motor activity testing revealed no effects that were considered treatment related.

Based on the absence of effects in this study, the NOAEL for spirodiclofen in rats is the limit dose of 2000 mg/kg bw (Sheets & Gilmore, 2000).

Table 30. Incidence of liver lobulation in fetal rabbits

Observation	Dose (mg/kg bw per day)			
	0	100	300	1000
Fetuses				
Number of fetuses examined	138	137	136	139
Distinct liver lobulation (*N*)	3 (2.2)[a]	0	1	14* (10.1)[a]
Historical control mean (range)[b] (%)	*0.5 ± 1.7 (0–7.1)*			
Historical control mean (range)[c] (%)	*2.2 ± 4.3 (0–11.6)*			
Historical control mean (range)[d] (%)	*1.1 ± 2.7 (0–11.6)*			
Litters				
Number of litters examined	21	22	20	20
Distinct liver lobulation (*N*)	2 (9.5)[e]	0	1	3 (15)[e]
Historical control mean (range)[b] (%)	*0.6 ± 1.9 (0–6.7)*			
Historical control mean (range) including pilot studies[f] (%)	*2.0 ± 7.0 (0–33.3)*			
Historical control mean (range)[c] (%)	*4.1 ± 8.0 (0–19)*			
Historical control mean (range)[d] (%)	*2.6 ± 6.6 (0–19)*			

From Holzum (1998)

* $P \leq 0.05$

[a] Percentage of fetuses affected in parentheses.

[b] Mean (± standard deviation) historical control incidence of liver lobulation from 18 studies conducted between 1991 and 1996.

[c] Mean (± standard deviation) historical control incidence of liver lobulation from nine studies conducted between 1997 and 1998 (Klaus, 2009).

[d] Mean (± standard deviation) historical control incidence of liver lobulation from all main studies (27 in total) conducted between 1991 and 1998.

[e] Percentage of litters affected in parentheses.

[f] Mean historical control incidence of liver lobulation from 18 main studies conducted between 1991 and 1996, as well as 5 pilot studies conducted in 1996, which used only three litters. Fetal incidence could not be calculated from the pilot studies, as the total number of fetuses examined was not provided.

Note: Positive control data, which were referenced but not included in the study report, were provided by the manufacturer in response to a request by the JMPR (Sheets, 1993).

In a GLP-compliant subchronic neurotoxicity study, spirodiclofen (purity 97.4–97.8%; mixed batch) was administered to groups of young adult Wistar rats (12 of each sex per dose) in the diet (in 1% corn oil) at doses of 0, 100, 1000 or 12 500 ppm (equal to 0, 7.2, 70.3 and 1088.8 mg/kg bw per day for males and 0, 9.1, 87.3 and 1306.5 mg/kg bw per day for females) for 13 weeks. Detailed clinical observations and body weight recordings were conducted weekly, and food consumption was determined every 1–2 days. Neurobehavioural assessments, which included FOB and motor activity testing (figure-eight maze), were performed in all 12 animals of each sex per group. Ophthalmological examinations were conducted on all animals at pretest and prior to terminal sacrifice. At study termination, all animals were subjected to gross necropsy and perfused, with six of each sex per dose subjected to histopathological evaluation of brain, peripheral nervous system tissues, eyes (with optic nerves) and skeletal muscle. Brain weights were also recorded for the animals selected for histopathological assessment.

There was no mortality at any dose level prior to scheduled terminal sacrifice. At 12 500 ppm, urine staining was observed in both sexes. High-dose females also showed oral staining and red-tinged stains at locations such as paws, snout, forelimbs and ears. Body weights were reduced for males (by 24%) and females (by 15%) at the high dose, but not at lower doses. Food consumption was reduced at the high dose for both sexes. For the FOB, there were compound-related effects in both sexes at the

high dose, but not at any lower doses. Observations associated with treatment were limited to various stains (including oral, urine, nasal, on paws and/or on ears) in both sexes, and the high-dose animals of both sexes tended to have slightly lower landing food splay and grip strength measurements on some test occasions. These small differences in grip strength and landing foot splay were attributed to the large decrease in body weight in these animals. No compound-related effects on motor and locomotor activity in the figure-eight maze were noted at any dose level for males. High-dose females showed a consistent slight decrease in motor and locomotor activity during week 4. Habituation was not affected by treatment. No changes were noted in ophthalmic findings. Compound-related gross lesions at terminal sacrifice were limited to increased incidence of ventral staining at the highest dose level in both sexes. Absolute brain weights were not affected at any dietary level. Relative brain weights were increased, which was likely due to the observed decrease in body weight at this dose level. No compound-related microscopic lesions were noted in either sex at the highest dose.

The NOAEL was 1000 ppm (equal to 70.3 mg/kg bw per day in males and 87.3 mg/kg bw per day in females) based on decreased body weights, decreased food consumption and increased urine staining in both sexes and decreased motor and locomotor activity for females (during week 4) at 12 500 ppm (Sheets & Gilmore, 2001).

Note: Positive control data, which were referenced but not included in the study report, were provided by the manufacturer in response to a request by the JMPR (Sheets, 1993; Sheets & Armintrout, 2000).

In a GLP-compliant developmental neurotoxicity study, spirodiclofen (purity 96.8–97.1%; mixed batch) was administered to pregnant Wistar Hannover rats (30 per dose) continuously from GD 0 to lactation day (LD) 21 in the diet at nominal doses of 0, 70, 350 or 1500 ppm (equal to 0/0, 6.5/14.0, 32.1/69.7 and 135.9/273.8 mg/kg bw per day [gestation/lactation]). On postnatal day (PND) 4, litters were standardized to four pups of each sex per litter; excess pups were killed and discarded. Pups were weaned on PND 21, after which time all animals received untreated diet. F_1 pups were assigned to subgroups (1 pup per litter per group [approximately 16 pups of each sex per dose]) in order to evaluate developmental landmarks (preputial separation or vaginal patency), brain weights, learning and memory (passive avoidance after weaning and an M-water maze task on PND 60), motor activity (in a figure-eight maze) and acoustic startle habituation. Serum cholesterol level was measured in the dams (LD 21) and offspring (PNDs 4 and 21), and neural tissues were collected from 10 rats of each sex per dietary level (representing approximately 20 litters) on PND 21 (brain only) and at study termination (approximately 75 days of age) for microscopic examination and morphometry.

No treatment-related effects were observed in the dams for mortality, clinical signs, FOB, serum cholesterol levels, reproductive performance or postmortem examinations. No treatment-related differences were noted in body weights, body weight gains or food consumption during the gestation period. During lactation, a statistically significant body weight decrease (of 5%) was observed in the 1500 ppm dams on LD 21. Food consumption was decreased (by 8%) in the 1500 ppm dams during LDs 7–14. However, body weight changes within each group did not show significant differences compared with the control group.

Treatment had no adverse effects on offspring survival, clinical signs or developmental landmarks. During preweaning, body weights were decreased in the 1500 ppm pups on PNDs 17 and 21 (by 5–8%). Body weight gains were decreased at 1500 ppm in both sexes at most intervals (by 6–18%), and overall (PNDs 0–21) body weight gain was decreased in each sex at 1500 ppm (by 9%). During post-weaning, body weights recovered in the 1500 ppm group. Post-weaning food consumption was similar between the treated and control males. No treatment-related effects were observed on FOB, motor activity or auditory startle reflex assessments. No treatment-related differences in the passive avoidance tests or the water maze task (Table 31) were reported at any dose by the study authors. For postmortem examination, no significant differences in absolute brain weight or

Table 31. Water M-maze performance for F_1 rats in the original developmental neurotoxicity study[a]

Session/parameter		Dietary concentration (ppm)			
		0	70	350	1500
Males					
Session 1 – Learning	No. of animals	16	16	16	16
	Trials to criterion	7.0 ± 2.1	7.2 ± 2.8	7.8 ± 2.9	6.9 ± 1.4
	Trial 1 – Errors	1.0 ± 1.0	0.6 ± 0.8	1.1 ± 1.1	0.8 ± 0.9
	Trial 1 – Duration (s)	19.4 ± 16.9	16.6 ± 14.0	21.6 ± 13.9	20.3 ± 14.2
	Trial 2 – Errors	0.3 ± 0.4	0.5 ± 0.7	0.6 ± 0.8 (2-fold increase)[b]	0.8 ± 1.0 (2.7-fold increase)[b]
	Trial 2 – Duration (s)	12.9 ± 7.0	12.2 ± 11.5	15.7 ± 15.1 (+18)[c]	17.3 ± 14.6 (+34)[c]
	Failed to meet criterion	1	0	0	0
Session 2 – Memory	No. of animals	15	16	16	16
	Trials to criterion	6.5 ± 2.8	5.6 ± 1.3	5.4 ± 0.7	5.3 ± 0.6
	Trial 1 – Errors	0.5 ± 0.7	0.4 ± 0.9	0.3 ± 0.7	0.3 ± 0.6
	Trial 1 – Duration (s)	9.7 ± 4.9	6.1 ± 4.0	8.9 ± 6.8	8.5 ± 5.5
	Trial 2 – Errors	0.3 ± 0.7	0.1 ± 0.3	0.3 ± 0.8	0.1 ± 0.3
	Trial 2 – Duration (s)	6.1 ± 3.8	3.5 ± 0.9	4.7 ± 3.8	4.9 ± 3.0
	Failed to meet criterion	0	0	0	0
Females					
Session 1 – Learning	No. of animals	16	16	16	16
	Trials to criterion	6.8 ± 2.5	7.1 ± 2.2	7.7 ± 2.6 (+13)[c]	7.6 ± 2.9 (+12)[c]
	Trial 1 – Errors	0.8 ± 1.3	0.8 ± 0.9	1.3 ± 1.0 (+62)[c]	1.1 ± 1.6 (+38)[c]
	Trial 1 – Duration (s)	15.3 ± 10.4	17.2 ± 13.9 (+12)[c]	22.6 ± 11.7 (+48)[c]	19.4 ± 14.0 (+27)[c]
	Trial 2 – Errors	0.3 ± 0.6	0.6 ± 0.6	0.4 ± 0.9	0.6 ± 0.6
	Trial 2 – Duration (s)	8.1 ± 3.0	10.5 ± 6.6 (+30)[c]	11.4 ± 8.8 (+41)[c]	12.1 ± 6.2 (+49)[c]
	Failed to meet criterion	0	0	0	1
Session 2 – Memory	No. of animals	16	16	16	15
	Trials to criterion	5.9 ± 1.9	8.5 ± 3.7* (+47)[c]	7.3 ± 2.3* (+26)[c]	8.9 ± 4.5 (53%)
	Trial 1 – Errors	0.1 ± 0.3	0.8 ± 1.1	0.3 ± 0.6	0.3 ± 0.5
	Trial 1 – Duration (s)	8.2 ± 6.1	12.3 ± 10.3	9.4 ± 7.8	7.5 ± 4.1
	Trial 2 – Errors	0	0.1 ± 0.5	0.1 ± 0.5	0.2 ± 0.6
	Trial 2 – Duration (s)	5.3 ± 1.9	6.6 ± 7.7	6.8 ± 4.7	5.2 ± 4.5
	Failed to meet criterion	0	0	0	4

From Sheets & Lake (2006)

* $P < 0.05$

[a] The water M-maze was repeated in a second, modified, developmental neurotoxicity study; results are presented in Table 33 below. Results are expressed as mean ± standard deviation.

[b] Value presented in parentheses represents increase compared with controls.

[c] Per cent change compared with controls given in parentheses.

Table 32. Selected microscopic brain measurements in F_1 rats (n = 10)[a]

Parameter	Brain measurement (mm)		
	Dietary concentration (ppm)		Historical control range[b]
	0	1500	
Males – PND 21			
Caudate putamen	3.05 ± 0.032	3.04 ± 0.118	2.96–3.30
Parietal cortex	1.88 ± 0.094	1.85 ± 0.077	1.81–2.03
Hippocampal gyrus	1.50 ± 0.070	1.46 ± 0.091	1.44–1.77
Males – Termination			
Caudate putamen	3.42 ± 0.182	3.29 ± 0.288 (−4)[c]	3.12–3.51
Parietal cortex	1.88 ± 0.088	1.75 ± 0.148* (−6)[c]	1.74–1.93
Hippocampal gyrus	1.62 ± 0.155	1.67 ± 0.148	1.57–1.74
Females – PND 21			
Caudate putamen	3.08 ± 0.078	3.00 ± 0.076* (−3)[c]	2.88–3.27
Parietal cortex	1.90 ± 0.138	1.89 ± 0.105	1.82–2.06
Hippocampal gyrus	1.45 ± 0.184	1.55 ± 0.114 (+7)[c]	1.38–1.69
Females – Termination			
Caudate putamen	3.38 ± 0.114	3.47 ± 0.063* (+3)[c]	3.21–3.55
Parietal cortex	1.82 ± 0.044	1.81 ± 0.061	1.76–1.88
Hippocampal gyrus	1.53 ± 0.176	1.62 ± 0.145 (+6)[c]	1.48–1.76

From Sheets & Lake (2006)
* $P < 0.05$
[a] Mean ± standard deviation.
[b] No details were provided regarding the source of the reference range data.
[c] Per cent change compared with controls given in parentheses.

cerebrum and cerebellum lengths were observed at any dose in either sex at any time point. There were no changes noted in morphometric measurements for day 21 male pups (Table 32). For day 21 females, there was a statistically significant decrease in the caudate putamen measurement. In day 70 pups, there was a statistically significant increase in the caudate putamen measurement in females and a decrease in the parietal cortex measurement in males. The morphometric changes were considered by the study authors to be unrelated to treatment, as the microscopy and gross measurements revealed no morphological changes in these areas, the changes were noted in one sex only and the changes were not noted in both time periods examined.

The study authors considered the NOAEL for maternal toxicity to be 350 ppm (equal to 32.1 mg/kg bw per day), based on decreased body weight, body weight gain and food consumption during lactation at 1500 ppm. However, the Meeting noted that although the body weight decrease in maternal animals may be treatment related, it was not considered biologically significant (decrease of 5%). Therefore, the Meeting concluded that the maternal NOAEL was 1500 ppm (equal to 135.9 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 350 ppm (equal to 32.1 mg/kg bw per day), based on decreased body weight during lactation at 1500 ppm (Sheets & Lake, 2006).

In a GLP-compliant modified developmental neurotoxicity study, spirodiclofen (purity 98.3–97.5%; mixed match) was administered to groups of pregnant Wistar Hannover rats (30 per dose) continuously from GD 0 to LD 21 in the diet at nominal doses of 0, 70, 350 or 1500 ppm (equal to 0, 5.4, 28.6 and 119.2 mg/kg bw per day during gestation and 0, 13.0, 65.7 and 262.1 mg/kg bw per day

during lactation). The United States Environmental Protection Agency and Health Canada (Pest Management Regulatory Agency) had reviewed the original developmental neurotoxicity study (Sheets & Lake, 2004; summarized above), requested that brain measurements be taken from low- and mid-dose animals and raised questions about the M-maze results for treated female offspring tested on PND 60. As the brains from the low- and mid-dose animals had been stored for an extended period in fixative and were therefore subject to shrinkage relative to tissues for control and high-dose animals, these tissues were considered by the manufacturer to be unsuitable for comparison. Consequently, the manufacturer chose to conduct a modified developmental neurotoxicity study. The purpose of this present study was to provide data needed to address the aforementioned issues without unnecessarily repeating tests that had been performed in the original developmental neurotoxicity study. On PND 4, litters with a minimum of seven pups, including at least three per sex, were culled to yield, as closely as possible, four males and four females. Subsets of surviving offspring, representing at least 18–20 litters per dietary level, were subjected to evaluation using the following observations and measurements: detailed clinical observations, body weight, tests of spatial learning and memory (i.e. M- and Cincinnati water mazes) beginning on PND 60 (±2 or 4 days, respectively) and an ophthalmic examination. The M-maze test was repeated under the same conditions as in the original study, and a separate group of animals was tested using the Cincinnati water maze, a test of spatial learning and memory with a more complex configuration. Whole-brain tissue was collected from 10 of each sex per dietary level (representing 20 litters) on PND 21 and at study termination (approximately 75 days of age) for morphometry.

