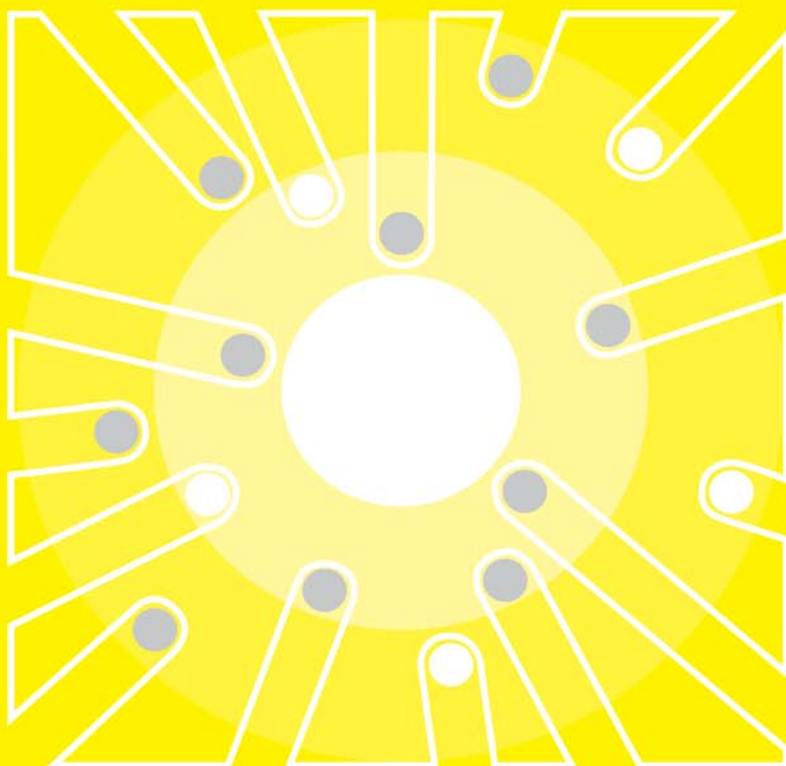
WHO FOOD ADDITIVES SERIES: 69

Toxicological evaluation of certain veterinary drug residues in food

Prepared by the
Seventy-eighth meeting of the Joint FAO/WHO Expert
Committee on Food Additives (JECFA)

Food and Agriculture Organization of the United Nations

World Health Organization



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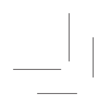
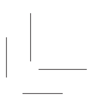
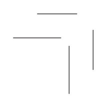
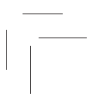

PREFACE

The monographs contained in this volume were prepared at the seventy-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 5–14 November 2013. These monographs summarize the data on the safety of residues in food of selected veterinary drugs reviewed by the Committee.

The seventy-eighth report of JECFA has been published by WHO as WHO Technical Report No. 988. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication; a summary of the conclusions of the Committee is given in Annex 4. Some of the substances listed in Annex 4 were considered at the meeting for residue evaluation only.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The toxicological monographs contained in this volume are based on working papers that were prepared by WHO experts (in the case of recombinant bovine somatotropins, a joint monograph was prepared by both WHO and FAO experts). A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by M. Sheffer, Ottawa, Canada.

Many unpublished proprietary reports are submitted to the Committee by various producers of the veterinary drugs under review and in many cases represent the only data available on those substances. The WHO experts based the working papers they wrote on all the data that were submitted, and all these reports were available to the Committee when it made its evaluations.

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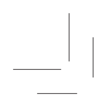
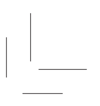
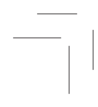
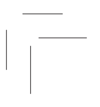
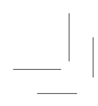
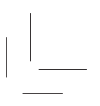
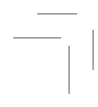
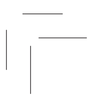
Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

RESIDUES OF VETERINARY DRUGS

GENTIAN VIOLET

First draft prepared by

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1. EXPLANATION

Gentian violet (Chemical Abstracts Service No. 548-62-9) has many common names, including CI Basic Violet 3, crystal violet and methyl violet 10B. It is a triphenylmethane dye with antibacterial, antifungal and anthelminthic properties. Gentian violet has been used for the treatment of fungal and parasitic infections in fish and topically for skin and eye infections in livestock. It was previously used in poultry feeds to inhibit the growth of mould and fungus; however, several countries have withdrawn approval or registration of this use.

In humans, gentian violet has been used as a hair dye, to treat gut parasites and for topical fungal treatment. It has also been used in human medicine to treat blood held for transfusions in order to prevent the transmission of Chagas disease caused by *Trypanosoma cruzi*. It also has activity as a topical antiviral agent.

Gentian violet is used in industrial processes for wood, leather, silk, nylon, paper and ribbon tapes and also as a biological stain. Contamination of the environment can occur, as about 10–15% of all dyes are lost directly to wastewater in the dyeing process. Gentian violet in water originating from contamination as a result of its industrial applications or from its illegal use in aquaculture is efficiently taken up from the water by fish.

Gentian violet has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). It was evaluated by the Committee at the current meeting at the request of the Twentieth Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2012), which asked for advice as to whether an acceptable daily intake (ADI) can be established and whether the continued use of gentian violet in food-producing animals is safe for humans.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Docampo & Moreno (1990) reviewed the metabolism and mode of action of gentian violet. No data were available on skin absorption of gentian violet in food-producing animals when the drug is applied topically, although Diamante et al. (2009) considered gentian violet to be poorly absorbed through human skin.

(a) Mice

Groups of 12 male and 12 female B6C3F1 mice were housed three per metabolism cage and given 14 doses of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpararosaniline) at 12-hour intervals by gavage. The total gentian violet doses were 5.6 mg/kg body weight (bw) (0.72 MBq/animal) and 7.1 mg/kg bw (0.72 MBq/animal) for males and females, respectively. The mice were killed 2 hours after receiving the final dose. Urine, faeces, liver, kidney, muscle, testes or ovaries and a fat sample were collected during the study, and radioactivity was measured (Table 1). The data show that gentian violet residues

Table 1. Disposition and excretion of multiple oral doses of ^{14}C-labelled gentian violet administered to mice

Sample	Gentian violet residues[a] (µg/g or µg/mL)	
	Males	Females
Liver	17.8 ± 2.6**	10.7 ± 3.4**
Kidney	1.6 ± 0.1**	2.7 ± 0.8**
Muscle	0.6 ± 0.4**	1.3 ± 0.7**
Gonad	0.49 ± 0.08	3.66 ± 1.08[b]
Fat	14.3 ± 3.0**	24.1 ± 7.0**
Urine	1.16 (5.9%)	1.58 (8.1%)
Faeces	12.89 (65.9%)	13.17 (67.4%)

**: $P < 0.01$ (student *t*-test for a significant sex difference)

[a] Values are means ± 1 standard deviation for 12 male and 12 female mice. Numbers in parentheses indicate the percentage of the total dose.

[b] Mean of eight mice.

Source: McDonald et al. (1984a); McDonald (1989)

were highest in adipose tissue (particularly in females), although a major portion (66–67%) was excreted in faeces (McDonald et al., 1984a; McDonald, 1989).

(b) Rats

Groups of three male and three female F344 rats were housed individually in metabolism cages and given a single dose by gavage of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpararosaniline). Gentian violet doses were 4.8 mg/kg bw (0.11 MBq/animal) and 5.2 mg/kg bw (0.34 MBq/animal) for males and females, respectively. Rats were killed 2, 4, 14 or 24 hours after dosing. Urine, faeces, liver, kidney, muscle, testes or ovaries and a fat sample were collected, and radioactivity was measured (Table 2). Half-lives of 14.5 and 14.4 hours were calculated following a single dose for the liver and kidney, respectively, for males and 17.0 and 18.3 hours, respectively, for females (McDonald et al., 1984a).

Groups of eight male and eight female F344 rats were housed individually in metabolism cages and given 14 doses of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpararosaniline) at 12-hour intervals by gavage. The total gentian violet doses were 3.5 mg/kg bw (5.2 MBq/animal) and 5.69 mg/kg bw (2.9 MBq/animal) for males and females, respectively. The rats were killed 2 hours after receiving the final dose. Urine, faeces, liver, kidney, muscle, testes or ovaries and a fat sample were collected, and radioactivity was measured (Table 3). As for mice, gentian violet residues concentrated in the adipose tissue of females; in males, the levels in liver and fat were similar. The percentages of administered gentian violet radioactivity excreted in the faeces of rats and mice were very similar, whereas considerably more was excreted in the urine of mice than in that of rats (McDonald et al., 1984a).

Two female bile duct–cannulated rats were administered a single dose of ^{14}C-labelled gentian violet (94.8% gentian violet and 5.2% pentamethylpararosaniline) by gavage at 300 µg (0.12 MBq) or 840 µg (0.34 MBq), and bile was

Table 2. Deposition and excretion of a single oral dose of ^{14}C-labelled gentian violet administered to rats[a]

Time after dose (h)	Tissue residue (µg/g or µg/mL)					Excretion (µCi)[b]	
	Liver	Kidney	Muscle	Testis/ ovary	Fat	Urine	Faeces
Males							
2	2.52 ± 0.75	0.48 ± 0.11	0.05 ± 0.01	0.03 ± 0.01	0.12 ± 0.05	0.045	0.001
4	3.51 ± 0.79	0.47 ± 0.04	0.05 ± 0.01	0.02 ± 0.02	0.12 ± 0.03	0.064	0.009
14	1.71 ± 0.15	0.22 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.50 ± 0.1	0.25	3.76
24	0.99 ± 0.14	0.13 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.66 ± 0.07	0.33	11.10
36	0.76 ± 0.12	0.10 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.72 ± 0.14	0.29 (2.2%)	9.55 (72.9%)
Females							
2	1.37 ± 0.28	0.48 ± 0.11	0.05 ± 0.01	0.03 ± 0.01	0.13 ± 0.01	0.025	0.001
4	2.84 ± 0.41	0.52 ± 0.11	0.15 ± 0.01	0.02 ± 0.02	0.42 ± 0.09	0.017	0.011
14	1.22 ± 0.19	0.23 ± 0.05	0.13 ± 0.05	0.04 ± 0.01	2.07 ± 0.36	0.11	4.39
24	1.11 ± 0.23	0.21 ± 0.06	0.16 ± 0.10	0.02 ± 0.01	3.30 ± 0.45	0.33	5.14
36	0.69 ± 0.15	0.14 ± 0.02	0.05 ± 0.01	0.02 ± 0.01	2.92 ± 0.77	0.20 (2.2%)	5.91 (63.8%)

[a] Values are means ± 1 standard deviation for three rats. Numbers in parentheses indicate percentage of the dose.
[b] 1 µCi = 37 kBq.
Source: McDonald et al. (1984a)

Table 3. Disposition and excretion of multiple oral doses of ^{14}C-labelled gentian violet administered to F344 rats[a]

Sample	Gentian violet residues (µg/g or µg/mL)	
	Males	Females
Liver	4.0 ± 0.6	3.7 ± 0.8
Kidney	0.7 ± 0.1**	2.9 ± 1.7**
Muscle	0.09 ± 0.03*	0.6 ± 0.5*
Gonad	0.08 ± 0.04	3.67 ± 0.76
Fat	3.2 ± 0.4**	20.2 ± 5.8**
Urine	3.18 (2.2%)	1.29 (1.6%)
Faeces	92.02 (65.5%)	58.04 (72.8%)

*: $P < 0.02$; **: $P < 0.01$ (student *t*-test for a significant sex difference)
[a] Values are means ± 1 standard deviation for seven male and eight female rats. Numbers in parentheses indicate the percentage of the total dose.
Source: McDonald et al. (1984a)

collected for 24 and 28 hours, respectively. The percentages of the oral dose collected from the two rats were 6.4% and 5.7% after 24 and 28 hours, respectively (McDonald et al., 1984a).

The authors concluded that orally administered gentian violet cation (which could be combined with a hydroxyl ion in the small intestine) with a relative molecular mass of 372 was absorbed to a greater extent than had been reported for other triphenylmethane dyes. The authors speculated that leucogentian violet, which is produced under anaerobic conditions by intestinal bacteria, may be absorbed and be preferentially taken up in the fat (McDonald et al., 1984a).

2.1.2 Biotransformation

(a) Bacteria

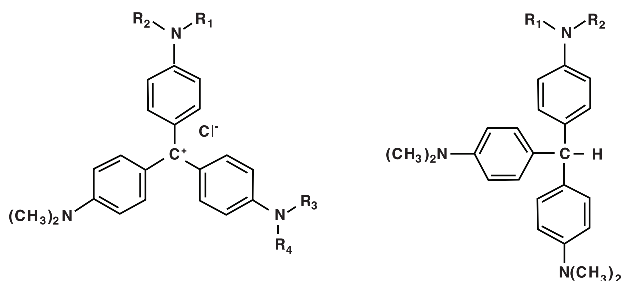
The biotransformation of gentian violet by cell suspensions of human, rat and chicken intestinal microflora and by 12 pure bacterial cultures has been studied. Incubations were carried out under anaerobic and aerobic conditions. All pure cultures and mixed intestinal microflora converted gentian violet to leucogentian violet. Gentian violet and leucogentian violet were identified in the incubation mixtures. The facultative anaerobes *Escherichia coli* and *Salmonella typhimurium* possessed little ability to reduce gentian violet under either anaerobic or aerobic conditions. Gentian violet at a concentration of 2.67 μg/mL of incubation medium was not toxic and did not inhibit bacterial growth when compared with control incubations (McDonald & Cerniglia, 1984).

(b) In vitro

The in vitro metabolism of gentian violet (*1a* in Fig. 1, hexamethylpararosaniline chloride, purity 98%) was studied in microsomes isolated from the livers of four strains of mice, three strains of rats, hamsters, guinea-pigs and chickens. Recovery of quantified products on a molar basis was only 30–35% from incubation mixtures with active microsomes containing gentian violet at 0.01 mmol/L. The gentian violet was demethylated to pentamethylpararosaniline chloride (*1b* in Fig. 1), *N*,*N*,*N*′,*N*′-tetramethylpararosaniline chloride (*1c* in Fig. 1) and *N*,*N*,*N*′,*N*″-tetramethylpararosaniline chloride (*1d* in Fig. 1). In general, microsomes from mice produced less demethylated products compared with microsomes from the other species. Microsomes from the guinea-pig produced less of *1c* and more of *1d* compared with microsomes from the other species. Demethylation differences between males and females were not apparent among the species (McDonald et al., 1984b; McDonald, 1989). The authors made no mention of leucogentian violet (*1e* in Fig. 1).

Gentian violet was metabolized under a nitrogen atmosphere by rat liver microsomes supplemented with reduced nicotinamide adenine dinucleotide phosphate (NADPH) to give a single-line electron spin resonance spectrum, which was considered to be from the tri-(*p*-dimethylaminophenyl) methyl radical. Elimination of the NADPH-generating system, use of heat-denatured microsomes or the presence of oxygen resulted in no electron spin resonance spectrum. This one-electron reduction to produce a carbon-centred free radical was inhibited approximately 50% by metyrapone and by an atmosphere of carbon monoxide (Harrelson & Mason, 1982).

Fig. 1. Structures of substituted pararosanilines



	R_1	R_2	R_3	R_4
1a	CH_3	CH_3	CH_3	CH_3
1b	CH_3	CH_3	CH_3	H
1c	CH_3	CH_3	H	H
1d	CH_3	H	CH_3	H

	R_1	R_2
1e	CH_3	CH_3
1f	CH_3	H

1a Gentian violet
1b Pentamethylpararosaniline chloride
1c *N*,*N*,*N′*,*N′*-tetramethylpararosaniline chloride
1d *N*,*N*,*N′*,*N″*-tetramethylpararosaniline chloride
1e Leucogentian violet
1f Leucopentamethylpararosaniline

(c) Mice

McDonald (1989) analysed the metabolites in tissues and faeces. Three demethylated metabolites (*1b*, *1c* and *1d* in Fig. 1) and two reduced metabolites, leucogentian violet (*1e*) and leucopentamethylpararosaniline (*1f*), were found in tissues and faecal extracts. Reduced metabolites (*1e* and *1f*) were predominant in tissues, and the parent compound was predominant in faeces.

(d) Rats

A female Fischer 344 rat was given 0.84 mg ^{14}C-labelled gentian violet (0.21 MBq) (94.8% gentian violet and 5.2% pentamethylpararosaniline) twice daily by gavage for 3 days, and faeces were collected for identification of metabolites. Leucogentian violet accounted for 11% of the radioactivity present in the 48- to 72-hour faeces collection (McDonald & Cerniglia, 1984).

McDonald (1989) also analysed the metabolites of gentian violet. In addition to the parent compound (*1a* in Fig. 1), demethylated metabolites (*1b*, *1c* and *1d*) and reduced metabolites (*1e* and *1f*) were identified. The highest metabolite concentrations observed were for reduced metabolites (*1e* and *1f*) in fat tissue. These metabolites were also found in other tissues. All five metabolites were also detected in faecal extracts, but the parent compound was more dominant in faeces than in other tissues.

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute oral toxicity of gentian violet has been reported by Hodge et al. (1972) and is summarized in Table 4.

Table 4. Results of studies of the acute oral toxicity of gentian violet

Species	Vehicle	LD_{50} (mg/kg bw)	Reference
Mouse	Water	405–570 (7-day follow-up)	Hodge et al. (1972)
Mouse	Propylene glycol	800 (7-day follow-up)	Hodge et al. (1972)
Rat	Propylene glycol	180 (7-day follow-up) 1 000 (24 h follow-up)	Hodge et al. (1972)
Guinea-pig	Propylene glycol	100–150	Hodge et al. (1972)
Rabbit	Propylene glycol	125–250	Hodge et al. (1972)
Cat	Propylene glycol	100–150	Hodge et al. (1972)
Dog	Propylene glycol	1 000	Hodge et al. (1972)

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

Hodge et al. (1972) also referred to unpublished results of Seppelin (1949), who identified an oral median lethal dose (LD_{50}) of 330 mg/kg bw in mice.

Hodge et al. (1972) reported that the most common sign of toxicity was lethargy, occurring approximately 1 hour after dosing, with anorexia in rats, rabbits, cats and dogs as the second most common sign. Ataxia occurred in mice, rats and guinea-pigs. Other signs seen, but not in all animals, were diarrhoea, excessive thirst, emesis and weight loss. Histological evidence of irritation, congestion and haemorrhage was also observed.

These animal data indicate that gentian violet is of moderate acute oral toxicity.

2.2.2 Short-term studies of toxicity

There are few published data describing short-term toxicity in laboratory animals.

(a) Rats

Littlefield et al. (1989) cited unpublished data from the United States Food and Drug Administration (USFDA, 1976) reporting on a 90-day study in rats that were fed gentian violet at levels up to 500 mg/kg in feed. Other than reporting a slight body weight loss, no other significant results were identified. No further details of the study were available.

(b) Dogs

Littlefield et al. (1989) cited the USFDA's (1976) unpublished data to report that a 90-day study in dogs was conducted by feeding gentian violet at levels up to 516 mg/kg in feed. Other than a liver weight increase, no other significant effects were identified. No other details of the study were available.

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a study compliant with good laboratory practice (GLP), 720 male and 720 female B6C3F1 (C57BL/6 × C3H) mice (approximately 4–5 weeks old) were fed gentian violet (99% gentian violet and 1% methyl violet) at a dietary concentration of 0, 100, 300 or 600 mg/kg feed (equal to 0, 10.7–14.3, 32.1–35.7 and 64.3 mg/kg bw per day for males and 0, 14.3, 35.7–39.3 and 71.4 mg/kg bw per day for females, respectively). The diets were certified to be within ±10% of target values. The allocation of the mice to the groups and sacrifice times are presented in Table 5. The mice were housed four per cage. Body weights, feed consumption and clinical signs were recorded weekly. The mice received a complete necropsy, histopathological examination and clinical chemistry analysis at the scheduled sacrifices after 12, 18 and 24 months of continuous dosing.

There was no effect on feed consumption or body weight gain; however, a dose-related effect was noted for mortality rates. Mortality (adjusted for sacrifices) in the controls of both sexes was less than 15% at 24 months, but was approximately 64% in the females and 23% in the males given the high dose. The histopathological findings are presented in Tables 6 and 7. Few dose-related non-neoplastic lesions were reported, but there were statistically significant dose-related increases in erythropoiesis in the spleen and atrophy of the ovaries in females at 24 months. A positive dose–response relationship for hepatocellular carcinoma was noted in males at 24 months and in females at 18 and 24 months. Statistical tests for dose-related trends with respect to 1) mortality due to liver neoplasms, 2) prevalence of liver neoplasms and 3) time to onset of liver neoplasms showed positive trends in both males and females (Table 8). Other dose-related toxicological responses, particularly in the female mice, included erythropoiesis in the spleen, atrophy of the ovaries, adenoma of the Harderian gland and the presence of type A reticulum cell sarcomas in the urinary bladder, uterus, ovaries and vagina.

Table 5. Experimental design for mice lifespan study

Dietary concentration (mg/kg feed)	Total number of mice		Interim sacrifice (12 months)		Interim sacrifice (18 months)		Terminal sacrifice (24 months)	
	Males	Females	Males	Females	Males	Females	Males	Females
0	288	288	48	48	48	48	192	192
100	144	144	24	24	24	24	96	96
300	144	144	24	24	24	24	96	96
600	144	144	24	24	24	24	96	96
Total	720	720	120	120	120	120	480	480

Source: Littlefield (1984); Littlefield et al. (1985)

Table 6. Microscopic histopathology summary of female mice

Site and lesion	12-month sacrifice[a]				18-month sacrifice[a]				24-month sacrifice[a]			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: benign neoplasm	0/48 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	3/47 (6%)	0/22 (0%)	3/24 (13%)	8/64 (33%)	8/185 (4%)	8/93 (9%)	36/93 (39%)	20/95 (21%)
Liver: malignant neoplasm	0/48 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	1/47 (2%)	0/22 (0%)	1/24 (4%)	3/24 (13%)	7/185 (4%)	5/93 (5%)	30/93 (32%)	73/95 (77%)
Uterus: RCS type A	0/47 (0%)	0/23 0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	0/22 (0%)	1/24 (4%)	1/24 (4%)	0/188 (0%)	2/95 (2%)	6/90 (0%)	12/93 (13%)
Uterus: RCS total	0/47 (0%)	0/23 (0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	1/22 (4%)	1/24 (4%)	1/24 (4%)	3/188 (2%)	2/95 (2%)	8/90 (9%)	14/93 (15%)
Bladder: RCS type A	0/48 (0%)	0/23 (0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	0/22 (0%)	1/24 (4%)	0/23 (0%)	0/188 (0%)	2/92 (2%)	3/89 (3%)	5/91 (6%)
Bladder: RCS total	0/48 (0%)	0/23 (0%)	0/24 (0%)	0/24 (0%)	0/47 (0%)	1/22 (5%)	1/24 (4%)	0/23 (0%)	13/18 (7%)	5/92 (7%)	6/89 (7%)	6/91 (7%)
Spleen: erythropoiesis	0/47 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	2/47 (4%)	1/21 (5%)	1/24 (4%)	0/23 (0%)	13/190 (7%)	15/96 (16%)	18/92 (20%)	42/95 (44%)
Ovaries: atrophy	0/47 (0%)	0/23 (0%)	0/22 (0%)	0/24 (0%)	0/45 (0%)	0/21 (0%)	0/22 (0%)	1/21 (5%)	11/178 (6%)	13/90 (15%)	25/89 (28%)	37/89 (42%)
Harderian gland: adenoma	2/48 (0%)	0/24 (0%)	1/24 (4%)	0/24 (0%)	2/46 (4%)	2/21 (10%)	3/23 (13%)	1/23 (4%)	8/186 (4%)	11/93 (12%)	18/89 (20%)	15/94 (16%)
Ovaries: RCS type A	0/47 (0%)	0/23 (0%)	0/22 (0%)	0/24 (0%)	0/45 (0%)	0/21 (0%)	0/22 (0%)	0/21 (0%)	0/178 (0%)	1/90 (1%)	3/89 (3%)	5/89 (6%)
Vagina: RCS type A	0/45 (0%)	1/23 (4%)	0/24 (0%)	0/23 (0%)	0/46 (0%)	0/22 (0%)	1/23 (4%)	0/22 (0%)	1/182 (0.5%)	1/90 (1%)	4/88 (5%)	8/87 (9%)
Vagina: RCS total	0/45 (0%)	1/23 (4%)	0/24 (0%)	0/23 (0%)	0/46 (0%)	1/22 (4%)	1/23 (4%)	0/22 (0%)	3/182 (2%)	1/90 (1%)	5/88 (6%)	10/57 (11%)

RCS: reticulum cell sarcoma

[a] Includes dead or moribund animals that were removed from the study prior to the scheduled sacrifice dates.

Source: Littlefield (1984); Littlefield et al. (1985)

Table 7. Microscopic histopathology summary of male mice

Site and lesion	12-month sacrifice[a]				18-month sacrifice[a]				24-month sacrifice[a]			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: benign neoplasm	0/48 (0%)	2/24 (8%)	0/24 (0%)	0/24 (0%)	3/48 (6%)	0/24 (0%)	2/24 (8%)	2/22 (9%)	17/183 (10%)	14/92 (19%)	20/93 (22%)	37/93 (38%)
Liver: malignant neoplasm	0/47 (0%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	5/48 (10%)	1/24 (4%)	2/24 (8%)	2/22 (9%)	27/183 (15%)	15/92 (17%)	17/93 (18%)	33/93 (35%)
Harderian gland: adenoma	1/48 (2%)	0/24 (0%)	0/24 (0%)	0/24 (0%)	2/47 (4%)	2/24 (8%)	2/23 (9%)	0/21 (0%)	7/187 (4%)	7/92 (7%)	10/94 (11%)	9/89 (10%)

[a] Includes dead or moribund animals that were removed from the study prior to the scheduled sacrifice dates.
Source: Littlefield (1984); Littlefield et al. (1985)

Table 8. Significance table for chronic toxicity and carcinogenicity study in mice[a]

	Females			Males		
	Mortality	Prevalence	Onset	Mortality	Prevalence	Onset
Mortality overall	0.000 05	–	–	0.012 88	–	–
Control vs 100 mg/kg feed	0.000 88	–	–	0.487 59	–	–
Control vs 300 mg/kg feed	0.000 79	–	–	0.100 99	–	–
Control vs 600 mg/kg feed	0.000 05	–	–	0.030 62	–	–
Liver: malignant neoplasms overall	0.000 05	0.000 05	0.000 05	0.013 54	0.000 45	0.000 05
Control vs 100 mg/kg feed	0.101 95	0.357 80	0.429 67	0.252 58	0.258 08	0.505 02
Control vs 300 mg/kg feed	0.008 18	0.000 05	0.000 05	0.245 63	0.433 69	0.306 65
Control vs 600 mg/kg feed	0.000 05	0.000 05	0.000 05	0.019 93	0.001 57	0.000 09
Liver: malignant neoplasms and benign tumours	0.000 05	0.000 05	0.000 05	0.013 54	0.000 05	0.000 05
Control vs 100 mg/kg feed	0.008 18	0.475 10	0.272 58	0.252 58	0.374 48	0.237 48

Table 8 (continued)

	Females			Males		
	Mortality	Prevalence	Onset	Mortality	Prevalence	Onset
Control vs 300 mg/kg feed	0.000 05	0.000 05	0.000 05	0.245 63	0.016 67	0.009 56
Control vs 600 mg/kg feed	0.000 05	0.000 05	0.000 05	0.019 93	0.000 05	0.000 05
Spleen: erythropoiesis	–	0.000 05	–	–	0.043 42	–
Control vs 100 mg/kg feed	–	0.017 38	–	–	0.007 43	–
Control vs 300 mg/kg feed	–	0.001 47	–	–	0.056 95	–
Control vs 600 mg/kg feed	–	0.000 05	–	–	0.022 48	–
Ovaries: atrophy	–	0.000 05	–	–	–	–
Control vs 100 mg/kg feed	–	0.014 90	–	–	–	–
Control vs 300 mg/kg feed	–	0.000 05	–	–	–	–
Control vs 600 mg/kg feed	–	0.000 05	–	–	–	–
Harderian gland: adenoma	–	0.001 10	–	–	0.061 59	–
Control vs 100 mg/kg feed	–	0.037 54	–	–	0.162 34	–
Control vs 300 mg/kg feed	–	0.000 05	–	–	0.030 70	–
Control vs 600 mg/kg feed	–	0.003 23	–	–	0.106 44	–

[a] Levels of significance (*P* values) for positive trends among dose groups for 1) mortality due to a specific disease, 2) prevalence (non-fatal) and (3) time to onset. Trend tests were performed across all dose groups and controls.

