BIXAFEN

First draft prepared by
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Explanation

Bixafen is the International Organization for Standardization–approved common name for *N*-(3′,4′-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide (International Union of Pure and Applied Chemistry) (Chemical Abstracts Service No. 581809-46-3), a novel fungicide from the pyrazole-carboxamide class. Bixafen exhibits broad fungicidal activity in various crops by inhibition of succinate dehydrogenase, an enzyme of complex II within the mitochondrial respiration chain.

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

All critical studies were certified as complying with good laboratory practice (GLP).

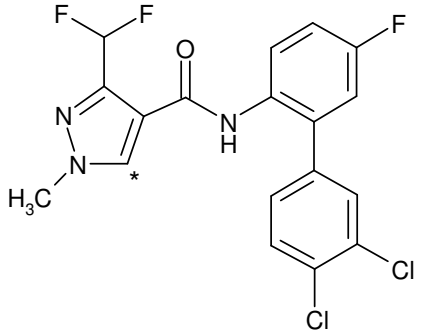
Evaluation for acceptable daily intake

Unless otherwise stated, the studies evaluated in this monograph were performed by laboratories that were certified for GLP and complied with the relevant Organisation for Economic Co-operation and Development (OECD) test guidelines or similar guidelines of the European Union or United States Environmental Protection Agency. As these guidelines specify the clinical pathology tests normally performed and the tissues normally examined, only significant exceptions to these guidelines are reported here, to avoid repetitive listing of study parameters.

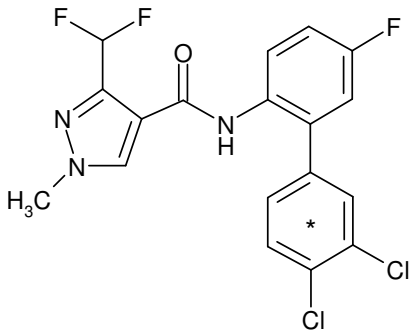
1. Biochemical aspects

The studies on absorption, distribution, metabolism and excretion (ADME) of bixafen in rats were conducted using the radiolabels shown in Fig. 1.

Fig. 1. Structure of bixafen and position of radiolabels



[pyrazole-5-^{14}C]bixafen



[dichlorophenyl-U-^{14}C]bixafen

1.1 Absorption, distribution and excretion

In an ADME study conducted according to OECD Test Guideline 417, groups of male and female Wistar Unilever HsdCpb:WU rats were administered by oral gavage a suspension of [dichlorophenyl-U-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 98%) in 0.5% aqueous tragacanth. The dosing regimens and sacrifice times are presented in Tables 1 and 2. The rats were sacrificed 3 days after dosing except for the animals used for the bile duct cannulation investigations, which were sacrificed 2 days after dosing. The total radioactivity, which included the test item and metabolites, was determined in plasma samples, bile, urine and faeces, as well as in organs and tissues at sacrifice. The metabolism was investigated by radio–high-performance liquid chromatography (HPLC) and spectroscopic methods in selected bile and urine samples and in faecal extracts (Bongartz, 2008).

Recovery. Between 93.5% (group 4) and 106.6% (group 7) of the administered dose was recovered by measurement of the total radioactivity in plasma samples, bile, urine and faeces, as well as in organs and tissues at sacrifice. The radioactivity recovered as a percentage of the administered dose is given in Table 3.

Absorption. The absorption of bixafen started immediately after administration, as was shown by the concentration of radioactivity in plasma. The maximum plasma concentration (C_{max}) was reached approximately 2–8 hours after the administration (T_{max}). The absorption rate of [dichlorophenyl-U-^{14}C]bixafen was calculated from the recoveries in bile, urine and body, excluding gastrointestinal tract, and was 86.3% of the recovered dose for males (group 5) and 82.9% for females (group 7), leading to the conclusion of an almost complete absorption (Table 4). It is assumed that the absorbed dose becomes systemically available.

Table 1. Treatment schedule in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Group	Dose (mg/kg bw)	No. of animals	Sex	Duration (h)	Treatment
1	2	4	Male	72	Single low dose
2	2	4	Female	72	Single low dose
3	50	4	Male	72	Single high dose
4	50	4	Female	72	Single high dose
5	2	5	Male	48	Single low dose/bile duct cannulation
6	2	4	Male	72	Pretreatment (14 × non-radiolabelled and 1 × radiolabelled test item)
7	2	5	Female	48	Single low dose/bile duct cannulation

ADME: absorption, distribution, metabolism, excretion; bw: body weight
Source: Bongartz (2008)

Table 2. Sampling details in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Group	Urine (h)	Faeces (h)	Bile (h)	Organs (h)	Blood (min or h)
1	4, 8, 12, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
2	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
3	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
4	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
5	4, 8, 24, 32 & 48	24 & 48	4, 8, 24, 32 & 48	48	Not performed
6	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
7	4, 8, 24, 32 & 48	24 & 48	4, 8, 24, 32 & 48	48	Not performed

ADME: absorption, distribution, metabolism, excretion
Source: Bongartz (2008)

Distribution and plasma kinetics. The distribution of bixafen from the central compartment to the organs and tissues was followed by measuring the concentration of the total radioactivity in plasma. The pharmacokinetic parameters (two- or three-compartment modelling using the TOPFIT software) are given in Table 5.

The maximum equivalent concentration in plasma (C_{max}) ranged between 0.4 and 6.6 µg/mL, depending on the dose. The maximum was reached approximately 2–4 hours after dosing for the low-dose groups and the test with pretreated rats. In the high-dose groups, the maximum plasma concentration was observed approximately 8 hours after dosing.

The radioactivity in the plasma declined to approximately 1% of the maximum value of radioactivity within 72 hours post-administration. That indicated no retention of the compound-related residues in the body of the animals.

Table 3. Recovery of radioactivity in the ADME rat study (as a % of administered dose) with [dichlorophenyl-U-^{14}C]bixafen

	Recovery of radioactivity (% of administered dose)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Urine	1.41	2.87	0.69	1.67	0.71	1.92	0.83
Bile	ND	ND	ND	ND	83.04	ND	55.93
Faeces	92.37	91.25	98.45	91.39	7.41	104.0	6.26
Total excreted	93.78	94.12	99.14	93.06	91.16	105.92	63.02
Skin	0.028	0.201	0.002	0.028	0.387	0.014	4.073
Sum in organs	0.300	1.373	0.105	0.174	2.348	0.165	28.03
Body, excluding gastrointestinal tract	0.328	1.573	0.106	0.202	2.734	0.179	32.11
Gastrointestinal tract	0.202	1.377	0.031	0.207	6.341	0.142	11.46
Total in body	0.530	2.950	0.137	0.409	9.075	0.321	43.57
Total recovery	94.31	97.07	99.28	93.47	100.2	106.2	106.6

ADME: absorption, distribution, metabolism, excretion; bw: body weight; ND: not determined
Source: Bongartz (2008)

Table 4. Recovery of radioactivity in the ADME rat study (as a % of total radioactivity recovered) with [dichlorophenyl-U-^{14}C]bixafen

	Recovery of radioactivity (% of total radioactivity recovered)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Urine	1.50	2.96	0.69	1.78	0.71	1.81	0.78
Bile	ND	ND	ND	ND	82.86	ND	51.47
Faeces	97.94	94.00	99.17	97.78	7.39	97.89	5.84
Total excreted	99.44	96.96	99.86	99.56	90.96	99.70	58.09
Skin	0.030	0.208	0.002	0.030	0.385	0.013	3.960
Sum in organs	0.318	1.416	0.105	0.187	2.334	0.156	26.71
Body, excluding gastrointestinal tract	0.348	1.624	0.107	0.217	2.719	0.169	30.67
Gastrointestinal tract	0.212	1.419	0.031	0.224	6.322	0.133	11.25
Total in body	0.560	3.043	0.138	0.441	9.041	0.302	41.92
Absorption (sum of urine, bile and body, excluding gastrointestinal tract)					86.29	ND	82.92

ADME: absorption, distribution, metabolism, excretion; bw: body weight; ND: not determined
Source: Bongartz (2008)

Compared with the low-dose tests, the plasma concentration curves of the high-dose groups and the test with pretreated rats featured a very fast elimination phase at the beginning of the test (= $t_{½\ elim\ 1}$) followed by a slower terminal elimination phase (= $t_{½\ elim\ 2}$). Thus, calculations of plasma concentrations for these tests were performed with a three-compartment model by TOPFIT. Plasma concentrations of both low-dose groups showed one terminal elimination phase. They were calculated with a two-compartment model by TOPFIT.

Table 5. Pharmacokinetic parameters in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Parameter	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 6 / male
T_{max} (h), measured	2.0	4.0	8.0	8.0	2.0
T_{max} (h), calculated	2.03	5.06	5.18	9.15	2.66
C_{max} (µg/mL), measured	0.49	0.56	6.55	5.39	0.42
C_{max} (µg/mL), calculated	0.51	0.54	5.72	5.56	0.42
$t_{½\ abs}$ (h)	0.46	0.61	0.52	0.05	0.19
$t_{½\ elim\ 1}$ (h)	8.42	9.36	3.48	2.87	0.95
$t_{½\ elim\ 2}$ (h)	ND	ND	21.7	6.31	25.0
$AUC_{0-\infty}$ (mg/L·h)	7.3	14.3	82.6	139.0	5.1
MRT_{tot} (h)	12.9	19.3	12.1	18.3	12.1
MRT_{abs} (h)	0.72	9.34	5.14	9.31	8.07
MRT_{disp} (h)	12.10	9.92	6.95	9.02	4.03

abs: absorption; ADME: absorption, distribution, metabolism, excretion; AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum equivalent plasma concentration; disp: disposition; elim: elimination; MRT: mean residence time; ND: not determined; $t_{½}$: half-life; T_{max}: time to reach C_{max}; tot: total

Source: Bongartz (2008)

Regarding all tests, there was a faster elimination of bixafen for males compared with females. Calculated area under the plasma concentration–time curve ($AUC_{0-\infty}$) values for females were approximately twice as high as those for males. This indicates a higher systemic exposure for female rats. Ten times higher $AUC_{0-\infty}$ values were calculated for the high-dose tests compared with the low-dose tests.

The mean residence time (MRT_{tot}) of bixafen-related radioactivity was short for all groups included in the study, ranging from 12 to 19 hours.

Excretion. For all treatments, the renal excretion was very low (< 2.9% of the administered dose). The major radioactivity (> 91% of the administered dose) was excreted via faeces. Bile duct–cannulated rats (low dose, 2 mg/kg body weight [bw]) showed a high excretion via bile, 83.0% for male rats and 55.9% for female rats (Table 3). For female bile duct–cannulated rats, a significant portion of radioactivity was still present in the body at the time of sacrifice (48 hours after administration).

The excretion was fast and almost complete 72 hours after administration. At this time, more than 93% of the administered dose had been excreted via urine and faeces in the tests with low, high and repeated doses. No significant dose-related differences were observed. During the first sampling period (0–24 hours), the excretion via faeces was approximately twice as fast for male rats as for female rats. Tests with bile duct–cannulated rats showed a total excretion of approximately 91% of the administered dose for male rats and approximately 63% for female rats (Tables 3 and 6).

Residues in organs and tissues at sacrifice. At sacrifice 72 hours after oral administration of bixafen (groups 1–4 and 6), between approximately 0.1% and 3.0% of the administered dose was found in the bodies (including gastrointestinal tract) of the rats. For bile duct–cannulated rats, approximately 9.1% (group 5, males) and 43.6% (group 7, females) were detected in the bodies (Table 3).

Table 6. Cumulative excretion of radioactivity in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Time (h)	Cumulative excretion of radioactivity (% of administered dose)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Urine							
4	0.21	0.12	0.10	0.09	0.04	0.24	0.03
8	0.42	0.37	0.17	0.19	0.15	0.54	0.12
12	0.61	ND	ND	ND	ND	ND	ND
24	1.19	1.67	0.57	0.85	0.47	1.59	0.36
32	ND	ND	ND	ND	0.53	ND	0.55
48	1.37	2.59	0.68	1.57	0.71	1.88	0.83
72	1.41	2.87	0.69	1.67	ND	1.92	ND
Faeces							
24	71.51	43.44	78.57	50.56	5.34	82.74	3.65
48	88.99	82.38	96.89	86.52	7.41	102.1	6.26
72	92.37	91.25	98.45	91.39	ND	104.0	ND
Bile							
4	ND	ND	ND	ND	11.05	ND	2.87
8	ND	ND	ND	ND	24.12	ND	7.05
24	ND	ND	ND	ND	67.23	ND	27.43
32	ND	ND	ND	ND	74.71	ND	36.27
48	ND	ND	ND	ND	83.04	ND	55.93
Total excreted	93.78	94.12	99.14	93.06	91.16	105.92	63.02

ADME: absorption, distribution, metabolism, excretion; bw: body weight; ND: not determined
Source: Bongartz (2008)

The highest equivalent concentrations were detected in the liver (approximately 0.05–0.84 µg/g) and the kidneys (approximately 0.01–0.20 µg/g) (Table 7). Higher concentrations were found in the perirenal fat and adrenal gland of female rats, especially in the high-dose tests (approximately 0.05–0.20 µg/g). The concentrations in the other organs and tissues were low and ranged between 0.001 and 0.09 µg/g for the low-dose tests and the test with pretreated rats and between 0.01 and 0.10 µg/g for the high-dose tests. From the renal and faecal excretion and from the elimination kinetics of total radioactivity from plasma, it was concluded that the small amounts of residual radioactivity in organs and tissues are subject to further elimination. Residues in the organs and tissues for female rats were in most cases higher than those for male rats (Table 7).

In a study on ADME conducted according to OECD Test Guideline 417, a group of four male Wistar Unilever HsdCpb:WU rats was administered by oral gavage a suspension of [pyrazole-5-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 99%) in 0.5% aqueous tragacanth at a single dose of 2 mg/kg bw. The rats were sacrificed 3 days after dosing. The total radioactivity, which included the parent compound and metabolites, was determined in plasma samples, urine and faeces, as well as in organs and tissues at sacrifice. The metabolism was investigated by radio-HPLC and spectroscopic methods in selected urine samples and in faecal extracts.

A total of 98.1% of the administered radioactivity was recovered from plasma, urine and faeces as well as organs and tissues at sacrifice (Table 8).

Table 7. Equivalent concentrations of residues in organs and tissues from the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

	Equivalent concentrations of residues (µg/g)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Erythrocytes	0.005 5	0.009 7	0.074 2	0.066 1	0.015 3	0.005 9	0.098 3
Plasma	0.002 7	0.009 8	0.053 9	0.050 6	0.017 6	0.001 8	0.135 7
Spleen	0.003 5	0.021 4	0.022 4	0.069 0	NA	0.003 0	NA
Liver	0.061 3	0.138 1	0.810 8	0.837 7	NA	0.052 3	NA
Kidney	0.012 3	0.053 5	0.099 6	0.203 1	NA	0.008 4	NA
Perirenal fat	0.005 0	0.052 0	0.024 9	0.115 5	NA	0.002 4	NA
Adrenal gland	0.008 7	0.088 2	0.051 9	0.199 3	NA	0.003 9	NA
Testis	0.001 8	NA	0.006 0	NA	NA	0.000 8	NA
Ovary	NA	0.038 5	NA	0.096 0	NA	NA	NA
Uterus	NA	0.019 8	NA	0.062 9	NA	NA	NA
Skeletal muscle	0.002 2	0.022 0	NC	0.043 1	NA	0.000 8	NA
Bone femur	0.001 9	0.008 4	NC	0.035 5	NA	0.000 9	NA
Heart	0.004 8	0.046 7	0.017 1	0.089 2	NA	0.001 9	NA
Lung	0.004 5	0.032 2	0.024 7	0.081 4	NA	0.002 2	NA
Brain	0.001 3	0.011 8	NC	0.025 1	NA	0.000 4	NA
Thyroid gland	0.009 9	0.040 0	NC	NC	NA	0.008 6	NA
Skin	0.002 2	0.017 3	NC	0.057 6	0.032 7	0.001 2	0.360 1
Carcass	0.004 2	0.033 6	0.012 1	0.065 6	0.074 1	0.001 4	0.879 5

ADME: absorption, distribution, metabolism, excretion; bw: body weight; NA: not analysed; NC: calculation of mean was not possible, as only one value was above the limit of detection
Source: Bongartz (2008)

Table 8. Recovery of radioactivity in the ADME study with [pyrazole-5-^{14}C]bixafen administered to rats at a dose of 2 mg/kg bw

	Recovery of radioactivity	
	% of dose administered	% of dose recovered
Urine	4.34	4.44
Faeces	93.37	95.20
Total excreted	97.71	99.64
Skin	0.030	0.031
Sum in organs	0.187	0.191
Body, excluding gastrointestinal tract	0.217	0.222
Gastrointestinal tract	0.134	0.137
Total in body	0.351	0.358
Balance	98.07	100

ADME: absorption, distribution, metabolism, excretion; bw: body weight
Source: Bongartz (2007)

The absorption of bixafen started immediately after administration, as shown by the concentration of radioactivity in the plasma. The C_{max} (0.42 µg/mL) was reached approximately 3 hours after administration (T_{max}). The distribution of the test substance from the central compartment to the different organs and tissues was followed by measuring the concentration of the total radioactivity in plasma. The pharmacokinetic parameters (two-compartment modelling using the TOPFIT software) are given in Table 9. There was a rapid elimination of the test item. The radioactivity in the plasma declined to approximately 0.5% of the maximum value of radioactivity within 72 hours post-administration. Plasma concentrations showed one terminal elimination phase (= $t_{½\ elim\ 1}$). Plasma concentrations were calculated with a two-compartment model by TOPFIT. The calculated $AUC_{0-\infty}$ value amounted to 6.5 h·mg/L. The mean residence time (MRT_{tot}) of bixafen-related radioactivity was short and amounted to 13 hours.

Table 9. Pharmacokinetic parameters in the ADME study with [pyrazole-5-^{14}C]bixafen administered to rats at a dose of 2 mg/kg bw

Parameter	Value
T_{max} (h), measured	3.0
T_{max} (h), calculated	2.4
C_{max} (µg/mL), measured	0.42
C_{max} (µg/mL), calculated	0.43
$t_{½\ abs}$ (h)	1.12
$t_{½\ elim\ 1}$ (h)	8.64
$AUC_{0-\infty}$ (h·mg/L)	6.5
MRT_{tot} (h)	13.3
MRT_{abs} (h)	0.88
MRT_{disp} (h)	12.5

abs: absorption; ADME: absorption, distribution, metabolism, excretion; AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum equivalent plasma concentration; disp: disposition; elim, elimination; MRT: mean residence time; $t_{½}$: half-life; T_{max}: time to reach C_{max}; tot: total
Source: Bongartz (2007)

The renal excretion was very low (4.34% of the administered dose). The major radioactivity (93.4% of administered dose) was excreted via faeces (Table 8).

Low amounts of residue were determined in organs and tissues of the animals at sacrifice, 72 hours after the oral administration of a single dose of 2 mg/kg bw. A negligible amount of radioactivity was found in the gastrointestinal tract, showing that the elimination of the compound-related radioactivity was nearly complete. The highest equivalent concentration was detected in the liver (0.03 µg/g).The concentrations in the other organs and tissues were low and ranged between 0.001 and 0.009 µg/g (Table 10). From the renal and faecal excretion and from the elimination kinetics of total radioactivity from plasma, it may be concluded that the small amounts of residual radioactivity in organs and tissues are subject to further elimination (Bongartz, 2007).

In a study conducted according to OECD Test Guideline 417, the distribution of [dichlorophenyl-U-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 98%) in male rats was investigated by quantitative whole-body autoradiography using the radioluminography technique. Eight male Wistar HsdCpb:WU rats were orally administered a target dose of 3 mg/kg bw of radiolabelled bixafen suspended in 0.5% aqueous tragacanth. One control animal was administered the same dose of unlabelled test material and sacrificed at 4 hours after dosing. The distribution of residues in tissues was determined at 1, 4, 8, 24, 48, 72, 120 and 168 hours after administration.

Table 10. Radioactivity residues in organs and tissues from the ADME study with [pyrazole-5-^{14}C]bixafen administered to rats at a dose of 2 mg/kg bw

Organ or tissue	Equivalent concentration (µg/g)	% of administered dose
Erythrocytes	0.001 9	0.001 6
Plasma	0.001 7	0.001 0
Spleen	0.002 6	0.000 3
Liver	0.026 6	0.071 5
Kidney	0.006 6	0.002 9
Perirenal fat	0.003 3	0.000 9
Adrenal gland	0.008 3	0.000 1
Testis	0.001 7	0.001 2
Skeletal muscle	0.002 3	0.001 0
Bone femur	0.001 9	0.000 3
Heart	0.004 2	0.000 9
Lung	0.003 5	0.001 3
Brain	0.001 2	0.000 5
Thyroid gland	0.009 3	< 0.000 1
Skin	0.002 2	0.030 0
Carcass	0.003 5	0.103 6

ADME: absorption, distribution, metabolism, excretion; bw: body weight
Source: Bongartz (2007)

Bixafen was absorbed quickly from the gastrointestinal tract. The maximum concentration of radioactivity in almost all organs and tissues was detected 1 hour after administration. The absorbed radioactivity was distributed rather quickly and evenly in the body, with a preference for liver, kidney, fat (brown and perirenal fat) and infraorbital, Harderian and adrenal glands at the early time points (Table 11).

Radioactive residues in all organs and tissues decreased rapidly between 8 and 48 hours. In nearly all organs and tissues, residues were below the limit of detection (LOD) or limit of quantification (LOQ) at later time points between 72 and 168 hours after dosing. At the end of the test period, only liver, kidney and nasal mucosa showed negligible residues, which were all below 0.05 mg/kg.

There was no sign of a significant retention of radioactivity in specific organs or tissues. Residues in glandular organs or tissues responsible for hormonal regulation (e.g. testis, adrenal or thyroid gland) were rapidly depleted in parallel with the depletion from the other organs and tissues. This is evident from the ratio of the maximum concentration in plasma to the concentration after 24 hours. In all cases – except in nasal mucosa and vitreal body – the ratio was greater than 5, indicating that bioaccumulation following daily administration of the product is unlikely.

Bixafen was rapidly eliminated from the body, predominantly via faecal excretion. Excretion was nearly complete 48 hours after administration. No significant expiration of ^{14}C-labelled volatiles was observed (Spiegel, 2007).

In a study conducted according to OECD Test Guideline 417, the distribution of [pyrazole-5-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 98%) in male rats was investigated by quantitative whole-body autoradiography using the radioluminography technique. Eight male Wistar HsdCpb:WU rats were orally administered a target dose of 3 mg/kg bw of radiolabelled bixafen suspended in 0.5% aqueous tragacanth. One control animal was administered

Table 11. Distribution of total radioactive residues in organs and tissues of rats after a single oral dose of [dichlorophenyl-U-^{14}C]bixafen

Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h	R/B	$C_{max}/C_{24\,h}$
Blood	0.499	0.413	0.357	0.063	0.013	0.008	< LOQ	< LOQ	1.000	7.955
Liver	4.843	4.141	3.104	0.623	0.105	0.067	0.031	0.016	9.703	7.771
Renal cortex	3.025	2.688	2.228	0.321	0.029	0.015	0.007	0.005	6.060	9.419
Renal medulla	2.389	2.303	1.507	0.259	0.031	0.015	0.007	< LOQ	4.786	9.217
Brown fat	5.231	4.616	4.031	0.580	0.020	#	#	#	10.480	9.025
Perirenal fat	3.965	4.837	3.611	0.270	0.012	< LOD	< LOD	#	9.691	17.913
Skeletal muscle	1.447	1.272	1.191	0.164	0.008	< LOD	< LOD	< LOD	2.899	8.846
Myocardium	3.188	2.816	2.415	0.380	0.018	< LOD	< LOD	< LOD	6.387	8.392
Lung	0.888	0.898	0.511	0.106	0.010	< LOQ	#	< LOD	1.799	8.459
Spleen	1.266	1.149	0.965	0.136	0.009	< LOQ	< LOQ	< LOD	2.536	9.295
Pancreas	3.364	3.369	2.413	0.385	0.018	< LOQ	< LOD	< LOD	6.750	8.762
Bone marrow	1.023	0.936	0.859	0.123	0.008	#	#	#	2.049	8.328
Testes	0.864	0.917	0.843	0.116	0.006	< LOD	< LOD	< LOD	1.838	7.881
Brain	1.691	1.105	0.825	0.098	< LOQ	< LOD	< LOD	< LOD	3.388	17.256
Spinal cord	1.977	1.313	0.998	0.109	< LOQ	#	#	#	3.960	18.112
Pituitary gland	2.023	1.703	1.493	0.210	0.012	#	#	#	4.052	9.611
Pineal body	1.994	1.857	1.474	0.186	#	#	#	#	3.995	10.739
Adrenal gland	4.160	3.915	3.155	0.412	0.025	0.010	< LOQ		8.334	10.102
Thymus	1.165	1.114	0.962	0.132	0.007	< LOD	< LOD	#	2.335	8.819
Thyroid gland	1.988	1.891	1.774	0.235	0.014	#	#	#	3.984	8.456
Salivary gland	3.021	2.716	2.293	0.347	0.018	< LOQ	#	#	6.052	8.713
Nasal mucosa	1.152	1.199	1.201	0.450	0.110	0.135	0.044	0.035	2.407	2.673
Skin	1.115	1.036	1.085	0.114	0.008	#	#	#	2.234	9.769
Vitreal body	0.095	0.181	0.139	0.043	0.009	0.008	#	#	0.362	4.165
Harderian gland	4.348	5.166	4.364	0.560	0.016	#	#	#	10.349	9.220
Infraorbital gland	4.691	4.906	4.897	0.615	0.028	#	#	#	9.829	7.975

#: organ or tissue usually visible in the rat sections, but not discernible in the radioluminograms; C_{max}: maximum equivalent concentration; $C_{24\,h}$: concentration after 24 h; LOD: limit of detection; LOQ: limit of quantification; R/B: ratio of maximum concentration in tissue to maximum concentration in blood
Source: Spiegel (2007)

the same dose of unlabelled test material and sacrificed at 4 hours after dosing. The distribution of residues in tissues was determined at 1, 4, 8, 24, 48, 72, 120 and 168 hours after administration.

Bixafen was absorbed quickly from the gastrointestinal tract. The maximum concentration of radioactivity in almost all organs and tissues was detected 1 hour after administration. The absorbed radioactivity was distributed rather quickly and evenly in the body, with a preference for liver, fat, infraorbital gland, Harderian gland and adrenal gland at the early time points (Table 12).

Radioactive residues in all organs and tissues decreased rapidly between 8 and 48 hours. In nearly all organs and tissues, residues were below the LOD or LOQ at later time points between 72 and 168 hours after dosing. At the end of the test period, only liver and nasal mucosa showed negligible residues above the LOD (LOQ). Both residues were below 0.05 mg/kg.

There was no sign of a significant retention of radioactivity in specific organs or tissues. Residues in glandular organs or tissues responsible for hormonal regulation (e.g. adrenal, testis or

thyroid gland) were rapidly depleted in parallel with the depletion from the other organs and tissues. This is evident from the ratio of the maximum concentration in plasma to the concentration after 24 hours. In all cases – except in nasal mucosa – the ratio was greater than 5, indicating that bioaccumulation following daily administration of the product is unlikely.

Bixafen was rapidly eliminated from the body, predominantly via faecal excretion. Excretion was nearly complete 48 hours after administration. No significant expiration of ^{14}C-labelled volatiles was observed (Spiegel, 2006).