No mortalities or significant treatment-related effects were observed in maternal animals during the study. Slight decreases in food consumption (of 4–5%) and body weight gain (of 4%) were noted during lactation; however, these findings were not considered to be adverse owing to the slight magnitude of the effects and lack of statistical significance. In addition, no treatment-related effects on reproductive performance were observed.

Offspring viability, litter size and sex ratio were not affected by exposure to the test substance. No treatment-related mortalities or adverse clinical signs of toxicity were observed in the offspring during lactation or post-weaning, nor were there any treatment-related ophthalmological findings. Decreased mean pup body weights in high-dose males and females were observed from PNDs 17 to 21 and were statistically significant at PND 21 only (decreased by 8%). Overall body weight gain was significantly decreased in high-dose males and females from PNDs 4 to 21 (by 9–10%). At post-weaning, mean body weight was decreased in high-dose males from PNDs 29 to 50 (by 5–6%), with statistical significance reached on PNDs 35 and 42. No significant body weight changes were observed during post-weaning (PNDs 28–70) for females.

In the M-maze (Table 33), control and treated animals of both sexes demonstrated acquisition during the learning phase. One control female, one low-dose male, one mid-dose female and one high-dose female failed to meet the criterion during the learning phase. In addition, one high-dose male was inadvertently not tested for acquisition. Animals were not included for testing in the retention phase if acquisition was not demonstrated. There were no compound-related effects on acquisition or retention in males or females at any dietary level. Furthermore, there were no statistical differences from control at any dietary level on either test occasion. In the Cincinnati water maze (Table 34), control and treatment group animals of both sexes demonstrated acquisition and retention. No significant treatment-related findings were observed in the Cincinnati water maze. Postmortem examination did not reveal any significant differences in absolute brain weight in males or females on PND 21 or at study termination. Relative brain weights were increased (by 9%) in high-dose males at study termination, which was considered to be due to the decrease in terminal body weight observed in this dose group, rather than a direct effect on the brain. There were no statistically significant changes in brain morphometric measurements (Table 35).

Table 33. Water M-maze performance for F_1 rats in the modified developmental neurotoxicity study[a]

Session/parameter		Dietary concentration (ppm)			
		0	70	350	1500
Males					
Session 1 – Learning	No. of animals	16	16	16	15
	Trials to criterion	6.4 ± 1.4	6.3 ± 2.5	7.9 ± 2.4 (+23)[b]	7.6 ± 2.1 (+19)[b]
	Trial 1 – Errors	0.6 ± 0.6	0.6 ± 0.9	0.6 ± 0.8	1.2 ± 1.1 (2-fold increase)[c]
	Trial 1 – Duration (s)	9.9 ± 4.2	13.6 ± 6.4 (+13)[b]	14.8 ± 7.8 (+50)[b]	15.9 ± 9.5 (+60)[b]
	Trial 2 – Errors	0.4 ± 0.5	0.3 ± 0.4	0.7 ± 0.8 (+75)[b]	1.0 ± 1.0 (2.5-fold increase)[c]
	Trial 2 – Duration (s)	10.4 ± 6.1	9.5 ± 5.6	16.2 ± 14.2 (+56)[b]	14.9 ± 9.9 (+43)[b]
	Failed to meet criterion	0	1	0	0
Session 2 – Memory	No. of animals	16	15	16	15
	Trials to criterion	5.4 ± 1.0	6.5 ± 2.4	5.6 ± 1.4	6.7 ± 2.6 (+24)[b]
	Trial 1 – Errors	0.2 ± 0.4	0.3 ± 0.5	0.3 ± 0.7	0.1 ± 0.3
	Trial 1 – Duration (s)	7.3 ± 4.0	8.6 ± 6.7	9.5 ± 7.3	6.2 ± 4.3
	Trial 2 – Errors	0	0	0	0
	Trial 2 – Duration (s)	5.1 ± 2.8	4.2 ± 2.2	4.5 ± 2.6	4.3 ± 1.6
	Failed to meet criterion	0	0	0	0
Females					
Session 1 – Learning	No. of animals	16	16	15	16
	Trials to criterion	7.6 ± 2.6	7.3 ± 2.1	8.1 ± 3.1	7.3 ± 2.9
	Trial 1 – Errors	0.8 ± 1.0	0.8 ± 1.1	0.9 ± 1.2	0.4 ± 0.8
	Trial 1 – Duration (s)	15.3 ± 10.4	12.3 ± 7.3	13.2 ± 8.1	12.6 ± 6.5
	Trial 2 – Errors	0.7 ± 1.1	0.9 ± 1.1	0.7 ± 1.3	0.5 ± 1.0
	Trial 2 – Duration (s)	12.7 ± 10.1	12.6 ± 7.0	10.7 ± 8.7	10.2 ± 6.5
	Failed to meet criterion	1	0	1	1
Session 2 – Memory	No. of animals	15	16	14	15
	Trials to criterion[c]	7.9 ± 3.6	8.4 ± 3.9	8.0 ± 3.8	8.9 ± 4.2 (+13)[b]
	Trial 1 – Errors	0.0 ± 0.0	0.4 ± 0.9	0.4 ± 0.6	0.1 ± 0.4
	Trial 1 – Duration (s)	4.9 ± 2.9	7.7 ± 9.4 (+57)[b]	9.1 ± 7.4 (+86)[b]	6.9 ± 4.9 (+41)[b]
	Trial 2 – Errors	0.2 ± 0.6	0.3 ± 0.6	0.1 ± 0.5	0.1 ± 0.4
	Trial 2 – Duration (s)	6.1 ± 7.3	5.7 ± 3.3	6.9 ± 9.3	4.9 ± 4.8
	Failed to meet criterion	0	1 (6.3)	2 (14)	3 (20)

From Gilmore, Sheets & Hoss (2007)

[a] Mean ± standard deviation.

[b] Per cent change compared with controls given in parentheses.

[c] Compared with controls.

[d] For females that failed to meet the criterion within 15 trials, a value of 15 was assigned for the purpose of calculating the mean number of trials for the group.

Table 34. Cincinnati water maze performance for F_1 rats[a]

Session		Dietary concentration (ppm)							
		0		70		350		1500	
		Latency	Errors	Latency	Errors	Latency	Errors	Latency	Errors
Males									
Learning phase	Day 1	300 ± 0	18.5 ± 3.1	286 ± 35	17.3 ± 3.8	300 ± 0	17.8 ± 4.2	289 ± 28	16.6 ± 3.9
	Day 2	241 ± 84	17.6 ± 7.8	227 ± 94	15.8 ± 5.8	217 ± 85	16.0 ± 6.4	269 ± 31	16.7 ± 3.9
	Day 3	175 ± 117	9.9 ± 7.6	118 ± 109	6.0 ± 5.1	104 ± 60	6.5 ± 4.9	171 ± 100	9.7 ± 5.7
	Day 4	80 ± 60	4.8 ± 5.5	100 ± 105	4.8 ± 7.2	48 ± 19	1.8 ± 1.8	96 ± 96	4.1 ± 5.6
	Day 5	52 ± 37	2.0 ± 3.3	63 ± 86	2.1 ± 4.2	48 ± 37	0.9 ± 1.5	44 ± 25	1.1 ± 1.1
	Overall[b]	169.6	10.6	158.8	9.2	143.4	8.6	173.8	9.6
Retention phase	Day 12	42 ± 19	1.0 ± 1.1	40 ± 26	1.9 ± 3.3	40 ± 16	0.4 ± 0.5	35 ± 21	0.5 ± 0.9
Females									
Learning phase	Day 1	278 ± 68	20.2 ± 6.4	280 ± 34	22.3 ± 4.0	296 ± 14	21.7 ± 5.9	282 ± 57	21.5 ± 5.8
	Day 2	242 ± 82	17.9 ± 6.0	218 ± 83	16.7 ± 6.3	224 ± 85	17.5 ± 8.2	178 ± 102	16.1 ± 9.4
	Day 3	154 ± 99	11.2 ± 7.6	112 ± 73	8.0 ± 7.0	110 ± 108	5.8 ± 6.1	85 ± 59	6.1 ± 5.3
	Day 4	81 ± 66	4.5 ± 5.5	42 ± 18	2.0 ± 1.4	66 ± 86	2.8 ± 4.7	52 ± 45	2.3 ± 2.8
	Day 5	41 ± 30	1.4 ± 1.9	24 ± 63	0.5 ± 0.7	57 ± 87	1.4 ± 2.0	35 ± 19	1.7 ± 1.2
	Overall[b]	159.2	11.0	135.2	9.9	150.6	9.8	126.4	9.5
Retention phase	Day 12	72 ± 69	4.9 ± 7.4	57 ± 50	1.8 ± 2.9	49 ± 46	2.2 ± 3.6	89 ± 85	5.0 ± 5.8

From Gilmore, Sheets & Hoss (2007)
[a] Mean ± standard deviation.
[b] Calculated by the reviewer.

Table 35. Selected microscopic brain measurements in F_1 rats from the modified developmental neurotoxicity study

Parameter	Brain measurement (mm)[a]				
	Dose (ppm)				Historical control[b]
	0	70	350	1500	
Males – PND 21 (n = 10)					
Caudate putamen	3.03 ± 0.141	3.04 ± 0.100	3.00 ± 0.100	2.93 ± 0.141 (−3)[c]	2.79–3.30
Cerebellum	5.16 ± 0.300	4.97 ± 0.374	5.02 ± 0.200	4.91 ± 0.283 (−5)[c]	4.03–4.90
Hippocampal gyrus	1.78 ± 0.100	1.71 ± 0.100	1.71 ± 0.200	1.76 ± 0.141	1.44–1.75
Parietal cortex	1.97 ± 0.100	2.00 ± 0.100	1.98 ± 0.100	1.90 ± 0.100	1.82–2.03
Frontal cortex	1.86 ± 0.100	1.92 ± 0.055	1.89 ± 0.141	1.78 ± 0.100	1.66–1.93
Males – PND 75 (n = 10)					
Caudate putamen	3.29 ± 0.100	3.28 ± 0.173	3.19 ± 0.173	3.32 ± 0.100	3.12–3.51
Cerebellum	5.05 ± 0.265	4.77 ± 0.374	4.78 ± 0.424	5.10 ± 0.173	3.83–4.96
Hippocampal gyrus	1.72 ± 0.100	1.76 ± 0.100	1.74 ± 0.100	1.67 ± 0.100 (−3)[c]	1.55–1.81
Parietal cortex	1.93 ± 0.055	1.96 ± 0.063	1.94 ± 0.045	1.97 ± 0.055	1.74–1.93
Frontal cortex	1.78 ± 0.100	1.79 ± 0.100	1.74 ± 0.100	1.82 ± 0.063	1.58–1.91

Table 35 (contd)

Parameter	Brain measurement (mm)[a]				
	Dose (ppm)				Historical control[b]
	0	70	350	1500	
Females – PND 21 (*n* = 10)					
Caudate putamen	2.93 ± 0.100	2.99 ± 0.200	2.96 ± 0.100	3.00 ± 0.100	2.83–3.23
Cerebellum	4.97 ± 0.346	4.76 ± 0.346	4.86 ± 0.283	4.90 ± 0.224	3.99–5.10
Hippocampal gyrus	1.69 ± 0.100	1.66 ± 0.141	1.64 ± 0.173	1.59 ± 0.100 (−6)[c]	1.38–1.74
Parietal cortex	1.96 ± 0.100	1.97 ± 0.100	1.96 ± 0.100	1.98 ± 0.055	1.70–2.02
Frontal cortex	1.84 ± 0.141	1.89 ± 0.063	1.87 ± 0.100	1.85 ± 0.063	1.64–1.96
Females – PND 75 (*n* = 10)					
Caudate putamen	3.22 ± 0.100	3.14 ± 0.200	3.17 ± 0.141	3.18 ± 0.100	3.21–3.55
Cerebellum	4.84 ± 0.200	4.85 ± 0.173	4.77 ± 0.245	4.64 ± 0.200 (−4)[c]	3.98–4.98
Hippocampal gyrus	1.75 ± 0.141	1.70 ± 0.100	1.71 ± 0.141	1.69 ± 0.173 (−3)[c]	1.48–1.76
Parietal cortex	1.84 ± 0.032	1.84 ± 0.100	1.88 ± 0.045	1.82 ± 0.045	1.68–1.88
Frontal cortex	1.75 ± 0.045	1.70 ± 0.100	1.73 ± 0.032	1.67 ± 0.100 (−5)[c]	1.65–1.84

From Gilmore, Sheets & Hoss (2007)

[a] Mean ± standard deviation.

[b] Historical control data were provided for 17 (terminal pup data) and 19 (PND 21 pup data) studies conducted between 2000 and 2006.

[c] Per cent change compared with controls given in parentheses.

Based on the results from the study, the NOAEL for maternal toxicity was 1500 ppm (equal to 119.2 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 350 ppm (equal to 28.6 mg/kg bw per day), based on decreases in preweaning body weight and body weight gain in both sexes and reduced post-weaning body weight in males at 1500 ppm (Gilmore, Sheets & Hoss, 2007).

Positive control data for validation of the developmental neurotoxicity studies (which were referenced, but not included, in the study report) were provided by the manufacturer (Sheets, 2001; Sheets & Lake, 2001). The data pertaining to the M-maze are summarized in Table 36. The Meeting noted that there was considerable variability in the M-maze data from both the validation studies as well as the spirodiclofen studies under review, which makes it difficult to clearly identify treatment-related changes in this parameter in any of these studies. Positive control data were also provided for the Cincinnati water maze (Table 37). However, data were provided only for males, and, as such, the study was not validated for females (Sheets, 2007).

The Meeting noted that small morphometric changes in the following brain regions were observed in the first study (Sheets & Lake, 2006) at the high dose (low and middle doses were not examined): caudate putamen, hippocampal gyrus and parietal cortex. In the second study (Gilmore, Sheets & Hoss, 2007), morphometry was performed at all dose levels, and small changes were noted at the high dose in the caudate putamen, cerebellum and hippocampal gyrus. Although some of the same brain regions appeared to be affected in both studies, the observed changes were small (3–7%), did not attain statistical significance in many cases and were not consistent between studies, and thus they were not considered to be related to treatment (see Table 35).