The lowest-observed-adverse-effect level (LOAEL) for non-carcinogenic effects was 14.3 mg/kg bw per day, the lowest dose tested. The authors concluded that under the conditions of the experiment, gentian violet appeared to be a carcinogen in mice at several different organ sites (Littlefield, 1984; Littlefield et al., 1985).

(b) Rats

In a GLP-compliant study, male and female weanling animals (F_0) were randomly divided into four groups and administered gentian violet (99% gentian violet and 1% methyl violet) in their feed at 0, 100, 300 or 600 mg/kg for at

least 80 days. While receiving dosed feed, the females were mated with males of the same dose level. Two males and two females were randomly selected from each litter (F_{1a} generation), and three animals per cage were allocated as weanlings to the chronic study. The F_{1a} animals continued on the same dose levels as their respective parents for the carcinogenicity studies (Littlefield et al., 1989).

In total, 570 male and 570 female F_{1a} Fischer 344 rats were fed gentian violet (99% gentian violet and 1% methyl violet) at 0, 100, 300 or 600 mg/kg diet (equal to approximately 0, 30, 80 and 160 mg/kg bw per day for males and 0, 40, 100 and 200 mg/kg bw per day for females, respectively) for 12, 18 and 24 months. The diets were certified to be within ±10% of target values. The allocation of the rats to the groups and sacrifice times are presented in Table 9. Feed consumption, body weights and clinical signs were recorded weekly. The rats received a complete necropsy, histopathological examination and clinical chemistry analysis at the scheduled sacrifices after 12, 18 and 24 months of continuous dosing.

The animals sacrificed after 12 months of dosing with gentian violet showed no dose-related pathology and are excluded from the results. Measurements of body weights, feed consumption and mortality and results of histopathological examinations were analysed statistically. Male and female rats fed 600 mg/kg feed for 24 months showed a decrease in body weight. Average feed consumption (based on grams of food per kilogram average body weight) was the same in all groups. Mortality rates at 24 months for the females were 33%, 38%, 60% and 66% for the control, low-dose, mid-dose and high-dose groups, respectively. For males, the same respective dose groups had mortality rates after 104 weeks of 33%, 33%, 48% and 39%.

The incidences of neoplastic lesions observed at the 18- and 24-month necropsies are presented in Tables 10 and 11. The majority of lesions were observed only at the 24-month necropsy, and incidences were mostly low.

Table 9. Experimental design of a chronic toxicity study carried out in Fischer 344 rats

Dietary concentration (mg/kg feed)	Total number of rats		Interim sacrifice (12 months)		Interim sacrifice (18 months)		Terminal sacrifice (24 months)	
	Males	Females	Males	Females	Males	Females	Males	Females
0	210	210	15	15	15	15	180	180
100	120	120	15	15	15	15	90	90
300	120	120	15	15	15	15	90	90
600	120	120	15	15	15	15	90	90
Total	570	570	60	60	60	60	450	450

Source: Littlefield et al. (1989)

Table 10. Incidence of neoplastic lesions in male Fischer 344 rats fed gentian violet in the diet for 18 or 24 months

Site and type of neoplastic lesion	Incidence of lesion							
	18 months				24 months			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: hepatocellular adenoma	0/15[a] (0%)[b]	1/15 (7%)	0/15 (0%)	0/14 (0%)	1/179 (0.5%)	1/90 (1%)	3/88 (3%)	4/89 (4%)
Testes: malignant mesothelioma	0/15 (0%)	0/15 (0%)	1/15 (7%)	1/15 (7%)	0/177 (0%)	0/90 (0%)	0/87 (0%)	1/90 (1%)
Thyroid: follicular cell adenocarcinoma	0/15 (0%)	0/15 (0%)	0/14 (0%)	0/13 (0%)	1/163 (1%)	4/84 (5%)	2/74 (3%)	5/79 (6%)
Thyroid: follicular cell adenoma	0/15 (0%)	0/15 (0%)	1/15 (7%)	1/15 (7%)	1/163 (1%)	0/84 (0%)	0/74 (0%)	2/79 (3%)
Thyroid: folliwcular cell adenoma and adenocarcinoma	0/15 (0%)	0/15 (0%)	1/15 (7%)	1/15 (7%)	2/163 (1%)	4/84 (5%)	2/74 (3%)	3/78 (9%)
Multiple organs: mononuclear cell leukaemia	6/15 (40%)	1/15 (7%)	3/15 (20%)	4/15 (27%)	104/180 (58%)	66/90 (77%)	69/90 (77%)	51/90 (57%)

[a] Incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats at risk.
[b] Values in parentheses represent the incidence of the neoplastic lesions as a percentage of the number of rats.
Source: Littlefield et al. (1989)

The incidences of follicular cell adenocarcinomas of the thyroid gland at 24 months for males in the 600 mg/kg feed group (6% versus 1% in controls) and females in the 300 and 600 mg/kg feed groups (5% and 8%, respectively, versus 1% in controls) were significantly increased compared with control group rats. The incidences of hepatocellular adenomas at 24 months were significantly increased in females in the 300 mg/kg feed group (2% versus 0% in controls), but not in the 600 mg/kg feed group, and in males in the 300 and 600 mg/kg feed groups (3% and 4%, respectively, versus 0.5% in controls), when compared with controls (Table 12). The incidence of mononuclear cell leukaemia appeared to be a time-related response; leukaemia showed a dose–response relationship in females at 18 months, but not at 24 months.

Table 11. Incidence of neoplastic lesions in female Fischer 344 rats fed gentian violet in the diet for 18 or 24 months

Site and type of neoplastic lesion	Incidence of lesion							
	18 months				24 months			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Liver: hepatocellular adenoma	0/15[a] (0%)[b]	0/11 (0%)	0/10 (0%)	0/14 (0%)	0/170 (0%)	1/90 (1%)	2/84 (2%)	1/87 (1%)
Heart: mononuclear cell leukaemia	0/15 (0%)	0/11 (0%)	0/10 (0%)	2/14 (14%)	27/169 (16%)	16/90 (18%)	19/83 (23%)	22/87 (25%)
Thyroid: follicular cell adenocarcinoma	0/15 (0%)	1/11 (9%)	0/10 (0%)	0/14 (0%)	1/159 (1%)	1/83 (1%)	4/76 (5%)	6/77 (8%)
Thyroid: follicular cell adenoma	0/15 (0%)	0/11 (0%)	0/10 (0%)	0/14 (0%)	1/159 (1%)	2/83 (2%)	3/76 (4%)	3/77 (4%)
Thyroid: follicular cell adenoma and adenocarcinoma	0/15 (0%)	1/11 (9%)	0/10 (0%)	0/14 (0%)	2/159 (2%)	3/83 (4%)	7/76 (9%)	9/77 (12%)
Multiple organs: mononuclear cell leukaemia	0/15 (0%)	2/11 (18%)	2/10 (20%)	6/14 (43%)	77/171 (45%)	38/90 (42%)	45/87 (52%)	40/87 (46%)

[a] Incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats at risk.

[b] Values in parentheses represent the incidence of the neoplastic lesions as a percentage of the number of rats.

Source: Littlefield et al. (1989)

The incidences of non-neoplastic lesions are presented in Table 13. There was a statistically significant increase in liver regeneration in all dose groups and statistically significant dose-related increases in eosinophilic foci in the liver in both sexes in the mid- and high-dose groups. For liver centrilobular necrosis, there was a dose-related increase, but statistical significance was seen only in the 300 mg/kg feed group in males and in the 600 mg/kg feed group in females. Female rats appeared to be more sensitive than males.

The LOAEL for non-neoplastic effects was 30 mg/kg bw per day, the lowest dose tested, based on the increase in liver regeneration observed in all dose groups. Gentian violet was carcinogenic in rats, with thyroid follicular cell adenocarcinomas and hepatocellular adenomas observed in both sexes (Littlefield et al., 1989).

In addition, Docampo & Moreno (1990) noted a report (National Toxicology Program, 1986) that the completely demethylated derivative of gentian violet, leucopararosaniline, is carcinogenic in rats, but no information was available on its potency. The tumours seen included the same thyroid tumours seen in the carcinogenicity study of gentian violet in rats (Littlefield et al., 1989).

Table 12. Mortality and incidence of specific neoplastic lesions expressed as levels of significance (P values) in Fischer 344 rats fed gentian violet in the diet for 24 months

	Significance levels (*P* values)[a]			
	Overall	Control vs 100 mg/kg feed level	Control vs 300 mg/kg feed level	Control vs 600 mg/kg feed level
Males				
Mortality	0.067	0.45	0.005 7	0.16
Liver: hepatocellular adenoma	0.009	0.069	0.004	0.008
Thyroid: follicular cell adenocarcinoma	0.004	0.017	0.066	0.002
Females				
Mortality	0.000 05	0.34	0.000 07	0.000 05
Liver: hepatocellular adenoma	0.092	0.083[b]	0.003	0.048
Thyroid: follicular cell adenocarcinoma	0.000 05	0.092	0.002	0.000 05
Multiple organs: mononuclear cell leukaemia	0.062	0.361	0.141	0.053

[a] Significant trend at 0.05 level for overall, 0.05/3 for control versus dose comparison (Bonferroni corrected). Significant trend at 0.01 level for overall, 0.01/3 for control versus dose comparison (Bonferroni corrected). Significant trend at 0.001 level for overall, 0.001/3 for control versus dose comparison (Bonferroni corrected).

[b] This significant value arises from the small number of tumours; the result was determined using Fisher's exact test.

Source: Littlefield et al. (1989)

2.2.4 Genotoxicity

Muller & Gauthier (1975) reported the binding of gentian violet with high preference to two adjacent A–T pairs and also a second deoxyribonucleic acid (DNA) interaction, which is much weaker and nonspecific. Using more sensitive methodology, Wakelin et al. (1981) showed that gentian violet binds externally to DNA, causing severe kinking and/or bending accompanied by a coupled unwinding of the Watson–Crick helix. The authors further concluded that the binding complexes with ribonucleic acid (RNA) were different and evidently a cooperative process.

The results of the assays to assess the genotoxic and mutagenic potential of gentian violet are presented in Table 14. The strong binding affinity and cellular toxicity of gentian violet have complicated the testing and interpretation of the assay results. Au et al. (1979) suggested that low levels of gentian violet were being inactivated in the test system by the 9000 × *g* supernatant fraction of rat liver homogenate (S9), whereas high levels were toxic to the organism.

Table 13. Incidence of non-neoplastic lesions in Fischer 344 rats fed gentian violet in the diet for 24 months

Site and type of lesion	Incidence of lesion[a]			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Males				
Liver				
Clear cell foci	6/179 (3%)	5/90 (6%)	5/88 (6%)	8/89 (9%)
Eosinophilic foci	7/179 (4%)	5/90 (6%)	20/88 (23%)	33/89 (37%)
Mixed foci	32/179 (18%)	26/90 (29%)	28/88 (26%)	47/89 (53%)
Regeneration	7/179 (4%)	11/90 (12%)	21/88 (24%)	15/89 (17%)
Centrilobular necrosis	5/179 (3%)	4/90 (4%)	8/88 (9%)	11/89 (12%)
Thyroid gland				
Follicular cysts	18/163 (11%)	7/84 (8%)	9/74 (12%)	17/97 (22%)
Spleen				
Red pulp hyperplasia	11/175 (6%)	7/88 (8%)	3/87 (3%)	15/86 (17%)
Lymph node	8/168 (5%)	9/86 (10%)	5/84 (6%)	11/81 (14%)
Females				
Liver				
Clear cell foci	1/170 (1%)	1/90 (1%)	3/84 (4%)	1/87 (1%)
Eosinophilic foci	0/170 (0%)	0/90 (0%)	6/84 (7%)	10/87 (11%)
Mixed cell foci	29/170 (17%)	32/90 (36%)	39/84 (46%)	30/87 (34%)
Regeneration	4/170 (2%)	9/90 (10%)	20/84 (24%)	18/87 (21%)
Centrilobular necrosis	7/170 (4%)	8/90 (9%)	6/84 (7%)	20/87 (23%)
Thyroid gland				
Follicular cysts	8/159 (5%)	9/83 (11%)	8/76 (11%)	7/77 (9%)

[a] Incidence is expressed as the number of rats with the identified non-neoplastic lesion divided by the number of rats at risk. Values in parentheses represent the incidence of the non-neoplastic lesions expressed as a percentage of the number of rats surviving.
Source: Littlefield et al. (1989)

Levin, Lovely & Klekowski (1982) studied the effect of light (plates containing gentian violet were irradiated at 23°C with a Sylvania tungsten/halogen lamp for 3 minutes at 20 cm) on the genotoxicity of gentian violet. In the Rosenkranz assay, a genotoxic effect was observed under conditions of dark and was enhanced by the irradiation. Harrelson & Mason (1982) reported that in the presence of NADPH and light, gentian violet was photoreduced to the same triarylmethyl free radical that is formed by enzymatic reduction. The presence of S9 had no effect on the genotoxicity of gentian violet. However, in the Ames test, where no mutagenic activity was observed but gentian violet was sufficiently toxic to sterilize the plate under conditions of dark incubation, the presence of S9 (active or thermally deactivated) virtually eliminated the toxicity of gentian violet under dark incubation and greatly decreased its toxicity under light conditions.

Table 14. Results of tests for genotoxicity and mutagenicity with gentian violet

Test system	Test object	Concentration	Results	Reference
In vitro				
Rosenkranz repairable DNA assay	*Escherichia coli* DNA polymerase-deficient strain	Not stated	Positive	Rosenkranz & Carr (1971)
Cytogenetic toxicity	CHO cells, human lymphocytes and HeLa and L cells, as well as *Peromyscus eremicus* and Indian Muntjac cell lines	0, 0.5 or 5 µg/mL	Positive (mitotic poison and clastogen)	Au et al. (1978)
Ames test[a]	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 1, 2 or 4 µg/plate	Negative, TA1535 equivocal	Shahin & von Borstel (1978)
	Saccharomyces cerevisiae XV185-14C	0, 2, 4, 6 or 8 µg/plate	Negative	
Chromosome breakage	CHO cells	0 or 10 µmol/L	Positive	Au & Hsu (1979)
Ames test[a]	*S. typhimurium* TA98, TA100, TA1535, TA1537	0, 0.1–50 µg	Negative, bactericidal ≥ 10 µg (no activation)	Au et al. (1979)
Rosenkranz repairable DNA assay[b]	*E. coli* W3110 pol A^+, mutant p3478 pol $A^=$	1, 10, 25 or 100 µg/plate	Positive	
Chromosome breakage[c]	CHO cells	5, 10 or 20 µg/mL	Positive, no activation	
Ames test[b]	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 0.1, 0.32, 1 or 3.2 µg/plate	Negative except for TA1535 only at 0.32 µg, and no activation	Bonin, Farquharson & Baker (1981)

Table 14 (continued)

Test system	Test object	Concentration	Results	Reference
Chromatid breakage	Human peripheral blood cells	20 µg/mL	Positive	Hsu, Cherry & Pathak (1982)
Ames test[b]	*S. typhimurium* TA98, TA100, TA1537	0, 1, 5, 10 or 50 µg/plate	Negative[c]	Levin, Lovely & Klekowski (1982)
Rosenkranz repairable DNA assay[b]	*E. coli* W3110 pol A^+, mutant p3478 pol $A^=$	0.1, 0.5, 1, 5, 7 or 10 µg/plate	Positive	
Ames test[b]	*S. typhimurium* TA1535	0, 0.025, 0.05, 0.1 or 0.5 µg/plate	Negative	Thomas & MacPhee (1984)
	E. coli DG1669	0, 25, 50, 75 or 100 µg/plate	Positive[d,e]	
Chromosome damage	Human lymphocytes[f,g]	1 µg/mL	Positive	Krishnaja & Sharma (1995)
Ames test	*S. typhimurium* TA97, TA98, TA100 *E. coli* WP2s	1–50 µg of metabolites/ plate for *Salmonella* 5 µmol/L concentration for *E. coli*	Negative (metabolites) in *Salmonella* test Positive in *E. coli* test, maybe metabolites positive as well	Hass, Heflich & McDonald (1986)
Ames test	*S. typhimurium* TA97, TA98, TA100, TA104	0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/plate with or without S9	Positive in TA97 with and without S9, positive in TA104 with S9, negative in others	Aidoo et al. (1990)
Mammalian cell mutagenicity	CHO-K1-BH_4 and CHO-AS52 cells	0–1.5 µg/mL with or without S9	Negative for CHO-K1-BH_4 cells, equivocal results for CHO-AS52 cells	

Table 14 (continued)

Test system	Test object	Concentration	Results	Reference
Lymphocyte DNA damage assay	B6C3F1 mouse	0, 0.2, 0.4, 0.6, 0.8 or 1.0 μg/mL	Positive for DNA damage	
Gene amplification test	SV40-transformed Chinese hamster cell line C060	0.02, 0.05 or 0.125 μg/mL	Dose-related weak SV40 DNA amplification	
In vivo				
Chicken embryo assay	Chicken embryos	0.5, 2, 5, 10, 20, 100, 1 000 or 2 000 μg/embryo	Toxicity ≥ 20 μg; no increase in sister chromatid exchange	Au et al. (1979)
Bone marrow assay	Mouse	Drinking-water 20 or 40 μg/mL for 4 weeks, calculated to be 4 and 8 mg/kg bw per day, respectively	No chromosome damage, decreased mitotic index	
Lymphocyte DNA damage assay	B6C3F1 mouse	Animals treated with 0, 2, 4 or 6 mg/kg bw as a single dose	Negative for DNA damage	Aidoo et al. (1990)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Without metabolic activation.

[b] With and without metabolic activation.

[c] At concentrations above 5.0 μg/plate, gentian violet was sufficiently toxic to sterilize the plates without S9 under conditions of dark incubation. The presence of S9 virtually eliminated the toxicity of gentian violet in the dark and greatly decreased the toxicity in the light.

[d] At gentian violet concentrations of 75 and 100 μg/plate with no S9, a large number of the cells were killed.

[e] With S9, all concentrations of gentian violet resulted in similar numbers of mutants.

[f] Cultured blood lymphocytes from β-thalassaemia traits and healthy individuals.

[g] The incidence of chromatid aberrations was not statistically different between normal and β-thalassaemia trait blood samples.

Thomas & MacPhee (1984) pointed out that all of the strains used by Au et al. (1979) and Bonin, Farquharson & Baker (1981) carried *rfa* mutations and were exceptionally sensitive to the toxic effects of gentian violet and thus not the most suitable strains to assess the mutagenicity of gentian violet, other than at very low dose levels. These authors reported negative results with *Salmonella typhimurium* strain TA1535 in the Ames assay using low doses (0.025–0.5 μg/plate) of gentian violet because of the toxic effects of gentian violet and thus disagreed with the positive results by Bonin, Farquharson & Baker (1981). However, the authors concluded that the positive results with DG1669 (an *Escherichia coli* K12 derivative that carries the *lac*Z(ICR24) frameshift marker and is DNA repair proficient) indicated that gentian violet is a direct-acting mutagen causing frameshift mutations in repair-proficient bacteria. Dose levels of 75 and 100 μg/plate were toxic when S9 was omitted, but not when S9 was present in the incubation mixture.

Aidoo et al. (1990) re-evaluated the genotoxicity of gentian violet (> 96% gentian violet, with the remainder being mainly methyl violet) by conducting mutagenesis and DNA damage experiments in both bacterial and mammalian cell systems. Mutagenicity of gentian violet in *Salmonella* was strain specific; it was mutagenic in TA97 and TA104 strains, but not in TA98 and TA100 strains. S9 tended to increase its mutagenicity. *N,N,N′,N″*-Tetramethylpararosaniline, a metabolite of gentian violet, was a weak mutagen in *Salmonella*. Gentian violet was not mutagenic in Chinese hamster ovary (CHO) cell line CHO-K1-BH_4, but equivocal results were obtained with CHO-AS52 cells. Gentian violet produced DNA damage in B6C3F1 mouse lymphocytes in vitro, but not in vivo. However, the dose levels used in these in vivo tests were much lower than those used in the carcinogenicity studies. The authors concluded that gentian violet is a point mutagen in bacteria and may be carcinogenic in mammalian cells by its clastogenic activity.

Gentian violet was found to break chromosomes in cultures of CHO cells (Au et al., 1978; Au & Hsu, 1979), human lymphocytes, HeLa and L cells and fibroblastic cell lines (Au et al., 1978).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

(i) Rats

In a three-generation reproductive toxicity study, gentian violet (99% gentian violet, 1% methyl violet) was administered in feed to Fischer 344 rats at a dose of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively). F_0 animals of both sexes were randomly allocated to treatment groups and fed the medicated ration for at least 80 days. Males and females of the same dose group were then caged together for 14 days for mating, after which males were returned to their own cages. Pups from this mating (F_{1a} generation) were used for a separate study. Following this, 90 rats of each sex for the control group and 45 rats of each sex for each treatment group were selected to continue in this study. F_0 animals

were mated a second time with animals of the same treatment group, as described previously. Following the births (F_{1b} generation), one male and one female from each litter were randomly selected for further study. At 100–140 days of age, F_{1b} generation females were randomly selected for mating with randomly selected males within the same dose group to produce the F_{2a} generation. A similar procedure was used to produce the F_{2b} generation. After 100–140 days, one male and one female per litter of the F_{2b} generation were randomly selected for mating to produce the F_{3a} generation. At weaning, two males and two females per litter of the F_{3a} generation were randomly selected for histopathology of a number of organs and tissues. The test substance was administered continuously in the diet of each treatment group (i.e. during mating, gestation, lactation and the interim rest periods). Brother–sister matings were avoided. Pups in each generation were examined for gross deformities.

A dose-related effect was noted on body weight in the 600 mg/kg feed group. Animals in this group had significantly lower body weights when compared with controls or the 100 and 300 mg/kg feed groups. Gentian violet had no effect on the number of pups per litter. The fertility index and number of stillborn animals compared across the generations or across doses did not exhibit a consistent trend. The number of animals not surviving to weanling age and sex ratio did not show significant dose or generation effects. No dose-related effects on the incidence of gross deformities were noted in examinations of pups of each generation. The only significant histopathological changes noted in the F_{3a} generation were a dose-related trend for focal dilatation of the renal cortex and tubules, a statistically significant dose-related trend for necrosis of the thymus ($P < 0.012$ for males and $P < 0.0001$ for females) and a statistically significant inverse dose–response relationship for red pulp haematopoietic cell proliferation of the spleen ($P < 0.001$ for males and $P < 0.000\,01$ for females).

The no-observed-adverse-effect level (NOAEL) for parental toxicity was 15 mg/kg bw per day, based on reductions in body weight at 30 mg/kg bw per day. A NOAEL for offspring toxicity could not be determined, as effects in the F_{3a} generation were present in all dose groups. The NOAEL for reproductive toxicity was 30 mg/kg bw per day, the highest dose tested (Littlefield, 1988).

(b) Developmental toxicity

(i) Rats

In a GLP-compliant study, groups (minimum of 20 animals) of pregnant CD rats were given 97.7% pure gentian violet by oral gavage at a dose of 0, 2.5, 5 or 10 mg/kg bw per day on gestation days 6–15. The vehicle was distilled water. Dams were weighed on gestation days 0, 6–15 and 20. Feed and water were available ad libitum. At sacrifice on gestation day 20, body, liver and gravid uterine weights and numbers of implantation sites, resorptions, and dead and live fetuses were recorded. Individual fetuses were weighed, sexed and examined for gross morphological abnormalities. All live fetuses were examined for visceral malformations using the Staples fresh tissue dissection method. Half of the fetuses were decapitated prior to dissection, and the heads were fixed in Bouin's solution for free hand sectioning and examination by Wilson's technique. All fetal carcasses were cleared and stained with alizarin red S and examined for skeletal malformations.

Three of 32 dams in the 10 mg/kg bw per day group died, whereas all other dams survived to day 20. Body weight gain was also significantly reduced in the 5 and 10 mg/kg bw per day dams. Clinical signs of toxicity (i.e. wheezing, lethargy, weakness, diarrhoea, lacrimation and rough coat) were observed to increase in a dose-related manner. There were no maternal signs of toxicity at 2.5 mg/kg bw per day. The Committee concluded that gentian violet treatment of the pregnant rats at 10 mg/kg bw per day resulted in a statistically significant increase in hydroureter, hydronephrosis and short ribs in the fetuses and clinical signs of maternal toxicity along with decreased body weight gain during treatment and the remainder of the gestation period. The fetal effects were seen only in conjunction with maternal toxicity and so were not seen at 2.5 mg/kg bw per day or at 5 mg/kg bw per day, where the maternal toxicity was limited. There were no gross malformations in any dose group.

The NOAEL for maternal toxicity was 2.5 mg/kg bw per day, based on clinical signs of toxicity at 5 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, based on fetal effects seen in conjunction with maternal toxicity at 10 mg/kg bw per day (Wolkowski-Tyl et al., 1982).

(ii) Rabbits

In a GLP-compliant study, groups of 30–40 artificially inseminated New Zealand White rabbits were given 97.7% pure gentian violet by oral gavage at a dose of 0, 0.5, 1 or 2 mg/kg bw per day on gestation days 6–19. At sacrifice, the numbers of pregnant dams in the groups were 26, 22, 21 and 23, respectively. The vehicle was distilled water. The does were weighed, prior to dosing, on days 0, 6–19 and 30 of gestation and observed for clinical signs of toxicity. At sacrifice on gestation day 30, body, liver and gravid uterine weights and numbers of implantation sites, resorptions, and dead and live fetuses were recorded. Individual fetuses were weighed, sexed and examined for gross morphological abnormalities. All live fetuses were examined for visceral malformations using the Staples fresh tissue dissection method. Half of the fetuses were decapitated after dissection, and the heads were fixed in Bouin's solution for free hand sectioning and examination by Wilson's technique. All fetal carcasses were cleared and stained with alizarin red S and examined for skeletal malformations.

Maternal mortality increased with dose (i.e. 0%, 7.4%, 15.4% and 22.6%, respectively). Maternal body weight gain was lower for all gentian violet–dosed does during treatment and gestation periods. Clinical signs, including wheezing, diarrhoea, congestion, wet nose, dyspnoea, lacrimation, anorexia and cyanosis, were observed in the dams in a dose-related manner. Fetal weights were significantly reduced in all gentian violet–treated groups compared with controls. No malformations unique to or with a higher incidence in any of the gentian violet–exposed groups were noted relative to the controls.