Table 12. Distribution of total radioactive residues in organs and tissues of rats after a single oral dose of [pyrazole-5-^{14}C]bixafen

Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h	R/B	$C_{max}/C_{24\ h}$
Blood	0.388	0.349	0.321	0.045	0.009	#	#	1.0	1.0	8.7
Liver	4.228	3.349	2.577	0.363	0.073	0.025	0.012	0.006	10.9	11.7
Renal cortex	2.457	2.144	1.936	0.221	0.035	0.007	< LOQ	< LOQ	6.3	11.1
Renal medulla	1.659	1.507	1.390	0.158	0.023	0.005	< LOD	< LOD	4.3	10.5
Brown fat	4.124	3.361	3.251	0.252	0.041	#	#	#	10.6	16.4
Perirenal fat	2.140	2.640	2.924	0.258	0.016	#	#	#	7.5	11.3
Skeletal muscle	1.196	1.130	1.078	0.126	0.014	#	#	#	3.1	9.5
Myocardium	2.925	2.465	1.819	0.252	0.027	#	#	#	7.5	11.6
Lung	0.844	0.776	0.784	0.064	0.014	#	#	#	2.2	13.2
Spleen	1.003	0.895	0.884	0.093	0.011	< LOD	< LOD		2.6	10.8
Pancreas	2.776	2.584	2.603	0.243	0.031	#	#	#	7.1	11.4
Bone marrow	0.900	0.725	0.695	0.084	0.010	#	#	#	2.3	10.8
Testes	0.687	0.719	0.606	0.077	0.008	< LOD	#	#	1.9	9.3
Brain	1.286	0.793	0.596	0.054	0.006	< LOD	#	#	3.3	23.8
Spinal cord	1.555	0.871	0.748	0.060	0.006	#	#	#	4.0	25.7
Pituitary gland	1.556	1.248	1.172	0.132	0.016	#	#	#	4.0	11.7
Pineal body	1.697	1.320	0.976	0.112	0.015	#	#	#	4.4	15.1
Adrenal gland	3.731	3.000	2.163	0.269	0.030	< LOQ	#	#	9.6	13.9
Thymus	0.977	0.878	0.735	0.088	0.009	#	#	#	2.5	11.1
Thyroid gland	2.156	1.519	1.367	0.167	0.020	#	#	#	5.6	12.9
Salivary gland	2.470	2.097	1.765	0.210	0.024	#	#	#	6.4	11.8
Nasal mucosa	1.358	1.084	0.708	0.411	0.172	0.064	0.074	0.032	3.5	3.3
Skin	0.876	0.667	0.635	0.094	0.012	#	#	#	2.3	9.4
Vitreal body	0.166	0.126	0.176	0.034	0.015	#	#	#	0.5	5.1
Harderian gland	2.816	3.842	3.140	0.379	0.043	#	#	#	9.9	10.1
Infraorbital gland	3.379	3.871	2.954	0.401	0.047	#	#	#	10.0	9.6

#: organ or tissue usually visible in the rat sections, but not discernible in the radioluminograms; C_{max}: maximum equivalent concentration; $C_{24\ h}$: concentration after 24 h; LOD: limit of detection; LOQ: limit of quantification; R/B: ratio of maximum concentration in tissue to maximum concentration in blood
Source: Spiegel (2006)

1.2 Biotransformation

For the investigation of the metabolism of [dichlorophenyl-U-^{14}C]bixafen, bile and urine samples as well as extracts of faeces were taken from the ADME study described above (Bongartz, 2008) and analysed and quantified by radio-HPLC. Faeces were conventionally extracted with

different mixtures of acetonitrile/water and pure acetonitrile followed by an exhaustive extraction (microwave assistance) with a mixture of acetonitrile/water (1 : 1). Approximately 90% of the faecally excreted radioactivity could be extracted.

Parent compound and metabolites were identified in bile (groups 5 and 7) and faecal extract (group 3) by spectroscopic investigations. The compounds in the other samples were assigned by comparison of the metabolite pattern and of the retention times of the identified metabolites. Unassigned (unidentified) metabolites were characterized by their extraction and chromatographic behaviour. An overview of the metabolites detected in bile, urine and faeces is given in Table 13.

Table 13. Metabolites detected in bile, faeces and urine in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Metabolite[a,b]	% of administered dose						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
B1	NC	NC	NC	NC	0.85	NC	0.65
B2	NC	NC	NC	NC	0.61	NC	0.86
B3	NC	NC	NC	NC	1.54	NC	0.93
B4 and B5	NC	NC	NC	NC	2.00	NC	13.24
B6, F1	0.74	0.34	0.99	NC	25.34	0.91	13.22
B8, F4	1.79	3.07	1.62	0.22	10.97	1.08	4.46
B10	NC	NC	NC	NC	2.67	NC	1.45
B12	NC	NC	NC	NC	1.17	NC	0.70
B13	NC	NC	NC	NC	5.88	NC	1.83
B15	NC	NC	NC	NC	2.85	NC	0.74
B16	NC	NC	NC	NC	4.30	NC	1.70
B18	NC	NC	NC	NC	1.50	NC	0.41
B19	NC	NC	NC	NC	1.24	NC	2.06
B20	NC	NC	NC	NC	12.24	NC	8.42
B21	NC	NC	NC	NC	2.53	NC	0.71
F8	1.23	2.56	0.89	1.16	NC	2.04	NC
F10	1.63	3.43	1.07	1.47	NC	2.70	NC
F11	2.23	1.66	0.90	NC	NC	1.89	NC
F12	NC	NC	NC	0.46	NC	1.76	NC
F13	2.93	2.80	1.10	1.00	NC	3.39	NC
F15	1.94	1.20	1.61	0.53	NC	2.46	NC
F17	1.73	0.86	1.05	0.40	NC	1.80	NC
F18	14.25	34.67	10.65	16.01	NC	14.73	NC
F19	7.41	5.44	3.51	1.84	NC	5.70	NC
F20	1.34	0.65	1.84	3.70	NC	1.79	NC
F21	2.30	0.18	2.66	2.94	NC	3.36	NC
F22	13.69	6.68	7.76	2.94	NC	12.80	NC
F25	2.38	NC	1.93	0.92	NC	2.83	NC
F26	10.96	11.91	7.07	6.26	NC	12.03	NC
F27	5.35	2.39	3.43	1.51	NC	3.81	NC

Metabolite[a,b]	% of administered dose						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
F29	4.06	2.01	45.96	44.19	NC	9.88	NC
Total identified	75.95	79.85	94.03	85.54	75.68	84.95	51.38
Total characterized[c]	7.55	4.29	2.07	1.88	7.36	7.23	4.55
Solids of faeces	8.88	7.11	2.35	3.96	NC	11.77	NC
Urine, not subquantified	1.41	2.87	0.69	1.67	0.71	1.92	0.83
Faeces, not analysed	NC	NC	NC	NC	7.41	NC	6.26
Total	93.78	94.12	99.14	93.06	91.16	105.87	63.02

ADME: absorption, distribution, metabolism, excretion; bw: body weight; NC: calculation of mean was not possible, as only one value was above the LOD

[a] Identified metabolites, peak identification and report name: B1: bixafen-desmethyl-hydroxy-5-hydroxyphenyl-6-glutathionyl (isomer 1); B2: bixafen-desmethyl-hydroxy-5-hydroxyphenyl-6-glutathionyl (isomer 2); B3: bixafen-desmethyl-5-hydroxyphenyl-6-cysteinyl-glucuronide; B4 and B5: bixafen-desmethyl-5-hydroxyphenyl-6-(glutathionyl-glutamic acid) and bixafen-desmethyl-5-hydroxyphenyl-6-glutathionyl; B6, F1: bixafen-desmethyl-5-hydroxyphenyl-6-cysteinyl; B8, F4: bixafen-5-hydroxyphenyl-6-cysteinyl; B10: bixafen-4-fluoro-5-hydroxyphenyl-glucuronide; B12: bixafen-6-hydroxyphenyl-glucuronide; B13: bixafen-desmethyl-*N*-*O*-glucuronide; B15: bixafen-*N*-*O*-glucuronide; B16: bixafen-4-hydroxyphenyl-glucuronide; B18: bixafen-3-hydroxyphenyl-glucuronide; B19: bixafen-desmethyl-hydroxypyrazole-glucuronide; B20: bixafen-desmethyl-*N*-glucuronide (isomer 1); B21: bixafen-desmethyl-*N*-glucuronide (isomer 2); F8: bixafen-desmethyl-5-hydroxyphenyl-6-(methylsulfinyl); F10: bixafen-desmethyl-5-hydroxyphenyl; F11: bixafen-desmethyl-6-fluoro-5-hydroxyphenyl; F12: bixafen-5-hydroxyphenyl-6-(methylsulfinyl); F13: bixafen-desmethyl-4-fluoro-5-hydroxyphenyl and bixafen-desmethyl-5-hydroxyphenyl-deschloro-(methylthio); F15: bixafen-5-hydroxyphenyl; F17: bixafen-6-fluoro-5-hydroxyphenyl; F18: bixafen-desmethyl-5-hydroxyphenyl-6-(methylthio); F19: bixafen-4-fluoro-5-hydroxyphenyl and bixafen-5-hydroxyphenyl-6-thiol-acetaldehyde; F20: bixafen-desmethyl-3-hydroxyphenyl; F21: bixafen-desmethyl-6′-hydroxy; F22: bixafen-5-hydroxyphenyl-6-(methylthio) and bixafen-4-hydroxyphenyl; F25: bixafen-3-hydroxyphenyl; F26: bixafen-desmethyl; F27: bixafen-*N*-hydroxy- and bixafen-desmethyl-hydroxypyrazole; F29: bixafen (parent compound).

[b] B indicates a metabolite in bile, F in faeces.

[c] Unidentified metabolites were characterized by their extraction and chromatographic behaviour: 8 metabolites in bile (B7, B9, B11, B14, B17, B22, B23, B24), 11 metabolites in faeces (F2, F3, F5, F6, F7, F9, F14, F16, F23, F24, F28).

Source: Bongartz (2008)

No significant sex-related differences were observed. Metabolites originating from molecular cleavage were not detected in this study. High amounts of parent compound were detected in faeces of rats in both high-dose tests and the test with pretreated rats.

The most important metabolic reaction was the demethylation in the pyrazole ring, forming bixafen-desmethyl. Females showed a higher demethylation rate compared with males, especially in the faeces. Hydroxylation of parent compound and bixafen-desmethyl led to hydroxylated compounds. The hydroxylation took place in different positions of the molecule. Elimination of the fluoro atom and a subsequent hydroxylation were also detected. Finally, an NIH shift of the fluorine atom was observed.

Most of the hydroxy compounds were conjugated with glucuronic acid. An *N*-conjugation of bixafen-desmethyl with glucuronic acid was also found. Conjugation with glutathione was a major metabolic reaction in the bile. Glutathione conjugates were further degraded to cysteine conjugates and methylthio, methylsulfinyl and thiol-acetaldehyde compounds.

A minor reaction was the elimination of one of the chloro atoms of bixafen-desmethyl-5-hydroxyphenyl and a further conjugation with methylthio (Bongartz, 2008).

In addition, for the investigation of the metabolism of [pyrazole-5-^{14}C]bixafen, urine samples as well as extracts of faeces were taken from the ADME study described above (Bongartz, 2007) and analysed and quantified by radio-HPLC. Faeces were conventionally extracted with different mixtures of acetonitrile/water and pure acetonitrile followed by an exhaustive extraction (microwave assistance) with a mixture of acetonitrile/water (1 : 1). Approximately 85% of the faecally excreted radioactivity could be extracted.

Parent compound and metabolites were assigned by comparison of the metabolite pattern and of the retention times of the metabolites, which were identified in the study with the dichlorophenyl label (Bongartz, 2008). Low amounts of label-specific metabolites were detected in urine samples. These metabolites were identified by spectroscopic investigations or by comparison with the non-radiolabelled reference compound. Unassigned metabolites were characterized by their extraction and chromatographic behaviour. An overview of the metabolites detected in urine and faeces is given in Table 14.

The main metabolic routes observed for male rats of the dichlorophenyl and the pyrazole labels are identical. Parent compound was detected in faeces only.

The most important metabolic reaction is the demethylation in the pyrazole ring, forming bixafen-desmethyl. Hydroxylation of parent compound and bixafen-desmethyl led to hydroxylated compounds. The hydroxylation took place in different positions of the molecule. Elimination of the fluoro atom and a subsequent hydroxylation were also detected. Finally, an NIH shift of the fluoro atom was observed. Conjugation with glutathione was a major metabolic reaction and led to an intermediate glutathione conjugate, which was further degraded to cysteine conjugates and methylthio, methylsulfinyl and thiol-acetaldehyde compounds.

A minor reaction, observed in urine, is the molecule cleavage forming pyrazole-4-carboxamide and desmethyl-pyrazole-4-carboxamide. An oxidation of bixafen-pyrazole-4-carboxamide led to bixafen-pyrazole-4-carboxylic acid. Another minor reaction is the elimination of one of the chloro atoms of bixafen-desmethyl-5-hydroxyphenyl and a further conjugation with methylthio (Bongartz, 2007).

In conclusion, the results obtained for male and female rats in the ADME study with the dichlorophenyl label (Bongartz, 2008) correspond well with the findings in the ADME study with the pyrazole label (Bongartz, 2007). Based on these results, the metabolic pathway as outlined in Figs 2 and 3 is proposed.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Results of studies of the acute toxicity of bixafen are summarized in Table 15.

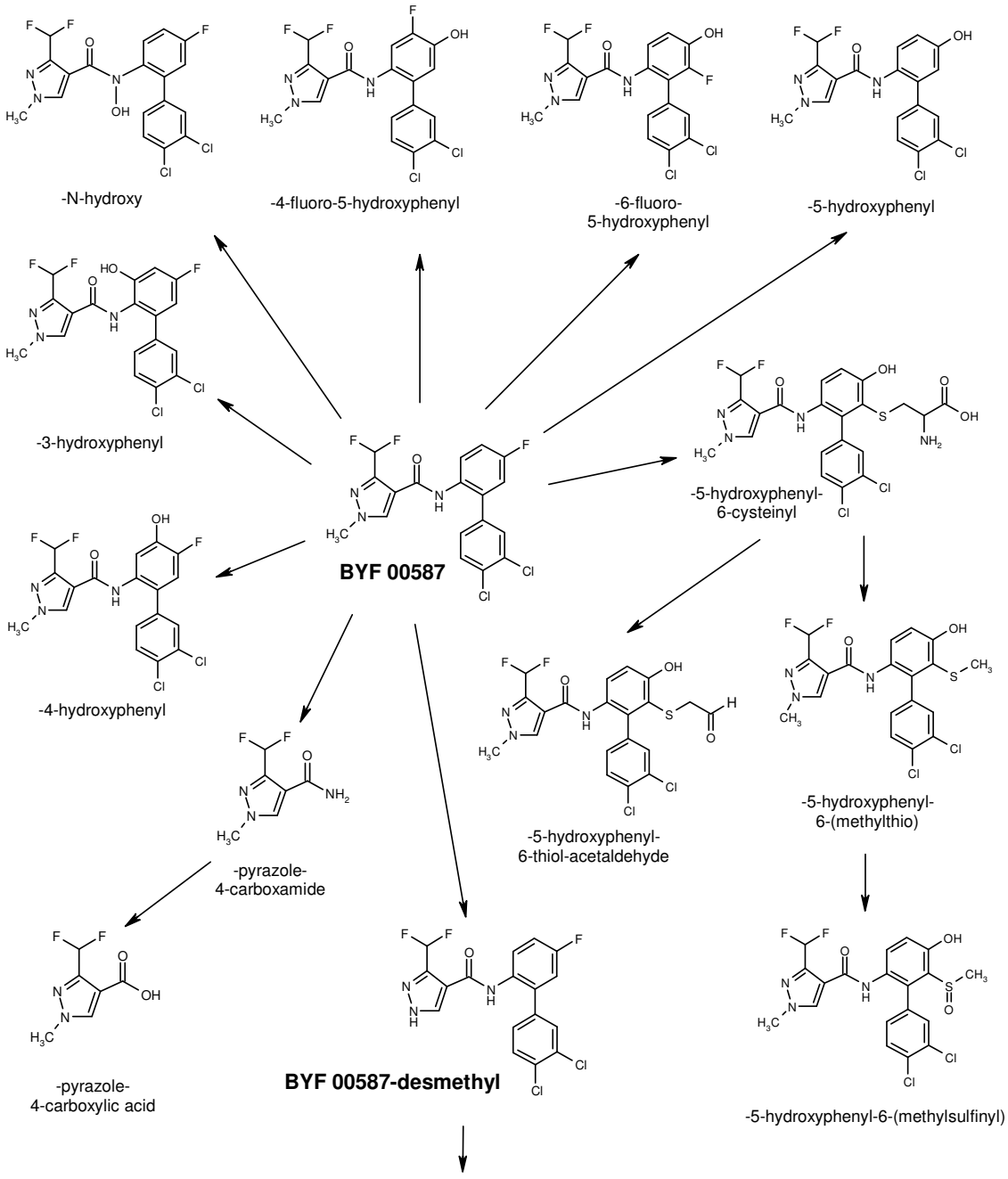
In an acute oral toxicity study conducted according to OECD Test Guideline 423, two groups of three fasted, young female Wistar rats (HsdCpd:Wu) received successively a single bixafen (purity 95.8%) dose of 2000 mg/kg bw by gavage in 2% Cremophor EL in demineralized water (as a vehicle and/or positive control) at a volume of 10 mL/kg bw. Clinical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days, and body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were killed, necropsied and examined for gross pathological changes.

Table 14. Metabolites detected in faeces and urine in the ADME rat study with [pyrazole-5-^{14}C]bixafen

Peak identification[a]	Metabolite report name	% of administered dose
U1	Bixafen-desmethyl-pyrazole-4-carboxamide	2.78
U3	Bixafen-pyrazole-4-carboxamide	0.97
U4	Bixafen-pyrazole-4-carboxylic acid	0.09
U2, U5–U17	Not identified	0.50
Total identified in urine	–	**3.84**
Total in urine	–	**4.34**
F1	Bixafen-desmethyl-5-hydroxyphenyl-6-cysteinyl	1.47
F4	Bixafen-5-hydroxyphenyl-6-cysteinyl	1.84
F8	Bixafen-desmethyl-5-hydroxyphenyl-6-(methylsulfinyl)	1.38
F10	Bixafen-desmethyl-5-hydroxyphenyl	2.31
F11	Bixafen-desmethyl-6-fluoro-5-hydroxyphenyl	1.53
F12	Bixafen-5-hydroxyphenyl-6-(methylsulfinyl)	0.43
F13	Bixafen-desmethyl-4-fluoro-5-hydroxyphenyl and bixafen-desmethyl-5-hydroxyphenyl-deschloro-(methylthio)	3.55
F15	Bixafen-5-hydroxyphenyl	1.92
F17	Bixafen-6-fluoro-5-hydroxyphenyl	1.99
F18	Bixafen-desmethyl-5-hydroxyphenyl-6-(methylthio)	14.13
F19	Bixafen-4-fluoro-5-hydroxyphenyl and bixafen-5-hydroxyphenyl-6-thiol-acetaldehyde	6.97
F20	Bixafen-desmethyl-3-hydroxyphenyl	1.55
F21	Bixafen-desmethyl-6′-hydroxy	2.19
F22	Bixafen-5-hydroxyphenyl-6-(methylthio) and bixafen-4-hydroxyphenyl	10.34
F25	Bixafen-3-hydroxyphenyl	1.94
F26	Bixafen-desmethyl	10.47
F27	Bixafen-*N*-hydroxy- and bixafen-desmethyl-hydroxypyrazole	2.83
F29	Bixafen (parent compound)	8.57
F2–F3, F5–F7, F9, F14, F24, F28	Not identified	9.60
Total identified in faeces	–	**75.42**
Total identified in urine and faeces	–	**79.27**
Total characterized in urine and faeces	–	**10.09**
Solids of faeces	–	6.36
Faeces not analysed[b]	–	1.98
Total in urine and faeces	–	**97.71**

[a] U indicates a metabolite in urine, F in faeces.
[b] Faecal samples 48–72 hours not analysed due to low amount of radioactivity in the pool.
Source: Bongartz (2007)

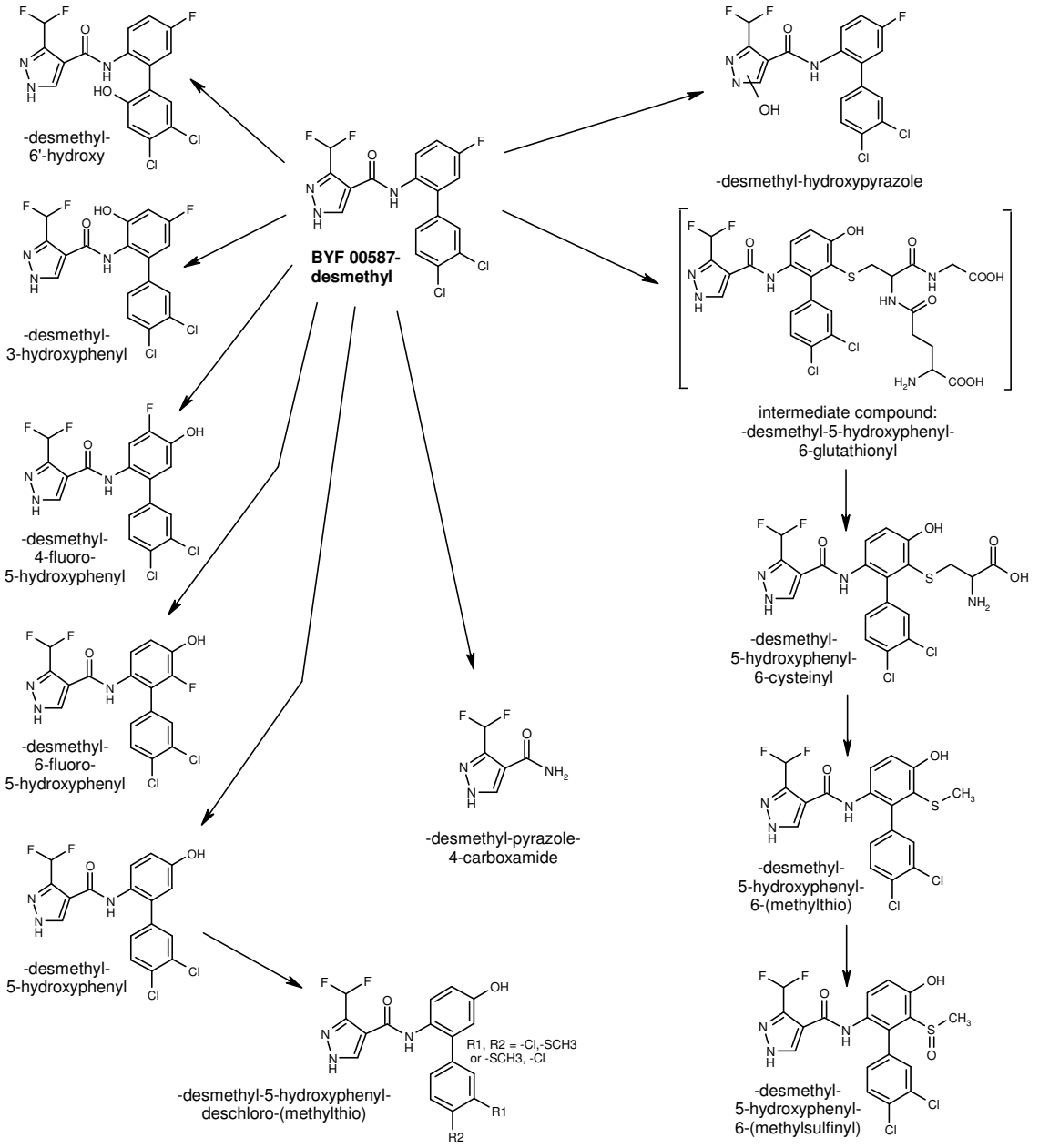
Fig. 2. Proposed metabolic pathway of [pyrazole-5-^{14}C]bixafen in rats



BYF 00587 = bixafen

Source: Bongartz (2007)

Fig. 3. Continued metabolic pathway of [pyrazole-5-^{14}C]bixafen in rats



BYF 00587 = bixafen
Source: Bongartz (2008)

Table 15. Summary of acute oral, dermal and inhalation toxicity of bixafen

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Rat	HsdCpd:Wu	F	Oral	95.8	> 2 000	Schüngel (2005a)
Rat	HsdCpd:Wu	M & F	Dermal	95.8	> 2 000	Schüngel (2005b)
Rat	HsdCpd:Wu	M & F	Inhalation	95.8	> 5.383	Pauluhn (2006)

bw: body weight; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male

No mortalities occurred at 2000 mg/kg bw, the only dose tested. The oral LD_{50} cut-off is greater than or equal to 5000 mg/kg bw, according to OECD Test Guideline 423. There was no toxicological effect on body weight or body weight gain. No clinical signs were observed, and no abnormalities were observed at gross necropsy (Schüngel, 2005a).

In an acute dermal toxicity study conducted according to OECD Test Guideline 402, groups of young adult Wistar rats (five of each sex) were administered bixafen (purity 95.8%) at a single dose of 2000 mg/kg bw. The pure solid test substance was applied semiocclusively for 24 hours to 10% of each animal's body surface moistened with distilled water. After an exposure period of 24 hours, the occlusion was removed, residual test material was removed with tepid water using soap and the area was gently patted dry. The rats were observed for clinical signs and mortality several times on the day of dosing and subsequently at least once daily, for an observation period of at least 14 days. Individual body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were terminated, necropsied and examined for gross pathological changes.

Bixafen was regarded as being of very low acute toxicity after dermal application. No clinical signs were observed in rats of either sex. Body weight and body weight gain were not affected by treatment. There were no gross pathological findings.

The dermal LD_{50} of bixafen was greater than 2000 mg/kg bw (Schüngel, 2005b).

In an acute inhalation toxicity study conducted according to OECD Test Guideline 403, groups of young adult Wistar rats (five of each sex) were exposed by the inhalation route to bixafen (purity 95.8%) in air for 4 hours at a concentration of 5.383 mg/L. Animals were exposed to the aerosolized test substance in Plexiglas exposure tubes applying a directed-flow nose-only exposure principle. A concurrent control group was exposed to an atmosphere using similar exposure conditions (15 L/minute; conditioned dry air). Rats were examined carefully several times on the day of exposure and at least once daily thereafter for 14 days. Visual placing response, grip strength on wire mesh, abdominal muscle tone, corneal and pupillary reflexes, pinnal reflex, righting reflex, tail pinch response and startle reflex with respect to behavioural changes stimulated by sounds (finger snapping) and touch (back) were the reflexes tested. The temperatures were measured shortly after cessation of exposure, and individual body weights were recorded before exposure and on days 3, 7 and 14. On day 15, surviving animals were terminated, necropsied and examined for gross pathological changes.

The method of dust generation employed a "Bayer Generator" system, which gave stable analysed concentrations in the range of 1500–20 000 mg/m^3. The bixafen concentration was determined by gravimetric analysis, where chamber samples were collected after the equilibrium had been attained in hourly intervals. Two samples during each exposure were also taken for analysis of the particle size distribution using an Andersen cascade impactor. At 5383 mg/m^3, the mass median aerodynamic diameter was 6.9 µm (standard deviation = 2.3 µm); 15.3% of the total particulate mass had an aerosol mass less than 3 µm, and 34.3% of the total particulate mass had an aerosol mass less than 5 µm.

Bixafen (solid aerosol) proved to be non-toxic to rats via the inhalation route. No mortality occurred up to the maximum technically attainable concentration. Clinical signs from exposure to bixafen were seen to include bradypnoea, laboured breathing patterns, reduced motility, piloerection, ungroomed hair-coat, limpness, giddiness, high-legged gait, flaccid paralysis of hindlegs and mydriasis. These effects were rapidly reversible, and all animals appeared normal by the 4th post-exposure day. Several animals displayed reduced tonus and horizontal grip strength. One female had an impaired righting reflex. Rectal temperature was lowered by approximately 20% in both sexes with the treatment. No significant treatment-related effects on body weight gain were noted. At necropsy, mild discolouration of the lung was observed, but this was considered not to be toxicologically relevant.

The acute inhalation LC_{50} of bixafen was in excess of 5.383 mg/L (Pauluhn, 2006).