In tests of learning and memory, the Meeting noted that females required an increased number of trials at all dose levels to meet the criterion during the memory phase of the original M-maze. In addition, four high-dose females failed to complete the retention task (five consecutive errorless trials

Table 36. M-maze performance[a] of rats from validation studies with methimazole[b] and scolopamine[c]

Session/parameter		Methimazole concentration (mg/ml)		Scopolamine concentration (mg/kg bw)	
		0	0.1	0	1.0
Males					
Session 1 – Learning	No. of animals	23	20	12	12
	Trials to criterion	7.3 ± 2.5	9.5 ± 3.3* (+30)[d]	7.8 ± 3.2	9.5 ± 4.1 (+22)[d]
	Trial 1 – Errors	0.9 ± 0.9	0.9 ± 0.7	0.9 ± 1.1	0.9 ± 0.5
	Trial 1 – Duration (s)	18.4 ± 11.9	24.3 ± 17.6 (+32)[d]	23.0 ± 17.7	28.4 ± 15.5 (+23)[d]
	Trial 2 – Errors	0.5 ± 0.8	0.8 ± 0.9 (+38)[d]	0.6 ± 0.9	0.6 ± 0.7
	Trial 2 – Duration (s)	15.0 ± 13.1	20.3 ± 12.0* (+35)[d]	20.3 ± 13.2	20.2 ± 14.0
	Failed to meet criterion	1 (4)[e]	2 (10)[e]	1 (8)[e]	3 (25)[e]
Session 2 – Memory	No. of animals	22	18	10	9
	Trials to criterion	5.8 ± 1.9	6.5 ± 2.3 (+12)[d]	6.8 ± 2.3	8.2 ± 2.8 (+21)[d]
	Trial 1 – Errors	0.2 ± 0.4	0.8 ± 1.2 (4-fold increase)[f]	0.2 ± 0.4	0.3 ± 0.5
	Trial 1 – Duration (s)	9.6 ± 11.7	17.3 ± 18.2 (+80)[d]	11.8 ± 6.1	15.1 ± 6.8 (+28)[d]
	Trial 2 – Errors	0 ± 0	0.2 ± 0.4	0.3 ± 0.7	0.3 ± 0.5
	Trial 2 – Duration (s)	4.2 ± 1.7	7.8 ± 6.7 (+86)[d]	8.9 ± 8.0	13.4 ± 8.7 (+50)[d]
	Failed to meet criterion	0	0	0	0
Females					
Session 1 – Learning	No. of animals	23	21	12	12
	Trials to criterion	8.1 ± 2.8	10.0 ± 3.0 (+23)[d]	10.1 ± 3.5	11.9 ± 3.0 (+18)[d]
	Trial 1 – Errors	0.7 ± 0.7	0.7 ± 0.7	1.2 ± 1.7	0.5 ± 0.5
	Trial 1 – Duration (s)	16.1 ± 9.5	17.6 ± 11.9 (+9)[d]	24.0 ± 14.6	18.4 ± 6.2*
	Trial 2 – Errors	0.6 ± 1.1	1.0 ± 1.5 (+67)[d]	0.7 ± 0.9	0.6 ± 0.7
	Trial 2 – Duration (s)	14.0 ± 13.1	19.7 ± 15.9 (+41)[d]	18.6 ± 14.6	19.1 ± 10.7
	Failed to meet criterion	0	2	2	5
Session 2 – Memory	No. of animals	23	19	10	6
	Trials to criterion	8.2 ± 3.7	9.5 ± 4.4 (+16)[d]	9.4 ± 3.9	11.2 ± 2.9 (+19)[d]
	Trial 1 – Errors	0.6 ± 1.0	0.5 ± 0.9	0.4 ± 0.7	0.5 ± 0.8
	Trial 1 – Duration (s)	10.3 ± 9.7	12.1 ± 10.3 (+17)[d]	14.0 ± 11.3	30.3 ± 18.5 (1.2-fold increase)[f]
	Trial 2 – Errors	0.1 ± 0.5	0.3 ± 0.6	0.3 ± 0.7	0.7 ± 0.8
	Trial 2 – Duration (s)	6.6 ± 5.0	8.1 ± 5.8 (+23)[d]	11.0 ± 9.2	14.0 ± 10.0 (+30)[d]
	Failed to meet criterion	3	3	0	0

From Sheets (2001); Sheets & Lake (2001)

* $P < 0.05$

[a] Mean ± standard deviation.

[b] Methimazole was administered to pregnant Wistar rats via drinking-water on GD 16 to LD 10; F_1 rats performed M-maze at beginning at PND 60. Doses of methimazole administered to maternal rats were calculated based on test substance intake and body weight data provided in the study report: days 0–7 (GD 16–22): 14.2 mg/kg bw per day; days 7–14 (LD 0–7): 18.9 mg/kg bw per day; days 14–21 (LD 10): 20.8 mg/kg bw per day (Sheets & Lake, 2001).

[c] Scopolamine was administered (subcutaneously) to 8-week-old Wistar rats 30–60 min prior to testing (Sheets, 2001).

[d] Per cent change compared with controls given in parentheses.

[e] Per cent of total given in parentheses.

[f] Compared with controls.

Table 37. Cincinnati water maze performance with scopolamine in males (n = 12)[a]

Session		Dose (mg/kg bw)							
		Control		0.3		0.6		1.2	
		Latency	Errors	Latency	Errors	Latency	Errors	Latency	Errors
Learning phase	Day 1	282 ± 42	20.3 ± 4.6	300 ± 0	40.5 ± 8.1	293 ± 24	38.6 ± 10.4	300.0 ± 0	45.2 ± 5.5
	Day 2	206 ± 83	14.0 ± 5.7	263 ± 69	30.0 ± 13.5	274 ± 48	37.4 ± 12.1	284 ± 55	45.1 ± 15.0
	Day 3	110 ± 82	6.4 ± 4.3	165 ± 100	15.3 ± 11.4	209 ± 117	22.9 ± 15.7	218 ± 99	29.2 ± 19.8
	Day 4	69 ± 48	3.0 ± 2.9	98 ± 84	6.0 ± 5.9	143 ± 107	14.2 ± 12.4	216 ± 98	24.6 ± 16.9
	Day 5	40 ± 23	1.2 ± 1.8	104 ± 106	6.8 ± 8.9	158 ± 96	14.8 ± 13.9	117 ± 118	20.8 ± 18.8
	Overall	141	9.0	186	19.7*	216*	25.6*	239*	33.0*
Retention phase	Day 12	76 ± 46	2.6 ± 3.0	129 ± 69	9.6 ± 6.3	139 ± 90	10.5 ± 7.8*	179 ± 82*	15.0 ± 9.7*

From Sheets (2007)
* $P < 0.05$
[a] Scopolamine was administered (by intraperitoneal injection) to Wistar rats 30 min prior to acquisition testing. Results are mean ± standard deviation.

during the memory phase) in this test (Sheets & Lake, 2006; see Table 31). In the second study (Gilmore, Sheets & Hoss, 2007), there was a slight increase in the number of trials to meet the criterion for high-dose females during the memory phase in the M-maze task, and there were a few females in the treated groups that failed to meet the criterion of five consecutive errorless trials in the retention phase (incidence of 0, 1, 2 and 3; see Table 33). It should be noted that in groups in which animals failed to complete the retention task, the values for mean trials to criterion are underestimated, as an arbitrary value of 15 trials was assigned for those animals that failed to complete the task within 15 trials. A Cincinnati water maze was also conducted in the second study. While there were no treatment-related findings noted in this maze, interpretation of these results was limited due to the novelty of this test to the conducting laboratory, the fact that the test was not validated for females and the considerable variability noted in the data in the current study for females, which led to uncertainty regarding the sensitivity of the test to detect treatment-related changes (see Table 34).

Overall, the Meeting concluded that the results of these studies did not indicate any treatment-related findings on neurotoxicity parameters in offspring, as the findings were inconsistent, and the considerable variability in the data limited their interpretation.

(b) Supplemental studies on mechanisms of endocrine effects

Several in vitro and short-term toxicity studies were conducted with spirodiclofen and its enol metabolites in order to further investigate the endocrine effects observed in the toxicology studies, which effects included vacuolation of steroid tissues (adrenal gland and Leydig cells) in mice, rats and dogs, signs of androgen deprivation in dogs (e.g. immature prostate and germinal epithelium, as well as oligospermia and aspermia), Leydig cell hypertrophy or hyperplasia in mice, rats and dogs, and neoplastic lesions in steroid-sensitive tissues, the testes and uterus, in the 2-year rat study (see sections 2.2(a), 2.3 and 2.5(a) above).

In vitro studies to investigate androgen and/or estrogen receptor-mediated mechanisms

In a non-GLP in vitro study, the effects of spirodiclofen and the enol (BAJ 2510) and 4-hydroxy-enol (at concentrations of 10^{-11}–10^{-5} mol/l) metabolites were investigated in a series of reporter gene and receptor binding assays. In the reporter gene assays, human androgenic (human prostate-derived PALM cells) or estrogenic (human breast cancer–derived MVLN cells) cell lines transfected with a

reporter gene (luciferase) coupled with a hormone response element were used in the presence or absence of estrogen (MVLN cells) or testosterone (PALM cells). The affinity of spirodiclofen and the metabolites for the estrogen receptor was tested in competitive receptor binding assays with human alpha and beta estrogen receptors with a fluorescence-labelled estrogen. In all test systems, estradiol or methyltestosterone was used as a positive control.

Spirodiclofen and the enol metabolite were not cytotoxic to either of the cell lines used, whereas the 4-hydroxy-enol metabolite showed slight cytotoxicity to MVLN cells. Spirodiclofen and its metabolites did not show any androgenic or antiandrogenic activity in PALM cells. In MVLN cells, spirodiclofen and the 4-hydroxy-enol did not show any estrogenic or antiestrogenic activity, whereas the enol metabolite showed some contradictory results. The effects of the enol metabolite appeared to be dependent on pH, such that estrogenic and antiestrogenic effects were noted at pH 6–6.5. In the receptor binding assays, none of the test compounds bound to either of the receptor subtypes under normal pH conditions (pH 7.5); however, at a lower pH (pH 6–6.5), the enol metabolite had a slight binding activity. The study author concluded that spirodiclofen and the enol and 4-hydroxy-enol metabolites did not display estrogenic, antiestrogenic, androgenic or antiandrogenic activity under conditions of physiological pH (Schmuck, 1999).

In vitro studies to investigate effects on cholesterol esterase

In a non-GLP in vitro radiometric assay, the possible inhibition of cholesterol esterase, an enzyme required for intestinal absorption and liberation of cholesterol from intracellular lipid pools, by spirodiclofen and the enol metabolite, BAJ 2510, was investigated. Based on findings in steroid-sensitive tissues noted in the in vivo studies, it was considered likely that steroidogenesis would be affected by treatment with spirodiclofen. One of the earliest steps in the synthesis of steroid hormones is the release of the precursor cholesterol from cholesterol ester stores by cholesterol esterase. The purpose of the current in vitro study was to investigate whether spirodiclofen or BAJ 2510 could inhibit steroid hormone synthesis at the level of this enzyme, by investigating its potential to inhibit the cholesterol esterase–catalysed release of radiolabelled fatty acid from a radiolabelled cholesterol ester. The potential for spirodiclofen to act as an alternative substrate for cholesterol esterase was also examined. Spirodiclofen (0.1–100 µmol/l) or BAJ 2510 (0.1–1000 µmol/l) was incubated with commercially available porcine pancreatic cholesterol esterase in the presence of radiolabelled cholesterol oleate for the cholesterol esterase inhibition experiments. Spirodiclofen (5 or 50 µmol/l) was also incubated in the absence of radiolabelled cholesterol oleate for the alternative substrate experiments.

A dose-dependent inhibition of cholesterol esterase was noted with spirodiclofen in two separate experiments (median inhibitory concentrations [IC_{50}s] of 12 and 43 µmol/l). BAJ 2510 inhibited the enzyme only at 1000 µmol/l. Spirodiclofen was not found to act as a substrate for cholesterol esterase, and thus inhibition of the enzyme by acting as a competing substrate could not be substantiated as the inhibitory mode of action. Results from in vivo studies demonstrated that spirodiclofen could not be detected in the plasma of spirodiclofen-treated animals (Wetzig & Ruehl-Fehlert, 2001). As BAJ 2510, the main plasma metabolite, displayed weak inhibitory potential on cholesterase esterase, interference with the synthesis of steroid hormones via effects on this enzyme was not supported by the results of this in vitro study (Freyberger, 2000).

In vitro studies to investigate effects on testosterone formation

In a non-GLP study, the effects of spirodiclofen and its enol metabolites (BAJ 2510, 3-hydroxy-enol and 4-hydroxy-enol) on testosterone secretion by rat testicular tissue maintained in dynamic organ culture were studied. Spirodiclofen (1.5–100 µmol/l) and the enol metabolites (1–1000 µmol/l) were incubated with rat testicular tissue in the presence of human chorionic gonadotrophin (hCG), dibutyryl-cyclic adenosine monophosphate (db-cAMP) or 25-hydroxycholesterol (25-OH-

cholesterol) and hCG, in order to investigate potential sites of action in the steroidogenic pathway of these test substances. 25-OH-cholesterol can enter the mitochondria without the need of active transport and thus can be used to circumvent potential inhibitory effects on cholesterol esterase and cholesterol transport into the mitochondria. Testosterone levels in the incubation medium were measured by radioimmunoassay.

Spirodiclofen suppressed hCG-stimulated testosterone formation in one of four repeat experiments and also suppressed cAMP-stimulated testosterone products (by 50% at 50 µmol/l). BAJ 2510 (≥10 µmol/l with hCG or db-cAMP; ≥100 µmol/l with hCG and 25-OH-cholesterol) strongly inhibited testosterone production under all conditions tested, whereas the hydroxy-enols produced moderate inhibition of hCG- and cAMP-stimulated testosterone at high doses only (1000 µmol/l). The fact that BAJ 2510 suppressed testosterone production even in the presence of 25-OH-cholesterol suggests that the site of inhibition is downstream of cholesterol transport into the mitochondria (Freyberger, 2001a).

In vitro studies to investigate effects on microsomal enzymes involved in testosterone synthesis

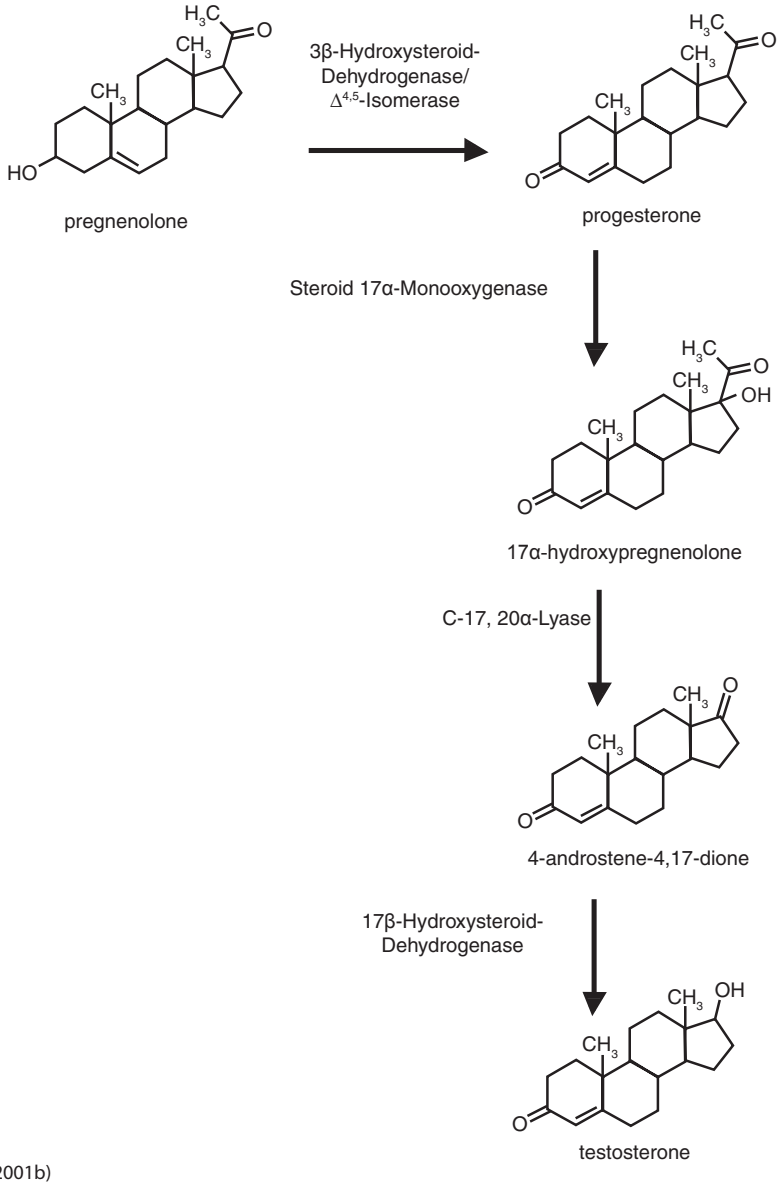
In order to investigate whether treatment with spirodiclofen interfered with microsomal cytochrome P450–dependent mono-oxygenases involved in steroid hormone synthesis, the effects of spirodiclofen and its metabolites BAJ 2510 (enol), 3-hydroxy-enol and 4-hydroxy-enol on steroid 17α-mono-oxygenase (involved in the conversion of progesterone to 17α-progesterone) and C-17,20-lyase (conversion of 17α-progesterone to androstenedione; Figure 2) were studied in a non-GLP in vitro study. Rat testicular microsomes served as an enzyme source. Microsomes were incubated with precursor steroids (progesterone or 17α-hydroxyprogesterone) in the presence of test compounds, and resultant products (17α-hydroxyprogesterone or androstenedione) were quantified by ultraviolet (UV) spectroscopy following HPLC separation from educts.