The authors concluded that there was no evidence of teratogenicity of gentian violet in this study with New Zealand White rabbits. Owing to the presence of maternal toxicity and significantly reduced fetal weights in all dosed groups, NOAELs could not be identified for maternal or embryo/fetal toxicity (Wolkowski-Tyl et al., 1983).

2.2.6 Special studies

Studies on mitochondria prepared from the livers of male Wistar rats provided evidence that gentian violet was a potent uncoupler of oxidative phosphorylation and that a free radical metabolite of gentian violet was not implicated in its mode of action (Moreno, Gadelha & Docampo, 1988). Gentian violet also uncouples oxidative phosphorylation in the mitochondria of unicellular protozoa, *Trypanosoma cruzi* (Gadelha et al., 1989).

Nussenzweig et al. (1953) observed that although gentian violet is a potent uncoupler of oxidative phosphorylation, this effect has not been seen in vivo in humans receiving blood that had been treated with gentian violet to protect against Chagas disease. Metabolic demethylation in the mammalian liver or species differences in the affinity of gentian violet for mitochondria were suggested as possible reasons for this different sensitivity to the uncoupling of oxidative phosphorylation.

In vitro, gentian violet has been shown to depress protein and RNA synthesis and oxygen consumption of fibroblasts (Mobacken, Ahonen & Zederfeldt, 1974). It is also shown to inhibit protein synthesis in *Trypanosoma cruzi*, probably due to inhibition of amino acid uptake in the cell (Hoffmann et al., 1995). By interacting with cell lipopolysaccharides, peptidoglycan and DNA, gentian violet damages both the bacterial and mitochondrial membrane by inducing permeability. This interferes with the electron transport mechanism at the cellular level, which could be the reason for its toxicity against bacteria and fungi. Many triphenylmethane dyes, such as malachite green, are known to inhibit the human glutathione *S*-transferase enzymes, although gentian violet is only a weak inhibitor of these enzymes (Glanville & Clark, 1997).

2.3 Observations in humans

Hodge et al. (1972) reported that the recommended therapeutic dose for anthelminthic treatment in adult humans is 2.1 mg/kg bw per day and that adverse effects were usually minimal and transient. Wright & Brady (1940) stated that the effects complained of in about one third of treated patients were gastrointestinal irritation, nausea, vomiting, diarrhoea and mild abdominal pain, which ceased on discontinuation of the treatment.

Epidemiological studies have indicated that the use of hair dyes (as a group) could be carcinogenic in humans, although these reviews could not establish a causal relationship (IARC, 1993; Rollison, Helzlsouer & Pinney, 2006; Baan et al., 2008). It is noted that the above-mentioned studies did not investigate the effects of gentian violet itself on human carcinogenicity, although gentian violet and related compounds can be used as non-oxidative direct hair dye ingredients in some countries. Diamante et al. (2009) reviewed the various reported toxicities of gentian violet in humans. Gentian violet is shown to cause dermal irritation/sensitization (Bielicky & Novák, 1969; Meurer & Konz, 1977; Lawrence & Smith, 1982), ocular irritation (Dhir et al., 1982), mucosal irritation (Slotkowski, 1957; Slotkowski & Redondo, 1966; John, 1968; Horsfield, Logan & Newey, 1976; Piatt & Bergeson, 1992) and bladder irritation (Walsh & Walsh, 1986; Kim et al., 2003; Diamante et al. 2009). An epidemic of nosebleeds in apple packers who used packing trays dyed with gentian violet has also been described (Quinby, 1968).

3. COMMENTS

The Committee reviewed studies submitted by a Member State as well as additional papers available in the published literature.

Gentian violet is structurally related to malachite green. The Committee evaluated malachite green in 2009 (Annex 1, reference *193*) and concluded that the use of malachite green in food-producing animals could not be supported. This was because its major metabolite, leucomalachite green, induces hepatocellular adenomas and carcinomas in female mice, and it could not be ruled out that this was by a genotoxic mode of action.

3.1 Biochemical data

Gentian violet is metabolized to leucogentian violet by isolated gut microflora from rats, chickens and humans. Strong binding of gentian violet to isolated gut bacteria and microsomal fractions of liver was demonstrated, and this is likely to affect the bioavailability of gentian violet. In studies in mice and rats using radiolabelled gentian violet, most of the administered dose is excreted in faeces, with urinary excretion being much less important. In mice, the excretion of gentian violet and its metabolites in urine is greater than in rats, but still represents less than 10% of the dose. Demethylation is the major metabolic pathway of biotransformation in liver microsomes, with mouse microsomes in vitro being less active than those from other rodents or chickens. In both rats and mice, the parent compound (gentian violet), its major metabolite leucogentian violet and their demethylated metabolites are found in tissues, urine and faeces.

Absorption of gentian violet from the gut is higher than that for other triphenylmethane dyes. Dosing mice and rats over 7 days demonstrated its distribution to fat, particularly in females.

3.2 Toxicological data

Critical studies are summarized in Table 15.

Table 15. Studies relevant to risk assessment

Species / study type (route)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Mouse				
Two-year study of toxicity and carcinogenicity (dietary)	Females: 0, 14.3, 35.7–39.3, 71.4	Erythropoiesis in spleen, atrophy of ovaries	–	14.3[a]
		Benign and malignant liver neoplasms (females)	–	**$BMDL_{10}$: 16.8**[b]

Table 15 (continued)

Species / study type (route)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Rat				
Two-year study of toxicity and carcinogenicity (dietary)	Males: 0, 30, 80, 160 Females: 0, 40, 100, 200	Increase in liver regeneration	–	30[a]
		Thyroid follicular cell adenocarcinoma (both sexes) and hepatocellular adenoma (males)	–	–
Three-generation study of reproductive toxicity, including developmental toxicity (dietary)	0, 5, 15, 30	Reproductive toxicity: No effects seen	30[c]	–
		Parental toxicity: Decreased body weight	15	30
		Offspring toxicity: Necrosis of thymus, focal dilatation of renal cortex and tubules, lowered red pulp haematopoietic cell proliferation in spleen	–	5[a]
Developmental toxicity study (gavage)	0, 2.5, 5, 10	Maternal toxicity: Reduced body weight gain, clinical signs	2.5	5
		Embryo and fetal toxicity: Increased hydroureter, hydronephrosis and short ribs	5	10
Rabbit				
Developmental toxicity study (gavage)	0, 0.5, 1, 2	Maternal toxicity: Increased mortality, decreased body weight gain, clinical signs	–	0.5[a]
		Embryo and fetal toxicity: Reduced fetal weight	–	0.5[a]

$BMDL_{10}$: lower 95% confidence limit on the benchmark dose for a 10% response; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] Lowest dose tested.

[b] Pivotal study value (Littlefield, 1984; Littlefield et al., 1985).

[c] Highest dose tested.

There were few data available on the acute and short-term toxicity of gentian violet, but the reported range of LD_{50}s, from 100 to 800 mg/kg bw, shows that it is of moderate acute oral toxicity. The most common sign of toxicity was lethargy, followed by anorexia and, in some animals, diarrhoea, excessive thirst, emesis and weight loss. In 90-day studies in rats and dogs, the only reported signs were slight body weight loss and increased liver weight, respectively.

In a 24-month study, gentian violet was given to mice in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equal to 0, 10.7–14.3, 32.1–35.7 and 64.3 mg/kg bw per day for males and 0, 14.3, 35.7–39.3 and 71.4 mg/kg bw per day for females, respectively). Few dose-related non-neoplastic lesions were reported, but there were statistically significant dose-related increases in erythropoiesis in the spleen and atrophy of the ovaries in females at 24 months. The LOAEL for non-carcinogenic effects was 14.3 mg/kg bw per day, the lowest dose tested. Significant, dose-related increases in neoplastic lesions were observed in both sexes, with the female mice being more sensitive. Hepatocellular adenomas and carcinomas were the most common lesions, with significant, dose-related increases found at 24 months in males and at both 18 and 24 months in females. Mortality due to liver neoplasms showed positive trends in both males and females, and there was a dose-related decrease in the time for the onset of liver neoplasms. The females also showed statistically significant dose-related increases in adenoma of the Harderian gland and in type A reticulum cell sarcoma in the urinary bladder, uterus, ovaries and vagina. The data clearly indicate that gentian violet is a multisite carcinogen in the mouse.

In a long-term study of toxicity, rats were exposed to gentian violet in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equal to approximately 0, 30, 80 and 160 mg/kg bw per day for males and 0, 40, 100 and 200 mg/kg bw per day for females, respectively). Gentian violet exposure of these animals was achieved by dosing the parents of the study animals prior to and during mating, with the same dose fed to the offspring from weaning up to 24 months of age. There was a statistically significant increase in liver regeneration in all dose groups and statistically significant dose-related increases in eosinophilic foci in the liver in both sexes in both the mid- and high-dose groups. For liver centrilobular necrosis, there was a dose-related increase, but statistical significance was seen only in the 300 mg/kg feed group in males and in the 600 mg/kg feed group in females. As in mice, female rats appeared to be more sensitive than males. The incidence of thyroid adenocarcinoma was increased in males, with statistical significance at the top dose only at 24 months. Females showed a statistically significant dose–response relationship for thyroid adenocarcinoma at 24 months. The incidence of hepatocellular adenomas showed a small but significant dose–response relationship in males and a significant increase in females at 300 mg/kg feed, but not at other doses. The data indicate a carcinogenic response to gentian violet in rats, although much weaker than the response in mice.

The data show that gentian violet binds to DNA, and this, together with the cellular toxicity of gentian violet, complicates both the testing of gentian violet in vitro and the interpretation of the results. The results are somewhat varied in *Salmonella typhimurium*, with positive responses in some strains but not in others. Gentian violet was clastogenic in vitro and positive in indicator tests for DNA damage. There are few in vivo tests on gentian violet. A single in vivo test for clastogenicity (mouse

bone marrow assay) showed no evidence of clastogenic activity, but the Committee noted that the gentian violet was given via the drinking-water at lower doses (4 and 8 mg/kg bw per day) than those used in the mouse cancer bioassay (ranging from 10 to 70 mg/kg bw per day). Similarly, the other in vivo test on DNA damage in mouse lymphocytes using single intravenous doses up to 6 mg/kg bw showed no effect. The Committee concluded that, overall, the data show that gentian violet is genotoxic.

In view of the carcinogenicity of gentian violet in the mouse and rat and evidence showing genotoxicity in a number of tests, the Committee concluded that gentian violet should be considered a carcinogen acting by a genotoxic mode of action.

In a multigeneration reproductive toxicity study, rats were given gentian violet in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively) over three generations. There were significant reductions in body weight in the top dose group in all generations. The NOAEL for parental toxicity was 15 mg/kg bw per day. In the F_{3a} generation, examined for histopathological effects, a dose-related trend for focal dilatation of the renal cortex and tubules, a statistically significant dose-related trend for necrosis of the thymus and an inverse dose–response relationship for red pulp haematopoietic cell proliferation of the spleen were seen. The effects in the F_{3a} generation were present in all dose groups, and a NOAEL for offspring toxicity could not be determined. Gentian violet had no effect on the number of pups per litter, fertility index, pup survival, sex ratio or number of stillborn animals. The NOAEL for reproductive toxicity was 30 mg/kg bw per day, the highest dose tested.

Two developmental toxicity studies were conducted in rats. In the first study, CD rats were given gentian violet at 0, 2.5, 5 or 10 mg/kg bw per day by oral gavage on days 6–15 of gestation. In the second study, the three-generation study in Fischer 344 rats described above, the F_{3b} generation was examined for teratogenic effects. In that study, gentian violet was given in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively). CD rats appeared to be more sensitive than Fischer 344 rats to the toxicity of gentian violet, with dose-related reductions in maternal weight gain at 5 and 10 mg/kg bw per day and increased clinical signs of toxicity, significant at 10 mg/kg bw per day and limited at 5 mg/kg bw per day (maternal toxicity NOAEL of 2.5 mg/kg bw per day). In Fischer 344 rats, reduction in body weight was seen only at 30 mg/kg bw per day and not at lower doses of 5 and 15 mg/kg bw per day (maternal toxicity NOAEL of 15 mg/kg bw per day). It was also noted that malformations (hydroureter, hydronephrosis and short ribs) were seen only in the CD rats. Effects on the fetus were seen only at doses that also caused maternal toxicity. The NOAEL for embryo and fetal toxicity in CD rats was 5 mg/kg bw per day.

In a developmental toxicity study, rabbits were given gentian violet at 0, 0.5, 1 or 2 mg/kg bw per day by oral gavage on days 6–19 of gestation. Maternal mortality was increased in a dose-related manner, and maternal body weight gain was decreased in all treated groups compared with controls. Fetal weights were significantly reduced in all treated groups compared with controls. There was no evidence of teratogenic effects. Owing to the presence of maternal toxicity and significantly reduced fetal weights in all dosed groups, NOAELs could not be identified for maternal or embryo/fetal toxicity.

In humans, case reports have shown that gentian violet has been associated with dermal irritation/sensitization, ocular irritation, mucosal irritation and bladder irritation following topical or employment-related exposure, but these are not relevant to the evaluation of the safety of gentian violet in food.

4. EVALUATION

The Committee concluded that it is inappropriate to set an ADI for gentian violet because it is genotoxic and carcinogenic. Gentian violet is widely used in various ways other than as an authorized veterinary drug, and there may be residues in fish from unauthorized use or from environmental exposures. Therefore, irrespective of whether it is used as a veterinary drug, the Committee agreed that some further guidance to risk managers was needed.

The Committee determined that the pivotal study for the evaluation of gentian violet is the carcinogenicity study in mice. Although it was not possible to add the adenomas and carcinomas in liver, the dose–response relationship for the two tumour types was very similar. Accordingly, a benchmark dose (BMD) evaluation was conducted using the data for the female mouse malignant liver neoplasms at the 24-month sacrifice.

The United States Environmental Protection Agency's (USEPA) BMD software (BMDS, version 2.2) was used for modelling the dose–response relationship for malignant liver neoplasms in gentian violet–treated female mice. The following dose–response models were fitted to the dose–incidence data and resulted in an acceptable fit: gamma, logistic, log-logistic, multistage, multistage cancer, probit, log-probit and Weibull. The BMD and lower 95% confidence limit on the benchmark dose (BMDL) values for an extra 10% risk compared with the modelled background incidence (BMD_{10} and $BMDL_{10}$) were estimated by performing 250 iterations.

Table 16. BMD_{10} and $BMDL_{10}$ calculations for gentian violet based on the incidences of malignant neoplasms in female mice

Model	AIC	*P* value	Scaled residual of interest	Accepted	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Gamma multi-hit	324.3	0.842	−0.151	Yes	24.0	18.8
Logistic	324.2	0.415	−1.016	Yes	22.3	19.5
Log-logistic	324.3	0.907	−0.093	Yes	24.7	19.6
Log-probit	324.4	0.775	0.231	Yes	25.2	19.8
Multistage	323.9	0.477	−1.03	Yes	19.9	16.8
Multistage cancer	323.9	0.477	−1.03	Yes	19.9	16.8
Probit	324.3	0.398	−1.125	Yes	20.3	17.8
Weibull	324.9	0.472	−0.562	Yes	22.9	17.8

AIC: Akaike's Information Criterion; BMD_{10}: benchmark dose for a 10% response; $BMDL_{10}$: lower 95% confidence limit on the benchmark dose for a 10% response

The BMD_{10} values from the accepted models ranged from 19.9 to 25.2 mg/kg bw per day, and the $BMDL_{10}$ values ranged from 16.8 to 19.8 mg/kg bw per day (Table 16). In order to be prudent, the Committee decided to use the more conservative lower end of this range of values for the evaluation and chose a $BMDL_{10}$ value of 16.8 mg/kg bw per day as the reference point for a margin of exposure (MOE) calculation.

The Committee estimated MOEs assuming a residue level of 0.5 µg/kg, which is a typical limit of quantification for gentian violet residues in foods, and a residue level of 5 µg/kg, which is 10 times the typical limit of quantification, as a hypothetical scenario. Assuming a daily consumption of 300 g of fish contaminated with gentian violet and its metabolites, the estimated theoretical exposures to gentian violet for a 60 kg person were 0.0025 and 0.025 µg/kg bw per day for the two residue levels, respectively. Comparison of these estimated exposures with the $BMDL_{10}$ of 16.8 mg/kg bw per day indicates MOEs of about 6.7×10^6 and 6.7×10^5, respectively. Based on considerations discussed at the sixty-fourth meeting of the Committee for unintended contaminants (Annex 1, reference *176*), these MOEs would be considered to be of low concern for human health.

However, the Committee noted that there were a number of uncertainties associated with the risk assessment, some of which were substantial. The uncertainties relate to two aspects of the data available for risk assessment. Firstly, there were insufficient residue data in food-producing animals or the environment from which to estimate dietary exposure to gentian violet, and hence assumptions had to be made. Secondly, there is very little information on the proportion of gentian violet and its metabolites in the total residue and on the carcinogenicity of the metabolites. For example, there is a published report that one of the possible metabolites of gentian violet, demethylated leucopararosaniline, is carcinogenic in rats, but no information is available on its potency. In addition, there is no information on the carcinogenicity of the major metabolite, leucogentian violet. The structure of gentian violet is similar to that of malachite green, and it is known that leucomalachite green is a more potent carcinogen than malachite green; therefore, it is possible that leucogentian violet is similarly a more potent carcinogen than gentian violet. Gentian violet and leucogentian violet are readily interconvertible in the body, and so it is likely that exposure to gentian violet will also result in exposure to leucogentian violet. Thus, there is inadequate information to determine the overall carcinogenicity of the metabolites of gentian violet (demethylated gentian violet, leucogentian violet and its demethylated metabolites).

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LASALOCID SODIUM

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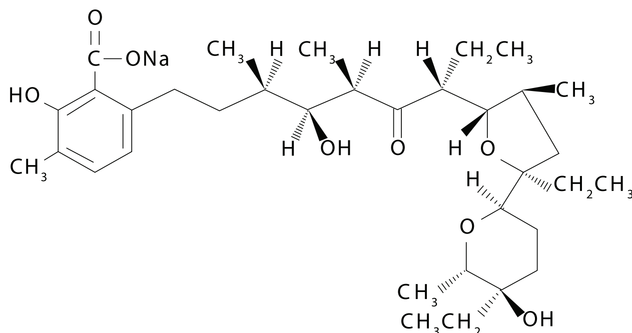
Fig. 1. Structure of lasalocid sodium (lasalocid A)



1. EXPLANATION

Lasalocid sodium (Chemical Abstracts Service No. 25999-20-6) is produced by *Streptomyces lasaliensis* and is a mixture of several closely related homologues: A, B, C, D and E. Lasalocid homologues B, C, D and E make up no more than a total of 10% of the total weight of the active substance.

Lasalocid sodium, a divalent polyether ionophore antibiotic, is approved for continuous use to control coccidiosis in poultry species at concentrations of 7.5–125 mg/kg feed. It is approved to protect against *Eimeria* species in broilers and replacement pullets, turkeys, pheasants and quails.

The mechanism of action of lasalocid and other ionophores has been extensively investigated and reported. Like other carboxylic polyether ionophores, lasalocid disturbs ionic homeostasis, leading to osmotic lysis of coccidia.

Lasalocid sodium has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated lasalocid sodium at the present meeting at the request of the Twentieth Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2012) with a view to establishing an acceptable daily intake (ADI) and recommending maximum residue limits (MRLs) in poultry tissues and eggs.

The structure of lasalocid sodium (International Union of Pure and Applied Chemistry name: sodium 6-[(3*R*,4*S*,5*S*,7*R*)-7-[(2*S*,3*S*,5*S*)-5-ethyl-5[(2*R*,3*R*,6*S*)-5-ethyl-5-hydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)]tetrahydro-3-methyl-2-furyl)]-4-hydroxy-3,5-dimethyl-6-oxononyl)]-2,3-cresotate) is shown in Fig. 1.

The present Committee considered data on pharmacodynamics, pharmacokinetics, short-term and long-term toxicity, genotoxicity, reproductive and developmental toxicity, carcinogenicity and microbiological safety. The original studies provided by the sponsor were all performed in the 1970s and 1980s; consequently, a number of them do not conform to the standards in force today. Several were performed before the introduction of good laboratory practice (GLP) and provide relatively limited reporting. Nevertheless, the overall package of data is considered sufficient to allow the derivation of a robust ADI. In addition

to a submission from the sponsor, a literature search was conducted using the Embase database and the following keywords: lasalocid, Ro 2-2985, X-537A, toxicity, cytotoxicity and neurotoxicity. Seventy-seven references were obtained and manually screened. Five of these reports are cited in the current monograph and were not included in the dossier provided by the sponsor.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Mice

In a study that pre-dated the implementation of GLP, 35 adult male Charles River CD-1 mice were administered a single dose of [^{14}C]lasalocid sodium at 1 mg/kg body weight (bw) by oral gavage. Groups of five animals were killed at 15 and 30 minutes and 1, 3, 6, 24 and 48 hours after dosing. Radioactivity was determined using liquid scintillation counting in whole blood, brain, carcass (i.e. all tissues not collected as individual tissues), faeces, fat, heart, kidney, large intestine contents, large intestine tissue, liver, lung, small intestine contents, small intestine tissue, spleen, stomach contents, stomach tissue, thymus and urine. Urine and faeces were collected as separate samples for each mouse over 24-hour periods.

Whole blood radioactivity levels were highest at 15 minutes after administration (at which point the levels accounted for 1.41% of the administered radioactivity). Radioactivity levels in whole blood were below the minimum detectable level at 24 hours and beyond. The half-life of elimination of radioactivity in whole blood was 3 hours.

Radioactivity was observed in all tissues examined, and levels were highest at 1 hour after administration. Radioactivity levels were highest in liver (at 1 hour after administration, residues in liver accounted for approximately 17.5% of the administered radioactivity). In all other tissues, radioactivity levels at 1 hour accounted for less than 0.5% of the administered dose and decreased in the order heart > lung > brain = kidney > spleen = thymus. Levels in fat were highest at 3 hours after administration (accounting for approximately 0.1% of the administered radioactivity). With the exception of liver, radioactivity levels were below the minimum detectable level in all of the above tissues by 24 hours after administration. Residues in liver remained detectable at 48 hours after administration.

Residues in the remaining carcass were detectable between 15 minutes and 6 hours after administration and peaked at 3 hours.

In the gastrointestinal tract tissues, radioactivity levels were highest in stomach contents 15 minutes after administration; highest in stomach tissue at 30 minutes after administration; highest in the small intestine contents between 15 minutes and 3 hours after administration; highest in small intestine tissue at 15 minutes after administration; highest in large intestine contents at 6 hours after administration; and fairly steady in large intestine tissue over the first 6 hours.

High levels of radioactivity were seen in the faeces from 6 to 24 hours after administration, with approximately 95% of radioactivity being excreted in faeces by 24 hours. Radioactivity was observed in urine between 3 and 24 hours after administration and accounted for less than 1% of the administered dose (Laurencot et al., 1979a).

In a study that pre-dated GLP implementation, 40 adult male Charles River CD-1 mice were administered seven daily doses of [^{14}C]lasalocid sodium at 1 mg/kg bw by oral gavage. Groups of five animals were killed at 15 and 30 minutes and 1, 3, 6, 24 and 48 hours after the last dose. Radioactivity was determined using liquid scintillation counting in whole blood, brain, carcass (i.e. all tissues not collected as individual tissues), faeces, fat, heart, kidney, large intestine contents, large intestine tissue, liver, lung, small intestine contents, small intestine tissue, spleen, stomach contents, stomach tissue, thymus and urine. Urine and faeces were collected as separate samples for each mouse over 24-hour periods. The remaining five animals were also killed at 48 hours after the last dose, but only their blood, urine and faeces were assayed.

Whole blood radioactivity levels were highest at 30 minutes after the last dose (at which point levels accounted for approximately 0.25% of the administered dose). Radioactivity levels in whole blood at 24 hours and beyond were below the minimum detectable level. The half-life of elimination of radioactivity in whole blood was 3 hours.

Radioactivity was observed in all tissues examined and was highest during the 3-hour period following the last administration. At 3 hours after administration, average radioactivity levels were highest in liver (accounting for almost 2% of the administered dose). In all other tissues, radioactivity levels at 3 hours accounted for less than 0.5% of the administered dose and decreased in the order heart > lung > brain > thymus > kidney > spleen > fat. With the exception of liver, levels of radioactivity were below the minimum detectable levels in all of the above tissues by 24 hours after administration. Residues in liver remained detectable at 48 hours after administration.

Residues in the remaining carcass were detectable between 15 minutes and 6 hours after administration and peaked at 3 hours.

In the gastrointestinal tract tissues, radioactivity was highest in stomach contents 15 minutes after the last administration; highest in stomach tissue at 15 minutes after the last administration; highest in the small intestine contents at 1–3 hours after the last administration; highest in small intestine tissue at 15 minutes after administration; highest in large intestine contents at 6 hours after the last dose; and highest in large intestine tissue at 6 hours after the last dose.

Radioactivity levels in faeces were roughly similar during each 24-hour period during the dosing period and for the 24 hours after the last administration. Approximately 95% of radioactivity was excreted in faeces by 24 hours after the last dose. Radioactivity levels in urine were also roughly similar during each 24-hour collection period during the dosing interval and during the 24 hours after the final dose. Excretion via the urine accounted for less than 1% of the total administered dose (Laurencot et al., 1980).

In a study reported to have been performed in compliance with GLP (a signed quality assurance statement was provided), 26 Charles River CD-1 mice (13 males and 13 females) were administered [^{14}C]lasalocid sodium as single oral (gavage) daily doses of approximately 1 mg/kg bw for 7 days. The drug was administered in 30% aqueous ethanol. Ten additional mice (five males and five females) served as untreated controls. Urine and cage wash were collected at 24-hour intervals for each cage during treatment and for up to 4 hours following the last dose. Faeces were collected from each cage at 24-hour intervals over the 7-day period. At 4 hours after the last dose, mice were killed. Radioactivity was determined in urine, cage wash, faeces and liver by liquid scintillation counting.

After the first oral dose, 96.68% of the administered radioactivity was excreted within 24 hours, with 89.75% detected in faeces, 1.44% in urine and 5.49% in cage wash. By 4 hours after the last dose, 77.08% of the dose was excreted in faeces, 1.01% in urine and a further 2.61% in cage wash (Hawkins, Elsom & de-Salis, 1987a).

Samples of pooled lyophilized livers and faeces were kept for metabolic profiling, which was undertaken as part of a separate study (Laurencot & Weiss, 1987) reported in section 2.1.2 below.

In a study that pre-dated GLP implementation, 10 adult Charles River CD-1 mice (5 males and 5 females) were administered [^{14}C]lasalocid sodium as a single oral (gavage) dose of 1 mg/kg bw in 10% ethanol. Urine and faeces were collected (separately) from individual animals over the following intervals: 0–4 hours, 4–8 hours, 8–12 hours, 12–24 hours, 24–48 hours and 48–72 hours. Radioactivity was determined using liquid scintillation counting.

In both male and female mice, 96% of the administered radioactivity had been excreted in faeces by 72 hours after dosing, with the bulk of it excreted within the first 24 hours (approximately 95% in males and 93% in females). Peak radioactivity levels were seen in faeces of males collected between 8 and 12 hours after administration and in faeces of females collected between 4 and 8 hours after administration. In both males and females, radioactivity in urine (over the entire 72-hour period) accounted for less than 1% of the administered radioactivity (Westheimer & Hutchinson, 1978a).