(b) Dermal and ocular irritation and dermal sensitization

Results of studies on dermal and eye irritation and dermal sensitization of bixafen are summarized in Table 16.

Table 16. Summary of irritation and skin sensitization potential of bixafen

Species	Strain	Sex	End-point	Purity (%)	Result	Reference
Rabbit	Crl:KBL(NZW)BR	F	Skin irritation	95.8	Not irritating	Schüngel (2005c)
Rabbit	Esd:NZW	F	Eye irritation	95.8	Not irritating	Schüngel (2005d)
Mouse	Hsd:Win NMRI	F	Skin sensitization (LLNA)	95.8	Not sensitizing	Vohr (2005)

F: female; LLNA: local lymph node assay; M: male

In a study of skin irritation potential conducted according to OECD Test Guideline 404, 0.5 g of pulverized bixafen (purity 95.8%) moistened with distilled water was applied to the shorn dorsal skin of three young adult female New Zealand White rabbits under a gauze patch (placed on the dorsolateral areas of the trunk of each animal and held in place with non-irritating tape for the duration of the exposure period). The contralateral skin area not treated with bixafen served as the control. In the first instance, only one rabbit was used, and three patches were applied successively to it for exposure periods of 3 minutes, 1 hour and 4 hours, respectively. The test was completed using two additional rabbits exposed for 4 hours. The responses were graded 1 hour later. Skin reactions were scored at 1, 24, 48 and 72 hours post-treatment.

No signs of systemic toxicity or mortality were observed during the study period. No erythema, eschar or oedema was seen in any animals at any time point. It was concluded that bixafen is non-irritating to rabbit skin (Schüngel, 2005c).

In a study of eye irritation potential conducted according to OECD Test Guideline 405, 0.1 mL of pulverized bixafen (purity 95.8%) was placed into the conjunctival sac of one eye of each of three male New Zealand White rabbits. The other eye, which was untreated, served as control. The treated eye was not rinsed for at least 24 hours following instillation. Ocular lesions were scored at 1, 24, 48 and 72 hours post-instillation.

No signs of systemic toxicity were observed during the study period. A slight redness of the conjunctivae was observed after 1 hour in all females and at 24 hours in one female (grade 1, having resolved by 2 days post-treatment). It was concluded that bixafen is not irritating to the eyes (Schüngel, 2005d).

In a study of skin sensitization potential conducted according to OECD Test Guideline 429, bixafen (purity 95.8%) was applied epicutaneously to the dorsal surface of each ear of female NMRI mice. Groups of six mice per dose were dosed once daily for 3 consecutive days with 25 µL of suspensions of bixafen at a concentration of 0%, 3%, 10% or 30% in dimethyl sulfoxide (DMSO). The rabbits were anaesthetized by inhalation of carbon dioxide and killed 1 day after the last application (day 4). The appropriate organs were then removed, and lymphatic organs (the auricular lymph nodes) were transferred into physiological (phosphate-buffered) saline. Animals were checked for mortality and clinical signs at least daily during the study. In particular, the site of application was examined for signs of local irritation. Individual body weights were measured at the start of the study and at scheduled termination.

No mortality or clinical signs were observed during the study. The mice did not show an increase in the stimulation indices for cell counts or for weight of the draining lymph nodes after application of bixafen. No substance-specific effects were determined for ear weights, and body weights of the animals were not affected by any treatment.

In conclusion, no activation of the cells of the immune system via the dermal route was seen by the local lymph node assay method. Thus, bixafen did not show any sensitization potential (Vohr, 2005).

2.2 Short-term studies of toxicity

Mice

In a range-finding study of toxicity not conducted according to GLP or OECD test guidelines, groups of five male and five female C57BL/6J mice were fed diets containing bixafen (purity 98.7%) at a concentration of 0, 100, 500 or 2500 parts per million (ppm) (equal to 0, 17, 81 and 305 mg/kg bw per day for males and 0, 21, 103 and 424 mg/kg bw per day for females, respectively) for 28 days. Morbidity/mortality checks were performed twice daily, and a detailed clinical examination was performed weekly. Body weights and feed consumption were recorded weekly throughout the study. Clinical chemistry analyses were performed at the end of the study. Any animals dying prematurely were necropsied, and selected tissues were examined microscopically. At study termination, all animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

At 2500 ppm, between days 7 and 14, all males were killed for humane reasons or were found dead, and 3/5 females were killed for humane reasons. Prior to necropsy, the majority of animals of both sexes had reduced motor activity, 2/5 males and 1/3 females had hunched posture, 3/5 males and 1/3 females were cold to touch, 2/3 females had tremors and piloerection, and there were isolated instances of slow respiration, half-closed eyes and wasted appearance. No mortalities or clinical signs occurred at 500 or 100 ppm.

Body weight at 2500 ppm was reduced by 18% in males due to a marked body weight loss during the 1st week of the study, whereas in females, body weight gain was reduced by 73% and 67% in weeks 1 and 3, respectively, and overall cumulative body weight gain was reduced by 25%. Feed consumption at 2500 ppm was reduced by 38% in males and by 16% in females during the 1st week of the study. Thereafter, feed consumption by females was reduced by 11% and 21% during weeks 3 and 4, respectively. No significant effects on body weight or feed consumption occurred at 500 or 100 ppm.

At the clinical chemistry assessment, treatment-related changes at 2500 ppm consisted of an increase in total cholesterol concentration (106%) in the two surviving females, whereas a reduced albumin concentration (22%) and an increase in alanine aminotransferase activity (371%) were observed in one of the two surviving females. Statistically significantly lower mean albumin concentrations were also noted at 500 ppm in both sexes (23% and 18% in males and females, respectively) and at 100 ppm in females (14%).

At necropsy, enlarged and dark livers and pallor in other organs were noted at 2500 ppm. Enlarged livers were also observed in 3/5 males and 2/5 females at 500 ppm. Minimal to slight hypertrophy of centrilobular hepatocytes was observed in the liver of the two surviving females at 2500 ppm and in 4/5 males and 4/5 females at 500 ppm. In addition, minimal or slight focal coagulative necrosis was seen in the two surviving females at 2500 ppm and in one male and all the females at 500 ppm (Table 17).

The no-observed-adverse-effect level (NOAEL) was 100 ppm (equal to 17 mg/kg bw per day for males and 21 mg/kg bw per day for females), based on liver toxicity (increased liver weight, clinical chemistry changes, focal coagulative necrosis) in both sexes at 500 ppm (equal to 81 mg/kg bw per day for males and 103 mg/kg bw per day for females) and above (Steiblen, 2004).

Table 17. Summary of selected findings in the 28-day mouse study

	Males				Females			
	0 ppm	100 ppm	500 ppm	2 500 ppm	0 ppm	100 ppm	500 ppm	2 500 ppm
Terminal body weight (g)	19.5	19.9	19.0	ND	15.2	15.7	15.7	16.2
Absolute liver weight (g)	0.85	0.96	1.11*	ND	0.70	0.76	0.88*	1.39*
Relative liver weight (% of body weight)	4.34	4.83	5.64*	ND	4.62	4.84	5.58	8.55*
Liver: centrilobular hypertrophy, minimal to slight	0/5	0/5	4/5	[1/5][a]	0/5	0/5	4/5	2/2 [3/3][a]
Liver: focal coagulative necrosis, minimal to slight	0/5	0/5	1/5	[3/5][a]	0/5	0/5	5/5	2/2 [1/3][a]

ND: not determined; ppm: parts per million; * P: < 0.05

[a] Premature deaths.

Source: Steiblen (2004)

In a study of toxicity conducted according to OECD Test Guideline 408, groups of 10 male and 10 female C57BL/6J mice were fed diets containing bixafen (purity 99.2%) at a concentration of 0, 50, 200 or 500 ppm (equal to 0, 8.5, 34.3 and 88 mg/kg bw per day for males and 0, 10.4, 42.9 and 110 mg/kg bw per day for females, respectively) for at least 90 days. Morbidity/mortality checks were performed twice daily, and a detailed clinical examination was performed weekly. Body weights and feed consumption were recorded weekly throughout the study. Clinical chemistry analyses were performed at the end of the study. Any animals dying prematurely were necropsied, and selected tissues were examined microscopically. At study termination, all animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

No treatment-related mortalities or clinical signs were noted during the study. One female at 500 ppm was killed for humane reasons; the moribund state of this animal was due to a hydrocephaly and was therefore not related to treatment. Body weight parameters and feed consumption were unaffected by treatment.

Treatment-related effects on clinical chemistry parameters involved an increase in alanine aminotransferase and alkaline phosphatase activity (72% and 28%, respectively) in males at 500 ppm and a decrease in total cholesterol concentration in males at 500 and 200 ppm (47% and 37%, respectively) (Table 18).

At necropsy at 500 ppm, mean absolute and relative liver weights were increased (21–23% in males, 16–20% in females). Enlarged livers were observed in 4/10 males and 4/9 females. At the histopathological examination, slight to moderate centrilobular to panlobular hepatocellular hypertrophy was noted in all males and in 8/9 females. A loss of centrilobular hepatocellular vacuolation was noted in all males, with a concomitant increase of diffuse hepatocellular vacuolation noted in 8/10 males. This change was interpreted to have occurred subsequent to the presence of centrilobular hepatocellular hypertrophy. In the stomach, a higher incidence of focal/multifocal squamous cell hyperplasia was noted in 3/10 males and 4/9 females; this finding was associated with a higher incidence of pustules noted in 3/10 males. At 200 ppm, necropsy revealed a slight increase in mean absolute and relative liver weight (11–12% in males, 8–10% in females). Histopathological examination showed minimal to slight centrilobular to panlobular hepatocellular hypertrophy in 7/10 males (Table 18).

The NOAEL was 50 ppm (equal to 8.5 mg/kg bw per day for males and 10.4 mg/kg bw per day for females), based on liver toxicity in males (increased weight, clinical chemistry changes and diffuse hepatocellular vacuolation) and focal/multifocal squamous cell hyperplasia of the stomach in both sexes at 200 ppm (equal to 34.3 mg/kg bw per day for males and 42.9 mg/kg bw per day for females) and above (Steiblen, 2005a).

Table 18. Summary of selected findings in the 90-day mouse study

	Males				Females			
	0 ppm	50 ppm	200 ppm	500 ppm	0 ppm	50 ppm	200 ppm	500 ppm
ALAT (IU/L)	43	41	38	74**	42	35	40	62
AP (IU/L)	94	101	104**	120**	149	129*	128*	145
Cholesterol (mmol/L)	1.79	1.49*	1.13**	0.94**	1.30	1.36	1.19	1.16
Albumin (g/L)	43	41	39**	38**	44	43	39**	40*
Terminal body weight (g)	23.3	23.2	23.1	23.4	118.6	18.6	18.9	19.1
Absolute liver weight (g)	0.96	0.96	1.07	1.18**	0.82	0.83	0.90	0.98**
Relative liver weight (% of body weight)	4.13	4.12	4.63*	5.06**	4.39	4.44	4.76	5.11**
Liver								
No. examined	10	10	10	10	10	10	10	9
Hepatocellular hypertrophy; centrilobular	0	0	7	10	0	0	0	8
Hepatocellular vacuolation; centrilobular	10	10	8	0	6	7	9	7
Hepatocellular vacuolation; diffuse	0	0	2	8	4	2	1	2
Stomach								
No. examined	10	10	10	10	10	10	10	9
Squamous cell hyperplasia	1	1	2	3	1	0	2	4

ALAT: alanine aminotransferase; AP: alkaline phosphatase; IU: international units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Steiblen (2005a)

Rats

In a range-finding toxicity study not conducted according to GLP or OECD test guidelines, groups of five male and five female Wistar Rj: WI (IOPS HAN) rats were fed diets containing bixafen (purity 99.7%) at a concentration of 0, 50, 350 or 2000 ppm (equal to 0, 3.5, 25 and 137 mg/kg bw per day for males and 0, 4.1, 28 and 138 mg/kg bw per day for females, respectively) for 28 days. Morbidity/mortality checks were performed twice daily, and a detailed clinical examination was performed weekly. Body weights and feed consumption were recorded weekly throughout the study. Haematology and clinical chemistry analyses were performed at the end of the study. On study day 29, all animals were necropsied, selected organs were weighed and a range of tissues was taken and fixed. Histopathological examinations were performed on all tissues from all the animals in the control and high-dose groups, whereas liver, kidney, lung and thyroid gland were also examined in all animals in the intermediate-dose group. Also at necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P450 content and specific cytochrome P450 isoenzyme profile.

There were no deaths and no treatment-related clinical signs at any dose level. Body weight gain was reduced at 2000 ppm in both sexes, by 21% (males: after 1–15 days) to 26% (females: after 1–22 days). Body weight was unaffected at 50 or 350 ppm in either sex. At 2000 ppm, feed intake was reduced by 19% in males from days 1 to 8, but returned to normal levels thereafter, whereas in females, there was a reduction in feed intake of 25% on days 1–8 and 9–13% from days 8 to 28. There was no impact on feed consumption in either sex at a dietary concentration of 350 or 50 ppm.

Haematological examination showed an increase in platelets of 21% and 22% in males and females, respectively, at 2000 ppm, the effect being statistically significant only in females. Males at 2000 ppm had a slightly longer mean prothrombin time of 32%, but this was not statistically

significant. Clinical chemistry findings included decreased bilirubin of 54% or 65% in males and females, respectively, at 350 ppm and of 85% and 59% in males and females, respectively, at 2000 ppm. Total serum cholesterol was elevated by 71% in females in the 2000 ppm dose group.

Terminal body weights were not affected by treatment. Liver weights were increased in the 350 and 2000 ppm dose groups in both sexes compared with controls, the effect being more pronounced in females (Table 19). At 350 ppm, females had an increase in liver weight of more than 25%, with 3/5 livers darkened and enlarged grossly. At 2000 ppm, dark livers were observed in 4/5 males. Dark and enlarged livers were observed in all females. Histologically, centrilobular hypertrophy (minimal to slight) was seen in all animals at the highest dose and in 1/5 females and 4/5 males in the intermediate-dose group. Minor, diffuse hypertrophy of the follicular cells in the thyroid gland was seen in 2/5 males at 2000 ppm.

A slight, dose-related increase in benzyloxyresorufin *O*-dealkylase (BROD) and pentoxyresorufin *O*-dependentylase (PROD) activities was observed in males, with no significant effect noted at 50 ppm. In females, BROD activity was considered slightly increased at 2000 ppm only; the per cent increase compared with the control female mean appeared high due to a low control value. No significant effect was noted in PROD activity for the females or in ethoxyresorufin *O*-deethylase (EROD) activity for both sexes (Table 19).

Table 19. Summary of selected findings in the 4-week rat study

	Males				Females			
	0 ppm	50 ppm	350 ppm	2 000 ppm	0 ppm	50 ppm	350 ppm	2 000 ppm
Total bilirubin (µmol/L)	1.3	0.9	0.6**	0.2**	1.7	1.2	0.6**	0.7**
Cholesterol (mmol/L)	1.7	1.6	1.6	2.1	1.8	1.9	2.2	3.1**
Terminal body weight (g)	393	377	390	379	231	234	234	225
Absolute liver weight (g)	10.7	10.3	11.6	12.8**	5.8	6.2	7.4*	9.0**
Relative liver weight (% of body weight)	2.71	2.74	2.98*	3.36**	2.52	2.65	3.15**	3.99**
Total P450 (nmol/mg protein)	1.05	1.23	1.30	1.34	0.74	0.74	0.81	1.00
EROD	60.7	53.4	56.6	50.1	60.5	62.6	41.1	47.8
BROD	10.1	11.8	47.2	58.7	1.6	3.4	6.4	15.6
PROD	4.8	5.7	13.5	17.1	3.0	3.6	3.5	5.3
Hepatocellular hypertrophy; centrilobular	0	0	4	5	0	0	1	5

BROD: benzyloxyresorufin *O*-dealkylase; EROD: ethoxyresorufin *O*-deethylase; ppm: parts per million; PROD: pentoxyresorufin *O*-dependentylase; *: $P < 0.05$; **: $P < 0.01$
Source: Langrand-Lerche (2004)

The NOAEL was 350 ppm (equal to 25 mg/kg bw per day for males and 28 mg/kg bw per day for females), based on reduced body weight gain, reduced feed consumption and liver toxicity (increased liver weight, increased cholesterol level) and thyroid effects (hypertrophy of follicular cells) at 2000 ppm (equal to 137 mg/kg bw per day for males and 138 mg/kg bw per day for females) (Langrand-Lerche, 2004).

In a study of toxicity conducted according to OECD Test Guideline 408, groups of 10 male and 10 female Wistar Rj: WI (IOPS HAN) rats were fed diets containing bixafen (purity 99.2%) at a concentration of 0, 50, 200, 800 or 2000 ppm (equal to 0, 3.2, 12.9, 50.4 and 130 mg/kg bw per day for males and 0, 3.9, 15.0, 59.2 and 153 mg/kg bw per day for females, respectively) for at least 90

days. An additional 10 males and 10 females fed test diet containing bixafen at a concentration of either 0 or 2000 ppm for at least 90 days were maintained on control diet for a further 28 days to examine the reversibility of any effects seen. Clinical signs were recorded daily, and body weight and feed consumption were measured weekly. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving animals (except for animals of the recovery groups) were subjected to a neurotoxicity assessment (motor activity, sensory reactivity and grip strength) during weeks 11–12 of the study. Additionally, during week 4 of the recovery phase, motor activity was recorded for all surviving animals. Ophthalmological examinations were performed on all animals during the acclimatization phase and on all surviving animals of the control and high-dose groups during week 13. Urine samples were collected overnight before necropsy on selected animals. A blood sample was collected on selected animals for haematology and clinical chemistry determinations before necropsy. In addition, a blood sample was collected on selected animals for triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) analysis during weeks 3–4 and 13 of the study and in week 5 of the recovery phase. All animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

No treatment-related mortalities or clinical signs (including the neurotoxicity assessment) were noted during the study. One female at 2000 ppm was terminated prematurely for humane reasons on day 73. Prior to necropsy, this animal had noisy respiration and a soiled nose. At necropsy, this animal was found to have a nasal cavity fracture, and histopathology revealed a minimal diffuse follicular cell hypertrophy in the thyroid gland.

Feed consumption of females at 800 and 2000 ppm was reduced by 8% or 11%, respectively, between days 1 and 8 and was comparable with that of the control group thereafter.

Clinical pathology determinations at 2000 ppm revealed an increase in the prothrombin time by 51% in males, an increase in total cholesterol concentration by 40% in females and a slight tendency towards higher gamma-glutamyltransferase activity in 5/9 females. Total bilirubin concentration was decreased by 50% in males and 43% in females at 2000 ppm, by 40% in males and 48% in females at 800 ppm and by 29% in females at 200 ppm (Table 20). Hormonal analysis revealed that TSH levels at 2000 ppm increased during weeks 3–4 by 64% in males and by 42% in females when compared with the controls. At week 13, no increase in TSH level was observed; only a slight (32%) increase in T_3 level in females was noted.

At necropsy, mean absolute and relative liver weights were increased by 18–24% in males and 33–41% in females at 2000 ppm and by 7–12% in males and 17–19% in females at 800 ppm (Table 20). At 2000 ppm, enlarged and/or dark and prominent lobulation of the liver was observed in some animals of both sexes and was associated with a minimal to slight centrilobular hepatocellular hypertrophy noted in all males and in 6/9 females at microscopic examination. Also at 2000 ppm, minimal to slight diffuse hypertrophy of the follicular cells was noted in the thyroid gland of 9/10 males and 5/9 females at scheduled termination. This finding was also observed in the female prematurely terminated due to accidental trauma.

At 800 ppm, enlarged and/or prominent lobulation of the liver was observed in some animals of both sexes and was associated with a minimal to slight centrilobular hepatocellular hypertrophy noted in 7/10 males at microscopic examination. Also at 800 ppm, minimal to slight diffuse hypertrophy of follicular cells was observed in the thyroid gland of 6/10 males and 2/10 females (Table 20).

The NOAEL was 200 ppm (equal to 12.9 mg/kg bw per day for males and 15.0 mg/kg bw per day for females), based on liver effects (enlarged livers, increased liver weight) and thyroid effects (hypertrophy of follicular cells) at 800 ppm (equal to 50.4 mg/kg bw per day for males and 59.2 mg/kg bw per day for females) and above (Steiblen, 2005b).

Table 20. Summary of selected findings in the 90-day rat study

	Males					Females				
	0 ppm	50 ppm	200 ppm	800 ppm	2 000 ppm	0 ppm	50 ppm	200 ppm	800 ppm	2 000 ppm
Body weight (g), day 90	553	537	540	534	540	295	292	296	297	288
Body weight gain (g), days 1–90	340	326	329	322	329	121	120	125	125	117
Feed consumption (g), day 8	25.6	25.7	25.6	25.7	25.1	19.9	21.1	18.9	18.3*	17.8**
Prothrombin time (s), day 90	15.0	14.7	16.0	16.4	22.7**	14.2	14.5	13.5	12.8*	12.5**
Total bilirubin (µmol/L), day 90	2.0	1.7	1.6	1.2**	1.0**	2.1	1.7	1.5**	1.1**	1.2**
Cholesterol (mmol/L), day 90	1.97	1.98	1.86	1.96	2.31	2.10	2.31	2.23	2.54	2.94**
TSH (ng/mL), weeks 3–4	6.1	5.7	6.7	6.7	10.0*	3.8	4.3	3.6	4.7	5.4
TSH (ng/mL), week 13	5.6	5.6	6.8	7.2	7.2	3.8	3.4	4.3	4.1	7.7
Terminal body weight (g)	521	514	512	506	505	286	277	279	281	271
Absolute liver weight (g)	11.5	11.7	11.9	12.2	13.5**	6.5	6.4	6.7	7.6**	8.7**
Relative liver weight (% of body weight)	2.19	2.28	2.32	2.41*	2.68**	2.28	2.31	2.40	2.71**	3.22**
Liver: enlarged	0/10	0/10	1/10	3/10	7/10	0/10	0/10	2/10	7/10	8/9
Liver: dark	0/10	0/10	0/10	0/10	3/10	0/10	0/10	0/10	0/10	2/9
Liver: prominent lobulation	0/10	1/10	0/10	3/10	4/10	0/10	0/10	0/10	3/10	2/9
Liver: centrilobular hepatocellular hypertrophy, diffuse										
- Minimal	0/10	0/10	0/10	6/10	4/10	0/10	0/10	0/10	0/10	5/9
- Slight	0/10	0/10	0/10	1/10	6/10	0/10	0/10	0/10	0/10	1/9
- Total	0/10	0/10	0/10	7/10	10/10	0/10	0/10	0/10	0/10	6/9
Thyroid: follicular cell hypertrophy, diffuse										
- Minimal	0/10	0/10	0/10	6/10	4/10	0/10	0/10	0/10	1/10	4/9
- Slight	0/10	0/10	0/10	0/10	5/10	0/10	0/10	0/10	1/10	1/9
- Total	0/10	0/10	0/10	6/10	9/10	0/10	0/10	0/10	2/10	5/9

ppm: parts per million; TSH: thyroid stimulating hormone; *: $P < 0.05$; **: $P < 0.01$
Source: Steiblen (2005b)

Dogs

In a study of toxicity conducted according to OECD Test Guideline 409, groups of four male and four female Beagle dogs were administered bixafen (purity 95.8%) in 0.5% aqueous carboxymethyl cellulose at a dosing volume of 5 mL/kg bw by oral gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day for 91–94 days. Clinical observations were conducted daily, feed consumption was measured daily and body weights were taken weekly. Clinical chemistry, urine analysis and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance. Following initiation of dosing, haematological, clinical chemistry and urine analysis data were collected from all animals during study weeks 5, 9 and 13.

Ophthalmic examinations were performed pre-exposure and pretermination. A gross necropsy was performed, organ weights were taken and tissues were examined microscopically.

One female in the high-dose group was sacrificed in extremis on study day 87 due to aspiration of the dosing material.

Pale faeces, observed in males and females in the high-dose group, were the only compound-related clinical finding. However, this finding was not of toxicological significance.

There were no treatment-related effects on body weight or feed consumption. No treatment-related ocular abnormalities were observed at ophthalmic examination.

Haematological examination showed a non-statistical decrease in red blood cell counts and statistically significant decreases in haemoglobin and haematocrit on days 58 and 86 in males in the high-dose group (Table 21); no effects were seen at 100 or 300 mg/kg bw per day.

There were no treatment-related clinical chemistry changes or urine analysis findings at any dose level.

Terminal body weights were not affected by compound administration. Compound-related increases in both absolute (non-statistically significant) and relative (statistically significant) liver weights occurred in males and females in the mid- and high-dose groups (Table 21).

Table 21. Summary of selected findings in the 90-day dog study

	Males				Females			
	0 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	1 000 mg/kg bw per day	0 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	1 000 mg/kg bw per day
Red blood cells (10^6/mL)								
- Day 58	6.91	6.41	6.51	5.99	6.72	6.76	6.60	6.68
- Day 86	7.19	6.86	6.68	6.10	7.02	6.84	6.62	7.06
Haemoglobin (g/dL)								
- Day 58	15.8	14.9	14.8	13.5*	15.1	15.4	14.7	15.3
- Day 86	16.6	16.1	15.5	13.9*	15.8	15.7	15.1	16.3
Haematocrit (%)								
- Day 58	46.2	43.4	43.4	39.5*	44.8	45.1	43.3	45.1
- Day 86	48.5	46.7	45.0	40.5*	46.3	45.7	43.7	47.9
Terminal body weight (kg)	11.15	10.08	11.10	10.43	8.03	7.76	8.08	7.55
Absolute liver weight (g)	351	337	415	432	258	280	316	309
Relative liver weight (% of body weight)	3.14	3.35	3.73*	4.15*	3.22	3.60	3.90*	4.09*
Liver: cytoplasmic vacuolization	0/4	0/4	0/4	2/4	0/4	0/4	0/4	3/4

bw: body weight; *: $P < 0.05$
Source: Sheets (2009)

Micropathological evaluation was conducted on all tissues from the control and high-dose groups. Based on increased liver weights in males and females in the mid- and high-dose groups and on micropathological changes noted in males and females in the high-dose group, livers from animals in the mid- and high-dose groups were also processed and evaluated microscopically. Compound-related micropathological lesions were limited to livers of two males and three females in the high-dose group. The compound-related morphological change, coded as "cytoplasmic vacuolization", consisted of enlarged hepatocytes, primarily centrilobular, with a vacuolated cytoplasm that did not displace the nucleus (Table 21). This cytoplasmic vacuolization would be more consistent with

glycogen than with fat. These enlarged hepatocytes correlate with the increased liver weights noted in the high-dose group.

The NOAEL was 100 mg/kg bw per day, based on an increase (> 20%) in absolute and relative liver weights of females at 300 mg/kg bw per day and above (Sheets, 2009).

In a study of toxicity conducted according to OECD Test Guideline 452, groups of four male and four female Beagle dogs were administered bixafen (purity 95.8%) in 0.5% aqueous carboxymethyl cellulose at a dosing volume of 5 mL/kg bw by oral gavage at a dose of 0, 10, 100 or 1000 mg/kg bw per day for 365–367 days. Clinical observations were conducted daily, feed consumption was measured daily and body weights were recorded weekly. Clinical chemistry, urine analysis and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance and during study weeks 14, 27, 40 and 52. Ophthalmic examinations were performed pre-exposure and pretermination. A gross necropsy was performed, organ weights were recorded and tissues were examined microscopically.

One male from the high-dose group was sacrificed prematurely for humane reasons on day 247. This animal had approximately 20 mL of blood-tinged fluid oozing from the mouth. Grossly, this animal was noted to have severe, bilateral, red-discoloured zones with clear foam in the lungs. The condition of this animal was attributable to interstitial pneumonia, as microscopic examination of the lungs revealed a mild degree of haemorrhage, oedema and inflammatory cells in the interstitium. The finding was not considered to be treatment related.