No inhibition of steroid 17α-mono-oxygenase occurred in the presence of spirodiclofen at 50 µmol/l (maximum non-precipitating concentration) or BAJ 2510 at 300 µmol/l. Slight inhibition of C-17,20-lyase was observed in the presence of spirodiclofen at 50 µmol/l and BAJ 2510 at 300 µmol/l, which produced 13% and 8% inhibition, respectively. Based on these results, it was concluded that inhibitory effects on these cytochrome P450–dependent microsomal mono-oxygenases in animals treated with spirodiclofen were unlikely to occur, as spirodiclofen is not detectable in plasma; although BAJ 2510 is present in micromole per litre concentrations, it did not significantly inhibit these enzymes in vitro. Further, in the in vitro experiments with rat testicular fragments, BAJ 2510 significantly decreased testosterone formation at concentrations as low as 10 µmol/l (Freyberger, 2001b).

In order to investigate whether treatment with spirodiclofen interfered with microsomal dehydrogenases involved in steroid hormone biosynthesis, the effects of spirodiclofen and its metabolites BAJ 2510 (enol), 3-hydroxy-enol and 4-hydroxy-enol on 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase (conversion of pregnenolone to progesterone) and 17β-hydroxysteroid dehydrogenase (conversion of androstenedione to testosterone; Figure 2) were studied in a non-GLP in vitro study. Rat testicular microsomes served as an enzyme source. Microsomes were incubated with precursor steroids (pregnenolone or androstenedione) in the presence of test compounds, and resultant products (progesterone or testosterone) were quantified by UV spectroscopy or liquid scintillation counting following HPLC separation from educts.

Spirodiclofen at 25 or 50 µmol/l inhibited the formation of progesterone by 20% and 22%, respectively, whereas BAJ 2510 (300 µmol/l), 3-hydroxy-enol (100 µmol/l) and 4-hydroxy-enol (100 µmol/l) failed to inhibit the formation of progesterone, demonstrating that spirodiclofen (at maximum non-precipitating concentrations), but not the enol metabolites, inhibited 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase by 20% in vitro. Spirodiclofen (50 µmol/l), BAJ 2510 (100 µmol/l), 3-hydroxy-enol (1000 µmol/l) and 4-hydroxy-enol (1000 µmol/l) failed to inhibit the transformation

Figure 2. Microsomal reactions of the testosterone-synthesizing pathway



From Freyberger (2001b)

of androstenedione to testosterone, indicating that none of the test compounds affect the 17β-hydroxysteroid dehydrogenase enzyme. Whereas spirodiclofen produced a moderate inhibition of 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase under the conditions of this study, the study author concluded that as plasma levels of the parent compound are not detectable in animals treated with spirodiclofen (Wetzig & Ruehl-Fehlert, 2001), it was unlikely that inhibition of this enzyme would occur in vivo (Freyberger, 2001c).

In vitro studies to investigate effects on mitochondrial enzymes involved in steroidogenesis

In a series of non-GLP in vitro assays, the effects of spirodiclofen and its metabolites, BAJ 2510 (enol), 3-hydroxy-enol and 4-hydroxy-enol, on the side-chain cleavage enzyme (cytochrome P450scc) were studied in freshly isolated rat testicular mitochondria. Pregnenolone formed by

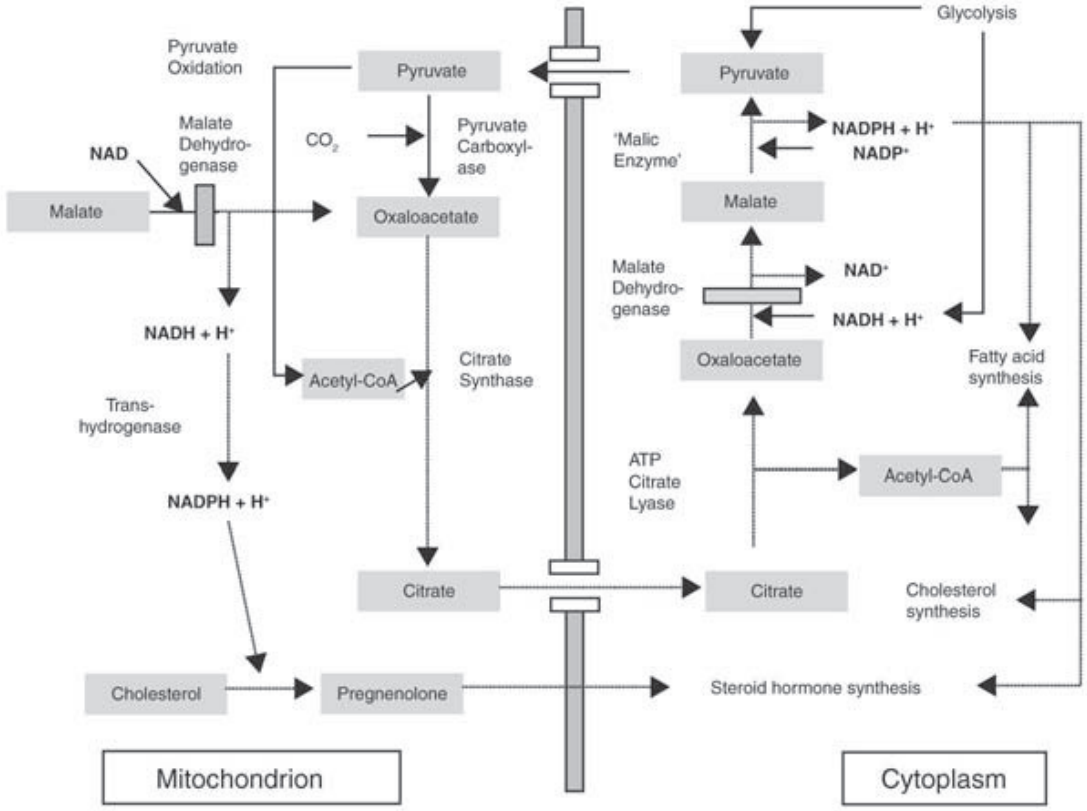
side-chain cleavage and released from mitochondria was enzymatically converted into progesterone and determined by HPLC. Effects on enzymes (malic enzyme [ME] and mitochondrial malate dehydrogenase [MD]) involved in the generation of reducing equivalents (reduced nicotinamide adenine dinucleotide phosphate [NADPH]) for the side-chain cleavage enzyme were studied as well using commercially available purified enzymes. Interactions with side-chain cleavage were also assessed in rat testicular tissue maintained in culture through measurements of progesterone and testosterone levels in the incubation medium. Finally, effects of BAJ 2510 on the availability of cytoplasmic reducing equivalents (produced through cytoplasmic MD) were studied. Cytoplasmic MD is part of the pyruvate/citrate shuttle that exports citrate from the mitochondria to the cytoplasm. There, citrate is degraded to acetyl coenzyme A (CoA) and oxaloacetate, which undergoes further reactions involving cytoplasmic MD, resulting in the formation of NADPH.

In the presence of 25-OH-cholesterol and malate, incubation of freshly isolated rat testicular mitochondria with BAJ 2510 at 100 µmol/l resulted in decreased side-chain cleavage (as evidenced by decreased progesterone formation). This inhibition could be lessened by increasing malate concentrations in the incubation medium and was almost reversed when malate was replaced by citrate. No interaction with the side-chain cleavage was observed in the presence of spirodiclofen at 50 µmol/l, 3-hydroxy-enol at 100 µmol/l or 4-hydroxy-enol at 100 µmol/l. These investigations suggested that BAJ 2510 affects steroidogenesis at the level of malate, which is involved in producing NADPH. As NADPH can be produced from malate through reactions involving either ME or mitochondrial MD, further assays were conducted to investigate which of these two enzymes might be affected by BAJ 2510. In these assays, BAJ 2510 inhibited mitochondrial MD, but not ME, as shown by a dose-dependent decrease in reduced nicotinamide adenine dinucleotide (NADH) oxidation by porcine heart mitochondrial MD in the presence of BAJ 2510. In cultured rat testicular tissue, BAJ 2510 decreased the hCG-stimulated synthesis of progesterone (50% inhibition with BAJ 2510 at 300 µmol/l), which was used as a measure of side-chain cleavage. Testosterone was also decreased by BAJ 2510, by a greater extent than was progesterone (90% inhibition with BAJ 2510 at ≥100 µmol/l). This suggests that BAJ 2510 may act at another site downstream of progesterone formation in the steroidogenic pathway. Similar findings were observed for ketoconazole, which is known to interfere with mitochondrial and microsomal target enzymes. Finally, in assays investigating potential effects on cytoplasmic MD, BAJ 2510 was found to inhibit this enzyme as well, as evidenced by a dose-dependent decrease in NADH oxidation by porcine heart cytoplasmic MD in the presence of BAJ 2510. This finding suggests that BAJ 2510 reduces the NADPH supply in the cytosol, resulting in strong inhibition of further NADPH-dependent reactions (Freyberger, 2001d).

The effect of BAJ 2510 on levels of reducing equivalents (NADH and NADPH) in rat testicular mitochondrial preparations was investigated in a series of non-GLP in vitro assays. As part of this study, a methodology to measure intramitochondrial NADH and NADPH was developed. Heat inactivation in the presence of detergent following addition of inhibitors of the respiratory chain/oxidative phosphorylation was used to suppress the decomposition of NADH and NADPH by mitochondrial lysates. NADH and NADPH levels in incubation medium were determined by reversed-phase ion pair chromatography and fluorescence detection.

In incubations with BAJ 2510 (300 µmol/l), statistically significant decreases in NADH concentrations were observed in the presence of varying concentrations of malate (decreases were 26–47%, 44–47% and 13–19% in the presence of malate at concentrations of 0.05 mmol/l, 0.5 mmol/l and 5 mmol/l, respectively). NADPH levels were also decreased by BAJ 2510, but less so, with increasing concentrations of malate (decreases were 26–41%, 25–31% and 2–6% in the presence of malate at concentrations of 0.05 mmol/l, 0.5 mmol/l and 5 mmol/l, respectively). Tartronate (5 mmol/l), a known inhibitor of mitochondrial ME, decreased NADH and NADPH levels to a similar extent (by approximately 25%), regardless of the concentration of malate used.

Figure 3. Proposed mode of action of the enol metabolite involving steroid inhibition[a]



Adapted from Kaune, Temerowski & Krautstrunk (2008)

[a] Consequences of mitochondrial MD inhibition are indicated by dotted lines; consequences of combined inhibition of mitochondrial and cytoplasmic MDs are indicated by dashed lines.

The study author concluded that these findings were consistent with the purported mechanism of action described for BAJ 2510, involving inhibition of intramitochondrial MD (Figure 3). It is thought that inhibition of this enzyme would result in decreased intramitochondrial NADH levels. Subsequently, less intramitochondrial NADPH would be formed from NADH (by transhydrogenation), and, finally, side-chain cleavage would be inhibited owing to a shortage of the relevant cofactor NADPH. As previously reported for the inhibition of side-chain cleavage by BAJ 2510, effects on NADPH and NADH levels also depended on malate concentrations, inhibition being most effective in the presence of low malate concentrations. The NADH levels tended to be more strongly inhibited by BAJ 2510 compared with the NADPH levels, which is in contrast to the findings with tartronic acid and again points to MD as the target of BAJ 2510. These findings further support the hypothesis of a competitive, reversible inhibition of MD by BAJ 2510 (Freyberger, 2002, 2003a).

In a second non-GLP study investigating the effect of BAJ 2510 on levels of reducing equivalents in the testes, varying concentrations of BAJ 2510 (purity 99.9%; 100, 200 or 300 µmol/l) were incubated with rat testicular mitochondrial preparations as described above (Freyberger, 2002) in the presence of malate (0.5 mmol/l). Tartronic acid was included as a positive control.

BAJ 2510 concentration-dependently decreased the overall amount of reducing equivalents (NADH + NADPH, statistically significant at all BAJ 2510 concentrations tested), NADH levels (statistically significant at BAJ 2510 concentrations of 200 and 300 µmol/l) and NADPH levels (statistically significant at all BAJ 2510 concentrations tested). In the presence of tartronic acid at

5 mmol/l, the overall amount of NADH + NADPH and NADPH concentrations were statistically significantly reduced by 30% and 34%, respectively, and NADH levels were non-significantly decreased by 28% (Freyberger, 2003b).

Short-term studies in rats

In a GLP-compliant subchronic toxicity study, spirodiclofen (purity 97.8%) was administered to groups of female Wistar rats (15 per dose) in the diet (containing 1% peanut oil) at dose levels of 0, 2500 or 10 000 ppm (equal to 0, 242.4 and 1209.6 mg/kg bw per day). Animals were dosed for 19 weeks and then monitored for a further 11 weeks of recovery with no test chemical. This study was conducted to investigate possible effects on circulating levels of hormones in female rats, in light of the increased incidence of uterine adenocarcinoma observed in the 2-year rat study (see section 2.3). Animals were inspected daily for mortality, and general clinical examinations, body weight measurements and feed intake determinations were conducted weekly. Blood samples were collected at weeks 7, 9, 11, 13, 17 and 19 during test substance administration and then at weeks 2 and 6 during the recovery period for determination of testosterone, estradiol, luteinizing hormone (LH) and progesterone. Vaginal smears were collected periodically throughout the study period for determination of estrous stage in order to attempt to collect blood samples when animals were in diestrus. All animals were subjected to gross pathological examination, and the liver, uterus, ovaries and adrenals were weighed. These tissues were fixed, but not subjected to histopathological examination.