(b) Rats

In a study that pre-dated GLP implementation, 35 adult male Charles River CD rats were administered a single dose of [^{14}C]lasalocid sodium at 1 mg/kg bw by oral gavage. Groups of five animals were killed at 15 and 30 minutes and 1, 3, 6, 24 and 48 hours after dosing. Radioactivity was determined using liquid scintillation counting in whole blood, brain, carcass (i.e. all tissues not collected as individual tissues), faeces, fat, heart, kidney, large intestine contents, large intestine tissue, liver, lung, small intestine contents, small intestine tissue, spleen, stomach contents, stomach tissue, thymus and urine. Urine and faeces were collected as separate samples for each rat over 24-hour periods.

Whole blood radioactivity levels were highest at 3 hours after administration (accounting for 0.122% of the administered radioactivity). Radioactivity levels in whole blood were below the minimum detectable level at 48 hours after administration. The half-life of elimination of radioactivity in whole blood was 4.8 hours.

Radioactivity was observed in all tissues examined. Average radioactivity levels were highest in liver (approximately 10% of the administered dose at 6 hours). In all other tissues, radioactivity levels accounted for less than 0.1% of the administered dose at all time points (levels decreased in the order lung > fat > kidney > spleen > heart > thymus > brain). Levels of radioactivity peaked in the above tissues between 15 minutes and 6 hours after administration. Low levels of radioactivity were still present in a number of tissues at 48 hours after administration.

Residues in the remaining carcass were detectable between 15 minutes and 6 hours after administration and peaked at 15 minutes.

In the gastrointestinal tract tissues, radioactivity levels were highest in stomach contents 15 minutes after administration; highest in stomach tissue at 30 minutes; highest in the small intestine contents at 3 hours after administration; highest in small intestine tissue also at 3 hours; highest in large intestine contents at 6 hours after administration; and highest in large intestine tissue also at 6 hours.

High levels of radioactivity were seen in the faeces at 24 hours after administration, with approximately 85% of radioactivity being excreted in faeces by 24 hours. A further 9% was excreted in faeces between 24 and 48 hours. Radioactivity was observed in urine between 15 minutes and 24 hours after administration and accounted for less than 1% of the administered dose (Laurencot et al., 1979b).

In a study that pre-dated GLP, 52 adult Charles River CD rats (26 males and 26 females) were administered unlabelled lasalocid sodium in the diet for 2 weeks. During the 1st week, the concentration in feed was 0.0080%, and during the 2nd week, it was 0.0070%. These dietary concentrations were equal to a daily dose of approximately 6.5 mg/kg bw for both male and female animals. The rats were then switched to a diet containing [^{14}C]lasalocid sodium at a concentration of 0.0080% for 3 days (equal to 6.2 ± 0.3 mg/kg bw for male rats and 6.9 ± 0.7 mg/kg bw for female rats). Rats were killed 0, 1, 3 (five males and five females per time point) and 5 days (six males and six females) after withdrawal of the medicated diet (no information is provided on the fate of the remaining 10 rats). The livers from all males at each time point were combined, and the livers from all females at each time point were combined. Each pool of livers was homogenized and fractionated into ethanol-soluble and ethanol-insoluble fractions. The ethanol-insoluble fraction was subjected to base hydrolysis and partitioned between water and ethyl acetate.

Five days after withdrawal of the [^{14}C]lasalocid sodium diet, radioactivity was present in the livers of both sexes. In male rats, radioactivity was present at higher levels in the ethanol-soluble fraction than in the ethanol-insoluble fraction at days 0 and 1 after withdrawal of the diet, whereas on days 3 and 5, levels in

the ethanol-insoluble fraction exceeded those in the ethanol-soluble fraction. In female rats, levels in the ethanol-soluble fraction exceeded those in the ethanol-insoluble fraction at all time points. In the female rats on day 0, radioactivity in the insoluble fraction was predominantly in the ethyl acetate fraction (approximately 70%), whereas in males, the reverse was true. At subsequent time points, radioactivity was generally present to a greater degree in the water fraction in both sexes.

This study was performed with a view to providing a comparison with results previously seen in female chickens (Laurencot et al., undated). In that study, 16 chickens were pretreated with unlabelled lasalocid sodium in feed at a concentration of 0.0075% for 16 days. Twelve of 16 birds then received one oral capsule containing 5.0 mg of labelled drug per day for 3 days. The remaining four birds served as controls. Whole blood, fat, kidney, liver, muscle, skin, urine and faeces were collected, and radioactivity was determined by liquid scintillation counting. Radioactivity levels were highest in liver and decreased in the order liver > kidney > fat > skin > muscle. In liver, radioactivity was highest in the ethanol-soluble fraction at 2 hours and 24 hours after the last administration, but at subsequent time points (i.e. up to 5 days after the last administration), radioactivity levels were higher in the ethanol-insoluble fraction. Ninety-five per cent of the radioactivity was excreted in urine and faeces by 24 hours after the last dose. Comparison with the rat study indicates that in male rats and in chickens, there is a transition of residues in liver over time from alcohol-soluble forms to alcohol-insoluble forms. Differences in the alcohol and water solubility of residues seen in male and female rats indicate increased hydrophilicity of residues in males, which may be related to the fact that, in the toxicity studies, females typically displayed increased sensitivity compared with males (Anonymous, ca 1980).

In a study reported to have been performed in compliance with GLP (a signed quality assurance statement was provided), 10 rats (5 males and 5 females; strain not reported) were administered [^{14}C]lasalocid sodium as single oral (gavage) daily doses of approximately 1 mg/kg bw for 7 days. The drug was administered in 30% aqueous ethanol. Six additional rats (three males and three females) served as untreated controls. Urine and cage wash were collected at 24-hour intervals for each cage during treatment and up to 4 hours following the last dose. Faeces were collected from each cage at 24-hour intervals over the 7-day period. At 4 hours after the last dose, rats were killed. Radioactivity was determined in urine, cage wash, faeces and liver by liquid scintillation counting.

After the first oral dose, 67.37% of the dose was excreted within 24 hours, with 66.7% in faeces and 0.67% in urine. By 4 hours after the last dose, 73.46% of the dose was excreted in faeces and 0.6% in urine. No significant radioactivity was seen in cage wash (Hawkins, Elsom & de-Salis, 1987b).

Samples of pooled lyophilized livers and faeces were kept for metabolic profiling, which was undertaken as part of a separate study (Laurencot & Weiss, 1987) reported in section 2.1.2 below.

In a study that pre-dated GLP implementation, 10 adult Charles River CD rats (5 males and 5 females) were administered a single oral (gavage) dose of [^{14}C]lasalocid sodium at 1 mg/kg bw in 10% ethanol. Urine and faeces were collected (separately) from individual animals over the following intervals: 0–4 hours, 4–8 hours, 8–12 hours, 12–24 hours, 24–48 hours and 48–72 hours. Radioactivity was determined using liquid scintillation counting.

In male rats, approximately 92% of the administered radioactivity had been excreted in faeces by 72 hours, with approximately 80% excreted within the first 24 hours. Peak radioactivity levels were seen in faeces collected between 12 and 24 hours after administration.

In female rats, approximately 86% of the administered radioactivity had been excreted in faeces by 72 hours, with approximately 59% excreted within the first 24 hours. Peak radioactivity levels were seen in faeces collected between 12 and 24 hours after administration.

In both males and females, radioactivity in urine (over the entire 72-hour period) accounted for less than 1% of the administered radioactivity (Westheimer & Hutchinson, 1978b).

In a study that pre-dated GLP implementation, five adult male Charles River CD rats had gastric catheters surgically inserted and their bile ducts cannulated. The rats were infused intragastrically with a solution of sodium taurocholate in physiological saline to ensure hydration and steady-state conditions of bile flow. After a 24-hour recovery period, rats were administered a single dose of [^{14}C]lasalocid sodium via the gastric cannula at 1 mg/kg bw.

Bile was collected separately for each rat at 6–24 hours predosing and at 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–30 and 30–48 hours after dosing. Urine and faeces were also collected as separate samples for each animal at 0–24 hours predosing and at 0–4, 4–8, 8–12, 12–24 and 24–48 hours after dosing. At 48 hours after the last dose, the rats were killed and radioactivity was assessed in gastrointestinal tract contents, gastrointestinal tract tissue, liver and the remaining carcass. Radioactivity was assessed in tissues and excreta using liquid scintillation counting.

Approximately 59% of administered radioactivity was recovered in bile during the 48 hours following administration. Radioactivity levels were fairly constant in the bile samples collected at 0–2, 2–4, 4–6, 6–8, 8–12 and 12–24 hours (between 7% and 11% of the administered radioactivity in each) and declined to 1–2% in each of the intervals from 24 to 30 hours and from 30 to 48 hours. Approximately 37% of the administered radioactivity was recovered in faeces over the 48 hours after administration, with a further 1% in urine. At 48 hours after administration, approximately 1% of the administered radioactivity remained in liver, approximately 0.5% in gastrointestinal tract contents and less than 0.1% in gastrointestinal tract tissue; levels were below the level of detection in the remaining carcass.

The total absorbed dose (i.e. the radioactivity seen in bile, urine, liver and gastrointestinal tract tissue) accounted for an average of approximately 61% of the administered dose, almost all of which was excreted via the bile (59% of the

administered dose). The unabsorbed dose (i.e. the radioactivity seen in faeces and the gastrointestinal tract contents) accounted for an average of approximately 37% of the administered dose. In total, approximately 98% of the administered radioactivity was recovered (Laurencot et al., 1978).

2.1.2 Biotransformation

A comparative metabolism study (GLP status not stated) was performed in which metabolite profiles were examined in lyophilized faeces and livers resulting from oral administration of [^{14}C]lasalocid sodium to mouse, rat, dog, turkey, swine and chicken. The study used faeces and livers sourced from other studies and so does not provide information on the doses administered to the test animals or the dosing regimens, although the report numbers for the studies from which the livers and faeces were sourced are provided. The mouse and rat faeces and livers were generated in the studies reported in Hawkins, Elsom & de-Salis (1987a, 1987b), both of which used seven daily lasalocid doses of 1 mg/kg bw. The reports referenced for the studies from which the livers and faeces of other species were sourced were not provided.

Lyophilized liver samples were extracted with methanol, which was then diluted with aqueous sodium chloride and extracted with hexane. The hexane extract was analysed by high-performance liquid chromatography (HPLC) with radioactivity detection. The remaining aqueous methanol fraction was further diluted with aqueous sodium chloride before undergoing extraction with chloroform followed by separation using preparative thin-layer chromatography and then resolution using HPLC with radioactivity detection.

Intact lasalocid in the liver accounted for 6.2% of the radioactivity in pig liver, 3.8% in turkey liver, 11.4% in chicken liver, 18.1% in dog liver, 31.9% in rat liver and 28.1% in mouse liver.

For dog, rat, mouse, chicken and turkey, the proportion of the total radioactive residues (TRR) in liver extracted into methanol was 67.8%, 84.6%, 75.8%, 50.3% and 47.5%, respectively. The proportion of the TRR extracted into the hexane fraction was 21.0%, 35.3%, 28.7%, 26.3% and 5.6% for dog, rat, mouse, chicken and turkey, respectively. The proportion of the TRR extracted into the chloroform fraction was 47.6%, 32.9%, 37.8%, 28.0% and 36.3% for dog, rat, mouse, chicken and turkey, respectively.

In the HPLC analysis of the hexane extract, lasalocid was the major peak in all species (11.2%, 25.4%, 23.1%, 8.8% and 11.2% of the TRR in liver from the dog, rat, mouse, chicken and turkey, respectively). All other peaks represented less than 5% of the TRR in liver in all species. In the HPLC analysis of the chloroform extract, all peaks, including lasalocid, represented less than 10% of the TRR in liver, and all non-lasalocid peaks represented less than 5% of the TRR in liver.

Lyophilized faecal samples were extracted and fractionated in the same way as the liver samples.

Intact lasalocid in faeces accounted for 33.2% of the radioactivity in pig faeces, 32.2% in dog faeces, 43.7% in rat faeces, 10% in turkey faeces and 12% in chicken faeces.

For dog, rat, mouse, chicken and turkey, the proportion of the TRR extracted into methanol was 79.3%, 82.1%, 72.2%, 67.8% and 77.6%, respectively. The proportion of the TRR in faeces extracted into the hexane fraction was 42.3%, 68.6%, 30.2%, 17.% and 28.4% for dog, rat, mouse, chicken and turkey, respectively. The proportion of the TRR extracted into the chloroform fraction was 21.1%, 10.2%, 36.3%, 44.7% and 43.5% for dog, rat, mouse, chicken and turkey, respectively.

In the HPLC analysis of the hexane extract, lasalocid was the major peak in all species (32.0%, 43.4%, 21.0%, 11.6% and 9.7% of the TRR in faeces from the dog, rat, mouse, chicken and turkey, respectively). All other peaks represented less than 10% of the TRR in faeces in these species. In the HPLC analysis of the chloroform extract, all peaks, including lasalocid, represented less than 1% of the TRR in faeces.

This study supports the position that lasalocid is the main residue present in liver and faeces. Although the other residues have not been identified, they are considered to be minor (Laurencot & Weiss, 1987).

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Oral administration

In a study that pre-dated GLP requirements, mice, rats and rabbits were administered lasalocid sodium as single doses by several routes. Neonatal rats were also tested, and a single dog was administered increasing doses of the substance. Lasalocid sodium was ground in a mortar and suspended in 5% gum acacia solution. Animals were observed for 5 days following administration of the test substance.

Groups of 10 CF-1s mice were administered lasalocid sodium by the oral (gavage) route at a dose of 125, 160 or 200 mg/kg bw, by the intraperitoneal route at a dose of 50, 63, 80 or 100 mg/kg bw or by the subcutaneous route at a dose of 100, 125, 160 or 200 mg/kg bw. Mice of both sexes were used, although the ratio of male to female animals was not specified.

Groups of 10 Wistar rats were administered lasalocid sodium by the oral (gavage) route at a dose of 80, 100, 160 or 200 mg/kg bw or by the intraperitoneal route at a dose of 15, 25 or 50 mg/kg bw. Rats of both sexes were used, although the ratio of male to female animals was not specified.

Groups of four albino rabbits were administered lasalocid sodium by the oral (gavage) route at a dose of 25, 36, 45 or 50 mg/kg bw.

Groups of 10 neonatal (< 24 hours old) rats were administered lasalocid sodium by the oral (gavage) route at a dose of 20, 32 or 40 mg/kg bw. Rats of both sexes were used, although the ratio of male to female animals was not specified.

A single female dog was administered daily doses of lasalocid sodium at increasing daily doses (the dose was doubled every day, with a break in dosing over the weekend). The starting dose was 2.5 mg/kg bw, and the highest dose

administered was 160 mg/kg bw. The substance was administered in gelatine capsules. Plasma glucose and cholesterol were determined predosing and at 1 hour after each dose, as well as 6 days after the final dose. Blood counts, haemoglobin, haematocrit and blood enzymes were determined predosing and 1 hour after the 40, 80 and 160 mg/kg bw doses.

In mice, deaths were observed at all doses and by all routes, with the number of deaths increasing in a dose-dependent manner. The median lethal dose (LD_{50}) was calculated to be 146 mg/kg bw for the oral route, 68 mg/kg bw for the intraperitoneal route and 140 mg/kg bw for the subcutaneous route. The only other sign of toxicity observed was tremors, noted only in the intraperitoneally dosed mice.

In rats, deaths were observed at all doses in the orally dosed animals and at the top two doses in the intraperitoneally dosed animals. The number of deaths increased in a dose-dependent manner in both the orally and intraperitoneally dosed animals. The LD_{50} was calculated as 122 mg/kg bw for the oral route and 26.5 mg/kg bw for the intraperitoneal route. Other observed signs of toxicity reported were cyanosis and respiratory depression in the orally dosed animals and cyanosis, respiratory depression and decreased motor activity in the intraperitoneally dosed animals.

In rabbits, deaths were observed at all except the second lowest dose, with the highest number of deaths seen at the highest dose. The oral LD_{50} was calculated to be 40 mg/kg bw. No other signs of toxicity were reported.

In neonatal rats, a number of treated animals were missing and presumed to have been cannibalized and so counted as having died. A single death was observed at the low dose, no deaths at the middle dose and six deaths at the high dose. The oral LD_{50} was calculated to be 33 mg/kg bw.

In the dog study, no symptoms of toxicity were observed at 2.5 or 5 mg/kg bw. Clinical signs of toxicity were seen at 10 mg/kg bw and above. At 10 mg/kg bw, only emesis was seen. At higher doses, clinical signs included partial paralysis of hind quarters (at ≥ 20 mg/kg bw), wobbly gait and loss of appetite (at ≥ 40 mg/kg bw), defecation, salivation and severe ataxia (at 160 mg/kg bw). The dog survived to the end of the 6-day post-dosing monitoring phase. Haematology results showed increased white blood cell counts (from 7300 to 15 300 per cubic millimetre) 1 hour after the 160 mg/kg bw dose, which returned to predosing levels in the 6-day post-dosing blood sample. Small changes were seen in a number of the blood biochemistry parameters; however, given that a single animal was sampled, it is not possible to comment on the significance of these.

This study is incompletely reported, and the post-dosing observation period in the rodent and rabbit studies was limited to 5 days (Pool, Hane & Suckow, 1972).

The oral LD_{50} values above suggest that there are marked species differences, with the rabbit being significantly more sensitive than the rat or mouse and with neonatal rats being more sensitive than adult rats. This is consistent with published literature, which reports considerable species differences in susceptibility

to lasalocid toxicity. Horses have been reported to be particularly more sensitive to lasalocid toxicity than any of the above species, with an oral LD_{50} of 21.5 mg/kg bw estimated by one group (Hanson, Eisenbeis & Givens, 1981), although it has been argued that the real oral LD_{50} may be considerably lower in this species (Kronfeld, 2002). In addition, neonatal bovine calves have been reported to be more sensitive to lasalocid toxicity than older calves (Benson et al., 1998). A review of the acute toxicity of lasalocid is provided by Galitzer (1984).

(b) Dermal application

A pre-GLP study was performed in rabbits to investigate acute dermal toxicity. Two New Zealand White rabbits of each sex were administered single dermal doses of lasalocid sodium at 500, 1000 or 2000 mg/kg bw. The hair was clipped prior to administration of the substance, and the skin of one animal of each sex was abraded. The substance was applied as a powder under a gauze patch, and a plastic lined sleeve was placed over the trunk of each rabbit and fastened in place. After 24 hours, the wrappings were removed. Test animals were observed for 14 days in total, and deaths were recorded.

Signs of toxicity reported were decreased motor activity, cowering in the cage and lacrimation. All animals in the 500 mg/kg bw group survived. In the 1000 mg/kg bw group, one animal with abraded skin died. In the 2000 mg/kg bw group, three animals died (one with abraded skin and two with intact skin). A dermal LD_{50} of 1400 mg/kg bw was calculated (Hane, 1977).

(c) Dermal and ocular irritation

A pre-GLP study was performed in rabbits to investigate skin and eye irritation.

For skin irritation testing, three New Zealand White rabbits were used (it was reported that rabbits of both sexes were used, although the numbers of each sex were not reported). The hair was clipped from the back of each animal, and 500 mg of powder moistened with distilled water was applied to intact and abraded sites on each animal. The test sites were covered first with gauze patches and then with plastic occlusive coverings. After 4 hours, the coverings and patches were removed, and irritation was evaluated. Further irritation readings were taken after 24 and 48 hours. No signs of irritation were reported in any of the tested animals (Hane, 1977).

For ocular irritation testing, three New Zealand White rabbits of each sex were used. Lasalocid powder (0.036 g) was instilled into the conjunctival sac of one eye of each of six rabbits. The eyes of three rabbits were washed with water 5 minutes after instillation. The eyes of the remaining three rabbits were washed after 24 hours. Ocular reactions were graded 14 days after instillation. Signs of corneal irritation, conjunctival redness and chemosis were seen in both groups in which the eyes had been washed 5 minutes and 24 hours after instillation (Hane, 1977).

(d) Dermal sensitization

A pre-GLP study was performed to investigate the skin sensitizing potential of lasalocid sodium in the guinea-pig maximization test following both an intradermal and a topical induction phase. Twenty female albino guinea-pigs per group were divided into two groups of 10. The test group received intradermal injections of 0.1 mL of Freund's complete adjuvant, 0.1 mL of liquid petrolatum containing 1% lasalocid sodium and 0.1 mL of 1% lasalocid sodium emulsified in Freund's complete adjuvant. Control animals received the same series of injections with only vehicles and not lasalocid sodium. One week later, test guinea-pigs received a topical application of lasalocid sodium dispersed in liquid petrolatum at a concentration of 25%, applied over the injection site on filter paper and wrapped in an occlusive cover for 48 hours. Control animals received a topical application of liquid petrolatum. Two weeks after the initial topical application, all guinea-pigs (test and controls) were challenged by topical application of 25% lasalocid sodium in 0.5 mL of liquid petrolatum, applied on filter paper and wrapped in an occlusive cover for 24 hours. Twenty-four hours after application, the coverings were removed, and the application sites were washed with water. Erythema and oedema were scored on a scale of 0–4, and areas involved in irritant responses were measured. Evaluation took place 24 and 48 hours after challenge.

Erythema was observed in both test and control animals, without notable differences between those animals that underwent induction and those animals that did not. No oedema was noted in either group. It is concluded that lasalocid sodium did not induce skin sensitization in the guinea-pig maximization test (Hane et al., 1977).

2.2.2 Short-term studies of toxicity

(a) Rats

In a pre-GLP study, groups of 16 Charles River CD rats (8 males and 8 females) were administered lasalocid sodium in the diet at an intended dose of 0 (control), 2, 5 or 20 mg/kg bw per day for 13 weeks. Dosing at the desired level was maintained by adjusting the concentration in the diet on a weekly basis based on the average feed consumption and body weight of each group. Animals were individually caged and allowed feed ad libitum. Average actual drug intake levels were calculated for each sex at each dose level for each week. In males, average doses were 2.1 (range 1.4–3.3), 5.2 (range 4.3–6.7) and 20.3 (range 17.1–27.8) mg/kg bw per day in the low-, mid- and high-dose groups, respectively; in females, they were 2.3 (range 1.5–3.6), 5.2 (range 4.2–6.2) and 19.7 (range 16.0–24.0) mg/kg bw per day, respectively.

Animals were observed daily for physical and behavioural signs of toxicity. Feed consumption and body weights were measured weekly. Neurological examinations (observations of gait, body position, muscle tone, movement of legs and reflexes) were performed prior to treatment and after 4, 8 and 12 weeks. Ophthalmic examinations (observations of eyelids, conjunctiva and sclera, examination of cornea, iris, lens and fundus with an ophthalmoscope) were performed at the onset of treatment, after 4 and 8 weeks and during the

12th week. Haematological examinations were performed on five rats of each sex per group prior to treatment and after 4, 8 and 12 weeks. Haematological examinations included measurement of the susceptibility of erythrocytes to haemolysis in hypotonic saline following collection of blood at necropsy. This was done because it was noted, while performing haemoglobin analyses, that some red blood cell samples required longer than expected to lyse. Heparinized whole blood was mixed with hypotonic saline (0.35%, 0.40%, 0.45% and 0.50% saline), and, after 30 minutes, intact cells were separated by centrifugation. Haemolysis was estimated in the supernatant by measuring light transmission. The per cent haemolysis was reported by comparison with measurements taken following mixing of whole blood with physiological saline. These measurements were also performed with blood from additional rats administered lasalocid sodium intravenously as a single dose of 5 mg/kg bw. Blood glucose was determined in samples from five rats of each sex per group after 4, 8 and 12 weeks. Blood biochemistry parameters were determined in samples collected at termination. Urine analysis was undertaken on urine collected individually from five rats of each sex per group prior to treatment and after 4, 8 and 12 weeks. At necropsy, organs were examined grossly, weighed and prepared for microscopic evaluation.

No compound-related mortality occurred during the study. Feed consumption and body weights were significantly reduced in females in the 20 mg/kg bw per day group over the entire dosing period (by week 13, body weights of females were 62% of those in the control group). The overall pattern of feed consumption and body weight in males in the 20 mg/kg bw per day group did not differ from that seen in control males, although average weekly feed intake and body weights were statistically significantly different between the two groups at a number of discrete time points. Feed consumption and body weights were reduced in females in the 5 mg/kg bw per day group compared with control females, although the effect did not reach statistical significance. No effects on feed consumption or body weight were seen in males in the 5 mg/kg bw per day group or in either sex in the 2 mg/kg bw per day group. No compound-related behavioural or neurological signs of toxicity were seen.

Haemoglobin and haematocrit levels were slightly (but statistically significantly) decreased in females at 20 mg/kg bw per day at the 4-week time point only. At other time points, haemoglobin, haematocrit, coagulation times and differential white blood cell counts showed only minor and transient variations across the dose groups, which were not considered to be drug related. In males and females in the 20 mg/kg bw per day group, mild variations in erythrocyte size and shape were noted, as well as some polychromatic and crenated erythrocytes and target cells. Investigations of the susceptibility of erythrocytes to haemolysis in hypotonic saline demonstrated reduced haemolysis in blood of female rats administered lasalocid sodium (e.g. after mixing with 0.40% saline, 92% haemolysis was seen for blood from control rats, compared with 68%, 44% and 29% for blood from female rats administered lasalocid sodium at 2, 5 and 20 mg/kg bw per day, respectively). An effect was also seen in male rats, although it was less marked, and the dose–response relationship was less clear (e.g. after mixing with 0.40% saline, 77% haemolysis was seen for blood of control rats, compared with 78%, 74% and 60% for blood from male rats administered lasalocid sodium at

2, 5 and 20 mg/kg bw per day, respectively). Haemolysis was also reduced in blood from male and female rats treated with a single intravenous dose of 5 mg/kg bw. The study authors argued that this effect should not be considered as adverse.

Blood glucose levels in males were unaffected. At 12 weeks after the start of dosing, blood glucose levels were statistically significantly reduced in female rats administered 2 and 20 mg/kg bw per day, but not 5 mg/kg bw per day. At 8 weeks after the start of dosing, blood glucose levels were statistically significantly reduced in female rats administered 20 mg/kg bw per day only. The effect was slight and not dose related and is not considered to be of biological significance. A statistically significant increase in serum alkaline phosphatase (AP) was seen in males and females in the 20 mg/kg bw per day group. Alanine aminotransferase (ALAT) levels were statistically significantly reduced in males in the 5 mg/kg bw per day group only and were statistically significantly decreased in females in the 5 mg/kg bw per day group and statistically significantly increased in females in the 20 mg/kg bw per day group. Aspartate aminotransferase (ASAT) levels were statistically significantly increased in males and females in the 20 mg/kg bw per day group. Blood urea nitrogen levels were statistically significantly decreased in males in the 20 mg/kg bw per day group. For many animals, the volume of serum available was too low to allow measurement of serum sodium, potassium, chloride and calcium levels. No clear pattern emerges from the available measurements.

There were no findings of note in the urine analysis.

The only lesion noted at gross pathology evaluation was an enlarged uterus in one rat in the 5 mg/kg bw per day group.

In females in the 20 mg/kg bw per day group, absolute lung, liver, kidney, adrenal gland, pituitary gland and ovary weights were decreased and relative (to body weight) heart, lung, liver, kidney, thyroid, adrenal, pituitary gland, spleen and brain weights were significantly increased. Ovary to body weight ratio was statistically significantly decreased.

In females at 5 mg/kg bw per day, liver to body weight ratios were increased.