The only compound-related clinical finding was pale faeces, observed in males and females in the high-dose group. However, this finding was not of toxicological significance, as the pale faeces were considered to be due to unabsorbed test compound.

There were no treatment-related effects on body weight or feed consumption. No treatment-related ocular abnormalities were observed at ophthalmic examination.

Treatment-related haematological changes were limited to red blood cell counts, haemoglobin and haematocrit values, which were statistically significantly decreased in males at 100 and 1000 mg/kg bw per day on days 92 and/or 183 (Table 22).

Treatment-related clinical chemistry changes were limited to alkaline phosphatase, which was increased in females at 1000 mg/kg bw per day (outside the historical control data range) and more subtly at 100 mg/kg bw per day (within the normal control range), and cholesterol levels, which were slightly increased in females at 100 and 1000 mg/kg bw per day (Table 22). None of the changes noted in the urine analysis parameters was considered to be treatment related.

Terminal body weights for males and females were not statistically significantly different from those of controls. However, there was a minimal trend of decreased mean terminal body weights (8.35%) in the high-dose females. Compound-related organ weight changes for the high-dose males and females were limited to increased relative liver weights with associated liver hypertrophy. Slight, centrilobular hypertrophy of the liver was present in one male and two females in the high-dose group (Table 22). These animals had increased absolute liver weights (non-statistically significant) and relative liver weights (statistically significant) at necropsy. The liver hypertrophy, coded as "centrilobular and midzonal hypertrophy, hepatocellular", was observed and was characterized by enlarged cells primarily involving the cytoplasm. The cytoplasmic appearance of hepatocytes varied from granular to intensively eosinophilic to pale. Often the intensively eosinophilic staining cytoplasmic organelles appeared to be compressed towards the outer edges of the cell.

The NOAEL was 10 mg/kg bw per day, based on haematological effects (decrease in red blood cell count, haemoglobin and haematocrit) in males and liver toxicity (increased liver weight, increased alkaline phosphatase and cholesterol levels) in females at 100 mg/kg bw per day (Eigenberg, 2008).

Table 22. Summary of selected findings in the 1-year dog study

	Males				Females			
	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	1 000 mg/kg bw per day	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	1 000 mg/kg bw per day
Red blood cells (10^6/mL)								
- Day 92	7.41	7.20	6.99	6.30*	6.94	7.21	6.37	6.84
- Day 183	7.82	7.24*	7.02*	6.50*	7.07	7.27	6.69	6.68
- Day 359	8.11	7.54	7.71	7.13	7.56	7.80	7.12	7.03
Haemoglobin (g/dL)								
- Day 92	17.3	17.1	16.4*	15.2*	16.2	16.6	15.4	16.9
- Day 183	18.6	17.6	16.7*	15.7*	17.0	16.9	16.5	16.6
- Day 359	19.1	18.2	18.1	17.1	18.1	18.1	17.2	17.4
Haematocrit (%)								
- Day 92	47.4	47.0	44.7*	41.3*	44.3	45.0	41.8	45.4
- Day 183	49.4	46.8	44.3*	42.0*	44.9	44.7	43.9	43.8
- Day 359	51.9	49.6	49.1	46.6	49.0	48.6	47.0	46.8
Alkaline phosphatase (U/L)								
- Day 183	59	89	102	112	66	74	84	152*
- Day 274	59	106	94	100	57	89	82	246*
- Day 359	53	98	90	105	42	77	87*	169*
Cholesterol (mg/dL)								
- Day 92	154	152	162	161	145	163	177*	187*
- Day 359	155	167	158	168	176	190	200	212
Terminal body weight (kg)	11.10	11.98	11.45	11.41	9.96	9.39	9.28	9.13
Absolute liver weight (g)	348	338	367	463	280	325	328	374
Relative liver weight (% of body weight)	3.14	2.81	3.22	4.04*	2.79	3.45*	3.55*	4.08*
Liver: hepatocellular hypertrophy	0	0	0	1	0	0	0	2
Liver: cytoplasmic pigmentation	1	2	4	3	1	2	1	3
Liver: cytoplasmic vacuolation	0	0	0	1	1	0	2	1

bw: body weight; U: units; *: $P < 0.05$
Source: Eigenberg (2008)

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a study of carcinogenicity conducted according to OECD Test Guideline 451, groups of 60 male and 60 female C57BL/6J mice were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 150 or 500 ppm (equal to 0, 6.7, 20.4 and 69.0 mg/kg bw per day for males and 0, 8.6, 25.5 and 85.0 mg/kg bw per day for females, respectively) for at least 78 weeks. An additional 10 animals of each sex per group, similarly treated, were killed after at least 52 weeks of treatment for interim evaluation. After 20 weeks of treatment, the rodent diet was found not to contain a sufficient level of vitamin K_3 and was consequently supplemented to ensure that a minimum level of 2.5 ppm vitamin K_3 was present in the diet for the remainder of the study. Mortality and clinical signs were checked daily. Additionally, detailed physical examinations, including palpation for masses, were performed weekly throughout treatment. Body weight and feed consumption were measured weekly for the first 13 weeks of the study, then approximately monthly thereafter. Haematological

determinations were performed at approximately 12 and 18 months on blood from designated animals. Where possible, blood smears were prepared from moribund animals just before termination. At scheduled termination, body weights were recorded prior to necropsy. All animals were subjected to necropsy, with selected organs weighed at scheduled interim kill and final termination. Designated tissues were fixed and examined microscopically.

There were no treatment-related effects on mortality, clinical signs, body weight parameters, feed consumption or haematological parameters in females at any dose level (Table 23). There was no evidence of a treatment-related effect on tumour incidence in either sex.

In males, during the first 20 weeks of treatment, a number of effects were observed at 500 and 150 ppm, consisting of increased mortality rate at 500 ppm, with eight premature deaths attributed to a haemorrhagic syndrome (as evidenced macroscopically/microscopically by red foci, blood clots, red liquid content, haemorrhage or haematoma in one or more organs), clinical signs at 500 ppm (wasted appearance, reduced motor activity, hunched posture, cold to touch, limited use of limbs, tremors, rapid respiration, prostration and/or absence of righting reflex) and a reduction in mean cumulative body weight gain from weeks 9 to 22 at 500 and 150 ppm. These effects were attributed to the treatment, but were most likely exacerbated by the abnormally low vitamin K_3 content in the rodent diet. This relationship was supported by the absence of persisting effects after the change to a vitamin K_3–supplemented diet, designed to ensure an adequate vitamin K_3 supply to all animals for the remainder of the study.

The cause of death in 3/5 males at 500 ppm allocated to the interim (12-month) kill phase of the study that died or were humanely killed during the first 20 weeks of treatment was attributed to a haemorrhagic syndrome. One male and one female that died before the interim (12-month) kill presented a centrilobular hepatocellular hypertrophy, similar to the treatment-related effect observed at the terminal kill. After the first 20 weeks of study, the mortality rate was not affected by treatment, and no treatment-related clinical signs were recorded in males.

At 500 ppm, body weight was decreased in males at most time points from weeks 9 to 62 by up to 6% ($P < 0.05$ or $P < 0.01$ on most occasions), compared with controls. Cumulative body weight gain was lower than that of controls by 10% ($P < 0.05$) between weeks 1 and 14 and by 33% ($P < 0.01$) between weeks 14 and 26. Thereafter, body weight gain was similar to or higher than that of controls throughout the study. Body weight on day 540, overall cumulative body weight gain between days 1 and 540 and feed consumption throughout treatment were similar to those of the controls in males (Table 23). At 150 ppm, body weight was decreased in males at most time points from weeks 9 to 62 by up to 4% ($P < 0.05$ or $P < 0.01$ on a number of occasions), compared with controls. Cumulative body weight gain was lower than that of controls by 7% between weeks 1 and 14 and by 29% ($P < 0.01$) between weeks 14 and 26. Body weight on day 540 and overall cumulative body weight gain between days 1 and 540 were similar to those of the controls in males.

Haematological evaluation in males at 500 ppm revealed slightly higher platelet counts at month 12 (+20%, $P < 0.01$) and month 18/19 (+22%, $P < 0.01$). In males also, higher haemoglobin concentration and higher mean corpuscular volume were noted for the two sampling periods when compared with the control group. As a consequence, haematocrit and mean corpuscular haemoglobin were higher than in the controls. These variations were slight, their magnitude often being lower at month 18/19 (4–6%, $P < 0.01$) than at month 12 (5–7%, $P < 0.01$). In males at 150 ppm, the same tendencies were observed; however, in view of their low magnitude (3–4%), they were considered not to be biologically relevant. There were no treatment-related changes in haematological parameters in females at any dose level.

At the 12-month interim kill, absolute and relative liver weights were increased by 17–23% in males and 28–31% in females ($P < 0.01$) at 500 ppm, when compared with controls, and by 9–13% in both sexes at 150 ppm, the effect being statistically significant in males only ($P < 0.05$ or $P < 0.01$). Minimal to moderate centrilobular hepatocellular hypertrophy was seen in 6/10 males and 2/10 females at 500 ppm, associated with a decreased incidence of diffuse hepatocellular vacuolation in 5/10 males, an increased incidence of hepatocellular single-cell degeneration/necrosis in 6/10 males,

Table 23. Summary of selected findings in the 78-week mouse study

	Males				Females			
	0 ppm	50 ppm	150 ppm	500 ppm	0 ppm	50 ppm	150 ppm	500 ppm
Unscheduled deaths								
- Week 53	3/60	5/60	3/60	15/60	6/60	5/60	5/60	7/60
- Week 78	8/50	7/50	9/50	12/50	10/50	8/50	12/50	13/50
Body weight (g)								
- Week 26	30.2	30.1	29.1**	28.9**	23.3	23.3	23.3	23.2
- Week 54	32.0	32.0	30.9*	30.9*	25.1	25.9*	25.7	26.1**
- Week 78	31.8	32.2	31.1	31.4	26.5	26.9	27.0	26.8
Terminal body weight (g), 12 months	27.1	28.0	27.6	26.1	22.0	22.1	21.6	22.3
Absolute liver weight (g), 12 months	1.18	1.22	1.31**	1.40**	1.00	1.04	1.11	1.30**
Relative liver weight (% of body weight), 12 months	4.36	4.35	4.75**	5.34**	4.54	4.71	5.12	5.81**
Microscopic findings at the 12-month kill								
No. of animals examined	10	10	10	10	10	10	10	10
Centrilobular hepatocellular hypertrophy, diffuse								
- Minimal	0	0	5	1	0	0	0	1
- Slight	0	0	0	3	0	0	0	1
- Moderate	0	0	0	2	0	0	0	0
- Total	0	0	5	6	0	0	0	2
Hepatocellular vacuolation, diffuse								
- Minimal	4	6	7	4	8	9	10	8
- Slight	5	2	3	1	0	0	0	0
- Moderate	1	0	0	0	0	0	0	0
- Total	10	8	10	5	8	9	10	8
Hepatocellular single-cell degeneration/necrosis, focal/multifocal								
- Minimal	0	0	0	5	0	0	0	0
- Slight	0	0	0	1	0	0	0	0
- Total	0	0	0	6	0	0	0	0
Multinucleated hepatocytes, focal/multifocal								
- Minimal	0	0	0	4	0	0	0	0
Hepatocellular brown pigment, focal/multifocal								
- Minimal	0	0	3	4	0	0	0	0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Langrand-Lerche (2008)

multinucleated hepatocytes in 4/10 males and hepatocellular brown pigment in 4/10 males. At 150 ppm, minimal centrilobular hepatocellular hypertrophy was seen in 5/10 males, associated with hepatocellular brown pigment in 3/10 males (Table 23).

The cause of death in 5/12 males and 1/13 females at 500 ppm allocated to the carcinogenicity phase (18 months) of the study that died or were humanely killed before the end of the study was attributed to a haemorrhagic syndrome. These deaths were confined to the first 20 weeks of the study. In addition, dark liver was noted in 4/12 males at macroscopic examination. At 150 ppm, no treatment-related cause of death was found for animals that died or were humanely killed before the end of the study.

At the 18-month terminal kill of the carcinogenicity phase of the study, absolute and relative liver weights were increased by 26–29% in males and 25–27% in females ($P < 0.01$) at 500 ppm, when compared with controls, by 19–20% in males and 12–14% in females ($P < 0.01$) at 150 ppm, and by 8–9% in females ($P < 0.01$) at 50 ppm. Also at 500 ppm, absolute and relative adrenal gland weights were higher by 27–32% in males ($P < 0.01$) when compared with controls (Table 24).

Macroscopic examination revealed dark liver in 16/38 males and 6/37 females at 500 ppm and enlarged liver in 13/38 males and 25/37 females at 500 ppm. Microscopic examination at 500 ppm revealed a minimal to marked centrilobular hepatocellular hypertrophy and a decreased incidence of diffuse hepatocellular vacuolation in both sexes and a higher incidence of focal/multifocal hepatocellular single-cell degeneration/necrosis, multinucleated hepatocytes, hepatocellular brown pigment, hepatocellular necrotic focus and interstitial mononuclear cell infiltrate in males. At 150 ppm, minimal to moderate centrilobular hepatocellular hypertrophy was seen in 37/50 males, associated with a decreased incidence of diffuse hepatocellular vacuolation in 27/50 males, and a higher incidence of hepatocellular single-cell degeneration/necrosis and multinucleated hepatocytes was seen in 5/50 males. At 50 ppm, minimal centrilobular hepatocellular hypertrophy was seen in 2/50 males.

In the thyroid gland, a higher incidence of focal/multifocal follicular cell hyperplasia was noted in both sexes at 500 ppm and in females at 150 ppm (Table 24), whereas in the adrenal gland, a decreased incidence of cortical atrophy was noted in males at 500 ppm.

The NOAEL for carcinogenicity was 500 ppm (equal to 69.0 mg/kg bw per day for males and 85.0 mg/kg bw per day for females), the highest dose tested. The NOAEL for toxicity was 50 ppm (equal to 6.7 mg/kg bw per day for males and 8.6 mg/kg bw per day for females), based on decreased body weights and liver toxicity (single-cell degeneration/necrosis) in males and thyroid effects (follicular cell hyperplasia) in females at 150 ppm (equal to 20.4 mg/kg bw per day for males and 25.5 mg/kg bw per day for females) and above (Langrand-Lerche, 2008).

Rats

In a combined study of chronic toxicity and carcinogenicity conducted according to OECD Test Guideline 453, groups of 70 or 80 female Wistar Rj:WI (IOPS HAN) rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 300 or 2000 ppm (equal to 0, 2.8, 17.4 and 117 mg/kg bw per day) for at least 24 months. After 13 weeks of exposure, 10 females allocated to the satellite groups in the controls and at the two highest dose levels were necropsied at the first scheduled interim kill and subjected to limited histopathological examination (liver, thyroid and macroscopic findings). After 52 weeks, 10 females from each group allocated to the chronic (12-month) phase were necropsied at the second scheduled interim kill. The remaining 60 females per group, allocated to the carcinogenicity (24-month) phase of the study, continued treatment until final termination after at least 104 weeks of treatment, when surviving animals were necropsied. Mortality and clinical signs were checked daily. Detailed physical examinations, including palpation for masses, were performed at least weekly throughout the study. Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Feed consumption was recorded twice weekly for the first 6 weeks of the study, approximately weekly from week 7 up to week 13, then every 4 weeks thereafter. Ophthalmological examinations were performed on all animals during

Table 24. Summary of selected findings in the 78-week mouse study at terminal sacrifice

	Males				Females			
	0 ppm	50 ppm	150 ppm	500 ppm	0 ppm	50 ppm	150 ppm	500 ppm
Terminal body weight (g), 18 months	28.1	28.4	27.5	27.5	23.5	23.7	23.8	23.4
Absolute liver weight (g), 18 months	1.18	1.23	1.41**	1.49**	1.18	1.29**	1.35**	1.48**
Relative liver weight (% of body weight), 18 months	4.22	4.33	5.07**	5.43**	5.03	5.43**	5.66*	6.30**
Absolute adrenal weight (mg), 18 months	4.4	4.7	4.7	5.6**	8.6	8.3	9.1	9.1
Relative adrenal weight (% of body weight, ×100), 18 months	1.56	1.65	1.71	2.05**	3.66	3.49	3.80	3.87
Liver, enlarged, 18 months	0/42	0/43	1/41	13/38	2/40	7/42	9/38	25/37
Liver, dark, 18 months	1/42	2/43	3/41	16/38	0/40	0/42	0/38	6/37
Microscopic findings at the 18-month sacrifice								
Liver								
No. of animals examined	50	50	50	50	50	50	50	50
Centrilobular hepatocellular hypertrophy; diffuse								
- Minimal	0	2	17	4	0	0	0	12
- Slight	0	0	18	8	0	0	0	3
- Moderate	0	0	2	20	0	0	0	0
- Marked	0	0	0	15	0	0	0	0
- Total	0	2	37**	47**	0	0	0	15**
Hepatocellular vacuolation, diffuse								
- Minimal	17	24	22	16	11	12	11	14
- Slight	14	9	5	0	15	22	25	6
- Moderate	0	0	0	0	11	4	2	1
- Total	31	33	27	16*	37	38	38	21**
Hepatocellular single-cell degeneration/necrosis, focal/multifocal								
- Minimal	0	0	4	23	0	1	0	0
- Slight	0	0	1	4	0	0	0	0
- Moderate	0	0	0	1	0	0	0	0
- Total	0	0	5*	28**	0	1	0	0
Multinucleated hepatocytes, focal/multifocal								
- Minimal	1	2	5	37	0	1	0	0
- Slight	0	0	0	2	0	0	0	0
- Total	1	2	5	39**	0	1	0	0

	Males				Females			
	0 ppm	50 ppm	150 ppm	500 ppm	0 ppm	50 ppm	150 ppm	500 ppm
Hepatocellular brown pigment, focal/multifocal								
- Minimal	0	0	0	14	0	0	0	0
- Slight	0	0	0	2	0	0	0	0
- Total	0	0	0	16**	0	0	0	0
Hepatocellular necrotic focus, focal/multifocal								
- Minimal	2	2	2	6	3	4	5	4
- Slight	2	0	2	3	1	0	0	0
- Moderate	0	0	0	0	1	0	1	1
- Total	4	2	4	9	5	4	6	5
Interstitial mononuclear cell infiltrate, focal/multifocal								
- Minimal	10	12	11	20	23	18	15	21
- Slight	2	2	1	3	4	5	9	4
- Moderate	1	0	0	1	0	0	1	1
- Total	13	14	12	24**	27	23	25	26
Thyroid								
No. of animals examined	48	50	50	50	48	50	50	50
Follicular cell hyperplasia, focal/multifocal								
- Minimal	4	3	5	11	11	8	15	26
- Slight	2	2	1	2	1	2	5	6
- Moderate	0	0	0	0	0	1	0	1
- Total	6	5	6	13*	12	11	20*	33**
Adrenal gland								
No. of animals examined	50	50	50	50	48	49	50	50
Cortical atrophy, focal/multifocal	42	45	35	14	0	0	1	0

ppm : parts per million ; *: $P < 0.05$; **: $P < 0.01$
Source: Langrand-Lerche (2008)

acclimatization and after approximately 12 and 24 months. Haematology and clinical chemistry determinations and urine analysis were performed during months 3/4, 6, 12, 18 and 24 on selected animals. In addition, coagulation parameters were measured in satellite groups in weeks 5 and 14. All surviving animals allocated to the chronic and carcinogenicity phases were subjected to necropsy after a minimum of 52 weeks or 104 weeks of treatment, respectively. Selected organs were weighed, and designated tissues were sampled and examined microscopically.

In the male rats originally included in the study, increased mortality (3/80, 0/70, 6/70, 9/80 and 28/80 at 0, 50, 150, 300 and 1000 ppm, respectively), elevated coagulation parameter times and haemorrhagic syndrome were observed (Tables 25 and 26). Therefore, all male animals were prematurely killed (males treated at 1000 ppm: on study day 169; males from all other groups: on study day 239) without full necropsy, and a separate study on the chronic toxicity and oncogenic potential of bixafen in male Wistar rats was conducted (Garcin, 2008; see below).

Table 25. Individual mortality in males from the initial chronic toxicity and carcinogenicity study in rats

Group	Animal no.	Day of death	Mode of death	Necropsy findings
Control	1684	104	Found dead	Stomach and/or intestines: dark contents
	1676	142	Found dead	Stomach and/or intestines: dark contents
	1711	146	Killed for humane reasons	–
150 ppm	2009	103	Found dead	Stomach and/or intestines: dark contents
	1984	104	Killed for humane reasons	–
	2010	111	Killed for humane reasons	Stomach and/or intestines: dark contents
	2032	145	Killed for humane reasons	Stomach and/or intestines: dark contents
	1979	176	Found dead	Stomach and/or intestines: dark contents
	1971	177	Found dead	–
300 ppm	2038	93	Found dead	Stomach and/or intestines: dark contents
	2047	124	Found dead	–
	2090	134	Killed for humane reasons	Stomach and/or intestines: dark contents
	2099	134	Found dead	Stomach and/or intestines: dark contents
	2059	142	Killed for humane reasons	Stomach and/or intestines: dark contents
	2053	148	Killed for humane reasons	Stomach and/or intestines: dark contents
	2087	160	Killed for humane reasons	–
	2061	165	Found dead	Stomach and/or intestines: dark contents
	2085	170	Found dead	Stomach and/or intestines: dark contents
1 000 ppm	2259	33	Found dead	–
	2197	34	Killed for humane reasons	–
	2218	44	Killed for humane reasons	Stomach and/or intestines: dark contents
	2246	75	Killed for humane reasons	–
	2235	84	Killed for humane reasons	Stomach and/or intestines: dark contents
	2244	84	Found dead	Stomach and/or intestines: dark contents
	2213	93	Found dead	Stomach and/or intestines: dark contents
	2216	93	Found dead	Stomach and/or intestines: dark contents
	2217	93	Found dead	Stomach and/or intestines: dark contents
	2226	96	Killed for humane reasons	Stomach and/or intestines: dark contents
	2273	96	Killed for humane reasons	Stomach and/or intestines: dark contents
	2263	97	Found dead	Stomach and/or intestines: dark contents
	2238	99	Found dead	Stomach and/or intestines: dark contents
	2224	107	Found dead	Stomach and/or intestines: dark contents
	2230	114	Killed for humane reasons	Stomach and/or intestines: dark contents
	2234	117	Found dead	–
	2205	118	Found dead	Stomach and/or intestines: dark contents
	2206	126	Found dead	Stomach and/or intestines: dark contents
	2267	138	Killed for humane reasons	Stomach and/or intestines: dark contents
	2269	138	Killed for humane reasons	–
	2207	139	Killed for humane reasons	Stomach and/or intestines: dark contents
	2225	140	Found dead	Stomach and/or intestines: dark contents
	2222	141	Killed for humane reasons	Stomach and/or intestines: dark contents

Group	Animal no.	Day of death	Mode of death	Necropsy findings
	2268	152	Killed for humane reasons	Stomach and/or intestines: dark contents
	2255	156	Killed for humane reasons	Stomach and/or intestines: dark contents
	2258	156	Killed for humane reasons	Stomach and/or intestines: dark contents
	2257	160	Killed for humane reasons	Stomach and/or intestines: dark contents
	2245	167	Killed for humane reasons	Stomach and/or intestines: dark contents

Source: McElligott (2008)

Table 26. Selected coagulation parameters from the initial chronic toxicity and carcinogenicity study in rats

	Males					Females			
	0 ppm	50 ppm	150 ppm	300 ppm	1 000 ppm	0 ppm	50 ppm	300 ppm	1 000 ppm
PT (s)									
- Week 4/5, satellite	19.4	ND	ND	26.7	40.2**	14.3	ND	14.6	14.0
- 3 months, main	21.0	18.8	27.1	28.5	46.1**	14.8	14.3	13.8*	14.1
- 3 months, satellite	24.1	ND	ND	33.5	45.8*	14.8	ND	13.8	14.3
- 6 months, main	17.2	17.3	17.0	17.0	ND	13.8	13.1	13.3	14.5
- 12 months, main	ND	ND	ND	ND	ND	16.3	15.6	15.9	15.5*
- 18 months, main	ND	ND	ND	ND	ND	15.7	15.2	15.5	14.4*
- 24 months, main	ND	ND	ND	ND	ND	15.6	15.6	15.2	14.9
APTT (s)									
- Week 4/5, satellite	27.9	ND	ND	38.9	51.8**	17.1	ND	17.7	20.2**
- 3 months, main	30.5	28.8	37.3	39.7	56.0**	16.2	ND	17.3	21.6**
- 3 months, satellite	32.7	ND	ND	41.5	58.8**	18.5	18.7	20.0	22.5**
- 6 months, main	18.8	18.1	18.9	19.3	ND	18.7	17.8	19.2	24.0*
- 12 months, main	ND	ND	ND	ND	ND	15.8	15.7	15.2	14.6*
- 18 months, main	ND	ND	ND	ND	ND	15.3	15.1	15.6	15.5
- 24 months, main	ND	ND	ND	ND	ND	14.6	14.4	14.5	14.4

APTT: activated partial thromboplastin time; ND: not determined; ppm: parts per million; PT: prothrombin time; *: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

As the mortality was unanticipated and there was evidence that the controls were also affected, albeit not to the extent of the treated animals, investigations into the reason for the mortalities commenced. One theory was that there may have been insufficient vitamin K in the diet. In fact, the vitamin K levels of the batches used in the earlier phase of this study (i.e. from 21 April 2005 to 5 October 2005) were not reported. However, analysis of the diet by the manufacturer showed that batch 50607, which was used from 6 to 12 October 2005 in the performing laboratory, contained less than 0.05 mg/kg of vitamin K_1 and less than 0.3 mg/kg of vitamin K_3. Thus, at least the vitamin K level of the diet from batch 50607 was significantly lower than the estimate of the requirement of 1 mg/kg for the Wistar rat. Discussions with the manufacturer of the diet revealed that it had moved manufacture of the diet in the middle of 2004 and had implemented new radiation and storage sterilization procedures, which temporally coincided with the deficit in vitamin K in the diet.

Based on the analyses, it was agreed with the manufacturers to supplement the diet with 15 mg/kg of the synthetic vitamin K_3 analogue menadione (batch 50720, used from 13 October 2005 to 28 December 2005, contained 15.7 mg/kg of menadione). The subsequent batches of diet used for the long-term study in female rats as well as in the supplementary long-term study in male rats (Garcin, 2008; see below) contained slightly lower amounts of menadione (7.1–10.6 mg/kg) than the target level of 15 mg/kg, but still adequate levels of the vitamin (for details, see section 2.6).

The effects of bixafen on blood coagulation parameters (prothrombin time, PT; activated partial thromboplastin time, APTT) of rats from the initial chronic toxicity and carcinogenicity study of rats receiving a vitamin K_3–deficient diet are shown in Table 26. After approximately 5–6 months of treatment, the male rats at 1000 ppm were taken from the study following significant mortality and signs of haemorrhage. After approximately 6 months of treatment, the diet was supplemented with 15 mg/kg of the synthetic vitamin K_3 analogue menadione.

In the female rats, no effect on mortality was observed at any dose level during the entire study. During the 1st year of treatment, the only treatment-related clinical sign recorded at 2000 ppm was a slightly higher incidence of hair loss compared with the control group. During the 2nd year of treatment, a slightly higher incidence of wasted appearance and genital discharge was observed at 2000 ppm in comparison with the controls; these nonspecific signs are commonly associated with ageing rats. At the ophthalmological examination at 12 months, no findings were observed at any dose. At 24 months, an increased incidence of posterior opacity of the lens was observed at all dose levels, although not in a dose-related manner, and a slightly increased incidence of abnormal colour (pale) of the retina fundus was also noted at all dose levels. In the absence of any indication of lens degeneration and in the absence of any increased retinal abnormality compared with the control group at the microscopic examination, these findings were considered not to be toxicologically relevant and not attributable to treatment (Table 27).