Clinical signs noted during the dosing period were restricted to evidence of light discoloured faeces in some low-dose (7/15) and all high-dose females. Clinical signs resolved during the 11-week recovery period. Decreased body weight gain was noted for low-dose (by 7%) and high-dose (by 24%) animals during the treatment period. Food consumption was increased in treated animals in a dose-dependent manner (by 10% at the low dose and by 37% at the high dose). High-dose animals had significantly reduced testosterone, estradiol and progesterone levels during the 19 weeks of treatment (Table 38). It should be noted that testosterone levels in many of the samples were below the limit of detection (0.025 ng/ml). The estradiol levels were depressed to a greater extent than the progesterone levels, resulting in an increased estradiol to progesterone ratio in high-dose animals during the treatment period. There were no statistically significant differences in hormone levels during the recovery period; however, estradiol levels remained depressed (by 13–15%) following 6 weeks of recovery from treatment. Liver weights (absolute and relative to body weight) were reduced in high-dose animals, but the reductions were not regarded as biologically significant, whereas adrenal and ovary weights (absolute and relative) were increased in high-dose animals. Four high-dose females had grossly enlarged ovaries; however, microscopic examinations were not conducted (Andrews, 2001).

Table 38. Circulating levels of hormones in female rats during treatment and recovery

	Testosterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)	Progesterone (ng/ml)	Estradiol/ progesterone
Treatment					
Week 7					
Control	0.02	92.20	1.19	8.36	11.02
2500 ppm	0.01	76.47	1.54	8.17	9.36
10 000 ppm	0.01	61.05***	0.84	5.47	11.16
Week 9					
Control	0.02	95.50	1.85	17.86	5.35
2500 ppm	0.01	84.73	1.59	10.36	8.19

Table 38 (contd)

	Testosterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)	Progesterone (ng/ml)	Estradiol/ progesterone
10 000 ppm	0.01	74.59*	1.75	4.45*	16.81
Week 11					
Control	0.02	97.21	1.46	9.78	9.94
2500 ppm	0.02	93.67	1.74	5.41	17.31
10 000 ppm	0.02	83.85	1.15	4.17	20.11
Week 13					
Control	0.02	130.88	1.13	18.67	7.52
2500 ppm	0.01	137.38	1.15	9.95	13.80
10 000 ppm	0.01	110.61	1.14	4.99*	25.97*
Week 17					
Control	0.04	95.37	1.98	25.17	3.79
2500 ppm	0.02	95.34	1.76	13.30	7.78
10 000 ppm	0.02	91.83	1.82	5.61**	16.31*
Week 19					
Control	0.02	109.72	1.18	8.03	13.67
2500 ppm	0.01	93.46	1.00	9.67	9.93
10 000 ppm	0.01	71.37***	0.87	3.62	19.73
Recovery					
Week 2					
Control	0.01	93.25	1.95	10.65	8.75
2500 ppm	0.01	91.83	2.24	14.11	6.51
10 000 ppm	0.01	79.46	2.18	15.33	5.18
Week 6					
Control	0.01	82.71	2.31	7.22	11.45
2500 ppm	0.01	84.30	1.88	7.10	11.87
10 000 ppm	0.01	71.92	2.57	9.42	7.29

From Andrews (2001)
* P <0.05; ** P < 0.001; *** P < 0.0001

Short-term studies in dogs

In a short-term toxicity study, spirodiclofen (purity 97.9–98.6%) was administered to groups of male Beagle dogs (five per dose) in the diet at dose levels of 0, 100 or 2000 ppm (equal to 0, 2.9 and 55.9 mg/kg bw per day) for 8 weeks. This study was intended to clarify testicular effects previously observed in Beagle dogs exposed to spirodiclofen for 14 weeks. Clinical observations were conducted twice daily, food and water intakes were assessed daily and body weights were recorded weekly. Reflexes were assessed and body temperature and pulse were recorded before the start of the study and at week 8. Blood was collected for haematological and clinical chemistry investigations at pretest and during week 8. Additionally, blood samples were collected on a weekly basis for measurement of cholesterol, triglycerides, testosterone and LH levels. Blood samples from week 8 were also analysed for levels of α-tocopherol and ubiquinone. At study termination, all animals were subjected to gross necropsy, and the following organs were weighed and examined histopathologically: brain, heart, liver, lungs, spleen, adrenals, kidneys, pancreas, thyroid, pituitary, testes, prostate gland and thymus.

Table 39. Mean LH and testosterone levels in the dog

Dietary concentration (ppm)	Hormone levels (ng/ml)								
	Week								
	1	2	3	4	5	6	7	8	All
LH									
0	3.94	2.60	2.06	2.82	3.74	5.60	9.42	4.82	4.37
100	4.14	2.68	3.12	4.42	5.52	7.14	4.30	5.18	4.56
2000	9.76	9.48	6.80	5.66	7.08	9.62	22.86	9.78	10.13
Testosterone									
0	0.60	0.63	0.35	0.52	0.87	0.50	0.93	1.37	0.72
100	0.74	0.79	0.87	0.84	0.79	1.01	1.01	0.82	0.85
2000	0.54	0.61	0.22	0.52	0.49	0.94	0.71	0.73	0.59

From Wetzig (2001); Wetzig & Hartmann (2001a)

The activities of the following enzymes were determined in liver tissue: cytochrome P450, cytochrome P450–dependent mono-oxygenases (ECOD, EROD, ALD), EH and the conjugation enzymes (GST, GLUT). Testosterone metabolism (hydroxylation) was also examined in liver tissue of control and high-dose animals by measuring 6α-, 6β-, 16α-, 2α- and 2β-hydroxytestosterone levels, as well as androstenedione levels. This study was generally conducted in compliance with GLP; however, the testosterone hydroxylase assay and hormone determinations were not conducted according to GLP requirements. Levels of tocopherol, ubiquinone and dolichol were also reportedly determined in blood samples; however, the results from these investigations were not included in the study report.

There were no compound-related effects on mortality, clinical signs, reflexes, body temperature, body weight, food consumption or haematological parameters examined. Clinical chemistry investigations revealed increased levels of ASAT, ALAT, AP and GLDH at 2000 ppm compared with the controls. Cholesterol levels were reduced at 2000 ppm, but triglyceride levels were not affected by the treatment. There were no treatment-related effects on serum testosterone levels. However, serum LH concentrations were significantly increased and at least doubled at several weeks during the treatment period in the 2000 ppm animals, providing some evidence that the hypothalamic–pituitary–gonadal axis was affected by treatment with spirodiclofen (Table 39). The measurement of microsomal enzyme activities in the liver tissue revealed an induction of phase I enzymes, especially ECOD and ALD, in the 2000 ppm dose group. The testosterone hydroxylation assay revealed a slight induction of 16α-hydroxylation in the 2000 ppm group, which the study authors noted correlates to cytochrome P450 enzyme subtype CYP2Bl and is also comparable to the induction of ALD. All other in vitro metabolic steps of testosterone remained unchanged.

There were no gross pathological findings attributable to treatment with spirodiclofen. Increases in absolute and relative liver and adrenal weights were observed in the 100 and 2000 ppm groups. Prostate weights were slightly decreased in both treatment groups (Table 40). The histopathological examination revealed treatment-related findings in the liver, adrenal glands and reproductive organs (Table 41). Hepatocellular cytoplasmic change was seen in three males dosed at 2000 ppm. This finding is considered to be the morphological correlate of an induction of hepatocellular enzyme systems, reflecting an adaptive response to the metabolism of the test compound. Furthermore, minimal to slight hepatocellular single-cell necroses, reflecting a mild toxic injury to the liver cells, were observed in three out of five males of the high dose group. In the testes of all high-dose animals, hypertrophy and vacuolation of the Leydig cells were found.

Table 40. Selected absolute and relative organ weights in the dog

Dietary concentration (ppm)	Absolute (g) or relative (to body) weight (g/kg)				
	Liver	Adrenals	Prostate	Thyroid	Pancreas
Absolute weight					
0	402.8	1.15	4.78	0.92	30.6
100	461.6	1.59	4.04	1.11	34.2
2000	499.8	1.72	4.10	1.09	35.8
Relative weight					
0	33.2	0.0955	0.380	0.0760	2.51
100	37.3	0.1292	0.320	0.0902	2.75
2000	41.1	0.1404	0.328	0.0904	2.93

From Wetzig (2001); Wetzig & Hartmann (2001a)

Table 41. Selected histopathological findings in the dog (n = 5)

Histopathological finding	Incidence (no. of animals with finding)		
	Dietary concentration (ppm)		
	0	100	2000
Adrenal – vacuolation/hypertrophy	0	4	5
Adrenal – mononuclear cell infiltrate	0	1	3
Testes – Leydig cell vacuolation	0	0	5
Testes – hypertrophy	0	0	5
Testes – degeneration of germinal epithelium	0	1	4
Liver – single-cell necrosis	0	0	3
Liver – cytoplasmic change	0	0	3

From Wetzig (2001); Wetzig & Hartmann (2001a)

Concurrently, a minimal to slight degeneration of the testicular germinal epithelium was observed in four high-dose animals. Increased vacuolation in the adrenal cortex was observed in all treated dogs except for one.

Based on the findings in the adrenal gland at both dose levels tested, no NOAEL was established. The LOAEL was the lowest dose tested, 100 ppm (equal to 2.9 mg/kg bw per day). The study authors suggested that the increased LH levels throughout the treatment period, as well as the subsequent Leydig cell hypertrophy, indicated that spirodiclofen causes an activation of the hypothalamic–pituitary–gonadal axis (Wetzig, 2001; Wetzig & Hartmann, 2001a).

Discussion of special studies on mechanisms of endocrine effects

Mechanistic studies were provided that suggested that the enol metabolite (BAJ 2510) may contribute significantly to the effects observed with spirodiclofen. In vitro studies provided evidence that BAJ 2510 disrupted the metabolism of cholesterol, which is a precursor to a variety of hormones. In vitro studies also confirmed that BAJ 2510 could inhibit mitochondrial and cytoplasmic MD in tissue culture, resulting in the reduction of reducing equivalents (in the form of NADPH) required by various cytochrome P450 mono-oxygenases involved in steroidogenesis, the downstream effect of which was ultimately predicted to be reduced hormone production (see Figure 3). In vitro studies

with BAJ 2510, as well as special in vivo studies with spirodiclofen, provided evidence of effects on steroid synthesis. Moreover, lesions observed in the reproductive and endocrine systems in the spirodiclofen database were consistent with perturbations in steroidogenesis. It was proposed by the manufacturer that the Leydig cell and uterine tumours observed in rats were related to these perturbations in steroidogenesis (Freyberger, 2007). Briefly, it was suggested that the inhibition of MD leads to a reduced capacity of cells to provide reducing equivalents necessary for steroid hormone biosynthesis. In response to decreased levels of steroid hormones, there is a prolonged stimulation and increased release of pituitary trophins such as LH. In male rats, it was suggested that such a prolonged stimulation by LH, brought about by a reduction of testosterone-mediated feedback inhibition, was likely responsible for the formation of the Leydig cell tumours observed in male rats. Levels of these hormones were investigated in an 8-week dog study. Treatment with spirodiclofen was associated with increased levels of plasma LH levels; however, decreases in testosterone were not shown. Although an in vitro study provided evidence that testosterone secretion by rat testicular fragments was suppressed by BAJ 2510, testosterone and LH levels were not investigated in the rat in vivo; thus, a direct link between perturbations of these hormones and the development of the Leydig cell tumours could not be made. In female rats, it was suggested that inhibition of ovarian steroid synthesis via the same mechanism involving inhibition of MD results in an imbalance in the estradiol to progesterone ratio, leading to a situation of estrogen dominance, with long-term estrogen stimulation eventually resulting in development of uterine neoplasia. In a 19-week dietary study in female rats, alterations in steroid levels were found to lead to such an altered estrogen to progesterone ratio. However, this effect was noted only at a dose (10 000 ppm; equal to 1209.6 mg/kg bw per day) that was almost 10-fold higher than that at which the uterine tumours were observed (2500 ppm; equal to 152.9 mg/kg bw per day), thus making it difficult to correlate these findings.

The Meeting noted that the Leydig cell and uterine tumours observed in rats are consistent with prolonged perturbations in steroidogenesis and are expected to be threshold mediated. However, a clear description of key events with dose–response relationships and temporal associations was not available, and the Meeting concluded that the data were not sufficient to develop a mode of action for the observed tumours.

3. Studies on metabolites

3.1 Acute toxicity studies

The results of the acute oral as well as dermal and eye irritation studies with metabolites of spirodiclofen are summarized in Tables 42 and 43. All studies complied with GLP.

3.2 Short-term studies of toxicity

Spirodiclofen (purity 98.9%) was administered to groups of Wistar rats (10 of each sex per dose) in the diet at dose levels of 0, 500, 2500 or 5000 ppm (equal to 0, 22.8/26.5, 115/136 and 358/413 mg/kg bw per day [males/females]) for 6 weeks. Additionally, BAJ 2510 (purity 99.5%) was administered to Wistar rats (10 of each sex per dose) in the diet at dose levels of 500, 2500 or 5000 ppm (equal to 37.1/43.2, 184/215 and 372/419 mg/kg bw per day [males/females]). The purpose of this study was to compare the subchronic toxicity of spirodiclofen with that of its enol metabolite (BAJ 2510). Physical examinations were performed weekly. Body weight was recorded once weekly. Food consumption was recorded twice weekly. Selected clinical chemistry parameters (total cholesterol, triglycerides) and hormone (progesterone, corticosterone, aldosterone) levels were determined once during the pretreatment period and at weeks 2, 4 and 6. All animals were necropsied, selected organs were weighed and a range of tissues were taken and fixed. Only the adrenal gland was examined microscopically.

Table 42. Acute oral toxicity of metabolites of spirodiclofen

Metabolite	Purity (%)	Vehicle	Species	Strain	Sex	LD_{50} (mg/kg bw)	Reference
BAJ 2510 (enol)[a]	98.0–99.9	0.5% aqueous carboxymethyl cellulose	Rat	Wistar (HsdCpb:Wu)	M & F	M: 500–1000 F: 300–500	Kroetlinger (2000a)
BAJ 2740-ketohydroxy[b]	98.5	Cremophor EL 2%	Rat	Wistar (HsdCpb:Wu)	M & F	>2500[c]	Kroetlinger (2000b)
BAJ 2740-MA-3-OH-cyclohexyl ester[d]	98.6	Cremophor EL 2%	Rat	Wistar (HsdCpb:Wu)	M & F	>2500[c]	Kroetlinger (2001)

F, female; M, male

[a] Clinical signs following administration included decreased motility in females at ≥200 mg/kg bw and decreased motility and reactivity, digging and cleaning gestures, laboured breathing, increased salivation and uncoordinated gait at ≥500 mg/kg bw. In animals that died, dark red discoloration of the liver and pale discoloration of the spleen were observed at necropsy.

[b] A soil metabolite.

[c] The reported LD_{50} was based on the OECD draft guideline "Acute Oral Toxicity—Acute Toxic Class Method" from 1995. Based on the revised OECD test guideline 423 (adopted in 2001), the company indicated that the interpretation would be as follows: LD_{50} oral >2000 mg/kg bw, equivalent to category "unclassified" of the Globally Harmonized System of Classification and Labelling of Chemicals, and LD_{50} cut-off of >5000 mg/kg bw.

[d] A plant metabolite, also referred to as M05 (2,4-dichloromandelic acid hydroxy-cyclohexyl ester).