In females in the 2 mg/kg bw per day group, uterus weights were statistically significantly decreased overall, although the organ weight was substantially increased in a single animal; uterus to body weight ratios were statistically significantly decreased. In the 5 mg/kg bw per day group, uterus weights were statistically significantly increased; uterus to body weight ratios were statistically significantly increased. In the 20 mg/kg bw per day group, uterus weights were statistically significantly decreased; uterus to body weight ratios were statistically significantly decreased. Whereas a decrease in uterus weight was seen in six of eight animals in the 20 mg/kg bw per day group, the effect in the 2 and 5 mg/kg bw per day groups was less clear and consistent. Based on the lack of a dose–response relationship and the absence of histopathological findings at all doses, the effect in the 2 and 5 mg/kg bw per day groups is not considered biologically significant.

In males in the 2 mg/kg bw per day group, only pituitary weights were slightly decreased. No effect on the pituitary was seen at higher doses; consequently, this

effect was not considered biologically significant. In males in the 5 mg/kg bw per day group, only testes weights were slightly decreased. No effect was seen on the testes at the higher dose. Liver to body weight ratios were slightly decreased at 5 mg/kg bw per day. No effect was seen at the higher dose. Based on the absence of a dose–response relationship, these effects were not considered drug related. In males in the 20 mg/kg bw per day group, only brain weights were slightly decreased, and heart to body weight ratio was slightly decreased. No effect on brain or heart weight was seen at lower doses.

Histopathological evaluation revealed increased haemosiderin in liver (Kupffer cells) and kidney epithelial cells in all females and in four and two males (livers and kidneys, respectively) at 20 mg/kg bw per day. Increased haemosiderin was also seen in the liver of a single female at 5 mg/kg bw per day. In six of eight females in the 20 mg/kg bw per day group, large vacuoles were noted in the pituitary. Small vacuoles in the myocardium were also noted in seven females and three males in the 20 mg/kg bw per day group, with similar vacuoles in skeletal muscle reported in five females in the 20 mg/kg bw per day group.

It is noted that the number of animals included in this study (eight of each sex per dose) was less than would be expected in a study performed for regulatory purposes today (Organisation for Economic Co-operation and Development [OECD] Test Guideline 408 specifies 10 of each sex per dose).

The only effect noted at the lowest dose of 2 mg/kg bw per day was a reduced susceptibility of erythrocytes to haemolysis in hypotonic saline. This effect is consistent with reports of studies performed with the divalent ionophore A23187. A number of authors (Reed & Lardy, 1972; White, 1974; Kuettner et al., 1977) have reported effects of A23187 in vitro, which has been observed to cause calcium-dependent loss of potassium, water and adenosine triphosphate from erythrocytes, as well as a reduced sensitivity to osmotic lysis. Kuettner et al. (1977) further reported that the increased calcium content of erythrocytes caused by A23187 induced morphological changes and reduced viscoelastic properties. Bogin et al. (1982) reported that A23187 reduced erythrocyte membrane fragility in the absence of calcium, but increased membrane fragility in the presence of calcium. It is notable that in the study under evaluation, effects on erythrocyte morphology were seen, although only at 20 mg/kg bw per day. The perturbation of ionic flux across the erythrocyte membrane induced by lasalocid has been specifically studied using nuclear magnetic resonance (Fernandez, Grandjean & Laszlo, 1987). It is not surprising that the ionophoretic effects of lasalocid should have an impact on (erythrocyte) membrane stability. The effect seen in this study was observed ex vivo and was not associated with a toxicological effect in vivo. Consequently, the increased resistance to lysis in hypotonic saline is not, in itself, considered to be an adverse effect.

The no-observed-adverse-effect level (NOAEL) was 2 mg/kg bw per day, based on reduced feed consumption, increased liver to body weight ratios and increased haemosiderin in the liver in females at 5 mg/kg bw per day (Pfitzer & Roberts, 1973).

In a pre-GLP study, groups of 80 Charles River CD weanling rats (40 males and 40 females) were administered lasalocid sodium in the diet at an intended dose of 0 (control), 1, 2, 3 or 10 mg/kg bw per day for 13 weeks. Dosing at the desired level was maintained by adjusting the concentration based on the average feed consumption and body weight of each group. Diets were adjusted for appropriate doses twice a week for the first 3 weeks (when feed consumption relative to body weight was rapidly decreasing) and weekly thereafter. Animals were caged individually and allowed feed ad libitum. Average actual drug intake levels were calculated for each sex at each dose level twice a week for the first 3 weeks and weekly thereafter. Average weekly group intakes (overall averages over the entire study period) were, for males, 0.72–1.11 (0.95), 1.35–2.56 (1.93), 2.25–3.34 (2.89) and 5.84–13.43 (9.70) mg/kg bw per day at 1, 2, 3 and 10 mg/kg bw per day, respectively; and for females, 0.66–1.21 (0.97), 1.44–2.54 (1.92), 1.79–3.84 (2.93) and 6.12–11.98 (9.39) mg/kg bw per day at 1, 2, 3 and 10 mg/kg bw per day, respectively.

Twenty male and 20 female rats from each group were killed after 8 weeks, and the remainder were killed at the end of 13 weeks. Animals were observed daily for physical and behavioural signs of toxicity. Feed consumption and body weights were measured weekly. Neurological examinations (observations of gait, body position, muscle tone, movement of legs and reflexes) were performed prior to treatment and after 4, 8 and 12 weeks. Ophthalmic examinations (observations of eyelids, conjunctiva and sclera, examination of cornea, iris, lens and fundus with an ophthalmoscope) were performed after 1 week of treatment and prior to necropsy at 8 and 13 weeks. Haematological examinations were performed on blood samples taken after 7 and 12 weeks. Blood biochemistry parameters were determined in samples taken from animals killed at 8 and 13 weeks. Urine analysis was undertaken on urine collected from each rat after 7 and 12 weeks. At necropsy, organs were examined grossly, weighed and prepared for microscopic evaluation.

No compound-related mortality occurred during the study. Body weights were significantly reduced in females in the 10 mg/kg bw per day group from weeks 8 to 13 (by week 13, body weights of females were 88.5% of those in the control group). Feed consumption in females in the 10 mg/kg bw per day group was also slightly reduced from weeks 9 to 13, although the effect did not reach statistical significance. Body weights in males in the 10 mg/kg bw per day group were slightly decreased in the last weeks of the study, but the effect did not reach statistical significance. No effect on feed consumption was evident in males in the 10 mg/kg bw per day group. No compound-related effects on body weight or feed consumption were seen in either sex in the other groups. No compound-related behavioural or neurological signs of toxicity were seen. No compound-related ophthalmic findings were noted.

Compared with controls, haematocrit and haemoglobin levels were statistically significantly decreased after week 7 in female rats in the 2, 3 and 10 mg/kg bw per day groups and after week 12 in female rats in the 3 and 10 mg/kg bw per day groups; haematocrit (but not haemoglobin) was statistically significantly decreased in females in the 2 mg/kg bw per day group at week 12. In male rats, haematocrit and haemoglobin were statistically significantly decreased

after both weeks 7 and 12 in the 1, 3 and 10 mg/kg bw per day groups but not in the 2 mg/kg bw per day group. Based on the lack of a dose–response relationship and on the small magnitude of the decreased haematocrit and haemoglobin levels seen in males in the 1 mg/kg bw per day group (96% or 97% of control values in all cases), the effect at this dose is not considered to be a drug effect. Coagulation times were slightly but statistically significantly reduced in both males and females in the 10 and 3 mg/kg bw per day groups after 12 weeks. A slight increase in coagulation time was seen in males in the 1 mg/kg bw per day group after 7 weeks, but was not considered to be drug related. Increased white blood cell counts were seen in females in the 10 mg/kg bw per day group after both 7 and 12 weeks, and increased white blood cell counts were seen in males in the 10 mg/kg bw per day group after 12 weeks only. Lymphocyte counts were statistically significantly decreased in females in the 10 mg/kg bw per day group and in females in the 3 mg/kg bw per day group at 12 weeks only. Neutrophil counts were statistically significantly increased in females in the 2, 3 and 10 mg/kg bw per day groups after both 7 and 12 weeks. In males, neutrophil counts were increased in the 3 and 10 mg/kg bw per day groups, but the effect did not reach statistical significance. Eosinophil counts were statistically significantly increased in females in the 2 and 3 mg/kg bw per day groups (but not in the 10 mg/kg bw per day group) after 12 weeks. The study report notes that morphological changes (target cells) were seen in a minority of erythrocytes after 12 weeks in all female rats in the 10 mg/kg bw per day group and in 7/20 females in the 3 mg/kg bw per day group; the finding was also noted in a small number of males at these doses. Small numbers of nucleated erythrocytes were noted in 3/20 females in the 10 mg/kg bw per day group and in 1/20 females in the 3 mg/kg bw per day group. Unlike in the previous study (Pfitzer & Roberts, 1973), susceptibility of erythrocytes to haemolysis was not specifically measured in this study. However, the study report indicates that in the haemoglobin analysis, increased resistance to lysis was noted at the 10 mg/kg bw per day dose in all females and in a number of males.

AP was statistically significantly increased in male rats in all dose groups at 13 weeks and in the 2 and 10 (but not the 1 and 3) mg/kg bw per day groups at 8 weeks. AP was statistically significantly increased in female rats in the 2, 3 and 10 mg/kg bw per day groups at both 8 and 12 weeks. The study authors argued that AP decreases markedly with age from around 400–500 International Units (IU) at weaning and that, consequently, the increased AP seen in this study represents a reduced decrease relative to controls (rather than an absolute increase). They further argued that, in this study, AP decreased particularly rapidly in the control group (control group averages at 8 and 13 weeks were reported to be 211.8 and 144.9 IU in males and 134.1 and 103.3 IU in females, respectively), whereas in a concurrent study using weanling rats, the values decreased less rapidly (control group averages at 8 and 13 weeks were reported to be 261.5 and 222.4 IU in males and 151.9 and 146.1 IU in females, respectively); on the basis of this information, the only biologically significant effect on AP was considered to occur in females in the 10 mg/kg bw per day group. This argument is not accepted. In the concurrent study, rats were treated with the same doses of lasalocid sodium, and measurements were taken at similar time points. However, it is noteworthy that the AP values reported in that study are higher in most dose groups, not only in the

control groups (suggesting that it is not appropriate to compare the control data from one study with the test data from the other study), although the difference in the values reported for the control groups in the two studies is a little more marked than in most other groups.

ASAT levels were statistically significantly increased in females in the 10 mg/kg bw per day group after 13 weeks only, and blood urea nitrogen levels were statistically significantly decreased in females in the 10 mg/kg bw per day group after 13 weeks and in males in the 3 and 10 mg/kg bw per day groups at 13 weeks.

Serum calcium levels were statistically significantly elevated in females at all doses at 8 weeks only and in males in the 2, 3 and 10 mg/kg bw per day groups at 8 weeks only. Serum sodium levels were statistically significantly increased in females in all dose groups at 8 weeks and in the 3 mg/kg bw per day group at both 8 and 13 weeks. In males, serum sodium levels were statistically significantly increased in the 2, 3 and 10 mg/kg bw per day groups at 8 weeks and in the 3 mg/kg bw per day group at 13 weeks also. Serum potassium levels were increased in females in the 10 mg/kg bw per day group at 8 weeks only and in males in the 3 and 10 mg/kg bw per day groups at 8 weeks only. Serum chloride levels were increased in males and females in the 10 mg/kg bw per day group at 13 weeks only. It is noteworthy that all changes in electrolyte levels seen in the 1, 2 and 3 mg/kg bw per day groups were within a few per cent of control values (the largest deviation was a value of 106% for calcium seen in females at 3 mg/kg bw per day at 8 weeks). Although the changes reached statistical significance on a number of occasions, whether or not the changes were drug related is questionable. At 10 mg/kg bw per day, some slightly larger variations were noted (the largest deviation from control was for serum potassium and calcium levels, which were both at 112% of control levels in females at 8 weeks).

There were no findings of note in the urine analysis.

No lesions considered to be compound related were noted at gross pathology.

In females, the following statistically significant organ weight changes were noted at 10 mg/kg bw per day: decreased absolute heart weight at week 13, decreased absolute and relative (to body weight) lung weights at weeks 8 and 13, increased relative liver weight at 13 weeks, increased relative kidney weight at weeks 8 and 13, decreased absolute ovary weight at week 8 and decreased absolute and relative ovary weights at week 13, increased relative thyroid weight at weeks 8 and 13, increased absolute adrenal weight at week 8, increased relative pituitary weight at week 8 and increased relative brain weight at week 8. At 3 mg/kg bw per day, the following statistically significant organ weight changes were noted: increased relative heart weight at week 8, decreased relative lung weight at week 13, increased relative liver weight at week 13, increased relative thyroid weight at week 13 and increased relative pituitary weight at weeks 8 and 13. In females in the 1 and 2 mg/kg bw per day groups, relative pituitary weights were statistically significantly increased at 13 weeks. Although relative kidney weights were not statistically different from controls in the 3 mg/kg bw per day group, they were statistically significantly different from controls in the 1 and 2 mg/kg bw per day groups at 8 weeks only.

In males, the following statistically significant organ weight changes were noted at 10 mg/kg bw per day: decreased absolute and relative heart weights at weeks 8 and 13 and decreased absolute and relative lung weights at week 13; at 3 mg/kg bw per day: decreased absolute and relative heart weights at week 13 and decreased relative heart weight at week 8; at 2 mg/kg bw per day: decreased absolute and relative heart weights at week 8; and at 1 mg/kg bw per day: decreased relative heart weight at week 8. A number of other statistically significant organ weight changes were seen, but were not considered compound related, as they occurred with no particular pattern.

A full histopathological examination was performed on five male and five female rats in the 10 mg/kg bw per day group and control group killed after 8 weeks. Histopathological evaluations focusing on liver, kidney and heart were performed on 15 additional male and female rats from the 10 mg/kg bw per day group and the control group as well as on 20 male and 19 female rats in the 3 mg/kg bw per day group. There was no histopathological examination of rats from the 1 and 2 mg/kg bw per day groups killed after 8 weeks.

After 8 weeks, vacuoles were noted in the myocardial fibres of females administered 10 mg/kg bw per day. The vacuoles were not stained by eosin or fat stains. The effect was not seen in animals administered 3 mg/kg bw per day. In the liver, haemosiderin was seen in Kupffer cells in the 3 and 10 mg/kg bw per day groups, with the effect being more pronounced at the higher dose. In the kidney, haemosiderin was moderately increased in some females administered 10 mg/kg bw per day. No differences compared with controls were noted in the 3 mg/kg bw per day group.

A full histopathological examination was performed on 20 male and 20 female rats in the 10 mg/kg bw per day group and control group killed after 13 weeks. Histopathological evaluations focusing on liver, kidney and heart were performed on 20 male and 20 female rats in the 2 and 3 mg/kg bw per day groups. There was no histopathological examination of rats from the 1 mg/kg bw per day group.

After 13 weeks, vacuoles were noted in the myocardial fibres of females administered 10 mg/kg bw per day. The vacuoles were not stained by eosin or fat stains. The effect was not seen in animals administered 2 or 3 mg/kg bw per day. In the liver, increased (relative to control) haemosiderin was seen in Kupffer cells in females and males in the 10 mg/kg bw per day group and in females only at 3 mg/kg bw per day, with the effect being more pronounced at the higher dose. In the kidney, haemosiderin was increased in some females in the 3 and 10 mg/kg bw per day dose groups, with the effect being more common at the higher dose. No differences compared with controls were noted in the 2 mg/kg bw per day group.

Based on increased AP levels seen in males at all doses at week 13, the lowest-observed-adverse-effect level (LOAEL) for this study was 1 mg/kg bw per day. No NOAEL was established (Pfitzer & Roberts, 1975a). It is noted, however, that the low-dose effect on AP seen in this study was not seen in other rat studies.

In a pre-GLP study, groups of 120 Charles River CD weanling rats (60 males and 60 females) were administered lasalocid sodium in the diet at an intended dose of 0 (control), 1, 2, 3 or 10 mg/kg bw per day for 13 weeks. The weanling rats were selected from offspring of rats that had been administered the same intended dose of lasalocid sodium in the diet. Parental animals were dosed for 3 weeks prior to mating and during mating (approximately 2 weeks), gestation and lactation (approximately 6 weeks). Weanling rats received lasalocid sodium in the diet immediately upon weaning and for 13 weeks thereafter. The present study (Pfitzer & Roberts, 1975b) detailed only the effects on weanling rats. In relation to effects on the parental generation, it reported that no effects were seen on the various parameters of fertility and reproductive performance except at the 10 mg/kg bw per day dose, at which there were reduced maternal and neonatal weight gains. The effects on reproductive parameters of the parental animals are reported in Hoar et al. (1974) and described in section 2.2.5 below.

Dosing of weanlings at the desired level was maintained by adjusting the concentration based on the average feed consumption and body weight of each group. Diets were adjusted for appropriate doses on a weekly basis. Animals were caged individually and allowed feed ad libitum. Average actual drug intake levels were calculated for each sex at each dose level on a weekly basis. Average weekly group intakes (overall averages over the entire study period) were, for males, 0.85–1.30 (0.96), 1.69–2.99 (1.95), 2.57–4.45 (2.99) and 8.37–13.03 (9.60) mg/kg bw per day at 1, 2, 3 and 10 mg/kg bw per day, respectively; and, for females, 0.79–1.24 (0.96), 1.65–2.54 (1.94), 2.51–5.63 (3.09) and 8.50–13.29 (9.80) mg/kg bw per day at 1, 2, 3 and 10 mg/kg bw per day, respectively.

Twenty male and 20 female rats from each dose group were killed after 4, 8 and 13 weeks. Animals were observed daily for physical and behavioural signs of toxicity. Feed consumption and body weights were measured weekly. Ophthalmic examinations (observations of eyelids, conjunctiva and sclera, examination of cornea, iris, lens and fundus with an ophthalmoscope) were performed after 1 week of treatment on all rats, after 3 weeks on 20 animals of each sex per dose, after 8 weeks on 20 animals of each sex per dose and after 11 and 13 weeks on the remaining animals. Haematological examinations were performed after 2, 7 and 12 weeks on 20 rats of each sex per group. Blood biochemistry parameters were determined in samples taken from animals killed at 4, 8 and 13 weeks. Urine analysis was undertaken on urine collected from rats after 2, 7 and 12 weeks. At necropsy, organs were examined grossly, weighed and prepared for microscopic evaluation. Only tissues from animals killed at 4 and 13 weeks were examined histologically.

No compound-related mortality occurred during the study. At the start of the 13-week dosing period, body weights of female weanlings in the 10 mg/kg bw per day dose group were statistically significantly reduced (compared with concurrent controls), but these had recovered by the end of the 1st week. Body weights were statistically significantly reduced in females in the 10 mg/kg bw per day group in weeks 12 and 13 (by week 13, body weights of females were 91.9% of those in the control group). Feed consumption of females in the 10 mg/kg bw per day group was reduced in weeks 11, 12 and 13 (down to 82.6% of that seen

in control females at 13 weeks). Feed consumption was also reduced in females in the 2 and 3 mg/kg bw per day groups in weeks 12 and 13, although the effect reached statistical significance only in the 2 mg/kg bw per day group at week 12. Body weights of male test animals were not statistically significantly different from those of control animals. Feed consumption was statistically significantly reduced in males in the 3 and 10 mg/kg bw per day groups. Differences in mean body weights and feed consumption occurred between controls and treatment groups at a number of other time points; however, due to their isolated occurrence, these are not considered biologically significant. No compound-related behavioural signs of toxicity were seen. Ophthalmic examination revealed increased levels of focal retinal degeneration in the 3 and 10 mg/kg bw per day groups. Focal retinal degeneration was also seen in rats in the 1 and 2 mg/kg bw per day groups, but the incidence was similar to that seen in the control group. The sponsor argued that the condition occurs spontaneously in the laboratory rat, that it has a multifactorial etiology (possibly including bacterial or viral infection) and that the increased incidence seen in the 3 and 10 mg/kg bw per day groups is likely to be an indirect effect resulting from increased overall stress in these groups and not a direct effect of the drug.

Compared with controls, haematocrit levels were statistically significantly decreased in females in the 10 mg/kg bw per day group after weeks 7 and 12; haemoglobin levels were statistically significantly decreased after week 12 only. Haematocrit levels were also decreased in female rats in the 2 and 3 mg/kg bw per day groups after week 2 only. In male rats, haematocrit and haemoglobin levels were statistically significantly decreased in the 10 mg/kg bw per day group after 12 weeks; after 2 weeks, haematocrit levels were statistically significantly decreased in the 1, 2 and 3 mg/kg bw per day groups (no effect seen after 7 or 12 weeks). All changes in haematocrit and haemoglobin levels were slight; additionally, some sporadic increases in haemoglobin were seen. Overall, the effect at 10 mg/kg bw per day (seen in both haematocrit and haemoglobin in both sexes at week 12) is considered possibly compound related. Other changes were seen only at interim time points and only in either haematocrit or haemoglobin and are not considered compound related. No compound-related changes in coagulation time occurred. Unlike in the Pfitzer & Roberts (1973) study, susceptibility of erythrocytes to haemolysis was not specifically measured in this study. However, the study report indicates that in the haemoglobin analysis, increased resistance to lysis was noted at the 10 mg/kg bw per day dose, particularly in females.

Statistically significantly increased white blood cell counts were seen in males and females in the 10 mg/kg bw per day group after 2, 7 and 12 weeks. Statistically significantly increased white blood cell counts were also seen in females in the 3 mg/kg bw per day group after 2 and 7 (but not 12) weeks, in females in the 2 mg/kg bw per day group after 2 weeks only, in males in the 3 mg/kg bw per day group after 7 weeks only and in males in the 2 mg/kg bw per day group after 2 weeks only. Lymphocyte counts were statistically significantly increased in females in the 10 mg/kg bw per day group at 2 weeks only and in males in the 10 mg/kg bw per day group at 12 weeks only. Lymphocytes were also statistically significantly increased in females at 2 mg/kg bw per day (2 weeks only) and 3 mg/kg bw per day (2 and 7 weeks). Neutrophil counts were statistically

significantly decreased in females in the 2 mg/kg bw per day group (2 weeks only), 3 mg/kg bw per day group (weeks 2 and 7) and 10 mg/kg bw per day group (week 2 only). In males, neutrophil counts were statistically significantly decreased in the 10 mg/kg bw per day group at 12 weeks. All of the above changes were slight, and only the increased white blood cell count at 10 mg/kg bw per day occurred at all time points. The study report noted that morphological changes (target cells) were seen in a minority of erythrocytes at 10 mg/kg bw per day in both sexes, although predominantly in females, after 2, 7 and 12 weeks. The report indicated that similar findings were seen in isolated individual rats at other doses, but the frequency and specific doses were not given.

AP was statistically significantly increased in female rats in the 10 mg/kg bw per day group after 4, 8 and 13 weeks. In male rats in the 10 mg/kg bw per day group, AP was increased, but only after 4 and 8 weeks. AP was also increased in males and females at 3 mg/kg bw per day, but only at interim time points. ASAT levels were statistically significantly increased in females in the 10 mg/kg bw per day group after 8 and 13 weeks. Other clinical biochemistry results differed significantly from controls only sporadically and are not considered compound related.

There were no findings of note in the urine analysis.

No lesions considered to be compound related were noted at gross pathology.

In females, the following statistically significant organ weight changes were noted at 10 mg/kg bw per day: increased relative heart weight at week 13, increased relative lung weight at week 13, increased relative liver weight at weeks 4 and 13, increased relative kidney weight at weeks 4, 8 and 13, decreased absolute ovary weight at week 13, increased absolute and relative uterus weights at week 8 (relative uterus weight was also increased at week 13, but the effect did not reach statistical significance), increased absolute and relative spleen weights at week 8 and increased relative spleen weight at week 13, increased relative adrenal weight at week 13 and increased relative brain weight at week 13; relative pituitary weights were increased at week 4, decreased at week 8 (along with absolute weight) and increased at week 13. At 3 mg/kg bw per day, the following statistically significant organ weight changes were noted: increased absolute and relative lung, liver and kidney weights at week 4 only, increased absolute and relative ovary weights at week 4, increased absolute and relative uterus weights at week 8 only, increased absolute and relative adrenal weights at week 4 only and increased absolute pituitary weight at week 8 only. At 2 mg/kg bw per day, the following statistically significant organ weight changes were noted: increased absolute and relative liver weights at week 4 only, increased absolute and relative ovary weights at week 4 only and increased absolute and relative adrenal weights at week 13. At 1 mg/kg bw per day, the following statistically significant organ weight changes were noted: increased absolute and relative liver weights at week 4 only and increased absolute and relative ovary weights at week 4 only. A small number of additional statistically significant deviations from control values were seen; however, due to their sporadic nature, they are not considered compound related. Many of the above changes, although occurring consistently across dose groups, occurred only at interim time points. The only findings seen after 13 weeks and considered possibly compound

related are those seen in the 10 mg/kg bw per day group (increased heart, lung, liver, kidney, adrenal, pituitary and brain weights and decreased ovary weight).

In males, the following statistically significant organ weight changes were noted at 10 mg/kg bw per day: decreased absolute heart weight at week 4 only, decreased absolute lung weight at week 4 only, decreased absolute testis weight at week 4 only, increased relative adrenal weight at weeks 4 and 13, increased absolute and relative pituitary weights at week 8 and increased relative pituitary weight at week 13, and increased relative brain weight at week 13. At 3 mg/kg bw per day, the following statistically significant organ weight changes were noted: decreased absolute heart weight at week 13, increased relative liver weight at week 4 only, increased absolute and relative adrenal weights at week 4 only and increased relative pituitary weight at week 13. At 2 mg/kg bw per day, the following statistically significant organ weight changes were noted: increased relative liver weight at week 13, increased absolute and relative adrenal weights at week 4 only and increased absolute and relative pituitary weights at week 13. At 1 mg/kg bw per day, the following statistically significant organ weight changes were noted: increased relative liver weight at week 4 only, increased relative adrenal weight at week 4 only and increased absolute and relative pituitary weights at week 13. A small number of additional statistically significant deviations from control values were seen, but, due to their sporadic nature, are not considered compound related. Many of the above changes, although occurring consistently across dose groups, occurred only at interim time points. The only findings seen after 13 weeks and considered possibly compound related are the increased adrenal weights seen at 10 mg/kg bw per day and the increased pituitary weights seen at all doses.

A full histopathological examination was performed on 20 male and 20 female rats in the 10 mg/kg bw per day group and control group killed after 4 weeks. Histopathological evaluations focusing on heart only were performed on 20 male and 20 female rats from the 3 mg/kg bw per day group. There was no histopathological examination of rats from the 1 and 2 mg/kg bw per day groups killed after 4 weeks.

After 4 weeks, vacuoles were noted in the myocardial fibres of females administered 10 mg/kg bw per day. The vacuoles were not stained by eosin or fat stains. The effect was not seen in animals administered 3 mg/kg bw per day. In the eye, focal loss of the epithelial layer and the layers of rods and cones was seen in one male in the 10 mg/kg bw per day group and in six controls.

A full histopathological examination was performed on 20 male and 20 female rats in the 10 mg/kg bw per day group and control group killed after 13 weeks. Histopathological evaluations focusing on liver, kidney and heart were performed on 20 male and 20 female rats in the 1, 2 and 3 mg/kg bw per day groups.

After 13 weeks, vacuoles were noted in the myocardial fibres of 13/20 females and 1/20 males administered 10 mg/kg bw per day. The vacuoles were not stained by eosin or fat stains. The effect was not seen in animals administered 2 or 3 mg/kg bw per day. In the liver, increased (relative to control) haemosiderin was seen in Kupffer cells in 17 females and 2 males in the 10 mg/kg bw per day group and in 5 females and 1 male at 3 mg/kg bw per day. The report

indicates that haemosiderin was also seen at 2 mg/kg bw per day, but that the amount was consistent with that seen in control livers. In the kidney, haemosiderin was increased in eight females and one male in the 10 mg/kg bw per day dose groups. In the eye, focal retinal lesions were seen in two rats in the 10 mg/kg bw per day group and in three controls. In a number of rats in which retinal degeneration was noted in the ophthalmoscopy examination, no histological effect was identified.