Treatment-related effects on body weight and body weight gain were observed at 2000 ppm throughout the study compared with the control group (Table 27). Body weight was decreased on day 8 by 3% ($P < 0.05$) and was reduced by 3%, 6%, 13%, 20% and 19% in weeks 14, 26, 54, 78 and 102, respectively; the effect was statistically significant ($P < 0.01$) for each interval. Cumulative body weight gain was also reduced at 2000 ppm throughout the study, with a reduction of 26% ($P < 0.01$) on week 1, and the overall cumulative body weight gain was reduced by 34% ($P < 0.01$) compared with the control group between day 1 and day 708. Feed consumption at 2000 ppm was reduced by 5%, 12%, 9%, 8% and 5% for the interval weeks 1–13, weeks 14–26, weeks 27–52, weeks 53–78 and weeks 79–104, respectively, in comparison with the control.

The APTT was increased at 2000 ppm for the satellite group at weeks 5 and 14 (+18% and +33%, respectively, $P < 0.01$) and for the chronic toxicity/carcinogenicity group in months 3/4 and 6 (+22% and +28%, respectively, $P < 0.01$). These changes were considered to be a consequence of the vitamin K_3 deficiency in the diet. Following the change to a vitamin K_3–adequate diet, no relevant change was observed at months 12, 18 and 24 (Table 27).

Increased total cholesterol concentrations were observed at 2000 ppm, with a decreasing magnitude from months 3/4 to 18 (from 68% to 36%, statistically significant for each time point), together with higher triglyceride concentrations at months 3/4 and 6 only (+74%, $P < 0.01$, and +27%, $P < 0.05$, respectively), whereas at 300 ppm, a significantly higher total cholesterol concentration (24% and 21%, respectively) was observed at months 3/4 and 6 only. Lower total bilirubin concentrations were noted at months 12, 18 and 24 at all dietary levels. The variations observed at months 3/4 and 6 were considered to be minimal in view of their low magnitude and the absence of a dose–effect relationship (Table 27). The slightly lower enzyme activities (aspartate aminotransferase, alanine aminotransferase and/or alkaline phosphatase) observed at 2000 and 300 ppm throughout the sampling periods were considered not to be adverse effects of the test substance.

Table 27. Selected findings in the 24-month rat study (females only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Mortality (no.)				
- Chronic toxicity and carcinogenicity phase, 12 months	2/80	3/70	1/80	2/80
- Carcinogenicity phase, 24 months	33/60	22/60	30/60	26/60
Hair loss (no.), chronic toxicity and carcinogenicity phase, 12 months	7/80	3/70	6/80	14/80
Posterior opacity of lens, 24 months	11/30	34/40	18/33	31/40
Retinal fundus abnormal colour (pale), 24 months	3/30	6/40	6/33	10/40
Body weight (g)				
- Day 1	195	195	194	194
- Day 8	214	216	211	208*
- Day 92	289	291	289	279**
- Day 176	321	321	316	301**
- Day 372	374	376	359	324**
- Day 540	425	419	399	342**
- Day 708	444	429	412	360**
Overall body weight gain (g), days 1–708	250	234	220	166**
Feed consumption (g/animal per day)				
- Weeks 1–13	19.2	19.9	20.1	18.3
- Weeks 14–26	18.7	18.9	18.3	16.4
- Weeks 27–52	19.1	19.3	19.2	17.4
- Weeks 53–78	20.1	19.9	20.0	18.4
- Weeks 79–104	22.2	21.6	22.6	21.1
APTT (s)				
- Week 5, satellite group	17.1	ND	17.7	20.2**
- Week 14, satellite group	16.2	ND	17.3	21.6**
- Month 3/4	18.5	18.7	20.0	22.5**
- Month 6	18.7	17.8	19.2	24.0**
- Month 12	15.8	15.7	15.2	14.6*
- Month 18	15.3	15.1	15.6	15.5
- Month 24	14.6	14.4	14.5	14.4
Cholesterol (mmol/L)				
- Month 3/4	1.75	1.80	2.17*	2.94**
- Month 6	1.92	1.96	2.33**	3.09**
- Month 12	2.16	2.31	2.43	3.19**
- Month 18	2.05	2.23	2.24	2.79*
- Month 24	2.33	2.27	2.52	2.60
Total bilirubin (µmol/L)				
- Month 3/4	4.0	3.4**	3.1**	3.6*
- Month 6	4.3	3.5**	3.6**	3.9*
- Month 12	2.6	1.9	1.1**	0.8**
- Month 18	2.8	1.9	1.2**	1.0**
- Month 24	1.7	1.2	1.0	0.7**

APTT: activated partial thromboplastin time; ND: not determined; ppm: parts per million: *: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

At the interim sacrifice of the 3-month satellite group, at 2000 ppm, absolute and relative liver weights were statistically significantly increased by 41–51% when compared with control animals. Macroscopic examination revealed a higher incidence of enlarged liver and/or dark liver compared with the controls, and at the microscopic examination, treatment-related effects were observed in the two organs examined (i.e. the liver and the thyroid gland). In the liver, a higher incidence and severity of diffuse centrilobular to panlobular hepatocellular hypertrophy were observed compared with the controls, and in the thyroid gland, minimal follicular cell hypertrophy was observed in some females (Table 28).

At the end of the chronic toxicity phase (12 months), at 2000 ppm, terminal body weight was lower (−8%, not statistically significant) in treated females compared with the controls. Absolute and relative liver weights were higher (by 31–42%, $P < 0.01$) when compared with controls. The macroscopic evaluation at 2000 ppm revealed enlarged liver and dark thyroid gland. At the microscopic examination, treatment-related effects were observed at 2000 ppm in the liver (minimal to moderate centrilobular to panlobular hepatocellular hypertrophy) and in the thyroid gland (higher incidence and severity of minimal to slight diffuse follicular cell hypertrophy and colloid alteration) (Table 28).

At the end of the carcinogenicity phase (24 months), terminal body weight was reduced at 2000 ppm by 18% ($P < 0.01$) and at 300 ppm by 7% (not statistically significant) when compared with controls (Table 29). Absolute and relative liver weights were increased by 17–41% ($P < 0.01$) at 2000 ppm, and relative liver weight was increased by 12% ($P < 0.01$) at 300 ppm. Also at 2000 ppm, relative thyroid weight was significantly higher (+41%; $P < 0.01$) compared with controls. At the macroscopic evaluation, higher incidences of enlarged and dark liver, dark thyroid and dark kidneys were observed at 2000 ppm compared with the controls.

Microscopic examination revealed treatment-related non-neoplastic changes in the liver and the thyroid gland at 300 and 2000 ppm and in the kidney at 2000 ppm. In the liver, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was observed, and a higher incidence and severity of hepatocellular brown pigments and multinucleated hepatocytes were noted. In the thyroid gland, minimal to slight follicular cell hyperplasia was observed. A higher incidence and/or severity of follicular cell hypertrophy and colloid alteration were observed together with a higher incidence of brown pigments in follicular cells. In the kidney, a higher incidence and severity of intratubular golden/brown pigments were observed. In the absence of any cellular alteration in the kidney, this finding was considered to be treatment related but not adverse (Table 29).

In the thyroid gland, two follicular cell carcinomas were observed at each of 50 and 2000 ppm. Also, benign follicular cell adenomas were observed in two decedent females at 2000 ppm, but not in any of the final kill animals at the end of the 24-month treatment period. The incidence in the current study was within the control range of the Registry of Industrial Toxicology Animals (RITA) database (version from 21 September 2007), based on the incidence data from 98 studies with 4823 female rats examined in total: thyroid follicular cell adenoma was observed in 84 animals (1.7%; range 0–10%), whereas thyroid follicular cell adenocarcinoma was observed in 38 animals (0.8%; range 0–6%).

The NOAEL for carcinogenicity was 2000 ppm (equal to 117 mg/kg bw per day for females), the highest dose tested. The NOAEL for toxicity was 50 ppm (equal to 2.8 mg/kg bw per day for females), based on liver effects (increased cholesterol, higher incidence and/or severity of hepatocellular brown pigments and multinucleated hepatocytes) and thyroid effects (higher incidence and/or severity of follicular cell hypertrophy and colloid alteration) at 300 ppm (equal to 17.4 mg/kg bw per day for females) and above (McElligott, 2008).

Table 28. Selected findings at the interim sacrifices in the 24-month rat study (females only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
3-month interim sacrifice				
Terminal body weight (g)	278.1	ND	276.3	269.5
Absolute liver weight (g)	6.17	ND	7.02	8.72**
Relative liver weight (% of body weight)	2.22	ND	2.54*	3.23**
Liver: large	0/10	ND	1/10	8/10
Liver: dark	2/10	ND	2/10	7/10
Liver: prominent lobulation	0/10	ND	7/10	7/10
Liver: centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	1/10	ND	0/10	6/10
- Slight	0/10	ND	0/10	2/10
- Moderate	0/10	ND	0/10	1/10
- Total	1/10	ND	0/10	9/10
Thyroid: follicular cell hypertrophy, diffuse; minimal	0/10	ND	0/10	3/10
12-month interim sacrifice				
Terminal body weight (g)	344.7	330.7	343.7	317.1
Absolute liver weight (g)	7.72	7.72	8.51	10.09**
Relative liver weight (% of body weight)	2.24	2.33	2.48*	3.19**
No. of animals examined macroscopically	10	10	10	9
Liver: obviously large	0	0	3	8
Thyroid: dark	0	0	0	3
No. of animals examined microscopically	10	10	10	10
Liver: centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	0	2
- Slight	0	0	0	5
- Moderate	0	0	0	2
- Total	0	0	0	9
Thyroid: follicular cell hypertrophy, diffuse				
- Minimal	1	1	1	5
- Slight	0	0	0	3
- Total	1	1	1	8
Thyroid: colloid alteration				
- Minimal	1	1	2	3
- Slight	0	1	0	2
- Total	1	2	2	5

ND: not determined; *: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

Table 29. Selected findings at the terminal sacrifice in the 24-month rat study (females only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Terminal body weight (g)	411.7	398.0	383.1	338.9**
Absolute liver weight (g)	9.89	9.80	10.39	11.57**
Relative liver weight (% of body weight)	2.43	2.46	2.73**	3.42**
Absolute thyroid weight (mg)	25.3	26.1	27.2	28.1
Relative thyroid weight (% of body weight, ×1 000)	6.00	6.54	7.11	8.43**
No. of animals examined macroscopically	27	38	30	34
Liver: obviously large	6	7	9	25
Liver: dark	1	0	1	23
Thyroid: dark	0	0	0	5
Kidney: dark	4	4	4	11
Liver				
No. of animals examined microscopically	60	60	59	60
Centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	11	23
- Slight	0	0	1	19
- Total	0	0	12**	42**
Hepatocellular brown pigments, focal/multifocal				
- Minimal	1	0	5	14
- Slight	0	0	0	5
- Total	1	0	5	19**
Multinucleated hepatocytes, focal/multifocal				
- Minimal	5	4	8	15
- Slight	0	0	0	3
- Total	5	4	8	18*
Thyroid				
No. of animals examined microscopically	57	60	58	59
Follicular cell hyperplasia, focal/multifocal				
- Minimal	0	0	0	5
- Slight	0	0	0	3
- Total	0	0	0	8**
Follicular cell hypertrophy, diffuse				
- Minimal	1	2	9	24
- Slight	0	0	0	10
- Total	1	2	9*	34**
Colloid alteration				
- Minimal	11	14	15	7
- Slight	3	3	17	30
- Moderate	0	0	2	14
- Total	14	17	34**	51**
Brown pigments, follicular cells; minimal	2	2	3	7
Follicular cell adenoma	0	0	0	2

	0 ppm	50 ppm	300 ppm	2 000 ppm
Follicular cell carcinoma	0	2	0	2
Follicular cell adenoma + carcinoma	0	2	0	4
Kidney				
No. of animals examined microscopically	60	60	60	60
Intratubular golden/brown pigments, focal/multifocal				
- Minimal	20	16	20	34
- Slight	0	2	0	5
- Total	20	18	20	39**

*: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

In a complementary combined study of chronic toxicity and carcinogenicity conducted according to OECD Test Guideline 453, groups of 70 male Wistar Rj:WI (IOPS HAN) rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 300 or 2000 ppm (equal to 0, 2.0, 12.1 and 80.5 mg/kg bw per day, respectively) for at least 24 months. After 54 weeks, the surviving 10 males from each group allocated to the chronic toxicity phase (12 months) were necropsied at the first scheduled interim kill. The remaining 60 animals of each sex per group, allocated to the carcinogenicity phase (24 months) of the study, continued treatment until final termination of the study after at least 104 weeks of treatment, when surviving animals were necropsied. Mortality and clinical signs were checked daily. Detailed physical examinations, including palpation for masses, were performed at least weekly throughout the study. Body weight was recorded weekly for the first 13 weeks, then approximately every 4 weeks thereafter. Feed consumption was recorded twice weekly for the first 6 weeks of the study, approximately weekly from week 7 up to week 13, then every 4 weeks thereafter. Ophthalmological examinations were performed on all animals during acclimatization and after approximately 12 and 24 months. Haematology and clinical chemistry determinations and urine analysis were performed during months 3, 6, 12–13, 18 and 24 on selected animals. At the scheduled chronic toxicity and carcinogenicity phase kill, selected organs were weighed, and designated tissues were sampled and examined microscopically.

The overall incidence and percentage of mortality were similar between the control and treated groups and showed no evidence of a treatment-related increase throughout the study. There were no treatment-related clinical signs observed during treatment. Up to and including the highest dose level tested (2000 ppm), there was no significant effect on feed consumption, except for a minimal decrease observed at 2000 ppm only during the 1st week of the study. No treatment-related changes were observed at ophthalmoscopy or at urine analysis. At the end of the 24-month treatment period, there was no evidence of treatment-related neoplastic findings.

Body weight gain was reduced at 2000 ppm compared with the controls on days 8 and 15 of the study, by 8% and 5%, respectively ($P < 0.01$ and $P < 0.05$, respectively). Following the first 2 weeks of treatment, body weight gain was essentially comparable to that of the controls for the remainder of the 24-month treatment period (Table 30).

Increased cholesterol concentrations were observed during the 1st year of treatment at 2000 ppm (20–27%; statistically significant for each time point) and at 300 ppm (17–25%; statistically significant at months 6 and 12/13). Also, slightly higher urea and inorganic phosphorus concentrations were observed until month 18 at 2000 ppm, and lower total bilirubin concentrations were observed throughout the sampling periods at 2000 and 300 ppm (Table 30). The slightly lower enzyme activities (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase) observed at 2000 ppm throughout the sampling periods and at 300 ppm at month 24 were considered not to be adverse effects of the test substance.

Table 30. Selected findings in the 24-month rat study (males only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Mortality (no.)				
- Chronic toxicity and carcinogenicity phase, 12 months	2/70	5/70	8/70	4/70
- Carcinogenicity phase, 24 months	35/60	40/60	43/60	38/60
Body weight (g)				
- Day 1	226	227	226	226
- Day 8	285	285	282	279
- Day 92	542	538	543	534
- Day 176	623	624	627	609
- Day 372	711	717	707	699
- Day 540	752	745	726	717
- Day 708	685	691	645	646
Body weight gain (g)				
- Days 1–8	59	59	57	54**
- Days 1–15	109	108	109	104*
Overall body weight gain (g), days 1–708	459	468	422	420
PT (s)				
- Month 3	17.3	16.3	16.2*	15.6**
- Month 6	17.0	16.9	16.1**	15.9**
- Month 12/13	16.2	16.3	16.1	15.0**
- Month 18	18.7	18.0	17.9	17.4**
- Month 24	18.9	17.7	17.7	16.7**
APTT (s)				
- Month 3	18.3	19.3	18.8	18.3
- Month 6	17.4	18.9**	18.2	18.1
- Month 12/13	17.9	18.0	18.1	18.2
- Month 18	15.7	15.3	15.4	14.5
- Month 24	14.4	14.1	14.0	13.5
Cholesterol (mmol/L)				
- Month 3	1.38	1.49	1.62	1.75*
- Month 6	1.69	1.86	2.08**	2.03*
- Month 12/13	1.90	2.07	2.37**	2.35**
- Month 18	2.48	2.55	2.68	2.65
- Month 24	2.22	3.15	2.59	3.14
Total bilirubin (µmol/L)				
- Month 3	1.0	0.6	0.5**	0.4**
- Month 6	1.8	1.3**	0.9**	0.8**
- Month 12/13	1.9	1.3*	1.0**	0.9**
- Month 18	2.2	1.6	1.2**	1.1**
- Month 24	1.6	1.5	1.0	0.8**

APTT: activated partial thromboplastin time; ppm: parts per million; PT: prothrombin time: *: $P < 0.05$; **: $P < 0.01$

Source: Garcin (2008)

At the end of the chronic toxicity phase (12 months), absolute and relative liver weights were increased by 34–37% at 2000 ppm and by 17–21% at 300 ppm, whereas absolute and relative thyroid weights were increased by 49–52% at 2000 ppm compared with the control group (Table 31). At the macroscopic examination, enlarged livers were observed in some animals at 2000 and 300 ppm, whereas dark thyroid glands were observed in 3/8 animals at 2000 ppm. These findings were correlated with microscopic changes. In the liver, a higher incidence of minimal to slight centrilobular to panlobular hepatocellular hypertrophy was observed at 2000 and 300 ppm, along with a higher incidence of eosinophilic and tigroid foci of hepatocellular alteration at 2000 ppm. In the thyroid gland, a higher incidence and severity of minimal to slight follicular cell hypertrophy and colloid alteration were observed at 2000 and 300 ppm.

Table 31. Selected findings at the 12-month interim sacrifice in the 24-month rat study (males only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Terminal body weight (g)	650.6	703.6	668.6	664.2
Absolute liver weight (g)	11.90	13.75*	14.30**	16.28**
Relative body weight (% of body weight)	1.83	1.95	2.14**	2.45**
Absolute thyroid weight (mg)	22.8	26.8	25.4	34.7**
Relative thyroid weight (% of body weight ×1 000)	3.52	3.80	3.80	5.25**
No. of animals examined macroscopically	9	8	8	8
Liver: enlarged	0	0	2	7
Thyroid: dark	0	0	0	3
No. of animals examined microscopically	9	8	8	8
Liver				
Centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	7	3
- Slight	0	0	0	5
- Total	0	0	7	8
Eosinophilic foci of hepatocellular alteration	4	4	3	7
Tigroid foci of hepatocellular alteration	3	2	3	7
Thyroid				
Follicular cell hypertrophy, diffuse				
- Minimal	0	0	2	5
- Slight	0	0	0	1
- Total	0	0	2	6
Colloid alteration				
- Minimal	3	3	4	6
- Slight	0	0	2	2
- Total	3	3	6	8

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Garcin (2008)

At the end of the carcinogenicity phase (24 months), absolute and relative liver weights were increased by 22–32% at 2000 ppm compared with controls, and at macroscopic examination, a higher incidence of enlarged and dark livers with a higher incidence of white foci was observed at 2000 ppm (Table 32). At the microscopic examination, a higher incidence of minimal to moderate centrilobular

Table 32. Selected findings at the terminal sacrifice in the 24-month rat study (males only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Terminal body weight (g)	642.7	639.0	598.3	597.4
Absolute liver weight (g)	12.72	12.76	12.91	15.62**
Relative liver weight (% of body weight)	1.99	1.99	2.16	2.63**
Absolute thyroid weight (mg)	51.6	41.2	35.5	41.1
Relative thyroid weight (% of body weight, ×1 000)	8.32	6.41	5.97	7.12
No. of animals examined macroscopically	25	20	17	22
Liver: enlarged	2	0	0	5
Liver: dark	0	0	0	9
Liver: foci, white	4	5	8	9
Liver				
No. of animals examined microscopically	60	60	60	60
Centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	13	20
- Slight	0	0	0	20
- Moderate	0	0	0	1
- Total	0	0	13**	41**
Hepatocellular brown pigments, focal/multifocal; minimal	0	0	1	9**
Eosinophilic foci of hepatocellular alteration				
- Minimal	41	39	35	39
- Slight	6	1	6	11
- Moderate	0	1	1	2
- Total	47	41	42	52*
Cystic degeneration, focal/multifocal				
- Minimal	1	5	0	6
- Slight	1	0	0	4
- Total	2	5	0	10*
Thyroid				
No. of animals examined microscopically	60	60	60	59
Follicular cell hyperplasia, focal/multifocal				
- Minimal	0	1	0	4
- Slight	1	0	1	1
- Total	1	1	1	5
Follicular cell hypertrophy, diffuse; minimal	1	0	1	5
Colloid alteration				
- Minimal	17	26	20	14
- Slight	7	8	14	33
- Moderate	0	0	2	5
- Total	24	34*#	36**	52**
Brown pigments, follicular cells; minimal	4	2	4	15**
Follicular cell adenoma	0	0	0	0
Follicular cell carcinoma	1	0	0	0

	0 ppm	50 ppm	300 ppm	2 000 ppm
Epididymides				
No. of animals examined microscopically	59	60	60	60
Oligospermia				
- Bilateral	5	7	12	11
- Unilateral	18	13	10	13
Testes				
No. of animals examined microscopically	59	60	60	60
Tubular degeneration, diffuse				
- Bilateral	4	7	9	10
- Unilateral	18	10	11	13

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; #: not statistically significant using a two-sided test
Source: Garcin (2008)

to panlobular hepatocellular hypertrophy was noted at 2000 and 300 ppm, whereas higher incidences of minimal hepatocellular brown pigments, of eosinophilic foci of hepatocellular alteration and of cystic degeneration were observed at 2000 ppm.

In the thyroid gland, higher incidences of focal/multifocal follicular cell hyperplasia, diffuse follicular cell hypertrophy and brown pigments in follicular cells were observed at 2000 ppm (Table 32). Also, a statistically significant higher incidence and/or severity of colloid alteration were observed at 50 ppm and above when applying a one-sided statistical test. However, the one-sided statistical analysis may be considered overly conservative when applied to non-neoplastic changes that could indeed be either increased or decreased by the treatment and are occurring with a high frequency in the control group. In such cases, a two-sided test would be more appropriate than a one-sided test. When applying the two-sided statistical analysis, the incidence of colloid alteration in the 50 ppm dose group was not statistically significant compared with the control group.

The alteration of colloid (stippled, granular or clumped colloid with variable staining characteristics) was considered not to be adverse at 50 ppm for the following reasons:

1) This finding is seen in the thyroid gland of control rats and reflects a normal age-related physiological process associated with the rapid turnover of colloid.
2) The female thyroid gland appeared to be more sensitive to bixafen, as evidenced by the occurrence of diffuse follicular cell hypertrophy at 300 ppm (9/58, $P < 0.05$) and 2000 ppm (34/59, $P < 0.01$) and of follicular cell hyperplasia at 2000 ppm (8/59, $P < 0.01$), but no difference in colloid alteration was observed in females at 50 ppm (McElligott, 2008).
3) The incidence was only slightly above the highest incidence seen in historical controls provided for five studies (colloid alteration was observed in 91/282 animals [32.3%], with a range from 11/50 to 28/58 [22–48%]), and there was no difference in severity when compared with controls.
4) Colloid alteration was not associated with any other findings indicative of stimulation (follicular cell hypertrophy or hyperplasia).

Slight trends towards increased incidences of bilateral tubular degeneration in the testis and of bilateral oligospermia in the epididymis observed at all dose levels were considered to be incidental and not related to treatment, as these findings also occurred in the control group, and the incidence of both findings in the treatment groups was within the range of historical control data provided for eight studies: bilateral diffuse tubular degeneration in the testis was observed in 83/464 animals (17.9%), with a range from 3/50 to 20/60 (6–33%), whereas bilateral oligospermia in the epididymis was observed in 101/464 animals (21.8%), with a range from 5/50 to 22/60 (10–37%).

At the end of the 24-month treatment period, there was no evidence of a test compound–related carcinogenic potential. All the neoplastic findings observed in treated animals were those commonly observed in this strain of rats kept under monitored environmental conditions and were considered to be incidental in origin.

The NOAEL for carcinogenicity was 2000 ppm (equal to 80.5 mg/kg bw per day for males), the highest dose tested. The NOAEL for toxicity was 50 ppm (equal to 2.0 mg/kg bw per day for males), based on liver effects (increased cholesterol levels, increased liver weights) and thyroid effects (higher incidence and/or severity of colloid alteration) at 300 ppm (equal to 12.1 mg/kg bw per day for males) and above (Garcin, 2008).

2.4 Genotoxicity

The results of the genotoxicity studies with bixafen are summarized in Table 33.

Table 33. Summary of genotoxicity studies with bixafen

End-point	Test object	Concentration or dose	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* (TA98, TA100, TA102, TA1535, TA1537)	±S9 mix: 0–5 000 µg/plate	95.8	Negative	Herbold (2005b)
Gene mutation (induced forward), *HPRT* locus	Chinese hamster V79 lung cells / HPRT	±S9 mix: 0–288 µg/mL	95.8	Negative	Herbold (2006b)
Chromosomal aberration	Chinese hamster V79 lung cells	±S9 mix: 0–240 µg/mL	95.8	Negative	Herbold (2006a)
In vivo					
Micronucleus induction	Male mice (Hsd/Win:NMRI) bone marrow erythrocytes	0, 125, 250 and 500 mg/kg bw; twice (24 h apart); intraperitoneal injection	95.8	Negative	Herbold (2005a)

HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

In a reverse gene mutation assay in bacteria conducted according to OECD Test Guideline 471, *Salmonella typhimurium* (strains TA98, TA100, TA102, TA1535 and TA1537) was exposed to bixafen (purity 95.8%) diluted in DMSO in the presence and absence of S9 metabolic activation, using the plate incorporation and preincubation methods. Doses were selected based on the results of a range-finding study. For the plate incorporation assay, doses of up to 5000 µg/plate were used, with three plates for each strain, condition and dose. The independent repeat was performed as a preincubation test using doses of up to 1581 µg/tube. Vehicle and controls (positive and negative) were included in each experiment. A reproducible and dose-related increase in mutant colonies of at least one strain was considered to be positive. In the main assay, bixafen did not influence the growth of any strain tested at dose levels of up to 5000 µg/plate. There was no indication of a bacteriotoxic effect of bixafen up to 5000 µg/plate. Total bacterial counts were either comparable with those of negative controls or differed only insignificantly. No inhibition of growth was noted. None of the strains, in both assays, showed a dose-related and biologically relevant increase in mutant colonies over those of the negative controls. Under the conditions of this study, bixafen did not induce gene mutations in any of the strains of *S. typhimurium* used (Herbold, 2005b).

In an in vitro mammalian cell gene mutation test conducted according to OECD Test Guideline 476, bixafen (purity 95.8%) was tested for its ability to induce forward mutations at the X-chromosome-linked hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) locus in Chinese hamster V79 lung (CHL) cells. Two independent sets of experiments were conducted in the presence and absence of S9 metabolic activation. Both negative and positive controls were used. After 5 hours of treatment with bixafen, cells were rinsed, trypsinized and transferred into growth media and further incubated for 6 days. CHL cells were exposed to bixafen concentrations of up to and including 288 µg/mL, in the absence and in the presence of S9 mix. At 144 µg/mL and above, the formulation in the vehicle began to form a precipitate. The means of the absolute cloning efficiency for the vehicle controls in the mutation experiments were 51.1% and 58.6% in the experiment without activation and 52.2% and 59.9% in the experiments with activation. In the mutation assay without metabolic activation, the mutant frequencies of the negative controls and of the vehicle controls were all within the normal range. The positive control ethyl methane sulfonate induced clear mutagenic and statistically significant effects in all trials. For bixafen-treated cultures, decreases in relative population growth were observed at 288 µg/mL. No relevant increases in mutant frequencies could be found in trials using bixafen. Furthermore, bixafen was evaluated as non-mutagenic in the non-activation trial. Similarly, in the case of the mutation assay with metabolic activation, the mutant frequencies of the negative controls were all within the normal range. The positive control 7,12-dimethylbenz[*a*]anthracene induced clear mutagenic and statistically significant effects in both trials. Bixafen-treated cultures showed no concentration-related decreases in relative survival or in relative population growth. However, precipitation was observed in the medium for the two highest tested concentrations. Relevant bixafen-induced increases in mutant frequencies could not be found. In addition, statistical analysis revealed no statistically significant increases. Therefore, bixafen was evaluated as non-mutagenic in the activation trial. Based on the results of the study, bixafen is considered not to be mutagenic in the V79/HPRT forward mutation assay (Herbold, 2006b).