Table 43. Summary of dermal and eye irritation studies with BAJ 2510 (enol)

Purity (%)	Route	Concentration (mg)	Species/strain	Sex	Result	Reference
99.9	Dermal	500[a]	Rabbit/ Himalayan	Male	No signs of dermal irritation	Leuschner (2002b)
99.9	Eye	100	Rabbit/ Himalayan	Male	No signs of ocular irritation	Leuschner (2002a)

[a] Test material was pulverized and moistened with water prior to application.

There were no treatment-related mortalities in the study. Treatment-related clinical signs were limited to soiling of the anogenital region or forelimbs, observed with both test substances at the middle and high doses. Body weights were decreased at the high dose (both sexes) with both test substances, and body weight gain per day was dose-dependently decreased at the middle and high doses (both sexes) with both test substances. Overall body weight gain per day was decreased at the middle and high doses for both sexes treated with spirodiclofen, in mid-dose females treated with BAJ 2510 and in high-dose animals of both sexes treated with BAJ 2510. Food consumption was decreased in mid- and high-dose animals treated with both test substances, with the most dramatic decreases occurring during the first week or two of treatment.

For animals treated with spirodiclofen, there were statistically significant increases in mean triglyceride levels (+48%) relative to the control group for females in week 6. The increases observed at weeks 2 and 4 were not considered to be treatment-related changes based on the variations observed in the pretreatment samples. No other treatment-related changes were observed (Table 44). For animals treated with BAJ 2510, although a statistically significant difference was noted for cholesterol concentration in males at 500 ppm in week 2, it was considered to be incidental. Throughout the study, mean triglyceride concentrations were higher in both sexes treated with BAJ 2510 at 5000 ppm and in males at 2500 ppm. Mean total cholesterol concentrations were slightly higher in females at 5000 ppm (both test substances) from week 2 onwards (Table 45). It was noted by the Meeting

Table 44. Triglyceride and cholesterol levels in rats administered spirodiclofen

Week	Mean concentration (mmol/l)[a]							
	Males				Females			
	Dietary concentration of spirodiclofen (ppm)							
	0	500	2500	5000	0	500	2500	5000
Triglycerides								
2	0.65 (—)	0.92 (+42)	0.86 (+32)	0.81 (+25)	0.56 (—)	0.62 (+11)	0.60 (+7)	0.78 (+39)
4	0.66 (—)	0.73 (+11)	0.72 (+9)	0.63 (−5)	0.45 (—)	0.46 (+2)	0.44 (−2)	0.57 (+27)
6	0.65 (—)	0.65 (0)	0.72 (+11)	0.59 (−9)	0.40 (—)	0.41 (+3)	0.39 (−3)	0.59** (+48)
Total cholesterol								
2	1.87 (—)	2.25* (+20)	1.80 (−4)	1.81 (−3)	1.84 (—)	1.98 (+8)	1.81 (−2)	1.99 (+8)
4	1.88 (—)	2.07 (+10)	1.79 (−5)	1.78 (−5)	1.77 (—)	1.95 (+10)	1.79 (+1)	2.10 (+19)
6	1.89 (—)	1.92 (+2)	1.92 (+2)	1.76 (−7)	1.66 (—)	1.87 (+13)	1.92 (+16)	1.99 (+20)

From Blanck (2007)
* $P \leq 0.05$; ** $P \leq 0.01$
[a] Per cent change compared with controls given in parentheses.

Table 45. Triglyceride and cholesterol levels in rats administered BAJ 2510

Week	Mean concentration (mmol/l)[a]							
	Males				Females			
	Dietary concentration of BAJ 2510 (ppm)							
	0	500	2500	5000	0	500	2500	5000
Triglycerides								
2	0.65 (—)	0.95 (+46)	1.24** (+91)	1.26** (+94)	0.56 (—)	0.63 (+13)	0.82 (+46)	1.22** (+118)
4	0.66 (—)	0.65 (−2)	1.10 (+67)	1.18* (+79)	0.45 (—)	0.50 (+11)	0.62 (+38)	0.82** (+82)
6	0.65 (—)	0.67 (+3)	1.13* (+74)	1.06 (+63)	0.40 (—)	0.41 (+3)	0.52 (+30)	0.81** (+103)
Total cholesterol								
2	1.87 (—)	2.10 (+12)	2.04 (+9)	1.97 (+5)	1.84 (—)	1.99 (+8)	2.04 (+11)	2.66** (+23)
4	1.88 (—)	1.89 (+1)	1.91 (+2)	1.83 (−3)	1.77 (—)	1.94 (+10)	1.92 (+8)	2.14* (+21)
6	1.89 (—)	1.83 (−3)	1.87 (−1)	1.75 (−7)	1.66 (—)	1.84 (+11)	1.94 (+17)	1.99* (+20)

From Blanck (2007)
* $P \leq 0.05$; ** $P \leq 0.01$
[a] Per cent change compared with controls given in parentheses.

that in the main toxicology database for spirodiclofen, decreases in cholesterol, often accompanied by decreases in triglyceride levels, were consistently noted in rats after as little as 4 weeks of treatment. The reason for the lack of consistency in these effects in the current study is not clear, but may be related to the use of a different anaesthetic (isoflurane) and the fact that animals were diet-fasted prior to blood sampling in the present study. Isoflurane has been associated with decreases in cholesterol levels in humans and experimental animals, and fasting would also be associated with lower cholesterol levels. It is possible that decreased basal cholesterol levels could mask decreases associated with treatment.

In females dosed with 5000 ppm spirodiclofen, decreases in mean progesterone concentrations from week 2 to week 6 (−40% to −51%), in mean aldosterone concentrations at week 6 (−33%) and in mean corticosterone concentrations at week 6 (−38%) were observed, compared with control values (Table 46). Hormone analysis in animals treated with BAJ 2510 (Table 47) revealed a decrease in mean progesterone concentrations from week 4 to week 6 (approximately −39%) in high-dose females compared with control values. It was noted that interpretation of the progesterone data was limited by the fact that animals were not in the same stage of estrus when blood was sampled.

Effects on the adrenal gland were observed in animals at all dose levels in both test substance groups. These included dose-related increases in absolute and relative weights (at the middle and high doses) (Tables 48 and 49), enlarged and/or pale adrenals (low-dose BAJ 2510 males and mid- and high-dose animals of both sexes treated with both test substances) (Table 50) and dose-related increases in minimal to marked vacuolation of the cortex in the zona fasciculata (Table 51). The adrenal vacuolation tended to be more severe for animals in the BAJ 2510 groups, compared with those treated with spirodiclofen. Other findings noted for mid- and high-dose males (both test

Table 46. Hormone levels in rats administered spirodiclofen[a]

Week	Hormone level (ng/ml or pg/ml)			
	Dietary concentration of spirodiclofen (ppm)			
	0	500	2500	5000
Males				
Progesterone (ng/ml)				
Pretreatment	6.91 ± 2.81	6.54 ± 3.21	6.30 ± 4.42	6.44 ± 2.92
2	3.17 ± 1.81	2.87 ± 1.72	2.81 ± 1.74	3.20 ± 1.11
4	1.68 ± 1.01	1.97 ± 0.92	2.42 ± 1.20	2.86 ± 1.58
6	2.65 ± 1.45	2.46 ± 1.15	2.68 ± 1.40	2.16 ± 0.79
Aldosterone (pg/ml)				
Pretreatment	293.87 ± 84.41	299.61 ± 96.41	254.81 ± 135.57	284.91 ± 92.26
2	166.37 ± 76.00	166.22 ± 95.90	158.20 ± 87.08	196.66 ± 62.51
4	120.63 ± 59.35	150.76 ± 62.36	170.04 ± 83.60	212.10 ± 103.55
6	180.54 ± 118.51	169.43 ± 79.04	171.10 ± 72.18	164.12 ± 56.21
Corticosterone (ng/ml)				
Pretreatment	315.60 ± 96.45	322.73 ± 127.02	260.50 ± 151.26	306.57 ± 114.16
2	195.08 ± 102.45	194.18 ± 114.52	171.13 ± 115.88	244.57 ± 86.64
4	109.09 ± 67.64	146.88 ± 73.66	167.42 ± 92.18	205.44 ± 121.37
6	181.92 ± 114.25	163.24 ± 83.03	187.55 ± 103.53	163.74 ± 76.50
Females				
Progesterone (ng/ml)				
Pretreatment	23.98 ± 5.41	21.38 ± 5.68	23.14 ± 8.64	19.14 ± 7.55
2	27.04 ± 10.69	22.82 ± 8.93	39.54 ± 23.67	16.22 ± 6.49* (−40)[b]
4	27.45 ± 7.28	27.09 ± 7.19	27.22 ± 10.37	13.96 ± 5.50** (−49)[b]
6	34.21 ± 10.90	30.86 ± 8.05	30.55 ± 8.30	16.71 ± 5.30** (−51)[b]

Table 46 (contd)

Week	Hormone level (ng/ml or pg/ml)			
	Dietary concentration of spirodiclofen (ppm)			
	0	500	2500	5000
Aldosterone (pg/ml)				
Pretreatment	471.87 ± 144.99	333.75 ± 135.69	401.97 ± 192.07	352.85 ± 124.96
2	457.74 ± 125.94	433.42 ± 184.85	525.03 ± 161.79	395.62 ± 189.75
4	438.15 ± 131.54	447.32 ± 168.42	471.99 ± 160.81	307.14 ± 136.25
6	400.21 ± 98.34	400.08 ± 167.17	405.27 ± 125.89	266.05 ± 79.49* (−34)[b]
Corticosterone (ng/ml)				
Pretreatment	474.19 ± 144.82	348.38 ± 116.79	415.23 ± 215.54	375.46 ± 166.30
2	495.35 ± 189.41	526.61 ± 314.54	648.76 ± 242.43	507.93 ± 404.77
4	486.29 ± 195.45	618.78 ± 346.11	692.78 ± 388.34	304.58 ± 180.31
6	503.57 ± 129.42	473.62 ± 182.99	547.91 ± 179.70	309.30 ± 107.99** (−39)[b]

From Blanck (2007)
* $P \leq 0.05$; ** $P \leq 0.01$
[a] n = 9–10; mean ± standard deviation.
[b] Per cent difference from controls is presented in parentheses.

Table 47. Hormone levels in rats administered BAJ 2510[a]

Week	Hormone level (ng/ml or pg/ml)			
	Dietary concentration of BAJ 2510 (ppm)			
	0	500	2500	5000
Males				
Progesterone (ng/ml)				
Pretreatment	6.91 ± 2.81	5.90 ± 3.01	6.75 ± 3.29	6.63 ± 4.14
2	3.17 ± 1.81	4.37 ± 1.91	3.69 ± 2.16	5.05 ± 2.73
4	1.68 ± 1.01	2.32 ± 1.49	2.15 ± 1.58	3.14 ± 1.90
6	2.65 ± 1.45	2.18 ± 1.70	3.01 ± 0.85	2.71 ± 1.46
Aldosterone (pg/ml)				
Pretreatment	293.87 ± 84.41	278.23 ± 90.64	293.70 ± 96.37	272.35 ± 127.93
2	166.37 ± 76.00	229.41 ± 79.21	218.52 ± 101.51	283.14 ± 124.64
4	120.63 ± 59.35	165.70 ± 94.88	159.83 ± 80.95	259.27 ± 180.22
6	180.54 ± 118.51	153.44 ± 95.93	240.19 ± 89.34	192.71 ± 100.84
Corticosterone (ng/ml)				
Pretreatment	315.60 ± 96.45	304.36 ± 97.14	317.05 ± 112.26	287.91 ± 162.53
2	195.08 ± 102.45	267.46 ± 103.61	241.20 ± 130.79	303.55 ± 137.46
4	109.09 ± 67.64	157.01 ± 104.60	129.33 ± 99.75	194.73 ± 125.17
6	181.92 ± 114.25	143.78 ± 108.07	212.90 ± 72.56	178.86 ± 103.40

Table 47 (contd)

Week	Hormone level (ng/ml or pg/ml)			
	Dietary concentration of BAJ 2510 (ppm)			
	0	500	2500	5000
Females				
Progesterone (ng/ml)				
Pretreatment	23.98 ± 5.41	24.40 ± 6.91	20.08 ± 7.65	21.10 ± 7.32
2	27.04 ± 10.69	27.18 ± 13.36	33.04 ± 21.66	22.78 ± 10.49
4	27.45 ± 7.28	30.03 ± 12.15	25.91 ± 10.12	16.84 ± 3.04* (−39)[b]
6	34.21 ± 10.90	40.09 ± 23.45	32.58 ± 13.46	20.69 ± 10.06* (−40)[b]
Aldosterone (pg/ml)				
Pretreatment	471.87 ± 144.99	460.98 ± 118.45	429.98 ± 126.87	424.19 ± 153.11
2	457.74 ± 125.94	468.67 ± 202.22	551.79 ± 186.62	478.41 ± 201.79
4	438.15 ± 131.54	520.64 ± 219.28	620.56 ± 291.04	373.27 ± 88.14
6	400.21 ± 98.34	449.72 ± 138.51	561.62 ± 388.19	297.57 ± 95.38
Corticosterone (ng/ml)				
Pretreatment	474.19 ± 144.82	450.64 ± 131.94	433.12 ± 147.38	448.60 ± 155.40
2	495.35 ± 189.41	751.62 ± 612.75	817.99 ± 493.10	598.83 ± 405.79
4	486.29 ± 195.45	866.46 ± 602.74	880.93 ± 513.05	413.10 ± 133.82
6	503.57 ± 129.42	515.61 ± 139.85	623.58 ± 393.27	329.50 ± 158.53

From Blanck (2007)
* $P \leq 0.05$
[a] $n = 9–10$; mean ± standard deviation.
[b] Per cent difference from controls is presented in parentheses.

substance groups) included decreased absolute and relative prostate weights and a slight increase in the incidence of atrophic/small prostates and/or seminal vesicles. These organs were not examined histopathologically.

A NOAEL was not established for either spirodiclofen or BAJ 2510. The LOAEL for spirodiclofen was 500 ppm (equal to 22.8 mg/kg bw per day for males and 26.5 mg/kg bw per day for females), based on decreased body weight gain per day and increased incidence of minimal to slight adrenal vacuolation observed in both sexes. The LOAEL for BAJ 2510 was 500 ppm (equal to 37.1 mg/kg bw per day for males and 43.2 mg/kg bw per day for females), based on decreased body weight gain per day and findings in the adrenal gland in both sexes (minimal to slight adrenal vacuolation in both sexes and decreased adrenal weight and increased incidence of paleness in males).

In conclusion, spirodiclofen and its enol metabolite were found to induce similar effects in the rat. There were some indications that the enol metabolite produced more severe effects than the parent compound. For example, the severity of vacuolation in the adrenal cortex was increased slightly in the BAJ 2510 group, indicative of some increase in relative toxicity. However, owing to issues with the stability of spirodiclofen, the adjusted doses of spirodiclofen (on a mg/kg bw per day basis) were lower than those for the enol metabolite. The study author noted that the higher polarity and water solubility of the enol may enhance its bioavailability compared with that of spirodiclofen and may account for some of the differences in comparative toxicity between the compounds. The study author also noted that because of the lower molecular weight of the enol metabolite (313.2) compared

Table 48. Adrenal weights in rats administered spirodiclofen

Parameter	Adrenal gland weight changes at terminal sacrifice[a]							
	Male (n = 10)				Female (n = 10)			
	Dietary concentration of spirodiclofen (ppm)							
	0	500	2500	5000	0	500	2500	5000
Mean absolute adrenal gland weight (g)	0.0577 (—)	0.0623 (+8)	0.0851** (+47)	0.0911** (+58)	0.0695 (—)	0.0717 (+3)	0.0802 (+15)	0.0851** (+22)
Mean adrenal gland to body weight ratio (%)	0.0155 (—)	0.0163 (+5)	0.0232** (+49)	0.0279** (+80)	0.0310 (—)	0.0319 (+3)	0.0363* (+17)	0.0406** (+31)

From Blanck (2007)
* $P \leq 0.05$; ** $P \leq 0.01$
[a] Per cent change compared with controls given in parentheses.