Based on increased haemosiderin seen in the liver of males and (predominantly) females at a dose of 3 mg/kg bw per day, the NOAEL was 2 mg/kg bw per day. In the absence of histopathological correlates, the organ weight effects seen at the lowest dose (increased absolute and relative pituitary weights) are not considered to represent an adverse drug effect (Pfitzer & Roberts, 1975b).

(b) Dogs

In a pre-GLP study, groups of six Beagle dogs (three males and three females) were administered lasalocid sodium in gelatine capsules as a single daily dose of 2, 5 or 10 mg/kg bw per day for 13 weeks. A further group of three males and three females acted as controls. Controls did not receive a placebo. Animals were caged individually and allowed feed ad libitum.

Animals were observed daily for physical and behavioural signs of toxicity as well as feed consumption. Body weights were measured weekly. Ophthalmic and neurological examinations (observations of posture, gait and reflexes) were performed on each dog prior to treatment and at approximately monthly intervals. Ophthalmic examinations included observations of eyelids, conjunctiva and sclera and examination of cornea, lens and fundus with an ophthalmoscope. Haematological examinations were performed prior to treatment and after 4, 8 and 12 weeks. Haematological examinations of 12-week blood samples included measurement of susceptibility of erythrocytes to haemolysis in hypotonic saline. Heparinized whole blood was mixed with various concentrations of hypotonic saline. After 30 minutes, intact cells were separated by centrifugation, and haemolysis was estimated in the supernatant by measuring light transmission using a photometer. Calculations of per cent haemolysis were based on tests with physiological saline (for 0% haemolysis) and distilled water (for 100% haemolysis).

Blood biochemistry parameters were determined in samples taken before the first administration and after 4, 8 and 12 weeks of administration. Urine analysis was undertaken on urine collected from each dog prior to the start of dosing and after the termination of the study. Electrocardiographic (ECG) examinations (lead II) were undertaken prior to the first administration and after weeks 4, 8 and 12. Complete necropsies were undertaken on all dogs after the 13-week dosing period. Organs were examined grossly, weighed and prepared for microscopic evaluation.

No compound-related mortality occurred during the study. No statistically significant differences in body weight were observed between the drug and control groups. A single female in the 10 mg/kg bw per day group was reported to have had reduced feed intake for a period of 10 days; this animal also displayed muscular weakness over the same period beginning in week 8. The study report indicated that

although other instances of poor appetite were observed on occasion throughout the study, the effect was not dose related. However, data on feed consumption were not included.

Two females in the 10 mg/kg bw per day group developed transient patterns of muscle weakness involving primarily the hindlimbs. In one of the females, the symptoms appeared in the 8th week and persisted for approximately 10 days, whereas in the other, symptoms appeared in the 10th week and lasted only 1 day. During the neurological examinations at 8 weeks, two males in the 10 mg/kg bw per day group were also noted to be suffering from abnormal muscle symptoms associated with the hind legs. One of these males was observed to have an awkward gait (associated with the hindlimbs), whereas the other demonstrated bilateral tremor of the hindlimbs. After the 12th week, there were no findings in the neurological examinations that distinguished drug from control groups.

ECG tracings (lead II) revealed no significant differences between test and control groups.

No significant differences were observed in haematocrit or haemoglobin levels in blood from test and control animals. After 12 weeks, mean prothrombin time was seen to be statistically significantly increased at all doses compared with controls, and mean coagulation time was seen to be increased in the 5 and 10 mg/kg bw per day groups compared with controls. After 8 weeks, mean prothrombin time was statistically significantly increased in the 10 mg/kg bw per day group, and mean coagulation time was increased only in the 2 mg/kg bw per day group. After 4 weeks, mean prothrombin time was statistically significantly decreased in the 2 mg/kg bw per day group and statistically significantly increased in the 10 mg/kg bw per day group, whereas coagulation time was statistically significantly increased in the 2 mg/kg bw per day group only. Prior to treatment, prothrombin time was statistically significantly increased in the 10 mg/kg bw per day group. All prothrombin and coagulation times are reported to be in the normal range. Overall, the effects on prothrombin and coagulation times appear to occur randomly across the groups and are not considered compound related. No effect of lasalocid sodium on haemolysis was seen. No compound-related effects were seen on differential white blood cell count.

Blood biochemistry revealed statistically significantly increased ALAT in the 10 mg/kg bw per day group after 4 and 8 weeks, but not after 12 weeks. The elevated group mean seen after 8 weeks was heavily influenced by the enzyme levels for the female that displayed muscle weakness at this time point; AP and ASAT levels were also increased in this female at this time point, but all had returned to control levels after 12 weeks. Blood urea nitrogen levels were statistically significantly decreased in the 5 mg/kg bw per day group prior to treatment, in the 2 mg/kg bw per day group after 4 weeks, in the 5 mg/kg bw per day group after 8 weeks and in the 10 mg/kg bw per day group after 12 weeks. Based on the inconsistent nature of these findings, the effects on blood urea nitrogen are not considered test compound related. The only other statistically significant effect was decreased serum chloride levels after 4 weeks in the 10 mg/kg bw per day group, after 8 weeks in all test groups and after 12 weeks in the 5 and 10 mg/kg bw per day groups. The study authors noted that serum chloride levels in the 5 and 10 mg/kg

bw per day groups were quite consistent over the study period, but that the control levels were increased after 8 and 12 weeks, resulting in relatively lower levels in the 5 and 10 mg/kg bw per day groups.The effect seen at 2 mg/kg bw per day was seen only at the 8-week time point and is not considered test compound related.

There were no findings of note in the urine analysis.

No lesions considered to be compound related were noted at gross pathology.

At necropsy, absolute spleen weights were statistically significantly increased in the 5 and 10 mg/kg bw per day groups, whereas relative spleen weights were statistically significantly increased only in the 5 mg/kg bw per day group. Absolute and relative uterus weights were statistically significantly increased in the 2 and 5 mg/kg bw per day groups, but not in the 10 mg/kg bw per day group. This effect on the uterus is not considered test compound related, as it was not dose related. Relative thyroid weights were statistically significantly increased in the 10 mg/kg bw per day group.

A full histopathological examination was performed on dogs from all groups killed after 13 weeks. Hepatic cells showed increased vacuolation in females in the 10 mg/kg bw per day group and to a lesser degree in females at 5 mg/kg bw per day; the effect was seen in three females at 10 mg/kg bw per day and in one female at 5 and 2 mg/kg bw per day as well as in the control group. The effect was not seen in males. The pathologist reported that the vacuolation was not due to lipid, glycogen or glycosaminoglycans, that it probably represents an intracellular accumulation of water, that such changes are generally reversible and that the effect was not associated with degenerative or inflammatory changes. It is noted that the effect seen in a single female at the middle dose was less pronounced than at the high dose and that in the control and 2 mg/kg bw per day animals in which vacuolation was seen, the extent of the effect was similar. This appears to suggest the occurrence of a dose–response relationship, with the greatest effect (number of animals affected and extent of the effect) seen in the high-dose animals. The pathologist concluded that the vacuolation effect is reversible and that it is therefore not biologically significant. However, as the reversibility was not demonstrated in this study, the effect at 5 mg/kg bw per day is considered to be a significant compound-related effect.

Congestion was observed in the spleen in all test animals in all groups: moderate congestion in all animals in the control and 2 mg/kg bw per day groups, moderate congestion in four animals and marked congestion in two animals at 5 mg/kg bw per day, and moderate congestion in two animals and marked congestion in four animals at 10 mg/kg bw per day. The possibility of a compound-related effect at 5 and 10 mg/kg bw per day cannot be ruled out.

Based on decreased serum chloride levels, increased spleen weights, increased congestion in the spleen and increased hepatocyte vacuolation at 5 and 10 mg/kg bw per day, the NOAEL was 2 mg/kg bw per day (Pfitzer & Swarm, 1973).

In a study reported to have been performed to GLP standards, groups of 20 Beagle dogs (10 males and 10 females) were administered lasalocid sodium in

feed at a concentration of 10, 35 or 180 mg/kg feed (equivalent to 0, 0.25, 1 and 5 mg/kg bw per day) for 24 months. A further group of 10 males and 10 females served as controls. Animals were caged individually. No formal GLP certification was included with the study report, although a quality assurance statement listing inspection dates was provided.

Animals were observed daily for physical and behavioural signs of toxicity as well as feed consumption. Body weights were measured weekly. Physical and neurological examinations (observations of the righting, patellar, flexor, extensor, visual placing response, corneal and papillary reflexes) as well as palpation for tissue masses were performed pretest and at monthly intervals. Ophthalmic examinations were performed pretest and after 2, 3, 4, 5, 6, 9, 12, 15, 18, 21 and 24 months. ECG examinations were performed pretest and after 2, 6, 7 (high dose and controls only), 12, 18 and 24 months. Haematological, blood biochemistry and urinary examinations were performed prior to treatment and after 3, 6, 9, 12, 15, 18, 21 and 24 months (biochemical analyses at 9 months were performed twice, as a new clinical chemistry analyser was introduced at that stage, and analyses were performed using both the automated and manual systems). Two animals of each sex per group were killed after 6 and 12 months, and all remaining surviving animals after 24 months. Organs were weighed and prepared for microscopic evaluation.

All animals survived until scheduled killing. No statistically significant effects on body weight were observed, and the pattern of body weights across the dose groups did not suggest a clear drug-related effect. Feed consumption was slightly reduced in the 180 mg/kg feed group over the first 8 weeks of drug administration, but the effect did not reach statistical significance. Sporadic statistically significant differences in feed consumption were seen between test and control groups, but as these occurred with no clear pattern across the doses or dosing period, they are not considered drug related.

Intermittent paralysis of the limbs was observed for a single day during week 21 in five animals (three males and two females) in the high-dose group. The animals appeared normal within 24 hours, and paralysis did not recur. Moderate tremors were reported in one female in the low-dose group for a single day during week 54 and again at week 100. As the tremors were seen in a single low-dose animal, they are not considered drug related. No other compound-related physical signs of toxicity were seen.

Ophthalmology revealed increased retinal lesions in two animals in the 180 mg/kg feed group compared with controls. Similar lesions were noted in the 10 mg/kg feed group, but not in the 35 mg/kg feed group. The lesions were first noted at interim time periods and did not progress over the course of the study. The lack of a dose–response relationship and the lack of progression over time suggest that the effect was not drug related. The reporting ophthalmologist concluded that the effects were the result of an unidentified inflammation event occurring early on in the study.

ECG examinations revealed changes at 6 months (increased PaVF amplitude and TaVF negativity); however, as similar changes were seen in animals from all groups, including controls, and as these returned to normal levels at

subsequent time points, the effect is not considered to have been drug related. Also at 6 months, one dog in the 180 mg/kg feed group displayed ECG evidence of myocardial damage. As the effect was seen in only one animal and as it was not seen at subsequent time points, it is not considered to have been drug related.

Haematology results included the following statistically significant changes from control values: decreased white blood cell counts seen in 180 mg/kg feed males at 3, 6 and 21 months (but not at 9, 12, 15, 18 or 24 months); decreased prothrombin time in 180 mg/kg feed males at 3 and 24 months only; decreased clotting time in 35 and 180 mg/kg feed females at 9 and 21 months only; and decreased prothrombin time in 35 and 180 mg/kg feed females at 24 months only. Statistically significant changes from control values were also noted at other time points, with no dose–response relationship. Overall, based on the lack of dose and time effects, none of the changes in haematological parameters is considered to represent a drug effect.

Blood biochemistry revealed statistically significantly increased AP in males at 9, 12, 18, 21 and 24 months (180 mg/kg feed). At 15 months, levels were increased, but the increase did not reach statistical significance. In females, AP was increased at 6, 9, 18, 21 and 24 months (180 mg/kg feed). At 12 and 15 months, levels were increased, but the increase did not reach statistical significance. ASAT levels were increased in 180 mg/kg feed males at 3 and 9 months only and in females at 3 months only. Glucose levels were decreased in 180 mg/kg feed females at 6 months and increased in 35 and 180 mg/kg feed females at 15 months. Glucose levels were decreased in 180 mg/kg feed males at 9 months only. Serum sodium levels were increased in 180 mg/kg feed males pretest and decreased in 180 mg/kg feed males at 6, 12 and 18 months and in 35 and 180 mg/kg feed females at 3 months. Serum calcium levels were decreased in 180 mg/kg feed males pretest and in 35 and 180 mg/kg feed females at 3 months and increased in 35 mg/kg feed females at 15 months and in 180 mg/kg feed females at 18 months. A number of other statistically significant deviations from control values were seen scattered across the dose groups and time points. Overall, the only blood biochemistry effect that occurred consistently over the study was the effect on AP, which was seen in both sexes at 180 mg/kg feed.

There were no findings of note in the urine analysis.

No lesions considered to be compound related were noted at gross pathology.

No statistically significant organ weight effects occurred with any pattern suggestive of a drug-related effect. The following organ weight changes were not statistically significant, but did occur with increasing dose and time: in males, increased absolute and relative testicular weights were seen at 180 mg/kg feed at 24 months, and decreased absolute and relative prostate weights were seen at 35 and 180 mg/kg feed at 24 months. Absolute and relative prostate weights were also decreased at 12 and 24 months, but results from these interim time points are based on examination of only two animals. In females, increased absolute and relative spleen weights were seen at all doses at 24 months and at 12 months (but only two females were examined at the interim time point), and increased absolute and relative thyroid weights were seen at all doses at 24 months.

Histopathological examination revealed only random changes, none of which was considered to be drug related. In the absence of any histopathological correlates, the organ weight findings are not considered to represent adverse drug effects.

Based on the transient intermittent paralysis of limbs seen in the 180 mg/kg feed group and on the increased AP levels seen at this dose, 180 mg/kg feed (equivalent to 5 mg/kg bw per day) is considered to be the LOAEL for this study. The NOAEL is therefore 35 mg/kg feed (equivalent to 1 mg/kg bw per day) (Hogan & Rinehart, 1980).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

A 2-year chronic toxicity study, reported to have been performed to GLP standards, was conducted in CD-1 mice. Lasalocid sodium was administered in feed at concentrations of 0 mg/kg feed (control group I and control group II), 10 mg/kg feed (low-dose animals were dosed with 20 mg/kg feed for the first 5 weeks of the study, after which the dose was adjusted down), 35 mg/kg feed (mid-dose animals were dosed with 60 mg/kg feed for the first 5 weeks, after which the dose was adjusted down) and 120 mg/kg feed (equivalent to 0, 1.5, 5.25 and 18 mg/kg bw per day, respectively, after week 5). The reason for the dose change at week 5 is not explained in the study report. Eighty mice of each sex were included in each group. Mice (5–6 weeks old at study initiation) were housed in groups of five same-sex animals. Feed and water were available ad libitum. No formal GLP certification was included with the study report, although a quality assurance statement listing inspection dates was provided.

Animals were observed daily for mortality and moribundity. Body weights, feed consumption, clinical signs and incidence/location of tissue masses were determined weekly for the first 13 weeks and biweekly thereafter. Ophthalmoscopic examinations were performed on all mice prior to initiation of the study, at week 6 and on all survivors at week 104. In addition, ophthalmoscopic examinations were performed on 10 mice of each sex per group every 3 months. Necropsies were performed on all mice found dead or killed in extremis, with gross observations recorded. After 104 weeks, all remaining mice were killed and necropsied, with gross observations recorded and tissues prepared for microscopic examination (brain, thoracic spinal cord, pituitary, thyroid, thymus, adrenals, heart, kidney, stomach, duodenum, jejunum, ileum, colon, caecum, colon, pancreas, ovaries, gall bladder, uterus, testes with epididymides, prostate, lung, spleen, liver, salivary glands, mesenteric lymph nodes, urinary bladder, nerve with muscle, bone marrow, rib junction, skin with mammary gland and any unusual lesions). Tissues from 20 males and 20 females in control groups I and II and in the 120 mg/kg feed group were examined microscopically, as were all tissues with masses or unusual lesions.

In males and females, overall survival at termination of the study was comparable in the test and control groups. In males, survival rates were comparable in all groups throughout the study. In females, reduced survival was noted in the 35 and

120 mg/kg feed groups at a number of interim time points, and survival over weeks 0–51 and 0–61 was statistically significantly reduced compared with control group II.

No effect on body weight or feed consumption considered to be associated with administration of the drug was seen.

The study report indicated that no treatment-related clinical signs were noted and that the incidence of tissue masses, nodules and wart-like lesions was comparable in control and test groups. Individual findings were not reported.

Ophthalmoscopic findings were unremarkable in all groups and were not suggestive of a compound-related effect.

Gross pathology examinations performed at terminal sacrifice and on animals that died or were killed in extremis during the study did not reveal findings suggestive of a compound-related effect.

Microscopic evaluation of tissues from animals killed at termination led to similar findings across control and test groups and was not suggestive of a drug effect. No evidence of a neoplastic effect of lasalocid was seen. Similarly, no drug effect was seen in the microscopic evaluation of tissues from males that died or were killed in extremis during the study. In females that died during the study or were killed in extremis, an increased incidence of lymphosarcoma was noted in the 10 and 120 mg/kg feed groups (3 cases seen in control group I, 5 cases in control group II, 9 cases in the 10 mg/kg feed group, 4 cases in the 35 mg/kg feed group and 10 cases in the 120 mg/kg feed group). As lymphosarcoma was not seen to occur with greater incidence in the test groups in animals killed at study termination and as a dose–response relationship was not evident in animals that died or were killed in extremis during the study, it is concluded that the increased incidence of lymphosarcoma noted in the 10 and 120 mg/kg feed groups did not represent a drug effect.

It is concluded that lasalocid sodium did not show evidence of tumorigenic potential in the mouse. The top dose of 120 mg/kg feed (equivalent to 18 mg/kg bw per day) was the NOAEL (Reno et al., 1980a).

(b) Rats

A 30-month toxicity and carcinogenicity study, reported to have been performed to GLP standards, was conducted in Fischer 344 rats. Lasalocid sodium was administered in feed at concentrations of 0 (control group I and control group II), 10, 35 and 120 mg/kg feed. Within each dose group, the dose administered varied considerably over the course of the study. For example, for males in the 120 mg/kg feed group, the mean initial dose level was calculated to be 11.3 mg/kg bw per day, whereas by 120 weeks, the dose was calculated to be 5.9 mg/kg bw per day. The average doses calculated from body weight and feed consumption were 0.5, 1.8 and 6.2 mg/kg bw per day for male rats and 0.6, 2.2 and 8.1 mg/kg bw per day for female rats, respectively. Eighty-five rats of each sex per group were included in control group I and in the lasalocid sodium groups, and 55 rats of each sex were included in control group II. Animals were caged individually except during the 3-week mating periods, when they were housed in pairs, and

during lactation, when offspring were housed with the mother. Feed and water were available ad libitum. No formal GLP certification was included with the study report, although a quality assurance statement listing inspection dates was provided. The study is reported to have continued for 30 months to meet the requirements of the United States Bureau of Foods (which required continuation until 30 months or 80% mortality).

The animals included in this study were selected at birth from litters derived from parental rats administered lasalocid sodium at the same doses from 1 week prior to breeding until weaning. When the youngest litter reached day 21, animals were randomly selected to enter into the lifetime carcinogenicity study (in which they received the same dose of lasalocid sodium as received by the parental animals during breeding, gestation and lactation).

Animals were observed daily for mortality and moribundity. Body weights, feed consumption, clinical signs and incidence/location of tissue masses were determined weekly. Neurological examinations (observations of the placement, righting, grasping and pupillary reflexes) were performed pretest and at intervals of approximately 4–5 weeks (examination of pupillary reflex was undertaken only from week 9 onwards). Ophthalmoscopic examinations were performed on all rats during week 1, on 10 rats per group at intervals of 3 months, on all rats sacrificed at 26 and 78 weeks as well as on all rats at terminal sacrifice and on any rats showing abnormal findings. Haematology (haematocrit, haemoglobin, coagulation time, total and differential leukocyte counts) and urinary examinations (appearance, pH, specific gravity, ketones, glucose, occult blood, total protein, bilirubin and microscopic examination of sediment) were performed on 10 rats of each sex per group at 3-month intervals and on all rats at 26 and 78 weeks as well as on all rats at terminal sacrifice. Clinical chemistry analyses (glucose, blood urea nitrogen, calcium, potassium, chloride, ALAT, ASAT and AP) were performed on 15 rats of each sex per group at 26, 78 and 104 weeks as well as on all rats at terminal sacrifice. Gross necropsy examinations were performed on all rats found dead or killed in extremis. Scheduled interim sacrifices were performed on 15 rats of each sex per group for all lasalocid sodium groups and for control group I at 26 and 78 weeks. All remaining rats were killed at 130 weeks. Gross pathology and histopathology examinations were undertaken at interim and terminal sacrifices. Organs were weighed (brain, heart, lung, liver, spleen, kidneys, testes with epididymis/ovaries, prostate/uterus, pituitary, thyroid, adrenals) and prepared for microscopic examination (brain, pituitary, thoracic spinal cord, eyes, salivary glands, thyroid, thymus, lungs, heart, liver, spleen, kidneys, adrenals, stomach, pancreas, duodenum, jejunum, ileum, colon, caecum, mesenteric lymph node, urinary bladder, testes with epididymis/ovaries, prostate/uterus, rib junction, bone marrow, nerve with muscle and any unusual lesions). Tissues from rats in control groups I and II and in the 120 mg/kg feed group were examined microscopically, as were tissues with masses or lesions from rats in the 10 and 35 mg/kg feed groups.

The percentage of animals surviving until week 130 was highest in the 120 mg/kg feed group, but was also increased in the 35 mg/kg feed group. The effect reached statistical significance in males in the 120 mg/kg feed group and was not considered adverse.

No effect on body weight or feed consumption considered to be associated with administration of the drug was seen, although statistically significant differences between test and control groups occurred at a number of interim time points. As these effects were transient and did not display a clear dose–response relationship, they are not considered to represent a drug effect.

The study report indicated that no treatment-related clinical signs were noted and that the incidence of tissue masses, nodules and wart-like lesions was comparable in control and test groups. Individual findings were not reported.

Haematology findings consisted of decreased haematocrit in males at 10, 35 and 120 mg/kg feed at week 130. The effect was statistically significant in the 35 and 120 mg/kg feed groups, but not in the 10 mg/kg feed group. Haematocrit was also statistically significantly reduced in all three test groups at week 26, but not at any of the seven intervening time points. Haemoglobin was also reduced in males in all test groups at week 130, but the effect reached statistical significance only in the 35 mg/kg feed group. In view of the absence of a consistent effect over time and the absence of a dose–response relationship, the effects on haematocrit and haemoglobin are not considered to be drug related. No effect considered to be drug related was seen on leukocyte counts or coagulation times.

Clinical chemistry revealed statistically significantly decreased ALAT in females in the 120 mg/kg feed group at weeks 26, 104 and 130. At week 78, ALAT was also reduced in females in the 120 mg/kg feed group, but the effect was not statistically significant. Blood urea nitrogen was statistically significantly reduced in females in all three test groups at weeks 26 and 78. No effect on blood urea nitrogen was seen in females at week 104 or 130. Blood urea nitrogen was also decreased in male rats in the 120 mg/kg feed group at weeks 26, 78 and 104, but not at week 130. Statistically significantly increased glucose levels were seen in males in the 35 and 120 mg/kg feed groups at week 26 only and in females in the 120 mg/kg feed group, also only at the 26-week time point. Chloride ion levels were statistically significantly decreased in females in the 120 mg/kg feed group at weeks 26, 78 and 104 (but not at week 130) and in the 35 mg/kg feed group at weeks 78 and 104 only. The clinical chemistry effects were not seen at all time points (and most were not seen at the final time point) and were not associated with histopathology. In light of this, they are not considered toxicologically relevant.

Urine analysis did not reveal any effects considered to be test item related.

Ophthalmoscopic examinations were unremarkable and did not reveal any effects suggestive of a drug effect.

Neurological examinations revealed an increased incidence of slow righting and grasping reflexes in females in the 120 mg/kg feed group in weeks 31–49 (e.g. the incidence of slow righting reflex at week 41 was 24/69 females compared with 2/70 females in control group I). The effect was not seen at earlier or later time points. A slightly increased incidence of these findings was also noted in the 35 and 10 mg/kg feed females at similar time points, although the number of animals affected was very low. No parallel effects were noted in males at any dose. In light of the low incidence of the findings at the low and middle doses and the absence of findings in males at any dose, the slow righting and grasping reflexes

in the 10 and 35 mg/kg feed groups are not considered drug related. No statistical analyses were performed on neurological data.

A number of organ weights were statistically significantly different from those seen in the control groups, but few differences appeared drug related. At 26 weeks, the following organ weight changes were noted: in males in the high-dose group, relative brain weight and relative testes plus epididymides weight were decreased, and absolute liver weight was increased. In females in the high-dose group, absolute and relative liver weights, absolute and relative spleen weights and absolute and relative adrenal weights were increased. Absolute and relative adrenal weights were also increased in females in the mid-dose group. At 78 weeks, the following organ weight changes were noted: in males in the high- and mid-dose groups, absolute and relative thyroid weights were decreased (the decrease in absolute thyroid weights in the mid-dose group was not statistically significant). In females, absolute and relative adrenal weights were increased in the mid- and high-dose groups. At 130 weeks, the following organ weight changes were noted: in males, absolute and relative testes plus epididymides weights were increased in the high-dose group. In females, absolute and relative liver weights were increased in the mid- and high-dose groups, absolute and relative adrenal weights were increased in the mid- and high-dose groups (although the effect was not statistically significant) and relative heart weights were increased in the high-dose group. The organ weight findings did not occur consistently over the course of the study, nor were they associated with histopathological findings. The organ weight effects are therefore not considered to be of toxicological significance.

No effects likely to be drug related were observed in the gross pathology examinations.

Microscopic examination revealed no findings suggestive of a drug effect.

In conclusion, lasalocid sodium did not demonstrate tumorigenic properties in this study. Based on a transient impairment of righting and grasping reflexes seen in females between weeks 31 and 49 at 120 mg/kg feed (equal to 8.1 mg/kg bw per day), the NOAEL was 35 mg/kg feed (equal to 2.2 mg/kg bw per day) (Reno et al., 1981).

2.2.4 Genotoxicity

Six in vitro studies were performed to examine the genotoxic potential of lasalocid sodium. The results are summarized in Table 1.

A non-GLP study was performed examining deoxyribonucleic acid (DNA) repair in *Bacillus subtilis* (rec-assay). Two strains of the bacteria were used: M45, a recombination deficient mutant (Rec–) strain derived from the wild-type H17 strain, and strain H17, with normal recombination capacity (Rec+). Agar plates were inoculated with the bacteria, covered with a paper disc containing 20 µL drug and incubated at 37 °C for 24 hours before the growth-inhibited zone was measured. Five plates were used for each dose. Lasalocid sodium (dissolved in dimethyl sulfoxide [DMSO]) was tested at concentrations of 1, 10 and 100 µg/plate, 4-nitroquinolone-*N*-oxide (the positive control, dissolved in DMSO) was tested at a concentration of 10 µg/plate and kanamycin sulfate (a negative control protein synthesis inhibitor substance, dissolved in distilled water) was tested at a concentration of 10 µg/plate.