In an in vitro mammalian chromosomal aberration test conducted according to OECD Test Guideline 473, the clastogenic potential of bixafen (purity 95.8%) was tested in CHL cells. Cultures were exposed in a pretest to bixafen at concentrations ranging from 1 to 600 µg/mL with or without S9 mix for 4 hours and harvested at 18 hours. In parallel, another group of cells was exposed from 1 to 200 µg/mL for 18 hours. Mitotic index was determined for all cultures. Concentrations that resulted in approximately 50% cytotoxicity were selected as the maximum concentrations for the main study. Osmolality and pH were unaffected by bixafen at concentrations up to 600 µg/mL. Based on the results of the pretests, the following concentrations were tested in the main study: 15–240 µg/mL for an exposure period of 4 hours and a harvest time after 18 hours; 60–240 µg/mL for an exposure period of 4 hours and a harvest time after 30 hours; and 1–16 µg/mL without S9 mix only for an exposure period of 18 hours and a harvest time after 18 hours. After 4 hours of treatment of CHL cells with bixafen, concentrations of 15, 30 and 60 µg/mL were used without S9 mix for assessment of the clastogenic potential of bixafen. With S9 mix, concentrations of 30, 60 and 120 µg/mL were employed. In addition, after 18 hours of treatment with bixafen, concentrations of 1, 4 and 8 µg/mL were selected for reading without S9 mix. None of the cultures treated with bixafen in the presence or absence of S9 mix showed statistically significant or biologically relevant increases in numbers of metaphases with aberrations. The positive controls mitomycin C and cyclophosphamide induced clear clastogenic effects and demonstrated the sensitivity of the test system. Based on the results of the study, bixafen is not considered to be clastogenic for mammalian cells in vitro (Herbold, 2006a).

In a mammalian erythrocyte micronucleus test conducted according to OECD Test Guideline 474, bixafen (purity 95.8%) was administered (by intraperitoneal injection) on two occasions, separated by 24 hours, to five male mice (Hsd/Win:NMRI) at a dose of 125, 250 or 500 mg/kg bw. Clinical signs were recorded for up to at least 24 hours after the second injection, and 4/10 mice in the 500 mg/kg bw group died. Both positive and negative control groups were used. The negative control group was treated with two intraperitoneal injections of 0.5% aqueous Cremophor emulsion, whereas the positive control group received only one intraperitoneal injection of cyclophosphamide at 20 mg/kg bw. Five additional animals were treated at 500 mg/kg bw of bixafen in case of mortality in the

initial group or a need for replacement slides due to pathological altered ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs). Bone marrow from at least one femur from each animal was sampled 24 hours after the last intraperitoneal injection. Slides of bone marrow cells were prepared and scored for the occurrence of micronucleated PCEs, the occurrence of micronucleated NCEs and PCE/total erythrocyte ratios. Two thousand PCEs were counted per animal. Four out of 10 treated mice died during the period, due to the acute intraperitoneal toxicity of 500 mg/kg bw of bixafen. Animals treated at 125, 250 and 500 mg/kg bw showed the following symptoms until their termination: apathy, staggering gait, weight loss, sternal recumbency, spasm, twitching, periodic stretching of body and difficulty breathing. There were no statistically significant changes in the mean PCE/total erythrocyte ratio observed for any of the bixafen-treated groups or the control group. There were no statistically or biologically significant variations in the incidence of micronucleated PCEs between the control and the bixafen-treated groups. No statistically significant variations in the number of micronucleated NCEs were observed between the negative control and bixafen-treated groups. The positive control, cyclophosphamide, caused a clear increase in the number of PCEs with micronuclei, but did not alter the ratio of PCEs to NCEs. Thus, from the study, there was no indication of a clastogenic effect of intraperitoneally administered bixafen in the micronucleus test in male mice (Herbold, 2005a).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a dose range–finding reproductive toxicity study, groups of 10 male and 10 female Wistar Crl: WI(Glx/BRL/Han)IGS BR rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 500, 1500 or 4500 ppm for 10 weeks before mating and during the mating period. Additionally, satellite groups of five males each were assigned to the control and 4500 ppm dose levels for additional blood collection for the evaluation of PT and APTT on day 29 of premating. The substance intakes at 500, 1500 and 4500 ppm during the premating period were equal to 36.2, 110 and 326 mg/kg bw per day for males and 41.3, 125 and 368 mg/kg bw per day for females, respectively. During lactation, the concentration of the test substance in the feed for the females was adjusted by 50%; the corresponding substance intakes were equal to 41.5, 123 and 371 mg/kg bw per day, respectively.

Body weight and feed consumption determinations and detailed clinical examinations of each animal were conducted weekly throughout the study. Blood samples were collected from adult females prior to termination and from adult males during week 13 (PT and APTT determinations). Reproductive parameters, such as mating, fertility and gestation indices, were evaluated. Litter parameters, such as litter size, sex ratio, pup weight, pup viability, body weight gain and clinical signs, were studied. All adult animals received a gross examination at necropsy. Selected organs (adrenals, brain, epididymides, kidney, liver, spleen, thyroid, ovaries, pituitary, prostate, seminal vesicles, testes, thymus and uterus) were weighed in adult rats. Offspring were subjected to a gross examination, and selected organs (brain, thymus, spleen and uterus) were removed and weighed. No microscopic examinations were performed in this study.

There were no compound-related mortalities or clinical observations observed during the course of this study at any dietary level tested.

P generation. A slight decrease in body weight at 4500 ppm was observed during premating in males and females, with decreases in body weight gain more pronounced in both sexes. A significant body weight decrease in females at 4500 ppm continued throughout gestation and lactation (Table 34). Also, terminal body weights at 4500 ppm were decreased in both males and females. At necropsy in males, liver and thyroid weights were increased at all dose levels, whereas thymus weight was decreased at 4500 ppm. In females, liver weight was increased at 1500 ppm and above, whereas reduced thymus, ovary and uterine weights were observed at 4500 ppm (Table 34).

Table 34. Summary of selected findings in parental animals of the range-finding reproductive toxicity study in rats

	Males				Females			
	0 ppm	500 ppm	1 500 ppm	4 500 ppm	0 ppm	500 ppm	1 500 ppm	4 500 ppm
Premating								
Body weight (g), week 10	409	441	411	392	244	244	238	232
Body weight gain (g), weeks 1–10	156	174	146	133	66	63	60	57
Feed consumption (g/animal per day), weeks 1–10	24.4	26.1	25.0	23.7	18.4	17.5	17.1	16.6
Gestation								
Body weight gain (g), days 0–20	ND	ND	ND	ND	105	109	98	72**
Feed consumption (g/animal per day), days 0–20	ND	ND	ND	ND	19.0	19.8	18.4	17.5
Lactation								
Body weight (g), day 21	ND	ND	ND	ND	292	299	295	248**
Feed consumption (g/animal per day), days 0–21	ND	ND	ND	ND	46.0	47.8	45.7	40.6
Terminal sacrifice								
Body weight (g)	449	485	455	419	292	294	296	248*
Absolute liver weight (g)	15.1	17.6*	17.9*	18.6*	14.4	14.5	16.8*	15.7
Relative liver weight (% of body weight)	3.36	3.63*	3.92*	4.43*	4.94	4.91	5.68*	6.34*
Absolute thyroid weight (mg)	28	33	35*	34	24	22	26	24
Relative thyroid weight (% of body weight ×1 000)	6.3	6.9	7.7*	8.1*	8.3	7.6	8.8	9.7
Absolute thymus weight (mg)	490	467	420	367*	265	250	250	139*
Relative thymus weight (% of body weight ×1 000)	109	96	92	87	91	86	85	55*
Absolute ovary weight (mg)	ND	ND	ND	ND	124	128	104	79*
Relative ovary weight (% of body weight ×1 000)	ND	ND	ND	ND	42	44	35	32*

ND: not determined; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2006)

The clinical chemistry determinations in control and 4500 ppm satellite males showed no compound-related effects on PT or APTT. In males bled after 13 weeks of exposure, there was no effect on PT, whereas the increased mean APTT level at 4500 ppm was within the range of historical control values. In females, no compound-related findings on PT or APTT were observed at any dietary level tested.

There were no treatment-related effects on reproductive performance (Table 35).

F_1 offspring. Pup body weight was significantly decreased by day 4, with increasing declines continuing throughout lactation, resulting in a significantly decreased pup body weight gain throughout lactation (Table 35). Also, absolute brain, thymus, spleen and uterine weights were decreased, with concomitant increased relative (organ to body weight ratio) brain and uterine weights and decreased relative spleen weight.

Table 35. Summary of selected findings on reproduction and offspring in the range-finding reproductive toxicity study in rats

	0 ppm	500 ppm	1 500 ppm	4 500 ppm
Mating index (%)	100	90	100	100
Fertility index (%)	100	100	100	100
Gestation index (%)	100	100	100	100
Days to insemination	2.4	2.3	2.7	1.9
Gestation length (days)	21.7	21.8	22.1	21.6
Mean no. of implantations per litter	12.6	11.9	11.6	10.7
Number born live	110	102	105	98
Number born dead	11	1	4	2
Birth index	95.7	96.4	93.1	93.5
Live birth index	88.8	99.1	95.7	98.2
Viability index	90.0	99.0	96.1	99.0
Lactation index	100	97.2	98.8	98.8
Offspring body weight (g)				
- Lactation day 0	5.5	5.9	6.0	5.5
- Lactation day 4 (before culling)	9.3	9.5	9.4	7.9*
- Lactation day 4 (after culling)	9.3	9.6	9.4	7.8*
- Lactation day 7	14.9	15.2	14.2	11.4**
- Lactation day 14	31.0	32.2	30.0	21.7**
- Lactation day 21	47.2	48.6	46.7	32.6**
Body weight gain (g), lactation days 0–21	41.6	42.8	40.7	27.1**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2006)

The NOAEL for reproductive toxicity was 4500 ppm (equal to 326 mg/kg bw per day for males and 368 mg/kg bw per day for females), the highest dose tested.

A NOAEL for parental toxicity could not be established, as increased liver and thyroid weights in males were observed at all dose levels tested. The lowest-observed-adverse-effect level (LOAEL) in males was 500 ppm (equal to 36.2 mg/kg bw per day).

The NOAEL for offspring toxicity was 1500 ppm (equal to 123 mg/kg bw per day), based on reduced body weight and body weight gain at 4500 ppm (equal to 371 mg/kg bw per day) (Young, 2006).

In a two-generation reproductive toxicity study conducted according to OECD Test Guideline 416, groups of 30 male and 30 female Wistar Han Crl: WI (HAN) rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 400 or 2500 ppm (equal to 0, 3.4, 27.3 and 169.2 mg/kg bw per day for P generation males [premating period] and 0, 3.9, 30.8 and 193.7 mg/kg bw per day for P generation females [premating period]) for 10 weeks prior to mating through to weaning of the F_1 offspring. Groups of 30 male and 30 female F_1 generation offspring were then similarly treated; the mean substance intakes at 0, 50, 400 and 2500 ppm were equal to 0, 3.3, 26.4 and 177.6 mg/kg bw per day for F_1 generation males (premating period) and 0, 4.0, 31.7 and 198.4 mg/kg bw per day for F_1 generation females (premating period). During the lactation period, the concentration of the test substance in the feed for the females was reduced by 50%; the corresponding substance intakes at 0, 25, 200 and 1250 ppm were equal to 0, 4.3, 33.3 and 207.5 mg/kg bw per day for P generation females and 0, 4.3, 35.9 and 226.0 mg/kg bw per day for F_1 generation females.

After a gestation period of about 22 days, litters were born, and 4 days after birth, the F_1 litters were reduced to eight pups (of equal sex ratio where possible). Pups found in a moribund state on day 4 were excluded from lactation. This was done to investigate possible malformations and to prevent cannibalism during the further rearing period. The selected F_1 pups were reared to an age of 4 weeks. At weaning, one male and one female per litter were chosen for further treatment and to produce the F_2 generation. Weanlings not chosen, as well as P generation females, were necropsied. P generation males were necropsied after the mating period, partly in the course of spermatological investigations. The weaned F_1 offspring were treated further with the test substance for at least 10 weeks (including a 3-week period for estrous cycle determination) and then co-housed for mating. The F_1 parental animals were killed as scheduled after their F_2 litters had been weaned at about day 28 postpartum.

Body weights, feed consumption and clinical signs were monitored regularly. Reproductive parameters, such as mating performance, fertility, duration of pregnancy, estrous cycling and sperm analyses, were examined in P generation and F_1 generation rats. Litter parameters, such as litter size, sex ratio, pup weight, pup viability, body weight gain and clinical signs, were studied in F_1 and F_2 offspring. Developmental milestones were evaluated in F_1 weanlings.

All parental animals were subjected to a gross examination at necropsy. Selected organs (brain, liver, kidneys, spleen, thyroid, thymus, adrenals, pituitary, testes, prostate, epididymides, seminal vesicles, coagulating glands, ovaries and uterus with cervix and oviducts) from adult rats were removed and weighed. For F_1 and F_2 pups, the brain, spleen, thymus and uterus from one male and/or one female pup per litter on day 21 were weighed. Microscopic examinations were performed on tissues from the control and 2500 ppm adult animals (brain, cervix, epididymides, gross lesions, kidney, liver, ovaries, pituitary, prostate, testes, seminal vesicles, coagulating glands, spleen, uterus with oviducts and vagina). Liver was also microscopically examined in the 50 and 400 ppm dose groups of the P generation and F_1 adults. Selected tissues (cervix, an epididymis, gross lesions, ovary, oviduct, prostate, seminal vesicles, coagulating glands, testes, uterus and/or vagina) from control and 2500 ppm 21-day F_1 and F_2 pups were examined microscopically. In F_1 females, ovarian follicle staging was evaluated in 10 randomized animals that had viable litters from the control and 2500 ppm groups.

There were no treatment-related mortalities or clinical signs during the course of the study in either generation.

The P generation males did not exhibit any test substance–related effects on body weight or body weight gain at any dietary level tested after 14 weeks of exposure to the test substance. In the F_1 generation males, absolute body weight was decreased by 6.2% at 2500 ppm, whereas body weight gain was unaffected after 14 weeks of treatment (Table 36). The lower body weight was considered to be due to lower pup weight in this group. There was a decrease in feed consumption in the 2500 ppm group P generation males in the 1st week of the study, thought to be due to taste aversion. Thereafter, feed consumption was unaffected by treatment at any dose level. In the F_1 generation males, feed consumption increased by 8.1% from week 5 of treatment in the 2500 ppm group.

In the P generation females at 2500 ppm, slight but significant decreases in body weight were seen during the premating period from weeks 7 through 10 (mean decline of 4.8%). Overall, the body weight gain in this group was reduced by 21.1%. Also at 2500 ppm, significant body weight declines were noted throughout gestation, with a reduction of body weight gain by 14.5%, as well as throughout the lactation period, with a significant reduction of body weight by 5.7%. Body weight effects were not observed at any other dietary level (Table 36).

F_1 generation females at 2500 ppm showed reduced body weights through the premating period (decline of 7.6%), with an overall reduction in body weight gain of 13.3%. The lower body weight was considered to be due to lower pup weight in this group. Also at 2500 ppm, significant body weight declines were noted throughout gestation, with a reduction of body weight gain by 18.2%, as well as throughout the lactation period, with a significant reduction of body weight by 8.3%. Body weight effects were not observed at any other dietary level (Table 36).

Table 36. Summary of selected findings in parental animals of the reproductive toxicity study in rats

	Males				Females			
	0 ppm	50 ppm	400 ppm	2 500 ppm	0 ppm	50 ppm	400 ppm	2 500 ppm
P generation								
Premating								
Body weight (g), weeks 14/10 (M/F)	448	448	453	447	244	243	242	232**
Body weight gain (g), weeks 1–14/1–10 (M/F)	198	199	198	194	60.2	58.1	56.5	47.5
Feed consumption (g/animal per day), weeks 1–10	23.5	23.5	24.0	23.5	17.4	17.1	16.9	16.6
Gestation								
Body weight (g), day 20	ND	ND	ND	ND	347	345	341	321**
Body weight gain (g), days 0–20	ND	ND	ND	ND	101.0	95.4	95.6	86.4**
Feed consumption (g/animal per day), days 0–20	ND	ND	ND	ND	19.7	19.2	19.0	18.6
Lactation								
Body weight (g), day 21	ND	ND	ND	ND	294	296	293	281*
Feed consumption (g/animal per day), days 0–21	ND	ND	ND	ND	45.0	44.5	47.5	44.0
Postmortem								
Body weight (g)	454	456	457	452	292	294	294	281*
Absolute liver weight (g)	15.9	16.5	17.2*	20.0*	14.4	14.8	15.3	18.0*
Relative liver weight (% of body weight)	3.51	3.61	3.77*	4.42*	4.92	5.03	5.21	6.39*
Liver: centrilobular hypertrophy	0/30	0/30	0/30	30*/30	0/30	0/30	0/30	28*/30
F_1 generation								
Premating								
Body weight (g), weeks 14/10 (M/F)	450	449	454	422*	245	245	245	226**
Body weight gain (g), weeks 1–14/1–10 (M/F)	159	161	157	154	54.8	57.0	54.2	47.5
Feed consumption (g/animal per day), weeks 1–10	24.5	24.2	24.4	23.8	17.2	17.4	17.2	16.0
Gestation								
Body weight (g), day 20	ND	ND	ND	ND	336	335	334	299**
Body weight gain (g), days 0–20	ND	ND	ND	ND	92.5	88.9	94.8	75.7**
Feed consumption (g/animal per day), days 0–20	ND	ND	ND	ND	17.8	18.2	18.6	16.7
Lactation								
Body weight (g), day 21	ND	ND	ND	ND	283	288	286	268*
Feed consumption (g/animal per day), days 0–21	ND	ND	ND	ND	44.2	44.7	43.6	40.5
Postmortem								
Body weight (g)	453	457	458	428	282	287	287	266*
Absolute liver weight (g)	15.6	16.2	16.7	18.5*	12.7	13.8*	14.2*	16.3*
Relative liver weight (% of body weight)	3.44	3.54	3.64*	4.31*	4.51	4.80*	4.95*	6.11*
Liver: centrilobular hypertrophy	0/30	0/30	2/30	28*/30	0/30	0/30	4/30	26*/30

F: female; F_1: first filial; M: male; ND: not determined; P: parental; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2007)

In P generation females, initial declines in feed consumption in the 1st week of the study were seen in both the 400 and 2500 ppm groups, considered to be a result of palatability of the test material. In the F_1 generation females, no effects on feed consumption were seen in any dose group.

At necropsy, treatment-related organ weight changes for the P generation were limited to kidney (increased at 2500 ppm in males), liver (increased at and above 400 ppm in males and at 2500 ppm in females), spleen (increased at 2500 ppm in males) and thymus (decreased at 2500 ppm in females). For the F_1 generation, treatment-related organ weight changes were limited to the increased liver weights in males at and above 400 ppm and in females at and above 50 ppm (Table 36). Treatment-related histopathological findings were limited to the liver. Minimal to slight, primarily centrilobular and/or diffuse hypertrophy of the liver was present in males and females of the P generation at 2500 ppm and in males and females of the F_1 generation at 400 and 2500 ppm (Table 36). This liver hypertrophy, coded as "centrilobular hypertrophy", was characterized by an enlarged cell, primarily involving the cytoplasm. Often the basophilic staining cytoplasmic organelles appeared to be compressed towards the outer edges of the cell. In general, the centrilobular hepatocytes were affected, with occasional involvement of the midzonal hepatocytes. The hypertrophy changes of the liver at 400 and/or 2500 ppm correlated well with the statistically significant liver weight increases present at these dietary levels.

Overall reproductive performance was not affected for any parameter (e.g. mating, fertility or gestation indices, days to insemination, gestation length or the median number of implantations) in either generation at any dietary level (Table 37).

Table 37. Summary of reproductive performance data in the reproductive toxicity study in rats

	0 ppm	50 ppm	400 ppm	2 500 ppm
P generation				
Co-housed females	30	30	30	30
Matings until day 0 post-coitum	30	29	29	30
Mating index	100	96.7	96.7	100
Fertility index	93.3	96.6	100	100
Gestation index	100	100	96.6	100
Gestation length (days)	22.0	21.9	21.9	21.9
Number of animals delivered	28	28	28	30
F_1 generation				
Co-housed females	30	30	30	30
Matings until day 0 post-coitum	30	30	30	30
Mating index	100	100	100	100
Fertility index	93.3	100	90.0	93.3
Gestation index	100	93.3	96.3	100
Gestation length (days)	21.9	21.9	21.8	21.8
Number of animals delivered	28	28	26	28

F_1: first filial; P: parental; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2007)

Results from the evaluation of vaginal smears in both the P and F_1 generation females did not indicate any test substance–related findings on estrous cycle length or periodicity at any dietary level tested.

There were no test substance–related effects observed on any sperm parameter evaluated at any dietary level tested for either generation.

The number of implantation sites, the live birth, viability and lactation indices, the number of pups born, the sex distribution, the litter sizes during lactation as well as prenatal losses were not altered by the treatment up to 2500 ppm.

A trend of an increase (non-statistically significant) in the incidence of early stillbirths (lungs did not float) occurred at 2500 ppm in the F_1 and F_2 generations (Table 38). Litters evaluated for early stillbirths (lungs did not float) were also non-statistically significantly elevated above controls at 2500 ppm in the F_1 and F_2 generations. These non-statistically significant increases in stillbirths in the F_1 and F_2 generations were considered to be secondary to the toxicity observed in dams at this dietary level and were within the range of historical control data for this finding. Historical control values were provided for eight one-generation studies conducted from 1998 to 2005 and for nine two-generation studies conducted from 1999 to 2005. In the one-generation studies, which included 7–10 litters, the number of stillborn pups ranged from 0/118 to 11/121 (0–9.1%) in 0/10 to 2/8 litters (0–25%). The control group with 11/121 stillborn pups was from the range-finding study for reproductive toxicity of bixafen (Young, 2006), including one dam delivering eight stillborn pups (out of nine pups in total). In the two-generation studies, which included 19–29 litters, the number of stillborn pups ranged from 0/306 to 6/291 (0–2.1%) in 0/28 to 2/24 litters (0–8.3%).

Table 38. Summary of selected litter data in the reproductive toxicity study in rats

	0 ppm	50 ppm	400 ppm	2 500 ppm
F_1 generation				
No. of litters	28	28	28	30
No. of implantations, total/per litter	342/12.2	318/11.4	332/11.9	338/11.3
No. of pups born	320	294	305	318
No. of pups missing/found dead/cannibalized	1/2/2	0/0/1	0/1/0	5/2/0
No. of stillborn pups	4	0	0	13
No. of pups born live	314	294	305	292
Litter size	11.4	10.5	10.9	10.6
No. of deaths, days 0–4/days 4–21	5/0	0/1	1/0	6/1
Pup weight (g), viable pups				
- Birth	5.9	6.0	6.0	5.8
- Day 4, pre-culling	9.6	9.9	9.8	9.2
- Day 7	15.4	15.9	15.5	14.1*
- Day 14	32.1	32.4	31.5	28.2**
- Day 21	48.8	49.5	48.0	43.2**
Pup weight gain (g), days 0–21	42.9	43.5	42.0	37.4**
Birth index[a]	93.1	92.1	92.2	93.4
Live birth index[b]	97.8	100	100	96.3
Viability index[c]	98.6	100	99.6	97.7
Lactation index[d]	100	99.6	100	99.5
F_2 generation				
No. of litters	28	28	26	28
No. of implantations, total/per litter	303/10.8	312/10.4	292/10.8	275/9.8
No. of pups born	290	296	276	265
No. of pups missing/found dead/cannibalized	4/0/0	0/3/0	4/1/6	3/5/0
No. of stillborn pups	1	6	2	6
No. of pups born live	289	290	274	259

	0 ppm	50 ppm	400 ppm	2 500 ppm
Litter size	10.4	10.6	10.6	9.5
No. of deaths, days 0–4/days 4–21	3/1	3/0	11/0	4/4
Pup weight, viable pups (g)				
- Birth	6.0	5.9	5.9	5.9
- Day 4, pre-culling	10.2	10.4	10.0	9.8
- Day 7	16.0	16.1	15.8	15.0
- Day 14	32.4	32.2	31.4	29.5**
- Day 21	48.4	49.1	47.3	44.5**
Pup weight gain (g), days 0–21	42.5	43.2	41.4	38.6**
Birth index[a]	95.3	89.0	91.6	96.1
Live birth index[b]	99.6	98.0	97.2	97.9
Viability index[c]	99.1	99.1	97.4	98.5
Lactation index[d]	99.6	100	100	98.2

F_1: first filial; F_2: second filial; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Birth index = Total no. of pups born per litter/Total no. of implantation sites per dam × 100.

[b] Live birth index = No. of live pups born per litter/Total no. of pups per litter × 100.

[c] Viability index = No. of live pups on day 4 pre-culling per litter/ No. of live pups born per litter × 100.

[d] Lactation index = No. of live pups on day 21 per litter / No. of live pups on day 4 post-culling per litter × 100.

Source: Young (2007)

There was no treatment-related effect on mean litter size or pup viability (survival) during lactation.

Offspring birth weights were not affected by treatment at any dose level, but there was a treatment-related decrease in offspring body weight and body weight gain during lactation for the F_1 and F_2 pups at 2500 ppm. This decrease in weight gain was apparent by day 4 postpartum and continued to intensify during lactation (Table 38).

The balanopreputial separation and vaginal opening in F_1 post-weanlings were unaffected up to 2500 ppm.

Offspring organ weights in the F_1 or F_2 pups at 2500 ppm that were statistically significantly different from those of controls included brain, spleen and thymus. As all these organ weight differences were associated with statistically significant deceases in body weights at day 21 of the lactation period, they were considered to be secondary to body weight changes in this group. No other dose groups exhibited any changes in organ weights compared with controls. There were no remarkable incidences of macroscopic or microscopic findings at necropsy in the F_1 or F_2 pups.

The NOAEL for reproductive toxicity was 2500 ppm (equal to 169.2 mg/kg bw per day for males and 193.7 mg/kg bw per day for females), the highest dose tested.

The NOAEL for parental toxicity was 400 ppm (equal to 26.4 mg/kg bw per day for males and 30.8 mg/kg bw per day for females), based on a reduction in body weight and liver effects (liver weight increase > 20%) at 2500 ppm (equal to 169.2 mg/kg bw per day for males and 193.7 mg/kg bw per day for females).

The NOAEL for offspring toxicity was 400 ppm (equal to 26.4 mg/kg bw per day for males and 31.7 mg/kg bw per day for females), based on a slight elevation in stillbirths and reduced pup weight/weight gain during lactation at 2500 ppm (equal to 177.6 mg/kg bw per day for males and 198.4 mg/kg bw per day for females) (Young, 2007).

(b) Developmental toxicity

Rats

In a prenatal developmental toxicity study conducted according to OECD Test Guideline 414, groups of 23 mated female Sprague-Dawley Crj:CD(SD) rats were administered bixafen (purity 95.8%) in 0.5% aqueous methyl cellulose at a dosing volume of 10 mL/kg bw by oral gavage at a dose of 0, 20, 75 or 250 mg/kg bw per day from gestation day (GD) 6 to GD 20. Clinical observations were recorded daily. Maternal body weights and feed consumption were recorded for all females at appropriate intervals from GD 0 to GD 21. At scheduled termination, on GD 21, the gravid uterine weight was recorded, and the dams were evaluated for number of corpora lutea and number and status of implantations (resorptions, dead and live fetuses). Live fetuses were removed from the uteri, counted, weighed, sexed and examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and stained for skeletal examination of bone and cartilage.