Table 49. Adrenal weights in animals administered BAJ 2510

Parameter	Adrenal gland weight changes at terminal sacrifice[a]							
	Male (n = 10)				Female (n = 10)			
	Dietary concentration of BAJ 2510 (ppm)							
	0	500	2500	5000	0	500	2500	5000
Mean absolute adrenal gland weight (g)	0.0577 (—)	0.0701 (+21)	0.1084** (+88)	0.1323** (+129)	0.0695 (—)	0.0746 (+7)	0.0820 (+18)	0.1037** (+49)
Mean adrenal gland to body weight ratio (%)	0.0155 (—)	0.0185 (+19)	0.0292** (+88)	0.0399** (+157)	0.0310 (—)	0.0333 (+7)	0.0376* (+21)	0.0493** (+59)

From Blanck (2007)
* $P \leq 0.05$; ** $P \leq 0.01$
[a] Per cent change compared with controls given in parentheses.

with that of spirodiclofen (411.4), an equal weight of the enol would represent approximately 1.3 times the number of BAJ 2510 molecules as the parent material, assuming complete conversion in the animal, which is thought to occur on the basis of the metabolism studies. Taken together, these considerations most likely account for the slight difference in response severity noted in the study. Overall, the two compounds were found to exert similar effects in the rat following repeated dosing under the conditions of this study, and it might be concluded that there is no indication in this study that BAJ 2510 produces a different spectrum of toxic effects or causes marked effects at lower doses compared with spirodiclofen (Blanck, 2007).

3.3 Genotoxicity

In vitro reverse mutation studies were conducted with metabolites of spirodiclofen to assess the potential for inducing gene mutation in vitro. All studies complied with GLP. The study results (summarized in the Table 52) were negative. None of the metabolites of spirodiclofen that were tested were found to demonstrate any genotoxic potential under the conditions tested.

3.4 Discussion of metabolites relevant for the dietary risk assessment

The following metabolites were identified only in plants: M05 (2,4-dichloromandelic acid hydroxy-cyclohexyl ester), M04 (2,4-dichloromandelic acid cyclohexyl ester glycosyl pentoside) and

Table 50. Incidence of gross pathology findings in rats

Observation	Incidence (no. of animals with finding)							
	Males (n = 10)				Females (n = 10)			
	Dietary concentration (ppm)							
	0	500	2500	5000	0	500	2500	5000
Spirodiclofen								
Pale adrenal	0	0	3	5	0	0	2	6
Enlarged adrenal	0	0	1	4	1	0	0	1
Seminal vesicles – small and/or atrophic	1	0	2	3	NA	NA	NA	NA
Prostate – small and/or atrophic	0	0	2	2	NA	NA	NA	NA
BAJ 2510								
Pale adrenal	0	3	9	10	0	1	3	10
Enlarged adrenal	0	0	6	9	1	0	1	7
Seminal vesicles – small and/or atrophic	0	0	0	4	NA	NA	NA	NA
Prostate – small and/or atrophic	0	2	0	1	NA	NA	NA	NA

From Blanck (2007)
NA, not applicable

Table 51. Incidence of vacuolation of the zona fasciculata in the adrenal gland of rats

Severity	Incidence (no. of animals with finding)							
	Males (n = 10)				Females (n = 10)			
	Dietary concentration (ppm)							
	0	500	2500	5000	0	500	2500	5000
Spirodiclofen								
Minimal	3	4	0	0	0	2	5	0
Slight	0	1	7	2	0	0	2	9
Moderate	0	0	2	5	0	0	0	1
Marked	0	0	0	3	0	0	0	0
Total	3	5	9	10	0	2	7	10
BAJ 2510								
Minimal	3	3	0	0	0	1	2	0
Slight	0	3	3	0	0	0	7	0
Moderate	0	0	6	1	0	0	0	8
Marked	0	0	1	9	0	0	0	2
Total	3	6	10	10	0	1	9	10

From Blanck (2007)

Table 52. Results of in vitro reverse mutation studies on metabolites of spirodiclofen

Metabolite (purity)	Test object	Concentration	Results	Reference
BAJ 2510 (enol) (98%)[a,b,c]	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	0–5000 µg/plate in DMSO with and without S9 mix	Negative	Herbold (1999)
BAJ 2740-ketohydroxy[d] (98.5%)[a,b,e]	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	0–320 µg/plate (preincubation) with and without S9 mix; in ethanol	Negative	Herbold (2001b)
BAJ 2740-MA-3-OH-cyclohexyl ester[f] (98.6%)[a,b]	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	0–5000 µg/plate in DMSO with and without S9 mix	Negative	Herbold (2001a)

DMSO, dimethyl sulfoxide; S9, 9000 × *g* rat liver supernatant

[a] Positive and negative controls included.

[b] Tested in triplicate, using both the standard plate method and the preincubation method.

[c] Cytotoxicity noted at 5000 µg/plate.

[d] A soil metabolite.

[e] Cytotoxicity noted at ≥500 µg/plate for plate incorporation method; ≥80 µg/plate for preincubation method.

[f] A plant metabolite, also referred to as M05 (2,4-dichloromandelic acid hydroxy-cyclohexyl ester).

M08 (2,4-dichloromandelic acid glucoside). The latter two metabolites are sugar conjugates of minor metabolites found in the rat. Limited toxicology data (acute oral and in vitro reverse mutation studies) were provided for M05, and no toxicology data were provided for the other two plant metabolites. The Meeting therefore concluded that the information available was not sufficient to conduct a hazard assessment for these metabolites. The enol metabolite was detected in plants and livestock matrices. As the enol metabolite was found to be of similar toxicity to the parent compound, the Meeting considered this metabolite to be toxicologically relevant for the dietary risk assessment.

4. Observations in humans

The results from routine medical surveillance of workers in a spirodiclofen production plant were provided for the years 2002–2005. Yearly medical examinations were performed on 90 workers who worked in the plant using the following Bayer CropScience personal protective equipment: full mask with filter (ABEK-P3), protective gloves and chemical-resistant suits. The medical tests included a recording of medical history, full physical examination with orientating neurological status (reflexes, sensibility and coordination) and skin status, as well as the following laboratory and technical examinations: erythrocyte sedimentation rate, full blood count, ASAT, ALAT, γ-glutamyl transferase, glucose, creatinine, cholesterol, urine status, lung function, electrocardiogram/ergometry, vision testing, audiometry, chest X-ray and sonography (if necessary).

Occupational medical surveillance of workers exposed to spirodiclofen, performed every year on a routine basis, not directly related to exposures, did not reveal any unwanted effects in the workers. Since 2002, no accidents with spirodiclofen occurred involving the workers, and no consultations of the medical department due to work or contact with spirodiclofen were required. In addition, no spirodiclofen-related allergenicity observations could be determined during that time period (Kehrig & Steffens, 2005).

Comments

Biochemical aspects

The absorption, distribution, metabolism and excretion of spirodiclofen were investigated in rats. Orally administered ^{14}C-labelled spirodiclofen was rapidly absorbed and eliminated. Blood

concentrations peaked at 3–4 h at low doses (1–2 mg/kg bw) and at ≥8 h at a higher dose (100 mg/kg bw). Decreased urinary excretion at the higher dose suggested saturation of absorption. Urine and faeces were the major routes of excretion. Retention in the carcass and organs was low (total body burden <1% of the administered dose within 48 h), and there was no evidence of bioaccumulation. Dietary pretreatment with non-labelled compound did not have a significant impact on absorption or elimination.

After oral administration, ^{14}C-labelled spirodiclofen was extensively metabolized in rats. Spirodiclofen appears to be rapidly metabolized to the enol metabolite (BAJ 2510). No parent compound was detected in the urine or bile, and up to 11 metabolites were identified, representing 59–90% of the administered dose. Up to 16% of the parent compound was detected in the faeces. The profile of metabolites was generally similar qualitatively in males and females, but varied quantitatively. The major urinary metabolite in females was the enol metabolite, BAJ 2510, whereas the major urinary metabolite in males was the 3-hydroxy-enol metabolite. The metabolic profile in the bile was similar to that observed in the urine; however, a hydroxylated glucuronide metabolite was found to be unique to the bile.

Toxicological data

Spirodiclofen is of low acute toxicity when administered via the oral, dermal and inhalation routes. The oral and dermal LD_{50} values in rats were >2500 and >2000 mg/kg bw, respectively. The inhalation LC_{50} in rats was determined to be >5.03 mg/l air. Spirodiclofen was not irritating to the eyes or skin of rabbits, but was found to be a dermal sensitizer under the conditions of the Magnusson and Kligman maximization test in guinea-pigs.

In short- and long-term studies of oral toxicity in mice, rats and dogs, the primary target organs of toxicity of spirodiclofen were the adrenal glands and testes. The predominant finding was vacuolation of the adrenal cortex, which was noted in the mouse, rat and dog and was often accompanied by increased adrenal weight. With extended duration of dosing, adrenal vacuolation was associated with adrenal hypertrophy in rats and adrenal enlargement and lymphocytic infiltration in mice. In rats, there were no adrenal findings after 4 weeks of dosing (NOAEL of 500 ppm, equal to 50 mg/kg bw per day, on the basis of changes in clinical chemistry parameters and induction of liver enzymes at 5000 ppm), and the overall NOAEL for adrenal effects from the 14-week and 2-year studies was 350 ppm, equal to 14.7 mg/kg bw per day from the 2-year study in rats. In mice, a NOAEL for adrenal findings of 100 ppm, equal to 30 mg/kg bw per day, was identified in the short-term study on the basis of adrenal findings at 1000 ppm in females. After long-term dosing in mice, a NOAEL for adrenal findings could not be identified, as there was an increased incidence of adrenal pigmentation and vacuolation in females at the lowest dose tested (25 ppm, equal to 5.1 mg/kg bw per day). The incidence of pigmentation was only slightly above the reported range for historical controls, and the increase in vacuolation was not statistically significant. However, as the adrenal gland is clearly a target organ, these findings were considered to represent a marginal LOAEL for females at this dose. Compared with other species, the adrenal effects were observed at the lowest doses in dogs. Although a NOAEL could not be established for adrenal findings in the special 8-week study in dogs (LOAEL of 2.9 mg/kg bw per day) or for females in the 14-week study in dogs (LOAEL of 8.4 mg/kg bw per day), an overall NOAEL for adrenal histopathology in the dog was identified from the 1-year study in dogs (NOAEL of 1.4 mg/kg bw per day). Reversibility of the adrenal effects was observed in the short-term study in rats during a 4-week recovery period. The Meeting noted that these adrenal findings are a consequence of repeated dosing, which is supported by the lack of adrenal findings in the 4-week study in rats, and are potentially associated with prolonged perturbation of steroidogenesis.

Testicular effects in mice included Leydig cell hypertrophy and vacuolation after short-term dosing and increased weight, discoloration, degeneration and interstitial cell hyperplasia in the long-term study. In the long-term study, an increase in epididymal aspermia was also noted at the highest dose. In the dog, Leydig cell vacuolation and hypertrophy, testicular immaturity and degeneration

of the germinal epithelium were noted in studies that ranged in duration from 4 weeks to 1 year. In the rat, Leydig cell hyperplasia was observed in the 2-year study. The overall NOAEL for testicular effects was approximately 4 mg/kg bw per day from the 1-year study in dogs (150 ppm), 2-year study in rats (100 ppm) and 18-month study in mice (25 ppm), although it should be noted that increased testes weights were noted at this dose in the 1-year study in dogs. The Meeting noted that when dosing in the dog began at a younger age, effects in reproductive tissues appeared to be more severe, and that the testicular effects were often accompanied by other effects, including immaturity of the prostate and oligospermia/aspermia of the epididymides, which were noted in the 4-week (at 10 000 ppm) and 14-week studies (at ≥630 ppm). The Meeting also observed that there was evidence that effects in male reproductive tissues progressed, and that NOAELs decreased, with increasing duration of exposure, and that the nature of the findings indicated that they were potentially a result of prolonged perturbation of steroidogenesis.

Other effects observed following short- or long-term oral exposure to higher doses of spirodiclofen included effects on the liver, cholesterol levels, thyroid, jejunum and thymus. Liver effects included hypertrophy, vacuolation and hepatocytomegaly in the mouse (at ≥1000 ppm, equal to 164 mg/kg bw per day); necrosis, cytoplasmic change, granulation and inflammatory infiltration in the dog (at ≥2000 ppm, equal to 84.7 mg/kg bw per day); and decreased concentrations of plasma proteins and tigroid basophilic foci in the rat (at 2500 ppm, equal to 110.1 mg/kg bw per day). Increased liver weight and enzyme induction occurring in mice and dogs at lower doses were considered to be an adaptive response to the administration of spirodiclofen. Decreased cholesterol concentrations, which were consistent with the proposed pesticidal mode of action of this chemical, were observed in the rat (at ≥110 mg/kg bw per day), dog (at ≥4 mg/kg bw per day) and mouse (at 1600 mg/kg bw per day) and were accompanied by decreased triglyceride concentrations in the rat. Vacuolation of the jejunum was observed in rats and dogs, and slight atrophy of the thymic cortex was also observed in dogs. Thyroid effects included decreased concentrations of T_4 in the dog (at 2000 ppm in the 4-week study) and an increase in concentrations of TSH (at ≥2500 ppm in the 14-week study) and colloidal alteration of the thyroid (at 2500 ppm in the 2-year study) in rats.

Spirodiclofen was adequately tested for genotoxicity in vitro and in vivo in a range of assays. It was not found to be genotoxic in mammalian or microbial systems.

The Meeting concluded that spirodiclofen was unlikely to be genotoxic.

In an 18-month study of oncogenicity in mice, administration of spirodiclofen at dietary concentrations of 0, 25, 3500 or 7000 ppm resulted in the development of late-onset hepatocellular adenomas and carcinomas in males and females at doses of ≥3500 ppm. Systemic toxicity was noted at the same doses, including changes in organ weights (liver, adrenal gland, testes and kidney) and histopathological findings in the adrenal gland (vacuolation and pigmentation), liver (hepatocytomegaly) and testes (hypertrophy and hyperplasia). As discussed previously, a NOAEL for systemic toxicity was not identified, on the basis of marginal effects on the adrenal gland in females at 25 ppm, equal to 5.1 mg/kg bw per day, the lowest dose tested. The NOAEL for carcinogenicity was 25 ppm, equal to 4.1 mg/kg bw per day, in this study. The Meeting noted that although preneoplastic lesions were not observed at lower doses than those at which the liver tumours were observed, this may have been due to the large dose spacing. Additionally, the Meeting noted that these tumours were observed only at high doses (≥3500 ppm), which also produced hepatotoxicity, and that the dose–response relationship for these tumours was likely to exhibit a threshold.