Table 1. Results of genotoxicity assays on lasalocid sodium

Test system	Test object	Concentration	Results	Reference
DNA repair deficiency (rec-assay)	*Bacillus subtilis*	1–100 μg/plate	Negative	Yamashita et al. (1977)
Ames test[a]	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538 *Escherichia coli* B/r WP2, WP2 hcr–	100–2 000 μg/plate	Negative	Yamashita et al. (1977)
Mutagenicity[a]	*Saccharomyces cerevisiae*	0.05–5.0 mg/mL incubation mixture	Negative	Albertini & Woreth (1988)
Forward mutation assay[a]	Chinese hamster lung V79 cells (*HGPRT* locus)	1–20 μg/mL incubation mixture without metabolic activation[b] 1–60 μg/mL incubation mixture with metabolic activation	Negative	Strobel (1989a)
Unscheduled DNA synthesis	Primary hepatocytes	0.5–12.5 μg/mL[c]	Negative	Strobel (1989b)
Chromosomal aberration test[a]	Cultured human peripheral blood lymphocytes	2–8 μg/mL without metabolic activation[d] 2–10 μg/mL with metabolic activation[e]	Negative	Dresp (1989)

DNA: deoxyribonucleic acid; HGPRT: hypoxanthine–guanine phosphoribosyltransferase; S9, 9000 × *g* supernatant fraction from rat liver homogenate

[a] Both with and without rat liver S9 fraction.

[b] At concentrations greater than 15.0 μg/mL, cytotoxicity did not allow meaningful evaluation.

[c] At concentrations greater than 3.0 μg/mL, cytotoxicity did not allow meaningful evaluation.

[d] At concentrations greater than 5.0 μg/mL, cytotoxicity did not allow meaningful evaluation.

[e] At concentrations greater than 6.0 μg/mL, cytotoxicity did not allow meaningful evaluation.

4-Nitroquinolone-*N*-oxide induced greater growth inhibition in the Rec− strain than in the Rec+ strain (the difference in growth inhibition between the two strains was reported as 7.0 mm). Kanamycin induced only slightly greater growth inhibition in the Rec− strain (the difference in inhibition between the two strains was reported as 1.7 mm). The difference in growth inhibition between the two strains seen in lasalocid sodium–treated plates was less than that seen with the negative control kanamycin: at lasalocid sodium concentrations of 1, 10 and 100 μg/plate, the difference in inhibition between the two strains was 0.2, 0.5 and 0.2 mm, respectively. The results indicate that lasalocid sodium was not DNA damaging at these doses (Yamashita et al., 1977).

A non-GLP study was performed examining lasalocid sodium's ability to induce mutations in *Salmonella typhimurium* strains TA100, TA1535 (base pair substitution–sensitive strains), TA98, TA1537 and TA1538 (frameshift mutation–sensitive strains) and in *Escherichia coli* strains B/r WP2 and WP2 hcr– (base pair substitution–sensitive strains, with the latter lacking excision repair capacity), both with and without metabolic activation.

Lasalocid sodium was tested at concentrations of 100, 200, 500, 1000 and 2000 µg/plate. Each concentration was tested in triplicate.

Positive controls were diethylsulfate for TA1535 and B/r WP2 (without metabolic activation), 9-aminoacridine for TA1537 (without metabolic activation), 2,4-dinitrophenylthiocyanate for TA1538 (without metabolic activation), 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide for TA98, TA100 and WP2 hcr– (without metabolic activation), 2-aminoanthracene for TA98, TA100, TA1535, TA1537 and WP2 hcr– (with metabolic activation) and 2-acetylaminofluorene for TA1538 (with metabolic activation). Kanamycin sulfate was used as a negative control for all strains. All drugs with the exception of kanamycin sulfate were dissolved in DMSO; kanamycin sulfate was dissolved in distilled water.

The number of revertants seen following incubation of tester strains with lasalocid sodium remained at background levels in all cases, whereas positive controls induced marked increases in the number of revertants.

Lasalocid sodium did not show evidence of mutagenic activity in this assay, although slight evidence of cytotoxicity was seen at concentrations above 500 µg/plate, limiting the strength of the conclusions that can be drawn in relation to the higher doses (Yamashita et al., 1977).

Lasalocid sodium was tested for mutagenic potential in yeast (*Saccharomyces cerevisiae* D7) with and without metabolic activation in a study reported to be GLP compliant. The induction of mitotic gene conversion was monitored by the appearance of tryptophan non-requiring colonies (on selective media), reverse mutation induction was monitored by the appearance of isoleucine non-requiring colonies (on selective media) and mitotic crossing-over was assessed by visual detection of pink and red colonies that occurred due to the formation of homozygous cells expressing the genotypes ade 2-40/ade 2-40 (red) and ade 2-119/ade 2-119 (pink) from the originally heteroallelic condition ade 2-40/ade 2-119, which forms white colonies. Induction of mitotic gene conversion and reverse mutation was assessed in both the logarithmic growth phase of cells and the stationary growth phase.

Lasalocid sodium was tested at concentrations ranging from 0.05 to 5.0 mg/mL incubation mixture. Positive controls were 4-nitroquinolone-*N*-oxide (at a concentration of 0.2 µg/mL incubation mixture) for systems without metabolic activation and cyclophosphamide (at a concentration of 0.5 mg/mL incubation mixture) for systems with metabolic activation. Lasalocid sodium and the positive controls were dissolved in DMSO.

Lasalocid sodium concentrations greater than 1.67 mg/mL incubation mixture caused the appearance of white flakes in the incubation mixture. The tested concentrations of lasalocid sodium did not induce gene conversion, reverse gene mutation or mitotic crossing-over, whereas the responsiveness of the system was demonstrated by positive results obtained with the positive controls. The study report indicated that the concentrations tested were selected based on a toxicity prescreen. The results of the toxicity prescreen were not provided, but it was noted that the number of colonies that grew in complete media was similar in the control and lasalocid sodium–treated groups. The results indicate that lasalocid sodium was not mutagenic in *Saccharomyces cerevisiae* (Albertini & Woreth, 1988).

Lasalocid sodium was tested in a forward mutation assay in Chinese hamster lung V79 cells with and without metabolic activation in a study reported to be GLP compliant. A mutation to the gene for the enzyme hypoxanthine–guanine phosphoribosyltransferase (*HGPRT*) allows cells to proliferate in the presence of the purine analogues 6-thioguanine and 8-azaguanine, which, in non-mutants, are converted into toxic nucleoside-5-monophosphates and inhibit growth.

Lasalocid sodium was tested at concentrations of 1, 5, 10, 15 and 20 µg/mL incubation mixture in experiments without metabolic activation and at concentrations of 1, 10, 20, 40 and 60 µg/mL incubation mixture in experiments with metabolic activation. Ethyl methanesulfonate (at a concentration of 80 µg/mL) and 2-acetylaminofluorene (at a concentration of 80 µg/mL) were used as positive controls in experiments without and with metabolic activation, respectively. All three drugs were dissolved in DMSO, which also served as the negative control. Experiments were performed twice.

In the absence of metabolic activation, a concentration of 15 µg/mL reduced cell viability to 43–49% of that seen in the absence of lasalocid sodium and was considered the highest concentration that could be evaluated. In the presence of metabolic activation, a concentration of 60 µg/mL reduced viability to 19–27%.

The frequency of mutations conferring 6-thioguanine resistance was similar in negative control and lasalocid sodium–treated cells, but was substantially increased in positive control groups both with and without metabolic activation. The results indicate that lasalocid sodium was not mutagenic in this study in mammalian cells (Strobel, 1989a).

Lasalocid sodium was tested in an unscheduled DNA synthesis assay that measured incorporation of ^{3}H-labelled thymidine into nuclear DNA of primary (metabolically active) hepatocytes isolated from male Fü-albino rats. The study is reported to have been performed to GLP standards.

Lasalocid sodium was tested in a first experiment at concentrations of 0.5, 1.0, 2.5, 5.0, 10 and 12.5 µg/mL and in a second experiment at concentrations of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL. 2-Acetylaminofluorene (0.2 µg/mL) was used as a positive control. Both drugs were dissolved in DMSO, which was also used as a negative control.

Isolated rat hepatocytes were exposed to lasalocid sodium or 2-acetylaminofluorene for 18 hours. Cells were fixed and subjected to autoradiography, after which cells were evaluated microscopically, and the number of silver grains in the nuclei of cells not in the S-phase was counted.

Concentrations of 4.0 μg/mL and higher were seen to be cytotoxic and did not leave a sufficient number of morphologically normal cells to allow evaluation. The level of ^{3}H-labelled thymidine incorporation into nuclear DNA following exposure of hepatocytes to concentrations of lasalocid sodium ranging from 0.5 to 3.0 μg/mL did not differ from that seen with the negative control (DMSO). The level of ^{3}H-labelled thymidine incorporation into nuclear DNA following exposure of hepatocytes to the pro-mutagen 2-acetylaminofluorene was elevated, demonstrating the functionality of the assay. The results indicate that lasalocid sodium did not induce DNA damage resulting in DNA repair in rat hepatocytes (Strobel, 1989b).

Lasalocid sodium was tested for its potential to induce chromosomal aberrations in cultured human peripheral blood lymphocytes, with and without metabolic activation. The study is reported to have been performed to GLP standards.

Lasalocid sodium was tested in three experiments at concentrations ranging from 2 to 8 μg/mL without metabolic activation and in one experiment at concentrations ranging from 2 to 10 μg/mL with metabolic activation. Bleomycin sulfate and cyclophosphamide were used as positive controls in experiments without and with metabolic activation, respectively. Lasalocid sodium was dissolved in DMSO, bleomycin sulfate in 0.9% saline and cyclophosphamide in distilled water. DMSO served as a negative control.

Mitogen (phytohaemagglutinin-M)–stimulated cells were incubated with lasalocid sodium or control substances with or without S9 mix and subsequently subjected to metaphase arrest (using colcemid). Cells were fixed and prepared for microscopic examination.

In each of the three experiments without metabolic activation, only the lowest lasalocid sodium concentration (4, 2 and 5 μg/mL in the first, second and third experiments, respectively) produced a sufficient number of analysable cells, due to cytotoxicity. The incidence of chromosomal aberrations at the lowest doses was similar to that seen in negative controls, whereas bleomycin sulfate induced a significant increase in chromosomal aberrations, demonstrating the functionality of the system. In the experiment with metabolic activation, the top concentration of lasalocid sodium (10 μg/mL) induced excessive cytotoxicity. At concentrations up to 8 μg/mL, the incidence of chromosomal aberrations in lasalocid sodium–treated cells was similar to that seen in negative controls, whereas cyclophosphamide induced a significant increase in aberrations. The results indicate that lasalocid sodium did not induce chromosomal aberrations in human peripheral lymphocytes in vitro (Dresp, 1989).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

In a pre-GLP study, groups of 288 Charles River CD rats (24 males and 24 females per group) were administered lasalocid sodium in the diet at an intended dose of 0 (control group 1 and control group 2), 1, 2, 3 or 10 mg/kg bw per day. Males were dosed from 21 days prior to mating through a 14-day mating period. Females were dosed from 21 days prior to mating through to weaning of pups. Weanling rats were selected for entry into a 13-week repeated-dose toxicity study (reviewed in section 2.2.2 above; Pfitzer & Roberts, 1975b). The present study (Hoar et al., 1974) detailed only the effects on the maternal F_0 generation as well as effects seen in the F_1 offspring up until weaning.

Dosing at the desired level was maintained by adjusting the concentration based on the average feed consumption and body weight of each group. Diets were adjusted for appropriate doses on a weekly basis. Average actual drug intake levels were calculated for each sex at each dose level on a weekly basis. Average weekly group intakes (overall averages over the entire study period) were, for males, 0.88–1.06 (0.96), 1.83–2.22 (1.92), 2.57–3.47 (2.91) and 9.07–11.48 (9.83) mg/kg bw per day at 1, 2, 3 and 10 mg/kg bw per day, respectively; and, for females, 0.85–1.23 (1.19), 1.74–2.48 (2.45), 2.81–3.95 (3.73) and 8.64–13.05 (12.38) mg/kg bw per day at 1, 2, 3 and 10 mg/kg bw per day, respectively.

Feed consumption and body weights were measured weekly. Females were also weighed at weekly intervals relative to day 0 of gestation. Litters were examined as soon as was practical after delivery for live and dead offspring and for external abnormalities. Pups were counted, weighed and sexed at birth and weighed again on days 4, 7, 14 and 21 of lactation. At day 4 of lactation, litters were culled to a constant maximum size of eight pups (four males and four females, where possible). At 21 days after delivery, pups were weaned, and 60 of each sex per dose were transferred to a 13-week repeated-dose toxicity study (see section 2.2.2 above; Pfitzer & Roberts, 1975b). Male F_0 animals were killed on day 4 of lactation, and female F_0 animals were killed after weaning and examined for implantation sites. Neither the male F_0 animals nor the pups were necropsied. Pups that died at birth or during lactation were examined for external abnormalities and prepared for detection of skeletal defects.

No compound-related effects on survival, body weight or fertility (i.e. number of pregnancies) were seen in male parental rats. In dams, body weights were statistically significantly reduced in the 10 mg/kg bw per day group only (compared with controls). No effects on reproductive parameters (length of gestation period, number of litters, litter size, number of implantation sites per litter, number of pups born alive per litter, sex distribution, percentage of pups alive on days 4 and 21 of weaning) considered to be drug related were seen. Average body weights of viable pups in the 10 mg/kg bw per day group were decreased at birth and up to weaning (21 days after birth) compared with those in the control group. Only pups that died at birth or during lactation were examined for visceral and skeletal abnormalities. A number of these animals did display abnormalities; however, as only small and

variable numbers of animals were examined in each group, it is not possible to comment on whether effects may have been compound related.

Owing to the limited range of parameters monitored, a NOAEL for parental toxicity cannot be established from this study. The only effect noted in the offspring was reduced body weights in the 10 mg/kg bw per day group, which may have been secondary to toxicity in the maternal animals. No effects on fertility or reproductive parameters were seen, and the NOAEL for reproductive toxicity in this study was therefore 10 mg/kg bw per day, the highest dose tested. However, this study can be considered as a preliminary study only, as it is not in line with accepted designs for multigeneration studies. In particular, males were not dosed over a full spermatogenic cycle (dosing for 70 days would be expected), and histopathological and functional observations on the male and female reproductive systems were not reported (Hoar et al., 1974).

A multigeneration reproductive toxicity study incorporating a teratology arm, reported to have been performed to GLP standards, was performed using Charles River CD rats. Lasalocid sodium was administered in feed at a concentration of 0 (control), 10, 35 or 120 mg/kg (equivalent to 0, 0.5, 1.75 and 6 mg/kg bw per day, respectively). Animals were caged individually except during the 3-week mating periods, when they were housed in pairs, and during lactation, when offspring were housed with the mother. Feed and water were available ad libitum. No formal GLP certification was included with the study report, although a quality assurance statement listing inspection dates was provided.

In the parental (F_0) generation, 70 rats (35 males and 35 females) per group were dosed. At weaning of the F_1 animals, 40 rats (20 males and 20 females) were randomly selected from each dose group to continue in the study and become the parents of the F_2 generation. This process was repeated at weaning of the F_2 animals, with a further 40 rats (20 males and 20 females) selected from each dose group to become the parents of the F_3 animals. The F_0 and F_1 generations were mated twice with the offspring from one of the matings used as a source of parental (F_1) animals for the subsequent generation. The additional mating of the F_0 generation provided females for use in teratology evaluations. Offspring from the second mating of the F_1 generation were discarded at weaning. The F_2 generation was mated 3 times with offspring from the first two matings killed at weaning. The third mating of the F_2 generation provided females for use in teratology evaluations.

Parental animals were observed daily for clinical signs of toxicity. Body weight, feed consumption, physical appearance and behaviour were recorded initially and at weeks 4 and 9 during the 9-week growth period. Animals were then housed in pairs for a 21-day mating period, after which males were returned to individual cages and females were transferred to nesting boxes for delivery of offspring and postnatal observation.

Naturally delivered offspring were examined, and the numbers of live and dead pups were recorded, along with body weights, sex and any abnormalities. On day 4, F_1 and F_2 litters were reduced to five males and five females (where possible). All other pups were killed and discarded without necropsy. At day 21,

approximately a third of the remaining pups from each litter were reported to have been killed and necropsied, with internal gross abnormalities recorded (however, necropsy data for F_1 and F_2 pups were not included in the study report). On the same day, 20 F_1 and F_2 pups of each sex per group were randomly selected to constitute the parental animals for the subsequent generation. Any remaining pups were killed and discarded without necropsy.

Parental males were killed and discarded after their last breeding cycle. Parental females were killed after weaning of their last litter (except for those in the teratology arm).

Following weaning of F_3 pups from one of the F_2 matings, 20 males and 20 females were randomly selected from each dose group except the 120 mg/kg feed group, from which only 14 males and 14 females were selected, killed and necropsied, with gross observations recorded. Tissues were prepared for microscopic examination.

Ten females from the F_0 generation and all females included in the third mating of the F_2 generation were entered into the teratology arm of the study. The day on which a vaginal plug or sperm in the vagina were observed was taken as day 0 of gestation. F_0 females in the teratology arm were observed on days 0, 6, 11 and 13 of gestation for appearance, behaviour and body weights. Parallel examinations of F_2 females in the teratology arm were performed on days 0, 6, 11, 15 and 19. Females in the F_0 generation were killed and caesarean sections performed on gestation day 13, and gestational parameters (numbers of corpora lutea, implantation sites, resorptions and live and dead fetuses) were recorded. Females in the F_2 generation were killed and caesarean sections performed on gestation day 19. In addition to the gestational parameters monitored in the F_0 generation, fetuses were examined externally, and weight, sex and crown–rump distance were measured. All fetuses from the F_0 and F_2 females were examined for external gross abnormalities. Approximately one third of fetuses from the F_2 females were examined for gross visceral abnormalities. The remaining two thirds of fetuses from the F_2 females were eviscerated and examined for skeletal abnormalities.

No treatment-related mortality was considered to have occurred in parental animals.

Body weights in F_0 parental animals were statistically significantly reduced in females in the 120 mg/kg feed group during the 9-week growth phase. A decrease (not statistically significant) in feed consumption was seen in these animals at week 9. Body weights in high-dose F_0 parental females were also reduced during gestation (measured from gestation days 0 to 13 in the teratology arm), but body weight gain during this period was not different in treated and control F_0 females. Feed consumption in high-dose F_0 females was also reduced during gestation, but statistically significantly only between days 0 and 6. In the F_1 and F_2 parental animals, body weights and feed consumption were not statistically significantly different in test and control groups during the growth phase, although reduced body weight was seen (not statistically significant) in F_2 females at 120 mg/kg feed. High-dose F_2 females in the teratology arm had reduced absolute body weights

throughout gestation (including on gestation day 0), and body weight gain during this period was also reduced, as was feed consumption.

With the exception of body weight effects, the study report indicated that no treatment-related clinical signs were observed in the parental generation (no log of clinical signs was provided in the study report).

The study report indicated that necropsy findings in parental animals did not suggest a treatment-related effect (no log of necropsy findings in parental animals other than those in the teratology arm was provided in the study report).

Pregnancy rates (number of pregnant females/number of females placed in breeding) and male fertility rates (number of males housed with females that became pregnant/number of males placed in breeding) were decreased in the high-dose group in all matings in all generations, but the effect was small except for in one of the matings in the F_2 generation, where it was statistically significant. No effect was seen on the number of females that delivered viable litters. The number of females with pups surviving to weaning was statistically significantly reduced in the high-dose group in one of the matings in the F_2 generation, but not in the other mating in the F_2 generation and not in other generations.

In the offspring, neonatal survival (survival up to day 4 of lactation) was decreased (not statistically significant) in F_1 animals in the high-dose group in one mating, but not in subsequent matings or generations. The percentage of pups surviving to weaning was statistically significantly reduced in high-dose F_3 animals from one mating of the F_2 generation, but the effect was not seen in pups from the other mating in this generation or in other generations. No effect on pup sex ratio was noted.

Pup body weight at 24 hours after birth was slightly reduced (not statistically significant) in the high-dose animals resulting from one of the F_0 matings, but not in pups from the second mating of F_0 animals or in subsequent generations. Pup body weight at 21 days after birth was slightly reduced in high-dose pups from all matings except one of the two F_0 matings, with a statistically significant effect seen in F_3 pups from one of the F_2 matings.

Clinical observations of pups did not reveal any differences between control and test groups, and neither did gross necropsy or histological examination of weanlings (gross necropsy and histological data were available for F_3 weanlings from one of the F_2 matings only).

In the teratology arm, pregnancy rates and male fertility were reduced in the F_2 generation in high-dose animals and to a lesser extent in low- and mid-dose animals. The effect was not statistically significant at any dose. No effects on pregnancy rate or male fertility rate were seen in the F_0 animals. The mean numbers of corpora lutea and implantations per pregnant dam were reduced in the high-dose group in the F_0 and F_2 generations and also in the mid-dose group in the F_2 generation. These reductions were not statistically significant. Implantation efficiency (number of implantations/number of corpora lutea) was statistically significantly reduced in high-dose F_0 dams. Implantation efficiency was also reduced in the mid-dose dams, but the effect was not statistically significant.

In F_2 dams, implantation efficiency was also reduced in the high-dose group, but not to a statistically significant degree. The incidence of resorptions, fetal deaths and fetal viability were similar in control and test groups in both F_0 and F_2 dams. Fetal body weights (measured in the F_3 generation only) were slightly reduced (not statistically significant) in the high-dose group. Fetal sex ratios and crown–rump distances were similar in all groups. The number and type of visceral abnormalities (hydronephrosis) were similar in control and treatment groups, and no skeletal abnormalities were seen in any group. The number of visceral variants (dilated renal pelvis and dilated brain lateral ventricles) was increased in the high-dose group (variants seen in 5/8 litters versus 4/15 litters in the control group). Similarly, the number of skeletal variants (delayed ossification) was increased in the high-dose group (variants in 4/9 litters versus 6/15 litters in the control group).

This is an old study, and consequently it does not meet all the requirements of current reproductive or developmental toxicity guidance (e.g. OECD Test Guideline 416 on the two-generation reproductive toxicity study or OECD Test Guideline 414 on the prenatal developmental toxicity study). In the high-dose group (120 mg/kg feed), reduced body weights were seen in parental females. The NOAEL for parental toxicity was 35 mg/kg feed (equivalent to 1.75 mg/kg bw per day).

The mean numbers of corpora lutea and implantations per pregnant dam were reduced in both the high-dose (120 mg/kg feed) and mid-dose (35 mg/kg feed) groups, resulting in decreased implantation efficiency in these groups. The high-dose group also showed decreased pregnancy and fertility rates. Based on these effects, the NOAEL for reproductive toxicity was 10 mg/kg feed (equivalent to 0.5 mg/kg bw per day).

In the high-dose group (120 mg/kg feed), the number of pups surviving to weaning and the body weights of pups surviving to weaning were reduced. In the high-dose group of the teratogenicity arm, fetal weights were slightly reduced, and the incidence of visceral and skeletal variants was increased. The NOAEL for offspring and embryo/fetal toxicity was therefore 35 mg/kg feed (equivalent to 1.75 mg/kg bw per day) (Reno et al., 1980b).

(b) Developmental toxicity

(i) Rats

The multigeneration study in rats included a teratogenicity arm. The study is described in the previous section (Reno et al., 1980b).

(ii) Rabbits

In a dose range–finding study reported to be GLP compliant, groups of six female New Zealand White rabbits were dosed orally by gavage over days 6–28 of gestation. Dose levels of lasalocid sodium made up in 0.5% carboxymethyl cellulose were 0 (controls), 1, 2 and 4 mg/kg bw per day. Control animals were dosed with 0.5% carboxymethyl cellulose. No formal GLP certification was included with the study report, although a quality assurance statement was provided.

Animals were examined daily for clinical signs of toxicity, excreta were checked at the start and end of each day for changes in output or quality of excreta, body weights were recorded (on days 4, 6, 9, 12, 15, 19, 22, 26 and 29) and feed consumption was recorded daily from day 4 of gestation. Surviving animals were killed on day 29 of gestation and subjected to gross necropsy of thoracic and abdominal contents. The reproductive tract was dissected out, and the number of corpora lutea graviditatis in the ovaries and the number and position of implantation sites in the uterus were recorded. Implantations were classified as live, a fetal death (death after approximately day 18 of gestation), a late embryonic death (embryonic remains are visible) or an early embryonic death (only placental remains are visible). Fetuses were examined externally for visible abnormalities, and the total weight of the live fetuses in the litter was recorded.

Analysed concentrations of the dosing formulations used in the initial days of the study were found to be up to 33% lower than the nominal concentrations in the 1 and 2 mg/kg bw per day groups but only up to 5% lower than the nominal concentration in the 4 mg/kg bw per day group. Dosing solutions were amended for the remainder of the study.

Reduced/pale faecal output was seen in 4/6, 5/6, 6/6 and 6/6 animals in the 0, 1, 2 and 4 mg/kg bw per day groups, respectively (possibly related to the dosing volume of 10 mL/kg bw), over the first days of administration and resolved before the termination of the study.

One animal in the 2 mg/kg bw per day group was killed prematurely after aborting its single fetus. This was not considered to be test substance related.

Body weight gains were reduced in the 1, 2 and 4 mg/kg bw per day groups in a dose-dependent manner (greater reduction in body weight gain at 4 mg/kg bw per day than at 2 mg/kg bw per day, and greater reduction at 2 mg/kg bw per day than at 1 mg/kg bw per day). Feed consumption was markedly reduced at 4 mg/kg bw per day and slightly reduced at 1 and 2 mg/kg bw per day. In all test groups, feed consumption was most markedly reduced on days 7 and 8.

No clear effect of lasalocid sodium on pregnancy rate or preimplantation loss was seen. At 4 mg/kg bw per day, there was a decrease in the number of mean live implantations resulting from an increased number of early and late embryonic deaths. No effect on the number of live implantations was seen at 1 or 2 mg/kg bw per day. Fetal weight was reduced at 2 and 4 mg/kg bw per day. Reduced fetal weights were also noted at 1 mg/kg bw per day, but the study authors argued that this may be a reflection of an increased litter size seen at this dose. One animal in the 4 mg/kg bw per day group produced a pup with a shortened tail with a small skin flap.

Overall, it is concluded that lasalocid sodium was associated with a dose-related decrease in body weight and feed consumption seen at all doses. At 4 mg/kg bw per day, there was a decreased number of mean live implantations (possibly secondary to maternal effects), and fetal weights were reduced at 1, 2 and 4 mg/kg bw per day (but at 1 mg/kg bw per day, these may have been a consequence of larger litter size). Based on the results of this dose range–finding

study, doses of 0, 0.5, 1 and 2 mg/kg bw per day were selected for the pivotal developmental toxicity study in rabbits (Clubb & Sutherland, 2003a).

In a developmental toxicity study reported to be GLP compliant, groups of 24 female New Zealand White rabbits were dosed orally by gavage over days 6–28 of gestation. Dose levels of lasalocid sodium made up in 0.5% carboxymethyl cellulose were 0 (controls), 0.5, 1 and 2 mg/kg bw per day. Control animals were dosed with 0.5% carboxymethyl cellulose. The dosing volume in all groups was 10 mL/kg bw. No formal GLP certification was included with the study report, although a quality assurance statement was provided.

Animals were examined daily for clinical signs of toxicity, excreta were checked at the start and end of each day for changes in output or quality of excreta, body weights were recorded (on days 4, 6, 9, 12, 15, 19, 22, 26 and 29) and feed consumption was recorded daily from day 4 of gestation. Surviving animals were killed on day 29 of gestation and subjected to gross necropsy. Premature decedents underwent necropsy at the time of death (fetuses of these animals were not weighed and were examined externally only).