At 250 mg/kg bw per day, one female was found dead on GD 20. Clinical signs for this female consisted of soiling around the nose and mouth on GD 18 and soiling of the fur in the head region on GD 19. This female lost 29 g in body weight between GD 6 and GD 8, which was associated with very low feed consumption. Body weight gain and feed consumption for this female were also reduced between GD 14 and GD 18, compared with the controls. Treatment-related clinical signs in surviving females at this dose level consisted of one female with soiling around the nose on GD 19, one female with soiling around the nose, mouth and abdomen on GD 21 and one female with piloerection on GDs 14 and 15. No premature deaths or treatment-related clinical signs were observed in dams at 75 or 20 mg/kg bw per day.

There was a marked loss in maternal body weight of 21.9 g on GDs 6–8 at 250 mg/kg bw per day, compared with a 5.7 g body weight gain in the controls over the corresponding period. On GDs 18–21, body weight gain was reduced by 21%. Overall, on GDs 6–21, maternal body weight gain was reduced by 17%, compared with the controls, at 250 mg/kg bw per day, and maternal corrected body weight change was 42% less than in the controls. At 75 mg/kg bw per day, there was a slight maternal body weight loss of 1.8 g on GDs 6–8, compared with a 5.7 g body weight gain in the control group. Thereafter, body weight gain was unaffected by treatment at 75 mg/kg bw per day, and maternal corrected body weight change was comparable with that of the control group (Table 39).

Mean maternal feed consumption at 250 mg/kg bw per day was reduced by 58% on GDs 6–8, 16% on GDs 8–10 and 11% on GDs 18–21, compared with the controls, the effect being statistically significant at each interval. At 75 mg/kg bw per day, maternal feed consumption was reduced by 21% on GDs 6–8, compared with the controls, the effect being statistically significant, but was comparable thereafter (Table 39).

No treatment-related maternal findings were observed at 20 mg/kg bw per day. No treatment-related findings were observed in dams at necropsy at any dose level.

There was no treatment-related effect on pregnancy rate. Pregnancy rate was 83% at 250 mg/kg bw per day, 96% at 75 mg/kg bw per day and 87% at 20 mg/kg bw per day and in the control group (Table 40).

Litter parameters, including number of live fetuses, early or late resorptions and dead fetuses, were unaffected by treatment. Mean fetal weight was reduced for both the combined and separate sexes by 6% at 250 mg/kg bw per day and by 2% at 75 mg/kg bw per day. There was no effect on mean fetal weight at 20 mg/kg bw per day (Table 40).

At least 18 litters with live fetuses were present in each group, and fetal evaluations were performed through external, visceral and skeletal examinations. No treatment-related external fetal observations were observed. The number of runt fetuses observed was very low, with no indication of a treatment-related effect (Table 41).

Table 39. Summary of selected maternal findings in a prenatal developmental toxicity study in rats

	0 mg/kg bw per day	20 mg/kg bw per day	75 mg/kg bw per day	250 mg/kg bw per day
Body weight (g)				
- GD 0	269.0	273.2	269.0	268.2
- GD 6	302.7	306.8	299.8	300.4
- GD 21	430.6	436.7	427.7	408.2
Body weight gain (g)				
- GDs 0–6	33.8	33.7	30.8	32.2
- GDs 6–8	5.7	4.6	−1.8**	−21.9**
- GDs 6–14	32.9	32.6	28.1	20.5**
- GDs 6–21	127.9	129.9	127.9	106.3*
Corrected body weight gain (g), GDs 0–21	60.4	59.1	56.3	34.9**
Feed consumption (g/day)				
- GDs 1–6	24.9	25.1	24.2	24.9
- GDs 6–8	24.9	24.0	19.7**	10.4**
- GDs 8–10	24.9	25.4	22.7	20.8*
- GDs 18–21	27.4	28.3	27.9	24.4*

bw: body weight; GD: gestation day; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2006)

Table 40. Summary of selected reproduction data in a prenatal developmental toxicity study in rats

	0 mg/kg bw per day	20 mg/kg bw per day	75 mg/kg bw per day	250 mg/kg bw per day
No. of animals mated	23	23	23	23
No. of animals pregnant	20	20	22	19
No. of animals with viable young at term	19	20	21	18
No. of animals with total resorption	1	0	1	0
No. of intercurrent deaths (with evidence of pregnancy)	0	0	0	1
No. of animals not pregnant	3	3	1	4
No. of females with implantations	20	20	22	19
No. of corpora lutea per dam	17.1	17.4	17.3	17.5
No. of implantations per dam	15.3	15.1	15.6	15.8
Preimplantation loss per dam (%)	10.0	14.4	8.9	8.6
No. of early/late resorptions per litter	0.5/0.1	0.7/0.1	1.0/0.0	0.9/0.2
No. of live fetuses per litter	14.7	14.3	14.6	14.7
No. of live fetuses	279	285	306	264
No. of litters with live fetuses	19	20	21	18
No. of dead fetuses/litters with dead fetuses	0/0	0/0	0/0	1/1
Postimplantation loss (% loss per female)	3.9	4.9	6.5	6.9
Male fetuses (%)	52.0	54.1	47.3	49.1
Male fetal weight (g)	5.72	5.65	5.58**	5.39**
Female fetal weight (g)	5.41	5.33	5.31*	5.09**

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2006)

Table 41. Summary of selected fetal findings in the prenatal developmental toxicity study in rats

	0 mg/kg bw per day	20 mg/kg bw per day	75 mg/kg bw per day	250 mg/kg bw per day
External findings				
No. of fetuses/litters examined	279/19	285/20	306/21	264/18
No. of runts	1/1	0/0	0/0	2/2
Visceral findings				
No. of fetuses/litters examined	135/19	137/20	148/21	127/18
Thymus misshapen	4/1	7/5	5/5	5/3
Ureter dilated	10/7	14/8	11/5	19/10
Renal pelvis dilated	4/3	4/3	5/4	9/4
Skeletal findings				
No. of fetuses/litters examined	144/19	148/20	158/21	137/18
Thoracic centrum: bipartite or dumbbell	1/1	1/1	3/3	3/3
5th sternebra: incomplete ossification, normal cartilage	20/9	21/13	25/13	36**/12
5th sternebra: unossified, normal cartilage	1/1	1/1	3/3	7*/3
14th thoracic rib(s), unilateral or bilateral: short	3/3	6/4	4/3	8/5
10th or 11th or 13th costal cartilage: discontinuous	3/2	3/2	4/2	6/5

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2006)

At the visceral examination, one fetus at 250 mg/kg bw per day was observed with the malformation diaphragmatic hernia. In isolation, this finding was considered to have occurred by chance. There was one fetus at 75 mg/kg bw per day and one fetus in the control group with the malformation renal papilla absent. In isolation and with one of the cases occurring in the control group, this malformation was considered to be fortuitous. No other malformations were noted at visceral examination. The incidence of the following variant findings was higher at both the fetal and litter levels than in the control group: misshapen thymus at all three dose levels, although not in a dose-related manner; and dilated ureter and dilated renal pelvis at 250 mg/kg bw per day (Table 41). However, as all three parameters were well within the in-house historical control range at both the fetal and litter levels, they were considered to be fortuitous findings.

At the skeletal examination, no malformations were observed. The incidence of the anomaly thoracic centrum: bipartite or dumbbell/dumbbell cartilage was higher at 250 mg/kg bw per day in terms of mean percentage of fetuses affected and percentage of litters affected and was outside the in-house historical control range for both parameters. To further evaluate this finding, statistical analysis was performed, using both the fetus and the litter as the statistical unit. The slightly higher incidence of this finding was found to be non-statistically significant at both the fetal and litter levels and was therefore considered to be a chance finding (Table 41).

The incidence of the following three variant findings was higher than in the control group at the specified dosage and was outside the in-house historical control range: 1) 5th sternebra: incomplete ossification/normal cartilage, at the fetal level, at 250 mg/kg bw per day; 2) 5th sternebra: unossified/normal cartilage, at both the fetal and litter levels at 250 mg/kg bw per day and at the litter level only at 75 mg/kg bw per day; and 3) 14th thoracic ribs (unilateral or bilateral): short, at both the fetal and litter levels at 250 and 20 mg/kg bw per day and at the fetal level only at 75 mg/kg bw per day. For the following variant finding, no in-house historical data exist, as this was the first study in which this combination of effects has been scored: 10th or 11th or 13th costal cartilage (unilateral or bilateral): discontinuous, higher at both the fetal and litter levels at 250 mg/kg bw per day compared with the controls. To further evaluate the relevance of these findings, statistical analysis was performed on the data using both the fetus and the litter as the statistical unit. The only findings where

a statistically significant effect was observed were 5th sternebra: incomplete ossification, normal cartilage and 5th sternebra: unossified, normal cartilage, at the fetal level only, at 250 mg/kg bw per day (Table 41). The two other findings were considered to have occurred by chance.

The NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain and feed consumption in the first days of treatment (i.e. GDs 6–8) at 75 mg/kg bw per day and above.

The NOAEL for embryo and fetal toxicity was 20 mg/kg bw per day, based on decreased fetal weights at 75 mg/kg bw per day and above (Wason, 2006).

Rabbits

In a prenatal developmental toxicity study conducted according to OECD Test Guideline 414, groups of 23 mated female New Zealand White rabbits were administered bixafen (purity 95.8%) in 0.5% aqueous methyl cellulose at a dosing volume of 4 mL/kg bw by oral gavage at a dose of 0, 25, 100 or 400 mg/kg bw per day from GD 6 to GD 28. Maternal body weights and feed consumption were recorded for all surviving females at suitable intervals from GD 3 to GD 29. Clinical observations were recorded daily. At scheduled sacrifice, on GD 29, the gravid uterine weight and the number of ribs were recorded, and the dams were evaluated for number of corpora lutea and number and status of implantations (resorptions, dead and live fetuses). The liver was weighed for all pregnant females and retained from all females. Live fetuses were removed from the uteri, counted, weighed and examined externally. The heads of fetuses from approximately half of each litter were immersed in Bouin's fluid, and the internal structures were examined after fixation. The bodies of all fetuses were dissected for soft tissue anomalies and sexed. Fetuses were eviscerated, skinned and fixed in absolute ethanol before staining, and a subsequent skeletal examination was performed.

At the highest dose level tested (400 mg/kg bw per day), eight females were humanely killed before the end of the study (Table 42). Three females were humanely killed after spontaneously aborting, one on GD 18 and two on GD 26. On the day of termination, the females had red traces and/or fetal/placental tissue on the tray. Two females had generalized hair loss with scab-like lesions on the skin of the neck area. The three females had a body weight loss of between 0.32 and 0.77 kg from GD 6 to termination, which was associated with very poor feed consumption several days before termination.

Five females at 400 mg/kg bw per day were humanely killed on GDs 11, 15, 16 and 22 (two females) without spontaneously aborting. Principal clinical signs observed in these females included no/few faeces, no urine, scabs/skin lesions and generalized hair loss on one or more occasions. The five females lost between 0.34 and 0.72 kg in body weight from GD 6 to the day of termination, which was associated with poor feed consumption. At necropsy, four of the eight females had white foci on the liver, and three showed prominent lobulation of the liver.

Treatment-related clinical signs in surviving females at 400 mg/kg bw per day consisted of no/few faeces noted for 9/15 females on one or more occasions, no urine in 4/15 females, scabs/skin lesions in 5/15 females and hair loss in 11/15 females, on two or more occasions. At 100 mg/kg bw per day, treatment-related signs were confined to 8/23 females with hair loss and with no or few faeces on one or more occasions, 2/23 with no urine on GDs 18–19 and 2/23 with a red vaginal discharge on GDs 28–29 (Table 42). At 25 mg/kg bw per day, vaginal discharge with red traces on the tray on GDs 26–27 was seen in 1/23 females, and 1/23 females had red traces on GD 28.

At 400 mg/kg bw per day, there was a body weight loss of 0.08 kg between GD 6 and GD 8 and a loss of 0.02 kg between GDs 10 and 14, compared with a body weight gain of 0.02 kg and 0.07 kg, respectively, over the corresponding period in the control group. Thereafter, body weight gain tended to be less at the high dose compared with the controls, resulting in an overall reduction in body weight gain of 59% by GD 29, the effect being statistically significant for periods of GDs 6–8 and GDs 18–22. Body weight change at 100 mg/kg bw per day was slightly less than that of controls over several periods, with a resulting overall reduction of 29% between GDs 6 and 28. At 25 mg/kg bw per

day, body weight gain was comparable with that of the controls. Maternal corrected body weight change was less in all three dose groups (−0.29, −0.25 and −0.23 kg, respectively, for the 400, 100 and 25 mg/kg bw per day groups) compared with the controls (−0.15 kg), but the effect was not statistically significant (Table 42).

Table 42. Summary of selected maternal findings in the prenatal developmental toxicity study in rabbits

	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day
No. of animals/no. of intercurrent deaths	23/0	23/0	23/0	23/8
No. of animals with hair loss	3	1	8	18
No. of animals with no faeces/few faeces	0/1	0/1	2/8	5/17
No. of animals with no urine	0	0	2	10
Body weight (kg)				
- GD 6	3.47	3.47	3.45	3.44
- GD 14	3.57	3.57	3.55	3.36
- GD 22	3.72	3.67	3.64	3.42
- GD 29	3.81	3.75	3.69	3.62
Body weight change (kg)				
- GDs 6–8	0.02	−0.01	−0.01	−0.08**
- GDs 6–14	0.10	0.10	0.10	−0.07*
- GDs 6–18	0.18	0.17	0.16	−0.05**
- GDs 6–26	0.31	0.25	0.21	0.08**
Corrected body weight change (kg), GDs 0–29	−0.15	−0.23	−0.25	−0.29
Feed consumption (g/day)				
- GDs 3–6	155	153	154	162
- GDs 6–8	165	161	163	120
- GDs 10–14	155	164	161	105
- GDs 14–18	155	158	134	85**
- GDs 18–22	163	154	143	108*
- GDs 22–26	130	119	102*	100*
Liver weight (g), pregnant females	92.8	91.2	102.1*	116.2**

bw: body weight; GD: gestation day; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2007)

Feed consumption at 400 mg/kg bw per day was reduced by 23–45% at each interval compared with the controls. The effect was most pronounced and statistically significant between GDs 14 and 18, GDs 18 and 22 and GDs 22 and 26. Feed consumption at 100 mg/kg bw per day was reduced at each interval between GD 14 and GD 26 by between 12% and 21%, but the effect was statistically significant only between GDs 22 and 26. Feed consumption was similar to that of controls at all intervals at 25 mg/kg bw per day.

At necropsy, liver weight was increased by 25% and 10% at 400 and 100 mg/kg bw per day, respectively, compared with controls (Table 42). In females at 400 mg/kg bw per day surviving to scheduled termination, white foci on the liver were observed in 4/15 females, with 1/15 females also having prominent lobulation and a depression in the median lobe of the liver. Enlarged urinary bladder containing purulent-like urine was noted for 1/25 females.

At 400 mg/kg bw per day, the percentage of dead fetuses was 8.1%, compared with 3.6% in controls. Also at 400 mg/kg bw per day, postimplantation loss was slightly higher (11.9%) than in the control group (8.4%), and combined fetal weights were decreased by 6%, which was statistically significantly lower than in controls. At 100 mg/kg bw per day, the only litter finding was a statistically significant lowering of male fetal weights by 5%, whereas female fetal weights were not different from those of the controls. No treatment-related litter findings occurred at 25 mg/kg bw per day (Table 43).

Table 43. Summary of selected reproduction data in the prenatal developmental toxicity study in rabbits

	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day
No. of animals mated	23	23	23	23
No. of animals pregnant	22	22	21	22
No. of animals reaching term with viable young	22	22	21	14
No. of animals reaching term with total resorption	0	0	0	1
No. of animals aborted	0	0	0	3
No. of intercurrent deaths with evidence of pregnancy	0	0	0	4
No. of animals reaching term/not pregnant	1	1	2	0
No. of intercurrent deaths with no evidence of pregnancy	0	0	0	1
No. of corpora lutea per dam	11.0	11.7	11.3	11.4
No. of implantation sites per dam	9.6	10.0	9.7	9.9
Preimplantation loss per dam (%)	12.6	14.7	14.8	12.0
No. of early resorptions per litter	0.4	0.5	0.3	0.4
No. of late resorptions per litter	0.1	0.0	0.0	0.1
No. of live fetuses per litter	8.8	9.4	9.0	8.6
No. of live fetuses	193	206	189	121
No. of litters with live fetuses	22	22	21	14
No. of dead fetuses	8	2*	8	12*
No. of litters with dead fetuses	4	2	4	5
Percentage of dead fetuses per litter	3.6	0.9	3.6	8.1
Postimplantation loss per litter (%)	8.4	5.7	6.1	11.9
Male fetuses (%)	53.8	56.3	49.7	57.7
Fetal weight (g)	37.6	37.6	36.3	35.2*
Male fetal weight (g)	38.6	37.8	36.5*	35.9
Female fetal weight (g)	36.4	37.4	36.0	34.4

bw: body weight; *: $P < 0.05$
Source: Wason (2007)

At caesarean section, there were 22, 22, 21 and 14 females with live fetuses in the control, 25, 100 and 400 mg/kg bw per day groups, respectively.

At the external examination, there were no treatment-related malformations at any dose level. The number of runt fetuses (body weight < 28 g) was increased at 400 mg/kg bw per day, where the mean percentage of fetuses classified as runts was 20% and the percentage of litters affected was 50%, compared with 6.3% and 27.3%, respectively, in the control group (Table 44).

Table 44. Summary of selected fetal findings in the prenatal developmental toxicity study in rabbits.

	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day
External findings				
No. of fetuses/litters examined	193/22	206/22	189/21	121/14
No. of runts	15/6	10/6	17/5	22/7
Visceral findings				
No. of fetuses/litters examined	193/22	206/22	189/21	121/14
Innominate artery: short	1/1	0/0	3/3	2/2
Subclavian artery (right): retro-oesophageal	1/1	2/2	3/2	6/4
Ureter (unilateral): convoluted/dilated	0/0	0/0	2/2	2/2
Renal pelvis (unilateral): dilated	0/0	0/0	0/0	3/2
Kidney: enlarged	0/0	0/0	0/0	3/3
Skeletal findings				
No. of fetuses/litters examined	193/22	206/22	189/21	121/14
Internasal or interfrontal suture: extra ossification site	0/0	0/0	0/0	3/2
Nasal split (unilateral)	1/1	0/0	1/1	2/2
At least one sternebra (except 5th or 6th sternebra) incompletely ossified	0/0	2/2	1/1	3/3
6th sternebra: unossified	6/4	4/2	8/6	11/6
1st metacarpal: unossified	9/5	4/2	3/2	10/8
1st proximal phalanx of the forepaw: unossified	0/0	0/0	1/1	2/2
Insertion point of pelvic girdle: unossified	1/1	2/1	1/1	6/3
Pubis (unilateral or bilateral): incompletely ossified	2/2	0/0	1/1	5/4
Pubis (bilateral): unossified	1/1	0/0	0/0	2/2
Astragalus (unilateral or bilateral): unossified	2/1	0/0	0/0	2/2

bw: body weight
Source: Wason (2007)

At the visceral examination, there were no treatment-related malformations at any dose level. At 400 mg/kg bw per day, a number of visceral findings occurred, including 1) innominate artery: short, 2) subclavian artery (right): retro-oesophageal; 3) ureter (unilateral): convoluted and dilated; 4) renal pelvis (unilateral): dilated (not severe); and 5) enlarged kidney (unilateral or bilateral). The small sample size in the high-dose group precluded a robust comparison (Table 44).

At 100 mg/kg bw per day, the only visceral anomaly that was increased over that of controls was ureter (unilateral): convoluted and dilated. However, statistical analysis found that this was not statistically significant at either the fetal or litter level, and therefore it was considered not to be treatment related. Also at 100 mg/kg bw per day, the variation innominate artery: short was increased over controls. However, the fetal incidence for this finding was within the in-house historical control range and only marginally outside the historical control range for litters, neither being statistically significant. Therefore, this variant finding was concluded to be unrelated to treatment.

At the skeletal examination, no malformations were observed. Several skeletal variations at 400 mg/kg bw per day were seen to be elevated in comparison with controls and historical control ranges. All of these variants related to ossification changes consistent with a delay in development. The findings included 1) internasal or interfrontal suture: extra ossification site, 2) nasal split, 3) at least one sternebra (except 5th or 6th sternebrae) incompletely ossified, 4) 6th sternebra unossified, 5) 1st metacarpal unossified, 6) 1st proximal phalanx of the forepaw unossified, 7) insertion point of

pelvic girdle unossified, 8) pubis unossified or incompletely ossified and 9) astragalus unossified. There were no treatment-related skeletal variations seen at 25 or 100 mg/kg bw per day (Table 44).

In conclusion, the numerous variants and anomalies observed at 400 mg/kg bw per day were considered to result from the severe maternal toxicity produced (32% mortality, from dramatic reductions in feed intake and body weight gain), which were clear signs that the maximum tolerated dose had been exceeded in pregnant rabbits.

The NOAEL for maternal toxicity was 25 mg/kg bw per day, based on clinical signs (hair loss, no or reduced excreta) and a reduction in body weight gain and feed consumption at 100 mg/kg bw per day and above.

The NOAEL for embryo and fetal toxicity was 25 mg/kg bw per day, based on reduced fetal weight at 100 mg/kg bw per day and above (Wason, 2007).

2.6 Special studies

(a) Analyses of diet for vitamin K_3 content

In the initial chronic toxicity and carcinogenicity study in rats, there was a significantly increased incidence of mortalities in the male rats at a dose level of 1000 ppm, with deaths occurring from study day 33 (McElligott, 2008). The analysis of the diet used in this study (batch 50607) showed that the level of vitamin K was very significantly lower than the estimate of the requirement of 1 mg/kg for the Wistar rat, and discussions with the manufacturer of the diet revealed that it had moved manufacture of the diet from Villemoisson to Augy in the middle of 2004 and had implemented new radiation and storage sterilization procedures, which temporally coincided with the deficit in vitamin K in AO4C-10P1 diet. Based on the analyses, it was agreed with the manufacturers to supplement the diet with 15 mg/kg of the synthetic vitamin K_3 analogue menadione. The analysed levels of vitamin K_3 are presented in Table 45. The batches of diet used for the 28-day and 90-day rat and mouse studies were not retrospectively analysed. For reference, analysis of the Purina diet, which was used for the multigeneration studies, showed the level of vitamin K_1 to be 0.65 mg/kg and that of vitamin K_3 0.8 mg/kg, indicating that there was sufficient vitamin K in the diet used for the multigeneration studies. These studies were performed in a laboratory in the United States of America (USA), and the diet supplier was different.

The developmental toxicity studies were performed using pelleted diet, and these were not retrospectively analysed, as no specific problems were encountered. This may be due to several factors. For the rabbit, the vitamin K requirements may be different from those of the rat, and the diet supplied to them, although from the same supplier (SAFE), was different from the diet supplied for the rat. In addition, the duration of treatment was shorter. For the rat, the treatment (which was shorter) was only in females, which are less susceptible to vitamin K problems; in addition, the strain was the Sprague-Dawley and not the Wistar.

(b) Mechanistic studies on blood clotting parameters

In a mechanistic study, the effects of bixafen on blood coagulation parameters of male rats receiving either a vitamin K_3–deficient diet or a diet supplemented with vitamin K_3 in excess were investigated. Forty male Wistar rats were taken from the initial chronic toxicity and carcinogenicity study of bixafen in rats (McElligott, 2008) after approximately 6 months on treatment with 1000 ppm bixafen in the diet. These rats all exhibited prolonged PT and APTT and were randomly placed into one of two groups of 20: group 1 with the existing diet (1000 ppm bixafen; vitamin K_3 concentration < 0.3 ppm), and group 2 given the same diet (1000 ppm bixafen; with 16 ppm vitamin K_3 added). The rats were placed on these regimens for 28 days. The mean achieved doses at the dietary concentration of 1000 ppm bixafen were 41.0 and 41.5 mg/kg bw per day for group 1 and group 2, respectively. Animals were observed daily for mortality and clinical signs. Physical examinations were performed weekly. Body weight and feed consumption were recorded once weekly. Haematology parameters were determined on study days 1 (before the change in diet) and 15 and at the end of the study. All

Table 45. Analyses of diet for vitamin K_3 content

Study (reference)	In-life dates	Diet	Batches of diet	When used	Vitamin K_3 concentration (mg/kg)
Mouse, 28 day (Steiblen, 2004)	02/2004–04/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Mouse, 90 day (Steiblen, 2005a)	06/2004–09/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Rat, 28 day (Langrand-Lerche, 2004)	12/2003–01/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Rat, 90 day (Steiblen, 2005b)	04/2004–08/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Mouse, carcinogenicity (Langrand-Lerche, 2008)	05/2005–12/2006	A04C-10P1	50107	30/05/05–24/07/05	Not analysed
			50322	25/07/05–18/09/05	Not analysed
			50607	19/09/05–13/11/05	< 0.3
			50720	14/11/05–08/01/06	15.7
			51027	09/01/06–05/03/06	8.2
			60118	06/03/06–20/08/06	9.6
			60323	21/08/06–12/12/06	7.1
Rat, chronic toxicity and carcinogenicity (McElligott, 2008)	03/2005–04/2007	A04C-10P1	41108/41123	21/04/05–01/06/05	Not analysed
			50107/50322	02/06/05–24/08/05	Not analysed
			50510	25/08/05–05/10/05	Not analysed
			50607	06/10/05–12/10/05	< 0.3
			50720	13/10/05–28/12/05	15.7
			51027	29/12/05–03/05/06	8.2
			60118	04/05/06–18/10/06	9.6
			60323	19/10/06–07/02/07	7.1
			60816	08/02/07–04/04/07	10.6
Rat, chronic toxicity and carcinogenicity (Garcin, 2008)	11/2005–12/2007	A04C-10P1	51027	08/12/05–01/03/06	8.2
			60118	02/03/06–27/09/06	9.6
			60323	28/09/06–14/12/07	7.1
			60816	15/12/07–01/08/07	10.6
Rat, reproductive toxicity (Young, 2006)	07/2005–11/2005	Purina Mills Rodent Lab CH Chow 5002 Meal	–	–	0.8
Rat, two-generation reproductive toxicity (Young, 2007)	01/2006–10/2006	Purina Mills Rodent Lab CH Chow 5002 Meal	–	–	0.8
Rat, mechanistic study with vitamin K_3–deficient and vitamin K_3–supplemented diet	10/2005–11/2005	A04C-10P1	50607	Batch used for the whole study	< 0.3
			50720	Batch used for the whole study	15.7

Study (reference)	In-life dates	Diet	Batches of diet	When used	Vitamin K_3 concentration (mg/kg)
(Steiblen, 2006)					
Rat, 28-day mechanistic study	10/2005–11/2005	A04C-10P1	50720	Batch used for the whole study	15.7
(Blanck, 2006)					

animals were necropsied, selected organs were weighed and a range of tissues was sampled and fixed, but not examined.

Nine animals fed with 1000 ppm bixafen in the vitamin K_3–deficient diet died before the end of the study. Eight animals were killed for humane reasons between study days 2 and 15, and one animal was found dead on day 15. During the study, 7/9 decedent animals had swollen eyelids, and 3/9 had protruding eyes on one or more occasions. In addition, half-closed right eye, ocular discharge, damaged eye(s) during the blood sampling, white area on the right eye, wasted appearance, focal swelling of the head, uncoordinated movements, soiled fur around the anogenital region or around the nose, cold to touch, reduced motor activity, general pallor and hunched posture were noted in one or two males on one or more occasions. Most of these animals had a loss of body weight on day 15 when compared with day 8, which was considered to be a consequence of the blood sampling session (at day 15). At necropsy, some animals were noted to have a pale appearance and soiled fur. Dark content in the stomach and/or in the intestines, haemorrhage(s) in the eye and haematoma in the Harderian gland were noted in some animals, whereas multifocal haemorrhage(s) in the submaxillary gland, exorbital gland, skeletal muscle, subcutis, blood clot at the surface of the brain and spinal cord, and red foci in the prostate or thymus were observed in one or two male animals. Haemorrhagic syndrome was considered to be the most probable cause of death or premature sacrifice. All these findings were considered to be related to the vitamin K_3 deficiency of this diet.