The toxicity and carcinogenicity of spirodiclofen were investigated in a 2-year dietary study in rats. The incidence of late-onset Leydig cell adenomas was increased in male rats at the highest dose tested (2500 ppm), preceded by an increased incidence of Leydig cell hyperplasia at ≥350 ppm. An increased incidence of uterine adenocarcinomas and uterine nodules was also observed in female rats at the highest dose that had died or were sacrificed before study termination. These adenocarcinomas were noted to have metastasized into various organs of the abdominal cavity, as well

as into the lung and bone marrow. Systemic toxicity was also noted at the highest dose, including increased mortality (females), decreased body weight (by 6–10%), increased levels of AP, decreased concentrations of cholesterol and triglycerides, and histopathological findings in the adrenal gland (vacuolation and hypertrophy; males only), ovary (increased portion of stroma), vagina (possible increase in the number of animals in estrus based on morphology of vaginal epithelium), jejunum (vacuolation), thyroid (colloidal alteration) and olfactory epithelium (atrophy/degeneration; males only). The NOAEL for systemic toxicity was 100 ppm, equal to 4.1 mg/kg bw per day, on the basis of the increased incidence of Leydig cell hyperplasia in males. The NOAEL for carcinogenicity was 350 ppm, equal to 14.7 mg/kg bw per day.

Several special studies were conducted with spirodiclofen and with three of the enol metabolites (BAJ 2510, 3-hydroxy-enol and 4-hydroxy-enol). Studies in vitro provided evidence that the enol metabolite (BAJ 2510) may contribute significantly to the effects observed with spirodiclofen via disruption of the metabolism of cholesterol, which is a precursor to a variety of hormones. Studies also confirmed that BAJ 2510 could inhibit the activity of MD in tissue culture, resulting in a decrease in reducing equivalents required by various cytochrome P450 mono-oxygenases involved in steroidogenesis, the downstream effect of which was ultimately predicted to be reduced hormone production. Studies with BAJ 2510 in vitro, as well as special studies with spirodiclofen in vivo, provided some evidence of effects on steroid synthesis. The Meeting noted that the increased incidence of Leydig cell and uterine tumours observed in rats was consistent with prolonged perturbations in steroidogenesis, and the dose–response relationship for these effects would be anticipated to exhibit a threshold. However, a clear description of key events, with dose–response relationships and temporal associations, was not available, and the Meeting concluded that the data were not sufficient to develop a mode of action for formation of the observed tumours by spirodiclofen.

The Meeting concluded that the relevance of the tumorigenic responses in rats and mice to humans could not be discounted. However, the Meeting noted that spirodiclofen was not genotoxic and that the dose–response relationship for the tumours would be anticipated to exhibit a threshold.

The effect of spirodiclofen on reproduction in rats was investigated in a two-generation study. Parental effects in both generations (F_0 and F_1) included vacuolation of the adrenal cortex and epithelium of the small intestine. Decreases in body weight and in concentrations of cholesterol, triglycerides and unesterified fatty acids were also observed in the F_1 generation (clinical chemistry evaluations were not performed for the F_0 generation). The NOAEL for parental toxicity was 70 ppm, equal to 5.2 mg/kg bw per day. Offspring toxicity included body weight loss and decreased body weight gain in the F_1 and F_2 pups at 350 ppm; the NOAEL for these findings in offspring was 70 ppm, equal to 5.2 mg/kg bw per day. Reproductive toxicity was observed in the F_1 generation only, at the highest dose tested (1750 ppm). Delayed sexual maturation was observed in male offspring, and increased severity of ovarian vacuolation/degeneration, decreased testes, spermatid and epididymal sperm counts, reduced size of testes and epididymides, as well as atrophy of the testes, epididymides and prostate were observed in some F_1 adults at the highest dose. The NOAEL for these findings in F_1 rats was 350 ppm, equal to 26.2 mg/kg bw per day. Although it is possible that the toxic effects on reproduction were associated with exposure in utero (as they were observed in the F_1 generation only), this remains uncertain, considering that F_1 rats began consuming treated diet at an earlier age, experienced a longer duration of dosing and were thus exposed to a higher overall average dose of spirodiclofen than the F_0 generation. The Meeting noted that this reproductive toxicity was potentially caused by sustained alteration of steroidogenesis.

The effect of spirodiclofen on developmental toxicity was investigated in rats and rabbits. In rats, there was no maternal toxicity noted (the NOAEL was 1000 mg/kg bw per day, the highest dose tested), although the Meeting noted that investigation of target organs was not conducted in maternal animals. In the fetus, marginal increases in the incidences of slight renal pelvis dilatation and asymmetrical fourth sternebrae were observed at the highest dose tested. However, as these findings

occurred at the highest dose tested (1000 mg/kg bw per day) and the incidences were within the range for historical controls, the Meeting considered that these effects represented a marginal LOAEL. The Meeting also noted that these findings would not be expected to occur after a single exposure (Solecki et al., 2003; Makris et al., 2009). The NOAEL for developmental toxicity in rats was 300 mg/kg bw per day on the basis of marginal findings at the highest dose tested. In the study in rabbits, maternal toxicity consisted of increased body weight loss and decreased food consumption at 300 mg/kg bw per day. The NOAEL for maternal toxicity in rabbits was 100 mg/kg bw per day, and the NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested.

The Meeting concluded that spirodiclofen was not teratogenic in rats or rabbits.

Neurotoxicity was investigated in a study of acute neurotoxicity, a short-term study of toxicity and studies of developmental neurotoxicity in rats. There was no evidence of neurotoxicity in the study of acute neurotoxicity, and the only evidence of neurotoxicity in the short-term study was decreased motor and locomotor activity in females at 12 500 ppm, equal to 1310 mg/kg bw per day (the limit dose), during 1 week of treatment. Two studies of developmental neurotoxicity were conducted. The second was a modified study, intended to clarify potential findings related to brain morphometry and learning and memory parameters in offspring in the first study. Effects in maternal animals were limited to small changes in body weight and/or food consumption at the highest dose tested, and these effects were not considered to be biologically relevant. The NOAEL for maternal toxicity in both studies was 1500 ppm, equal to 119 mg/kg bw per day, the highest dose tested. In offspring, observed morphometric changes were small (3–7%), did not attain statistical significance in many cases and were not consistent between studies, and thus they were not considered to be related to treatment. In tests of learning and memory, the findings were also inconsistent, and the considerable variability in the data limited their interpretation. Overall, the Meeting considered that these studies did not indicate any treatment-related findings on neurotoxicity parameters in offspring. The NOAEL was 350 ppm, equal to 28.6 mg/kg bw per day, on the basis of decreases in body weight and body weight gain in offspring at 1500 ppm.

Studies of acute toxicity, short-term studies of toxicity and studies of genotoxicity were conducted with some of the metabolites of spirodiclofen, including BAJ 2740-ketohydroxy (a soil metabolite) and BAJ 2740-MA-3-OH-cyclohexyl ester (a plant metabolite)—neither of which were detected in the studies of metabolism in rats——as well as the enol metabolite (BAJ 2510). BAJ 2740-ketohydroxy and BAJ 2740-MA-3-OH-cyclohexyl ester were both of low acute toxicity when administered orally. BAJ 2510 was moderately toxic via the oral route (LD_{50}, 300–500 mg/kg bw per day) and was not irritating to the eyes or skin. A 6-week dietary study comparing the relative toxicities of spirodiclofen and the enol metabolite was conducted. Effects noted with both test substances included decreased body weight and food consumption, increased adrenal weights, and enlargement and vacuolation of the adrenal gland. Both were also associated with decreased progesterone concentrations in females. In this short-term study, spirodiclofen and the enol metabolite were found to exert similar effects in rats given repeated doses under the conditions of the study, and there was no indication that BAJ 2510 produced a different spectrum of toxic effects or caused marked effects at lower doses than did spirodiclofen. Studies of reverse mutation in vitro were conducted with three metabolites of spirodiclofen (BAJ 2510, BAJ 2740-ketohydroxy and BAJ 2740-MA-3-OH-cyclohexyl ester) to assess potential for inducing gene mutation in vitro. None of these metabolites of spirodiclofen were found to demonstrate any mutagenic potential under the conditions tested.

The following metabolites were identified only in plants: M05 (2,4-dichloromandelic acid hydroxy-cyclohexyl ester), M04 (2,4-dichloromandelic acid cyclohexyl ester glycosylpentoside) and M08 (2,4-dichloromandelic acid glucoside). The latter two metabolites are sugar conjugates of minor metabolites found in the rat. Limited toxicology data were provided for M05, and no toxicology data were provided for the other two plant metabolites. The Meeting therefore concluded that the information available was not sufficient to conduct a hazard assessment for these metabolites. The enol

metabolite was detected in plants and livestock matrices. As the enol metabolite was found to be of similar toxicity to the parent compound, the Meeting considered this metabolite to be toxicologically relevant for the dietary risk assessment.

There were no reports of adverse health effects in manufacturing plant personnel or in operators and workers exposed to spirodiclofen formulations.

The Meeting concluded that the existing database on spirodiclofen was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.01 mg/kg bw per day based on the NOAEL of 1.4 mg/kg bw per day identified on the basis of adrenal effects in both sexes and increased relative testes weights in males at 4.3 mg/kg bw per day in the 1-year study in dogs, and with a safety factor of 100. This ADI provides adequate protection for the marginal adrenal effects noted in females at the lowest dose in the 18-month study in mice. The ADI provides a margin of at least 410-fold relative to the NOAEL for liver tumours in mice and 1470-fold relative to the NOAEL for Leydig cell and uterine tumours in rats, and thus the Meeting considered that spirodiclofen was not likely to pose a carcinogenic risk to humans at dietary levels of exposure.

The Meeting noted that spirodiclofen was not acutely toxic after short-term dosing, that there were no adverse findings in a study of acute neurotoxicity and that there were no developmental toxicity findings that were expected to occur after a single dose in studies in rats or rabbits. The Meeting also noted that findings in the male reproductive system (observed in dogs, rats and mice) would not be caused by a single dose. Consequently, the Meeting determined that an acute reference dose (ARfD) was unnecessary.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	—	25 ppm, equal to 5.1 mg/kg bw per day[b]
		Carcinogenicity	25 ppm, equal to 4.1 mg/kg bw per day	3500 ppm, equal to 610 mg/kg bw per day
Rat	Two-year studies[a]	Toxicity	100 ppm, equal to 4.1 mg/kg bw per day	350 ppm, equal to 14.7 mg/kg bw per day
		Carcinogenicity	350 ppm, equal to 14.7 mg/kg bw per day	2500 ppm, equal to 110.1 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Parental toxicity	70 ppm, equal to 5.2 mg/kg bw per day	350 ppm, equal to 26.2 mg/kg bw per day
		Reproductive toxicity	350 ppm, equal to 26.2 mg/kg bw per day	1750 ppm, equal to 134.5 mg/kg bw per day
		Offspring toxicity	70 ppm, equal to 5.2 mg/kg bw per day	350 ppm, equal to 26.2 mg/kg bw per day
	Developmental toxicity[c]	Maternal toxicity	1000 mg/kg bw per day[e]	—
		Embryo/ fetotoxicity	300 mg/kg bw per day	1000 mg/kg bw per day
	Developmental neurotoxicity[a,d]	Maternal toxicity	1500 ppm, equal to 119 mg/kg bw per day[e]	—
		Offspring toxicity	350 ppm, equal to 28.6 mg/kg bw per day	1500 ppm, equal to 119 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
Rabbit	Developmental toxicity[c]	Maternal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
		Embryo/ fetotoxicity	1000 mg/kg bw per day[e]	—
Dog	Eight-week study of toxicity[a,f]	Toxicity	—	100 ppm, equal to 2.9 mg/kg bw per day[b]
	Fourteen-week study of toxicity[a]	Toxicity in males	200 ppm, equal to 7.7 mg/ kg bw per day	630 ppm, equal to 26.6 mg/kg bw per day
		Toxicity in females	—	200 ppm, equal to 8.4 mg/kg bw per day[b]
	One-year study of toxicity[a]	Toxicity	50 ppm, equal to 1.4 mg/kg bw per day	150 ppm, equal to 4.3 mg/kg bw per day

[a] Dietary administration.
[b] Lowest dose tested.
[c] Gavage administration.
[d] Two studies combined.
[e] Highest dose tested.
[f] Study conducted with males only.

Estimate of acceptable daily intake for humans

0–0.01 mg/kg bw

Estimate of acute reference dose

Unnecessary

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

Results from epidemiological, occupational health and other such observational studies of human exposure

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} of 3–4 h) and extensive (up to 76%; based on renal excretion data)
Distribution	Widely distributed; highest concentrations in liver and kidneys
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapidly excreted; 90% eliminated at 48 h
Metabolism in animals	Extensively metabolized; no parent compound detected in urine or bile. Quantitative differences in metabolic profile between sexes.
Toxicologically significant compounds (animals, plants, environment)	Parent compound and enol metabolite
Acute toxicity	
Rat, LD_{50}, oral	>2500 mg/kg bw
Rat, LD_{50}, dermal	>2000 mg/kg bw
Rat, LC_{50}, inhalation	>5.03 mg/l air
Rabbit, dermal irritation	Not an irritant
Rabbit, ocular irritation	Not an irritant
Guinea-pig, dermal sensitization	Sensitizing (maximization test)

Short-term studies of toxicity	
Target/critical effect	Adrenal gland (cortical vacuolation and increased weight)
Lowest relevant oral NOAEL	50 ppm (equal to 1.4 mg/kg bw per day; 1-year study in dogs)
Lowest relevant dermal NOAEL	1000 mg/kg bw per day (28-day study in rats)
Genotoxicity	
	No genotoxic potential
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Adrenal gland (cortical vacuolation and increased weight)
Lowest relevant NOAEL	50 ppm (equal to 1.4 mg/kg bw per day; 1-year study in dogs)
Carcinogenicity	Tumours in livers (mice), testes (rat) and uterus (rat) at doses that caused target organ and/or systemic toxicity. NOAELs identified; unlikely to pose a risk to humans at levels of dietary exposure.
Reproductive toxicity	
Reproduction target/critical effect	Delayed sexual maturation, decreased spermatid and sperm counts, atrophy of male sex organs, and ovarian vacuolation/degeneration in F_1 animals
Lowest relevant reproductive NOAEL	350 ppm (equal to 26.2 mg/kg bw per day)
Developmental target/critical effect	Renal pelvis dilatation and asymmetric fourth sternebrae
Lowest relevant developmental NOAEL	300 mg/kg bw per day (rat)
Neurotoxicity/delayed neurotoxicity	
Acute neurotoxicity	No evidence of neurotoxicity; NOAEL: 1000 mg/kg bw per day
Subchronic neurotoxicity	Decreased motor and locomotor activity (females only); NOAEL: 87 mg/kg bw per day
Developmental neurotoxicity	No evidence of developmental neurotoxicity
Other toxicological studies	
Mechanism studies	Possible effect on steroidogenesis by the enol metabolite via effects on MD
Studies with metabolites	
Acute toxicity	BAJ 2510 was moderately acutely toxic via the oral route (LD_{50} 300–500 mg/kg bw); BAJ 2740-ketohydroxy and BAJ 2740-3-OH-cyclohexyl ester were of low acute oral toxicity (LD_{50} >2500 mg/kg bw)
Short-term toxicity	Similar results following short-term dosing with BAJ 2510 and spirodiclofen: decreased body weight and adrenal effects
Genotoxicity	BAJ 2510, BAJ 2740-ketohydroxy and BAJ 2740-3-OH-cyclohexyl ester were not mutagenic in vitro
Medical data	
	No occupational or accidental poisoning reported

Summary

	Value	Study	Safety factor
ADI	0–0.01	Dog, 1-year	100
ARfD	Unnecessary	—	—

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