Two male animals fed with 1000 ppm bixafen in the vitamin K_3–supplemented diet died before the end of the study. One animal was found dead at the beginning of the study (day 2). Prior to necropsy, this animal had right eyelid swollen and half-closed eye on day 1. At necropsy, this animal was noted with a red focus in the kidney, mottled red thymus, dark content in the stomach and damaged right eye due to blood sampling. This death probably occurred due to the previous exposure to the vitamin K_3–deficient diet, as this animal died at the beginning of the study. Another animal was killed for humane reasons on day 15, prior to necropsy; this animal had protruding eyes, swollen eyelids, focal swelling of the head, white area on the left eye and damaged eyes due to blood sampling on day 15. In addition, this animal was noted to have a body weight loss on day 15 when compared with day 8, which was considered to be a consequence of the blood sampling session (at day 15). At necropsy, damaged eyes due to blood sampling and haematoma in the Harderian gland were noted. This last finding is likely to be a consequence of the blood sampling procedure and was therefore considered to be incidental.

Most of the clinical signs described in the decedent animals were also found in the surviving animals. Some surviving animals fed with vitamin K_3–deficient diet were noted to have ocular discharge and swollen right eyelid on one or two occasions. In addition, one male was noted to have a protruding eye on one occasion. Some surviving animals fed with vitamin K_3–adequate diet had right eye half-closed, ocular discharge of the right eye and swollen right eyelid on one or two occasions. In this study, all the clinical observations described in the two groups were considered to be a consequence of the repeated blood sampling conducted in the present study and the previous study (McElligott, 2008). Most of the surviving animals of the two groups had a body weight loss on day 15 when compared with day 8, which was considered to be a consequence of the blood sampling session (at day 15).

On day 1 (before the change of diet), similar values were noted for PT and APTT between the two groups. In both groups, the mean values of the two parameters were markedly increased when

compared with the normal physiological range in this species kept under similar conditions. On days 15 and 29, after feeding with vitamin K_3–adequate diet, mean PT and APTT values were back to normal values for rats of this strain and age, whereas they remained significantly elevated in the group receiving the vitamin K_3–deficient diet (Table 46).

Table 46. Selected findings in the 28-day rat study (males only) with vitamin K_3–deficient diet or vitamin K_3–supplemented diet

	Group 1 (1 000 ppm) (< 0.3 ppm vitamin K_3)	Group 2 (1 000 ppm) (16 ppm vitamin K_3)
Premature deaths	9/19	2/17
PT (s)		
- Day 1	43.0	45.1
- Day 15	37.8	15.3**
- Day 29	34.0	16.0**
- Historical control data: mean (range)	14.48 (9.2–19.1)	
APTT (s)		
- Day 1	65.4	68.1
- Day 15	67.6	17.8**
- Day 29	57.4	18.7**
- Historical control data: mean (range)	23.16 (15.7–32.4)	

APTT: activated partial thromboplastin time; ppm: parts per million; PT: prothrombin time; *: $P < 0.05$; **: $P < 0.01$
Source: Steiblen (2006)

In conclusion, the deficiency of vitamin K_3 in the diet in combination with the administration of bixafen was considered to be the cause of mortalities, elevated coagulation parameter times and haemorrhagic syndrome observed in male rats from the chronic toxicity and carcinogenicity study, as reported above (Steiblen, 2006).

A further mechanistic study was conducted to determine if bixafen dietary exposure in male rats results in changes to blood clotting parameters or significantly affects coagulation factors when the vitamin K_3 level in feed is 16 ppm. Groups of 10 male Wistar Rj: WI (IOPS HAN) rats were fed diets containing bixafen (purity 95.8%) at a concentration of 0, 2000, 4500 or 10 000 ppm (equal to 0, 162, 375 and 828 mg/kg bw per day) for 28 days. Animals were observed daily for clinical signs and twice daily for mortality. Physical examinations were performed weekly. Body weight and feed consumption were recorded at least weekly. Haematology and clinical chemistry parameters (including coagulation parameters) were determined at the end of the study. All animals were necropsied, selected organs were weighed and a range of tissues was taken and fixed. Histological examinations were not performed.

There were no mortalities during the study. Clinical signs consisted of nasal discharge on day 2 only for 3/10 animals at 10 000 ppm and for 2/10 animals at 4500 ppm. No treatment-related clinical signs were noted at 2000 ppm.

Body weights at 10 000 ppm were reduced by 13% throughout the study, and the body weight gain was reduced by 31%. At 4500 ppm, body weights were reduced by 8–9%, with body weight gain reduced by 19%. At 2000 ppm, body weights were reduced by 2–3%, with body weight gain reduced by 8% (Table 47).

Feed consumption was reduced by 9–26% at 10 000 ppm during the course of the study and by 20% at 4500 ppm for the 1st week of treatment.

The haematological examinations revealed no toxicologically significant variations for the parameters assayed. At 10 000 and 4500 ppm, lower total leukocyte counts (−21% and −24%, respectively) were noted, with lower lymphocyte counts (−24% and −23%, respectively). However, no clear dose–effect relationship was observed; therefore, these slight changes were considered not to be toxicologically relevant. Concerning the coagulation parameters, there was no evidence for an increase in PT. The slight statistically significant decreases observed at 4500 and 10 000 ppm were not dose related and not believed to be toxicologically meaningful. The statistically significant increases of very low amplitude of factors IX and XII, observed at 2000, 4500 and 10 000 ppm, carry no known toxicological consequence (Table 47).

Table 47. Selected findings in the 28-day rat study (males only) with vitamin K_3–supplemented diet

	0 ppm	2 000 ppm	4 500 ppm	10 000 ppm
Body weight (g)				
- Day 1	211	213	212	214
- Day 8	264	256	240**	230**
- Day 15	310	304	284**	273**
- Day 22	352	343	323**	306**
- Day 28	381	370	349**	331**
Body weight gain (g)				
- Days 1–8	53	43*	28**	16**
- Days 1–15	99	91	72**	59**
- Days 1–22	141	130	111**	92**
- Days 1–28	170	157	137**	118**
Feed consumption (g/animal per day)				
- Day 8	25.3	23.1	20.3**	18.6**
- Day 15	27.5	25.5	25.9	24.2*
- Day 22	28.9	26.5	26.3	24.9**
- Day 28	28.3	26.6	26.7	25.8
White blood cell count (10^9/L)	16.8	14.0	12.8**	13.2**
Lymphocyte count (10^9/L)	13.4	10.9	10.3**	10.2**
PT (s)	14.70	13.85	13.34*	13.67*
Factor IX (s)	34.55	36.19**	36.69**	36.58**
Factor XII (s)	25.35	26.31*	26.51**	26.64**
Glucose (mmol/L)	5.93	5.02	4.47**	4.50**
Absolute liver weight (g)	10.70	13.09**	13.07**	13.39**
Relative liver weight (% of body weight)	3.06	3.82	4.04**	4.32**
Absolute thyroid weight (mg)	19.7	22.1	23.8	24.3
Relative thyroid weight (% of body weight, ×1 000)	5.65	6.50	7.37*	7.85**
Liver: enlarged	0/10	6/10	9/10	10/10
Liver: dark	0/10	4/10	7/10	3/10

ppm: parts per million; PT: prothrombin time; *: $P < 0.05$; **: $P < 0.01$
Source: Blanck (2006)

At necropsy at 10 000 ppm, liver weights were increased by 25–41%, and relative thyroid weight was increased by 39%. Enlarged livers were seen in all 10 animals, and dark livers in 3/10

animals. At 4500 ppm, liver weights were increased by 22–32%, with relative thyroid weight increased by 30%. Enlarged and dark livers were found in 9/10 and 7/10 animals, respectively. At 2000 ppm, absolute and relative liver weights were increased by 22% and 25%, respectively, and relative thyroid weight was increased by 15%. Dark and enlarged livers were seen in 4/10 and 6/10 animals, respectively (Table 47).

A NOAEL could not be identified, as decreased body weight gain and liver toxicity (increased [> 20%] liver weight, enlarged liver, dark liver) were observed at all doses. The LOAEL was 2000 ppm (equal to 162 mg/kg bw per day). The NOAEL for blood coagulation effects in male rats fed a vitamin K_3–supplemented diet was 10 000 ppm (equal to 828 mg/kg bw per day), the highest dose tested (Blanck, 2006).

(c) Mechanistic study on thyroid hormone levels

A mechanistic study was conducted to investigate the thyroid changes induced by bixafen in the rat by measuring plasma thyroid hormone levels (TSH, T_3 and T_4) and liver enzyme induction following continuous oral administration (gavage) for up to 14 days. Groups of 15 male and 15 female Wistar Rj: WI (IOPS HAN) rats were treated orally by gavage for 1, 3, 7 or 14 days with vehicle (aqueous 0.5% methyl cellulose 400) or bixafen (purity 95.8%) at a dose of 150 mg/kg bw per day. Clinical observations were performed daily, and body weight and feed intake were measured weekly for animals treated for 7 or 14 days only. A detailed physical examination was performed weekly during the treatment period, commencing on day 1. Animals were killed 24 hours after 1, 3, 7 or 14 daily doses. On each day of sacrifice, blood samples were taken for analysis of thyroid hormone (TSH, T_3 and T_4) concentrations in the plasma. On the last day (day 15), all animals were killed, the livers were sampled and weighed, and the livers from six animals of each sex per group were used to prepare microsomes for the assessment of hepatic cytochrome P450 content and P450 isoenzyme and uridine diphosphate–glucuronosyltransferase (UDPGT) activities. Terminal body weights were also recorded.

There were no mortalities, treatment-related clinical signs or effects on body weight in either sex. During the 1st week of treatment, feed consumption was reduced by 19% in females.

Hormonal investigations showed that treatment increased plasma TSH levels in females on days 3 (+70%), 7 (+93%) and 14 (+151%) and in males on day 14 (+81%). In addition, a slight transient reduction in T_3 levels was seen in females on days 3 (−23%) and 7 (−10%), and a tendency towards lower mean T_4 levels was seen in males on day 14 (−12%) (Table 48).

After 14 days of exposure, a dose of 150 mg/kg bw per day increased liver weight by 22–24% in both sexes. Total hepatic P450 content was increased by 17–20% in both sexes, EROD activity was increased by 30% in males and PROD activity was increased by 73–123% in both sexes. A considerable increase in BROD activity was seen in males (562%) and in females (451%) when compared with controls. A slight increase was also observed in mean UDPGT activity (55–60%) in both sexes (Table 48).

In summary, these data indicate that bixafen after 14 days at 150 mg/kg bw per day induces phase I and II hepatic enzymes (Rascle, 2008).

3. Observations in humans

There were no reports of adverse health effects in manufacturing plant personnel. Also, there were no reports of poisonings with bixafen.

Table 48. Selected findings in the 14-day mechanistic study in rats

	Males		Females	
	0 mg/kg bw per day	150 mg/kg bw per day	0 mg/kg bw per day	150 mg/kg bw per day
T_3 (nmol/L)				
- Day 1	0.895	0.894	0.884	0.845
- Day 3	0.829	0.779	0.946	0.724**
- Day 7	1.107	1.033	1.143	1.031
- Day 14	1.052	1.005	1.027	1.043
T_4 (nmol/L)				
- Day 1	55.5	53.4	35.4	33.2
- Day 3	45.8	45.4	34.4	31.1
- Day 7	50.7	45.4	36.5	32.7
- Day 14	48.9	43.1*	31.8	32.3
TSH (ng/mL)				
- Day 1	2.55	2.89	1.00	0.87
- Day 3	3.46	4.19	1.17	1.99**
- Day 7	3.81	4.64	1.31	2.53**
- Day 14	3.65	6.61**	1.46	3.67**
Absolute liver weight (g)	14.81	18.10**	8.79	10.91**
Relative liver weight (% of body weight)	3.46	4.11**	3.40	4.28**
Total P450 content (nmol/mg protein)	1.15	1.38*	1.11	1.30
BROD (pmol/min per milligram protein)	8.28	54.79**	2.70	14.87**
EROD (pmol/min per milligram protein)	26.32	34.37*	32.68	34.46
PROD (pmol/min per milligram protein)	5.65	12.62*	2.89	5.01*
UDPGT (nmol/min per milligram protein)	11.28	17.44**	7.12	11.40**

BROD: benzyloxyresorufin *O*-dealkylase; bw: body weight; EROD: ethoxyresorufin *O*-deethylase; PROD: pentoxyresorufin *O*-dependentylase; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; UDPGT: uridine diphosphate–glucuronosyltransferase; *: $P < 0.05$; **: $P < 0.01$
Source: Rascle (2008)

Comments

Biochemical aspects

In rats given [dichlorophenyl-U-^{14}C]bixafen orally by gavage, absorption was rapid and accounted for at least 83% of the total administered radioactivity after a single low dose (2 mg/kg bw). The maximum plasma concentrations of radioactivity were reached approximately 2–4 and 8 hours after administration of the low and high doses (2 and 50 mg/kg bw), respectively. Radioactivity was widely distributed throughout the body. Elimination of the radioactivity was mainly via faeces (> 91%), whereas elimination via urine accounted for 1–3% of the administered dose. In bile duct–cannulated rats, extensive biliary excretion (up to 83%) was demonstrated. Elimination of the radioactivity from the body was rapid, with a half-life in plasma of 8–9 hours and a mean residence time of 13–19 hours (for the low dose). Residues in tissues at 72 hours after a single oral dose as well as after repeated oral dosing accounted for 0.1–3% of the administered radioactivity, with liver and kidneys containing the highest concentrations of residues.

Metabolism of bixafen in rats was extensive, and more than 30 metabolites were identified. The main metabolic routes included demethylation, hydroxylation of the parent and bixafen-

desmethyl, and conjugation with glucuronic acid or glutathione. A minor metabolic reaction was the cleavage of the amide bridge of bixafen.

Toxicological data

The LD_{50} in rats treated orally or dermally with bixafen was greater than 2000 mg/kg bw, and the inhalation LC_{50} in rats was greater than 5.38 mg/L. Bixafen was not a skin irritant in rabbits, was not irritating to the eye of rabbits and was not a skin sensitizer in the local lymph node assay in mice.

Following repeated administration of bixafen, the liver was the primary target organ in mice, rats and dogs. Increased liver weights and hepatocellular hypertrophy were observed in all species tested and were considered to reflect hepatic microsomal enzyme induction. Also, in several studies, there was evidence for liver toxicity based on clinical chemistry changes (increased serum alkaline phosphatase and cholesterol, decreased serum albumin) and histopathological changes (hepatocellular pigmentation, degeneration and necrosis). In mice and rats, the thyroid was an additional target, which was considered to be secondary to the enhanced hepatic clearance of thyroid hormones. This suggestion was supported by a 14-day mechanistic study in rats in which a marked induction of phase I and II hepatic enzymes, a slight reduction of thyroid hormone (T_3, T_4) levels and a significant increase of TSH levels were observed at 150 mg/kg bw per day, the only dose tested.

In a 4-week study in mice using dietary concentrations of 0, 100, 500 and 2500 ppm (equal to 0, 17, 81 and 305 mg/kg bw per day for males and 0, 21, 103 and 424 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 17 mg/kg bw per day), based on liver toxicity (increased liver weight, clinical chemistry changes, focal coagulative necrosis) at 500 ppm (equal to 81 mg/kg bw per day) and above. In a 13-week study in mice using dietary concentrations of 0, 50, 200 and 500 ppm (equal to 0, 8.5, 34.3 and 88 mg/kg bw per day for males and 0, 10.4, 42.9 and 110 mg/kg bw per day for females, respectively), the NOAEL was 50 ppm (equal to 8.5 mg/kg bw per day), based on liver toxicity in males (increased liver weight, clinical chemistry changes, diffuse hepatocellular vacuolation) and focal/multifocal squamous cell hyperplasia of the stomach in both sexes at 200 ppm (equal to 34.3 mg/kg bw per day) and above.

In a 4-week study in rats using dietary concentrations of 0, 50, 350 and 2000 ppm (equal to 0, 3.5, 25 and 137 mg/kg bw per day for males and 0, 4.1, 28 and 138 mg/kg bw per day for females, respectively), the NOAEL was 350 ppm (equal to 25 mg/kg bw per day), based on reduced body weight gain, reduced feed consumption, liver toxicity (increased liver weight, increased cholesterol level) and thyroid effects (hypertrophy of follicular cells) at 2000 ppm (equal to 137 mg/kg bw per day). In a 13-week study in rats using dietary concentrations of 0, 50, 200, 800 and 2000 ppm (equal to 0, 3.2, 12.9, 50.4 and 130 mg/kg bw per day for males and 0, 3.9, 15.0, 59.2 and 153 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 12.9 mg/kg bw per day), based on liver effects (enlarged liver, increased liver weight) and thyroid effects (hypertrophy of follicular cells) at 800 ppm (equal to 50.4 mg/kg bw per day) and above.

In a 13-week study in dogs testing dose levels of 0, 100, 300 and 1000 mg/kg bw per day by oral gavage, the NOAEL was 100 mg/kg bw per day, based on an increase (> 20%) in absolute and relative liver weights of females at 300 mg/kg bw per day and above. In a 1-year study in dogs testing dose levels of 0, 10, 100 and 1000 mg/kg bw per day by oral gavage, the NOAEL was 10 mg/kg bw per day, based on haematological effects (decrease in red blood cell count, haemoglobin and haematocrit) in males and liver toxicity (increased liver weight, increased alkaline phosphatase and cholesterol levels) in females at 100 mg/kg bw per day and above.

Long-term studies of toxicity and carcinogenicity were conducted in mice and rats. As a result of technical problems in the production of the feed, the vitamin K_3 level of the diet (< 0.3 ppm) used in the first 5–6 months of the studies was significantly lower than the estimated requirement for mice and rats (approximately 1 ppm), with the consequence of a haemorrhagic syndrome and increased mortality, especially in the male animals from the high-dose group of mice and the mid-dose and high-dose groups of rats. After approximately 6 months of treatment, the diet was supplemented with 7–16 ppm of the synthetic vitamin K analogue menadione, and the studies were completed as

scheduled, with the exception of the male rats, which were prematurely terminated after approximately 6–8 months of treatment. An additional study of chronic toxicity and carcinogenicity was therefore conducted in male rats (see below).

In the 78-week study of carcinogenicity in mice using dietary concentrations of 0, 50, 150 and 500 ppm (equal to 0, 6.7, 20.4 and 69.0 mg/kg bw per day for males and 0, 8.6, 25.5 and 85.0 mg/kg bw per day for females, respectively), there was no evidence for carcinogenicity up to the highest dose tested (500 ppm, equal to 69.0 mg/kg bw per day). The NOAEL for toxicity was 50 ppm (equal to 6.7 mg/kg bw per day), based on thyroid effects (follicular cell hyperplasia) in females and decreased body weights and liver toxicity (single-cell degeneration/necrosis) in males at 150 ppm (equal to 20.4 mg/kg bw per day) and above.

In the initial 24-month study of toxicity and carcinogenicity in rats, which was completed as planned for females only, dietary concentrations of 0, 50, 300 and 2000 ppm (equal to 0, 2.8, 17.4 and 117 mg/kg bw per day, respectively) were tested. There was no evidence for carcinogenicity up to the highest dose tested (2000 ppm, equal to 117 mg/kg bw per day). The NOAEL for toxicity was 50 ppm (equal to 2.8 mg/kg bw per day), based on liver effects (increased cholesterol, higher incidence and/or severity of hepatocellular brown pigments and multinucleated hepatocytes) and thyroid effects (higher incidence and/or severity of follicular cell hypertrophy and colloid alteration) at 300 ppm (equal to 17.4 mg/kg bw per day) and above. In the complementary 24-month study of toxicity and carcinogenicity in male rats using a vitamin K_3–supplemented diet (7–11 ppm) and dietary concentrations of bixafen of 0, 50, 300 and 2000 ppm (equal to 0, 2.0, 12.1 and 80.5 mg/kg bw per day, respectively), there was no evidence for carcinogenicity up to the highest dose tested (2000 ppm, equal to 80.5 mg/kg bw per day). The NOAEL for toxicity was 50 ppm (equal to 2.0 mg/kg bw per day), based on liver effects (increased cholesterol levels, increased liver weights) and thyroid effects (higher incidence and/or severity of colloid alteration) at 300 ppm (equal to 12.1 mg/kg bw per day) and above.

The Meeting concluded that bixafen is not carcinogenic in mice or rats.

Bixafen was tested for genotoxicity in vitro and in vivo in an adequate range of assays. There was no evidence of genotoxicity.

The Meeting concluded that bixafen is unlikely to be genotoxic.

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

In a two-generation study of reproductive toxicity in rats using dietary concentrations of 0, 50, 400 and 2500 ppm (equal to 0, 3.3, 26.4 and 169.2 mg/kg bw per day for males and 0, 3.9, 30.8 and 193.7 mg/kg bw per day for females, respectively), the NOAEL for reproductive toxicity was 2500 ppm (equal to 169.2 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 400 ppm (equal to 26.4 mg/kg bw per day), based on a reduction in body weight and liver effects (liver weight increased > 20%) at 2500 ppm (equal to 169.2 mg/kg bw per day). The NOAEL for offspring toxicity was 400 ppm (equal to 26.4 mg/kg bw per day), based on a slight elevation in stillbirths and reduced pup weight/weight gain during lactation at 2500 ppm (equal to 177.6 mg/kg bw per day).

In a developmental toxicity study in rats testing dose levels of 0, 20, 75 and 250 mg/kg bw per day, the NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain and feed consumption at 75 mg/kg bw per day and above in the first days of treatment (i.e. GDs 6–8). The NOAEL for embryo and fetal toxicity was 20 mg/kg bw per day, based on decreased fetal weights at 75 mg/kg bw per day and above.

In a developmental toxicity study in rabbits testing dose levels of 0, 25, 100 and 400 mg/kg bw per day, the NOAEL for maternal toxicity was 25 mg/kg bw per day, based on clinical signs (hair loss, no or reduced excreta) and a reduction in body weight gain and feed consumption at 100 mg/kg bw per day and above. The NOAEL for embryo and fetal toxicity was 25 mg/kg bw per day, based on reduced fetal weight at 100 mg/kg bw per day and above.

The Meeting concluded that bixafen is not teratogenic in rats or rabbits.

A study using high-dose male rats from the initial 24-month study of toxicity and carcinogenicity provided evidence that the low vitamin K_3 level of the diet (< 0.3 ppm) was the cause of the haemorrhagic syndrome, as the prolonged blood coagulation time could be reversed by a vitamin K_3–supplemented diet (16 ppm). This conclusion was supported by the fact that no adverse effects on blood coagulation were observed in the multigeneration studies using diets with an adequate level of vitamin K (vitamin K_1: 0.65 ppm; vitamin K_3: 0.8 ppm).

In a 28-day study in male rats using a vitamin K_3–supplemented diet (16 ppm) and dietary concentrations of bixafen of 0, 2000, 4500 and 10 000 ppm (equal to 0, 162, 375 and 828 mg/kg bw per day, respectively), the NOAEL for effects of bixafen on blood coagulation parameters was 10 000 ppm (equal to 828 mg/kg bw per day), the highest dose tested.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. Also, there were no reports of poisonings with bixafen.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for bixafen of 0–0.02 mg/kg bw, based on the NOAEL of 2.0 mg/kg bw per day for liver and thyroid effects observed at 12.1 mg/kg bw per day in the 24-month study of toxicity and carcinogenicity in male rats. A safety factor of 100 was applied.

The Meeting established an acute reference dose (ARfD) for bixafen of 0.2 mg/kg bw, based on the NOAEL of 20 mg/kg bw for decreased body weight gain and feed consumption observed in the first days of treatment at 75 mg/kg bw in a developmental toxicity study in rats. A safety factor of 100 was applied.

As the estimated exposures to M18, M20, M44, M45 and M47 are below the respective acute and chronic thresholds of toxicological concern for Cramer class III compounds, there is no concern for these metabolites. Bixafen-desmethyl has been tested in rodents through its formation from the parent compound and is therefore covered by the ADI for bixafen. For M25 and M26, their structural similarity to bixafen-desmethyl is such that the Meeting concluded that they would also be covered by the ADI for bixafen.

Levels relevant to risk assessment of bixafen

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 6.7 mg/kg bw per day	150 ppm, equal to 20.4 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 69.0 mg/kg bw per day[b]	–
Rat	Two-year studies of toxicity and carcinogenicity[a,c]	Toxicity	50 ppm, equal to 2.0 mg/kg bw per day	300 ppm, equal to 12.1 mg/kg bw per day
		Carcinogenicity	2 000 ppm, equal to 80.5 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	2 500 ppm, equal to 169.2 mg/kg bw per day[b]	–
		Parental toxicity	400 ppm, equal to 26.4 mg/kg bw per day	2 500 ppm, equal to 169.2 mg/kg bw per day
		Offspring toxicity	400 ppm, equal to 26.4 mg/kg bw per day	2 500 ppm, equal to 177.6 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	20 mg/kg bw per day	75 mg/kg bw per day
		Embryo and fetal toxicity	20 mg/kg bw per day	75 mg/kg bw per day
Rabbit	Developmental toxicity study[d]	Maternal toxicity	25 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	25 mg/kg bw per day	100 mg/kg bw per day
Dog	One-year study of toxicity[d]	Toxicity	10 mg/kg bw per day	100 mg/kg bw per day

bw: body weight; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level; ppm: parts per million

[a] Dietary administration.
[b] Highest dose tested.
[c] Two studies combined.
[d] Gavage administration.

Estimate of acceptable daily intake

0–0.02 mg/kg bw

Estimate of acute reference dose

0.2 mg/kg bw

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 bixafen

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; ≥ 83%
Dermal absorption	No data
Distribution	Widely distributed; highest concentrations in liver and kidneys
Potential for accumulation	None
Rate and extent of excretion	> 93% within 72 h (> 91% in faeces, including up to 83% in bile; 1–3% in urine)

Metabolism in animals	Extensive (> 30 metabolites identified); demethylation, hydroxylation of parent and bixafen-desmethyl; conjugation with glucuronic acid and glutathione; cleavage of the amide bridge of bixafen as a minor metabolic reaction
Toxicologically significant compounds in animals, plants and the environment	Bixafen
Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.38 mg/L (4 h, nose-only exposure)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Mouse, dermal sensitization	Not sensitizing (local lymph node assay)
Short-term studies of toxicity	
Target/critical effect	Liver in mice, rats and dogs; thyroid in rats
Lowest relevant oral NOAEL	8.5 mg/kg bw per day (mouse)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver and thyroid in mice and rats
Lowest relevant NOAEL	2.0 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	26.4 mg/kg bw per day
Lowest relevant offspring NOAEL	26.4 mg/kg bw per day
Lowest relevant reproductive NOAEL	169.2 mg/kg bw per day, the highest dose tested
Developmental toxicity	
Target/critical effect	Reduced fetal weights, visceral or skeletal variations at maternally toxic dose (rats and rabbits)
Lowest relevant maternal NOAEL	20 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	20 mg/kg bw per day (rat)
Neurotoxicity	
Acute and subchronic neurotoxicity	No specific data, but no indications from repeated-dose studies
Other toxicological studies	
Study on blood coagulation	Vitamin K_3–deficient diet contributed to prolonged blood coagulation times and haemorrhagic effects in male rats
Mechanistic study on thyroid effects	Induction of phase I and II hepatic enzymes was likely the cause of the observed thyroid hormone changes
Medical data	
	No adverse health effects reported in manufacturing plant personnel

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Two-year study of toxicity and carcinogenicity in rats	100
ARfD	0.2 mg/kg bw	Developmental toxicity study in rats (maternal toxicity)	100

ADI: acceptable daily intake; ARfD: acute reference dose; bw: body weight

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