In scheduled necropsies, thoracic and abdominal contents were examined macroscopically. The reproductive tract was dissected out, and the number of corpora lutea graviditatis in the ovaries and the number and position of implantation sites in the uterus were recorded. Implantations were classified as live, a fetal death (death after approximately day 18 of gestation), a late embryonic death (embryonic remains are visible) or an early embryonic death (only placental remains are visible). Fetuses were killed, and each live fetus was identified within the litter and its weight recorded. Fetuses were examined externally for visible abnormalities, including macroscopic examination of eyes and cranial bones (following removal of skin over these areas).

Each fetus was dissected and examined for abnormalities of the thoracic and abdominal viscera. The cranium was sectioned to allow inspection of the brain. The sex of each fetus was recorded. Skeletons were prepared and examined for skeletal abnormalities and for the extent of ossification.

Reduced/altered (size and colour) faecal output was seen in 3/24, 9/24, 8/24 and 20/24 animals in the control, 0.5, 1 and 2 mg/kg bw per day groups, respectively. Red staining was noted in the cages of three animals in the 2 mg/kg bw per day group, although there were no obvious effects on pregnancy outcome. One animal in the control group was found dead (cause of death unknown), and one animal in the 2 mg/kg bw per day group was killed (the animal had not eaten since 2 days before the start of the study); neither of the deaths was attributed to treatment.

Body weight gains were reduced (compared with controls) in the 0.5, 1 and 2 mg/kg bw per day groups in a dose-dependent manner. In the 2 mg/kg bw per day group, body weight gain was reduced over the entire dosing period (with mean body weight on day 29 being similar to that seen on day 6). In the 1 mg/kg bw per day group, body weight gain was reduced, particularly over the initial 6 days of

treatment, and in the 0.5 mg/kg bw per day group, body weight gain was slightly lower than that of controls, particularly during early gestation. Feed consumption was reduced in all test groups in a dose-related manner.

No clear effect of lasalocid sodium on pregnancy rate was seen. At 2 mg/kg bw per day, there was a small increase in preimplantation loss (17% versus 9% in the control group). Preimplantation loss was also slightly increased in the 1 mg/kg bw per day group (13%) and the 0.5 mg/kg bw per day group (11%), but the increases seen in these dose groups are too small to be concluded to be test substance related. The absolute number of live implantations as well as the percentage of live implantations were reduced in the 2 mg/kg bw per day group (72% versus 89% in the control group), with a parallel increase in the number of early embryonic deaths (19% versus 7% in the control group) and late embryonic deaths (4% versus 1% in the control group). In the 1 and 0.5 mg/kg bw per day groups, the percentage of live implantations was marginally different from that seen in controls (84%, 85% and 89% at 1, 0.5 and 0 mg/kg bw per day, respectively). Mean fetal weight was reduced in all test groups (43.0, 41.8, 38.0 and 33.9 g in the 0, 0.5, 1 and 2 mg/kg bw per day groups, respectively), although the reduction at 0.5 mg/kg bw per day was small and is not considered to represent a drug effect.

A greater incidence of fetuses with forelimb flexure was noted in the 1 and 2 mg/kg bw per day groups (three occurrences in two litters at 2 mg/kg bw per day, a single occurrence at 1 mg/kg bw per day and none at 0.5 mg/kg bw per day or in controls). A single occurrence of enlarged bile duct and absent spleen and gall bladder was noted at 2 mg/kg bw per day.

An increased incidence of corneal opacity was seen in fetuses in the 2 mg/kg bw per day group (four occurrences in four litters versus a single occurrence in the control group). An increased incidence of bifurcated/misshapen gall bladders was noted in all test groups (five occurrences in four litters at 2 mg/kg bw per day, two occurrences in two litters at 1 mg/kg bw per day, four occurrences in three litters at 0.5 mg/kg bw per day and none in the control group). The abnormalities in the gall bladder did not occur in a dose-related manner, and the study report indicated that they are common (although they were not seen in concurrent controls in this study) and should not be considered to represent a test-related effect. Historical control data from the laboratory were provided. These consisted of the incidence of bilobed/bifurcated/clubshaped/misshapen gall bladders in one study per year between 2000 and 2012. The incidence of the abnormality varied from zero occurrences per study to four occurrences in two litters in one study (in which 140 fetuses from 18 litters were examined, producing an incidence of 2.86% of fetuses or 11% of litters). The incidence of the abnormality in the current study was 3.47% of fetuses (17.4% of litters) at 2 mg/kg bw per day, 1.28% of fetuses (10% of litters) at 1 mg/kg bw per day and 2.45% of fetuses (13.6% of litters) at 0.5 mg/kg bw per day. The incidence rates for the 0.5 and 1 mg/kg bw per day doses are considered to be comparable with that seen in the historical control data. However, the incidence seen at 2 mg/kg bw per day exceeds that seen in historical controls, and consequently the possibility that it may have been drug

related cannot be ruled out. No other visceral abnormalities were considered to have been possibly test substance related.

A number of minor skeletal abnormalities/variants were noted at 1 and 2 mg/kg bw per day, as well as an increased incidence of incomplete ossification at 2 mg/kg bw per day.

Minor skeletal abnormalities/variants included, in fetuses in the 1 and 2 mg/kg bw per day groups, increased incidence of jugal(s) connected/fused to maxilla (10 occurrences in five litters at 2 mg/kg bw per day, 7 occurrences in four litters at 1 mg/kg bw per day, a single occurrence at 0.5 mg/kg bw per day and 4 occurrences in four litters in the control group). An increased incidence of complete 13th supernumerary rib was seen at 1 and 2 mg/kg bw per day (86 occurrences in 22 litters at 2 mg/kg bw per day, 61 occurrences in 17 litters at 1 mg/kg bw per day, 50 occurrences in 14 litters at 0.5 mg/kg bw per day and 52 occurrences in 15 litters in controls). An increased incidence of displaced pelvic girdle was also noted at 2 mg/kg bw per day (17 occurrences in 22 litters at 2 mg/kg bw per day versus 37 occurrences in 10 litters in controls). An increased incidence of unossified/incompletely ossified cranial bones, hyoid, odontoid process, pubes, digital bones, epiphyses and astragalus was seen at 2 mg/kg bw per day.

A NOAEL for maternal effects could not be established from this study, as effects on faeces, body weight gain and feed consumption were seen at all doses. This is likely to be a result of the known sensitivity of rabbits to antibacterial effects on the microflora of the gastrointestinal tract. In relation to embryo/fetal toxicity, the LOAEL was considered to be 1 mg/kg bw per day, based on decreased litter weights, increased incidence of forelimb flexure and minor skeletal abnormalities/variants at this dose. While the possibility that these effects may have been secondary to maternal toxicity is acknowledged, based on the available data, the NOAEL for embryo/fetal toxicity was established as 0.5 mg/kg bw per day (Clubb & Sutherland, 2003b).

2.2.6 Special studies

(a) Cardiovascular and respiratory end-points

Original studies specifically examining cardiovascular and respiratory end-points were not provided. While literature reports demonstrate that lasalocid has a positive ionotropic effect on in vitro muscle preparations and in anaesthetized dogs (Pressman, 1976; Bukoski, Seidel & Allen, 1979), at the doses used in the repeated-dose and chronic toxicity studies evaluated previously in this report, no evidence of cardiovascular or respiratory toxicity was seen. An ADI derived from the toxicity studies evaluated in this report will therefore be protective against potential cardiovascular and respiratory effects.

(b) Neurological/behavioural end-points

No original studies dedicated specifically to the evaluation of the neurotoxic potential of lasalocid have been provided. However, a number of literature reports relating to the neurotoxicity of ionophores, including lasalocid, are available, and a

number of the repeated-dose and chronic toxicity studies evaluated above included an analysis of neurological effects.

Literature reports indicate lasalocid to be neurotoxic in a number of species. Safran, Alzenberg & Bark (1993) reported a cluster of 10 cases of neurotoxic findings in dogs, 8 of which were suspected to have consumed dog food contaminated with lasalocid. Clinical signs are reported to have included

> progressive, bilaterally symmetrically ascending muscle weakness which progressed from the hind to the forelimbs followed by quadriplegia, hyporeflexia, and hypotonia. In 7 dogs, the respiratory muscles were affected to various degrees, causing dyspnoea and apnea in the most severe case. The mental status and cranial nerve function appeared to be normal. Pain perception was maintained. The dogs' ability to wag their tails was not affected. These neurological signs were consistent with a generalised lower motor neuron deficit.

To support their suspicions that the effects were the result of lasalocid poisoning, the authors purposely exposed two healthy dogs to lasalocid (at 10 and 15 mg/kg bw) via the diet. Starting 6–8 hours after ingestion, the dogs exhibited a gradual onset of clinical signs consistent with those seen in the dogs admitted to their clinic.

A further instance of suspected lasalocid poisoning in dogs was reported by Espino et al. (2003). Three bloodhounds demonstrated the following neurological symptoms:

> tetraparesia, hyporeflexia and hypotonia. The dogs' ability to wag their tails was not affected. Mental status and cranial nerve function appeared normal. Pain perception was maintained. The neurological signs were consistent with a generalised lower motor neuron deficit.

The dogs had eaten six dead broilers 1 day before symptoms emerged, and the presence of lasalocid in the broilers' food (150 mg/kg food) was subsequently confirmed. Clinical signs resolved between 8 and 12 days after exposure.

Similar clinical signs were reported in cats concluded to have been exposed to cat food containing the ionophore salinomycin (Van der Linde-Sipman et al., 1999). Histology revealed abnormalities in the peripheral nerves of the forelimbs and hindlimbs, with lesions localized in the axons, myelin sheath and Schwann cells (destruction of myelin sheath with formation of digestion chambers, collapsed axonal sheath filled with foamy macrophages and swollen Schwann cells). The cat food was found to have been contaminated at levels of 16–21 mg/kg. Assuming a body weight of 2 kg and consumption of 100 g food per day, this would represent a dose of 0.8–1.05 mg/kg bw, although the period over which the cats were exposed is not clear from the report.

The nature of the neurological findings reported in the repeated-dose and chronic toxicity studies evaluated previously in this report shows consistency with those presented in the literature reports referred to above. It is notable that in the repeated-dose and chronic toxicity studies, effects on the respiratory system were not noted, possibly because of exposure to lower doses. In addition, it is notable

that all symptoms of neurotoxicity noted in the repeated-dose studies were transient and resolved without removal of the drug.

The neurological symptoms reported in the literature reports and in the study reports evaluated previously in this monograph are consistent with those reported for other ionophores (Novilla et al., 1994).

The mechanism of action underlying the neurotoxic effects of lasalocid may not be limited to its ability to transport cations across membranes. Lasalocid (and other ionophores) is also reported to stimulate catecholamine release, even in the absence of Ca^{2+}. Perlman, Cossi & Role (1980) examined the ability of lasalocid, ionomycin (a divalent cation ionophore) and monensin (a monovalent cation ionophore) to release noradrenaline from phaeochromocytoma cells in vitro. All three stimulated the release of noradrenaline in the presence of Ca^{2+}, but lasalocid and monensin also stimulated release independently of Ca^{2+}. While the stimulation of noradrenaline release by monensin was greatly reduced in Na^{+}-free media, the removal of Na^{+} had a much less marked effect on lasalocid-stimulated noradrenaline release. Decreasing pH from 7.4 to 6.5 potentiated the effect of lasalocid. Lasalocid is a weak acid and in its protonated form is uncharged and so will have increased lipid solubility, which would facilitate its entry into cells. The authors concluded that catecholamine secretion by ionophores may take place by several mechanisms. Lasalocid may stimulate catecholamine release by Ca^{2+}-dependent, Na^{+}-dependent and pH-dependent mechanisms.

Consistent with the above, lasalocid has been reported to stimulate 5-hydroxytryptamine release from platelets in vitro in both the presence and absence of Ca^{2+}, by directly forming a lipophilic complex with 5-hydroxytryptamine and transporting it across the platelet membrane. This was observed at concentrations below those that stimulate Ca^{2+} uptake by cells. Lasalocid acts as a transporter of biogenic amines as well as a Ca^{2+} transporter. By stimulating Ca^{2+}-mediated exocytosis, it can stimulate the release of acetylcholine release at the neuromuscular junction (Wörner & Brossmer, 1975; Pressman, 1976).

Lasalocid has also been reported to induce morphological changes and degeneration in neuronal cells in cerebral cultures at concentrations that do not damage non-neuronal (glial) cells. Lasalocid was observed to induce Ca^{2+} influx in the neuronal cell. The effects were blocked by MK-801, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, suggesting involvement of the NMDA receptor/ channel in lasalocid neurotoxicity (Safran et al., 1993).

(c) Microbiological effects

A JECFA decision-tree approach that was adopted by the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and that complies with International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) Guideline 36 (GL36) was used by the Committee to determine the need to establish a microbiological ADI for lasalocid sodium (VICH, 2004). The decision-tree approach initially seeks to determine if there may be microbiologically active lasalocid sodium residues entering the human colon. If the answer is “no” to the questions in any of the first three steps, then no

microbiological ADI is necessary. However, should such residues be present, then two end-points of public health concern are to be considered: (1) disruption of the colonization barrier and (2) increase of the population(s) of resistant bacteria. At step 4 of the decision-tree process, it is possible to provide scientific justification to eliminate testing (i.e. the need for a microbiological ADI) for either one or both end-points. Step 5 is where a microbiological ADI would be determined. Should a microbiological ADI not be necessary, then the toxicological ADI would be used. The Committee evaluated studies on minimum inhibitory concentration (MIC) susceptibility, faecal binding interaction and biological activity of lasalocid sodium and the potential for the development of lasalocid sodium resistance and used the decision-tree to answer the following questions in the assessment of lasalocid sodium.

Step 1: Are residues of the drug and/or its metabolites microbiologically active against representatives of the human intestinal flora?

Yes, lasalocid sodium is microbiologically active against some bacterial genera and species representative of the human intestinal flora.

Lasalocid sodium is a polyether ionophore antibiotic used in veterinary medicine as a feed additive to treat poultry for the prevention of coccidiosis caused by *Eimeria* spp. Lasalocid sodium is not used in human medicine. Lasalocid sodium is highly active against Gram-positive bacteria and some obligate anaerobes. Lasalocid sodium is inactive against many Gram-negative aerobic and facultative anaerobic enteric bacteria, including Enterobacteriaceae. The antimicrobial spectrum of activity for lasalocid sodium against bacteria representative of the human intestinal microflora has been reported. The minimum concentrations of lasalocid sodium required to inhibit the growth of 50% of organisms (MIC_{50} values) have been determined on several different culture media against 84 bacterial strains, many of which are representative of the human gastrointestinal tract microflora (Table 2). The methodology employed was standard MIC agar dilution, as described by the National Committee for Clinical Laboratory Standards (NCCLS, 2004) for anaerobic bacteria. The MIC_{50}s of lasalocid sodium for a wide range of bacteria were as follows: *Bifidobacterium* spp., 0.25 µg/mL; *Eubacterium* spp., 0.125 µg/mL; *Clostridium* spp., 0.125 µg/mL; *Peptostreptococcus* spp., 0.063 µg/mL; *Lactobacillus* sp., 0.125 µg/mL; *Enterococcus* spp., 0.5 µg/mL; *Streptococcus* spp., 0.063 µg/mL; *Bacteroides* sp., 4 µg/mL; *Escherichia coli*, > 128 µg/mL; *Proteus* spp., > 128 µg/mL; and *Salmonella* spp., > 128 µg/mL. There were differences between MIC_{50} values for the different media that could be explained by binding of lasalocid sodium to proteins. The Wilkins-Chalgren medium not supplemented with blood was found to be the most appropriate for the assessment of the MIC_{50} of lasalocid sodium. Some tests were done with Wilkins-Chalgren medium supplemented with sheep's blood on different agar media. The MIC_{50} values were lower when blood was not added to the Wilkins-Chalgren medium to support growth (Table 2) (McConville, 1998).

Table 2. MIC_{50}s of lasalocid sodium against 84 strains of bacteria, many representing the normal human intestinal microbiota, by culture medium

Genus	Species	ATCC No.	MIC_{50} (µg/mL)			
			WC agar	WCB	RCA	TJA
Bifidobacterium	*adolescentis*	15703	0.5	2	–	–
		15704	0.25	1	–	–
		15705	< 0.063	0.5	–	–
		15706	0.25	0.5	–	–
	infantis	15697	0.5	1	–	–
		15702	0.125	1	–	–
		25962	< 0.063–0.25	0.2–4	–	–
	breve	15698	0.5	1	–	–
		15700	0.125	1	–	–
		15701	0.5	2	–	–
Eubacterium	*cylindroides*	25728	0.25	4	–	–
		27803	0.125	1	–	–
		27804	0.063	0.25	–	–
		28705	0.125	0.5	–	–
	lentum	43055	0.25	2	–	–
		25559	0.50	2	–	–
	dolichrum	29143	0.125	1	–	–
		29144	0.125	1	–	–
		33656	0.063–0.25	8	–	–
	rectale	35183	0.25	2	–	–
Clostridium	*difficile*	43594	0.063–0.25	2	–	–
		43596	0.063–0.125	2	–	–
		43597	0.063–0.125	2	–	–
		43598	0.063–0.125	2	–	–
	perfringens	9081	0.25	8	–	–
		12918	–	4	8	–
		12919	0.125–0.25	4	–	–
	breve	12920	0.125–0.5	8	–	–
		19574	0.125–0.25	8	–	–
		43150	0.125–0.5	2–8	NR	–

Table 2 (continued)

Genus	Species	ATCC No.	MIC_{50} (µg/mL)			
			WC agar	WCB	RCA	TJA
Peptostreptococcus	*hydrogenalis*	49630	< 0.063	0.5	–	–
	magnus	14955	0.125	1	–	–
		15794	0.125	1	–	–
		29328	< 0.063	1	–	–
		53516	< 0.063	1	–	–
	prevotii	9321	0.125	2	–	–
		14952	< 0.063	1	–	–
	productus	27340	< 0.063	0.5	–	–
		35244	< 0.063	0.5	–	–
		43917	< 0.063	0.25–0.5	–	–
Lactobacillus	*acidophilus*	4356	0.125	0.5	–	–
		4357	0.125	NR	–	–
		33200	NR	4	–	–
Enterococcus	*faecalis*	19083	0.5–1	16	–	–
		19948	0.25–0.5	–	–	–
		23241	0.5–1	–	–	–
		27274	0.5–1	–	–	–
		29212	0.5–1	–	–	–
		49532	0.5	–	–	–
		49533	0.125–0.5	–	–	–
		49757	0.25–0.5	–	–	–
	faecium	6569	0.5–1	–	–	–
Streptococcus	*agalactiae*	624	0.063–0.25	4	–	–
		NCTC 8542	0.125	–	–	–
		NCTC 9409	0.125	–	–	–
		NCTC 9412	0.063–0.125	–	–	–
	bovis	43143	0.25	4	–	–
		43144	0.125–0.25	8	–	–
		49133	0.125–0.25	4	–	–
	pyogenes	8058	< 0.063	4	–	–
		19615	< 0.063	2	–	–
		21059	< 0.063	1	–	–

Table 2 (continued)

Genus	Species	ATCC No.	MIC_{50} (µg/mL)			
			WC agar	WCB	RCA	TJA
Bacteroides	*fragilis*	29768	4	–	–	–
		43858	4	–	–	–
		43860	4	–	–	–
Fusobacterium	*prausnitzii*	27766	NR	NR	NR	–
		27768	NR	NR	> 128	–
	nucleatum	NCTC 10562	–	16	16	–
		NCTC 11326	–	NR	64	–
	ulcerans	NCTC 12111	–	128	128	–
		NCTC 12112	–	8	–	–
	varium	NCTC 10560	–	> 128	> 128	–
Escherichia	*coli*	884	> 128	–	–	–
		8739	> 128	–	–	–
		10798	> 128	–	–	–
Proteus	*mirabilis*	7002	> 128	–	–	–
		8427	> 128	–	–	–
	vulgaris	33420	128	–	–	–
Salmonella	*enteriditis*	4931	> 128	–	–	–
		13076	> 128	–	–	–
		31194	> 128	–	–	–
	typhimurium	NCTC 73	> 128	–	–	–
		NCTC 74	> 128	–	–	–
		NCTC 8298	> 128	–	–	–

ATCC: American Type Culture Collection; MIC_{50}: minimum concentration required to inhibit the growth of 50% of organisms; NCTC: National Collection of Type Cultures; NR: no result obtained; RCA: reinforced clostridial agar; TJA: tomato juice agar; WC: Wilkins-Chalgren agar; WCB: Wilkins-Chalgren agar containing 5% weight per volume defibrinated sheep's blood
Source: McConville (1998)

In a more recent GLP-compliant study (Pridmore, 2004a), the MIC of lasalocid sodium was determined against 30 bacterial strains, comprising 10 isolates from each of three groups of *Bacteroides*, *Fusobacterium* and *Peptostreptococcus* spp. genera representing the normal human intestinal microbiota. All

strains were sourced from the faecal microbiota of healthy unmedicated humans. The test system was standardized agar dilution MIC methodology using quality control strains as described in the NCCLS guidelines, now called the Clinical and Laboratory Standards Institute (CLSI) guidelines. To assess the effect of bacterial density on lasalocid sodium activity, each MIC was determined using two inoculum levels, 10^5 and 10^9 colony-forming units (cfu)/mL, for each strain. Lasalocid sodium activity against each bacterial group is summarized in Table 3. MIC_{50}, MIC_{90} and geometric mean of MIC values were calculated for each bacterial group (Table 4). In tests using the higher bacterial inoculum density, lasalocid sodium exerted little or no antibacterial activity against *Bacteroides* spp. (MIC_{50} value of 128 μg/mL). This is consistent with the known spectrum of activity for lasalocid sodium, which has low activity against Gram-negative bacteria. Lasalocid sodium activity was clearly demonstrable against the other two bacterial groups tested at the higher inoculum density. *Fusobacterium* was the most susceptible group (MIC_{50} of 1 μg/mL), whereas *Peptostreptococcus* was less susceptible, with a MIC_{50} of 4 μg/mL. At the low inoculum density, lasalocid sodium MICs against *Bacteroides* spp. (MIC_{50} of 32 μg/mL) and *Peptostreptococcus* spp. (MIC_{50} of 2 μg/mL) were lower than those obtained using the higher inoculum density. *Fusobacterium* spp. did not grow well when tested at a lower inoculum density. Thus, lasalocid sodium MIC_{50} values were not obtained for this microorganism when tested at 10^5 cfu/mL.

Table 3. MICs of lasalocid sodium against 30 strains of anaerobic bacteria representing the normal human intestinal microbiota

Strain description	DWC code[a]	High inoculum density		Low inoculum density	
		Lasalocid sodium MIC (μg/mL)	Inoculum density (cfu/mL)	Lasalocid sodium MIC (μg/mL)	Inoculum density (cfu/mL)
Bacteroides vulgatus	9795	128	9×10^8	64	9×10^4
Bacteroides merdae	9863	64	4×10^9	16	4×10^5
Bacteroides uniformis	9865	128	2×10^9	32	2×10^5
Bacteroides eggerthil	9888	64	5×10^9	32	5×10^5
Bacteroides stercoris	9891	> 128	3×10^9	64	3×10^5
Bacteroides caccae	9898	> 128	2×10^9	64	2×10^5
Bacteroides vulgatus	9900	128	5×10^8	32	4×10^4
Bacteroides ovatus	9907	> 128	2×10^9	64	2×10^5
Bacteroides distasonis	9912	64	3×10^9	16	3×10^5
Bacteroides uniformis	9923	128	2×10^9	64	2×10^5
Fusobacterium nucelatum	9861	1	1×10^7	No growth	1×10^3
Fusobacterium nucelatum	9854	1	8×10^8	No growth	8×10^4
Fusobacterium necrogenes	9885	8	6×10^8	4	6×10^4
Fusobacterium necrophorum	9890	2	1×10^9	No growth	1×10^5
Fusobacterium necrogenes	9895	1	2×10^9	No growth	2×10^5

Table 3 (continued)

Strain description	DWC code[a]	High inoculum density		Low inoculum density	
		Lasalocid sodium MIC (μg/mL)	Inoculum density (cfu/mL)	Lasalocid sodium MIC (μg/mL)	Inoculum density (cfu/mL)
Fusobacterium necrophorum	9902	1	2×10^7	No growth	2×10^3
Fusobacterium nucelatum	9903	64	7×10^8	64	7×10^4
Fusobacterium nucelatum	9908	128	8×10^9	64	8×10^5
Fusobacterium necrophorum	9914	4	2×10^9	No growth	2×10^5
Fusobacterium nucelatum	9921	1	2×10^9	No growth	2×10^5
Peptostreptococcus anaerobius	9791	4	1×10^8	2	1×10^4
Peptostreptococcus asaccharolyticus	9776	4	2×10^9	2	2×10^5
Peptostreptococcus asaccharolyticus	9783	4	3×10^9	2	3×10^5
Peptostreptococcus asaccharolyticus	9786	2	5×10^8	2	4×10^4
Peptostreptococcus asaccharolyticus	9787	4	4×10^8	2	4×10^4
Peptostreptococcus magnus	9781	4	4×10^8	2	4×10^4
Peptostreptococcus magnus	9792	2	6×10^8	2	6×10^4
Peptostreptococcus micros	9774	1	6×10^8	0.5	6×10^4
Peptostreptococcus magnus	9780	2	1×10^9	2	1×10^5
Peptostreptococcus sp.	9624	8	2×10^9	4	2×10^5

cfu: colony-forming unit; MIC: minimum inhibitory concentration
[a] Code identifying strains held within the Don Whitley Scientific Ltd culture collection.
Source: Pridmore (2004a)

To determine the microbiological ADI, both the McConville (1998) (Table 2) and the Pridmore (2004a) (Table 3) MIC data were used in the evaluation of the minimum inhibitory concentration derived from the lower 90% confidence limit for the mean MIC_{50} of the relevant genera for which the drug is active (MIC_{calc}).

The genera with a MIC_{50} – *Eubacterium* (0.125 μg/mL; McConville, 1998), *Bacteroides* (32 μg/mL; Pridmore, 2004a), *Bifidobacterium* (0.25 μg/mL; McConville, 1998), *Fusobacterium* (1 μg/mL; Pridmore, 2004a), *Peptostreptococcus* (2 μg/mL; Pridmore, 2004a), *Clostridium* (0.125 μg/mL; McConville, 1998), *Enterococcus* (0.5 μg/mL; McConville, 1998) and *Lactobacillus* (0.125 μg/mL; McConville, 1998) – were used to determine the MIC_{calc}.

Table 4. Summary of MIC parameters for lasalocid sodium against bacterial genera tested in the study by Pridmore (2004a)

Bacterial genus	MIC parameter	High inoculum density	Low inoculum density
Bacteroides	MIC range (µg/mL)	64 to > 128	16–64
	MIC_{50} (µg/mL)	128	32
	MIC_{90} (µg/mL)	> 128	64
	Geometric mean (µg/mL)	104	39
Fusobacterium	MIC range (µg/mL)	1–128	[Not determined: only three results available]
	MIC_{50} (µg/mL)	1	
	MIC_{90} (µg/mL)	64	
	Geometric mean (µg/mL)	3.7	
Peptostreptococcus	MIC range (µg/mL)	1–8	0.5–4
	MIC_{50} (µg/mL)	4	2
	MIC_{90} (µg/mL)	4	2
	Geometric mean (µg/mL)	3	2

MIC: minimum inhibitory concentration; MIC_{50}: minimum concentration required to inhibit the growth of 50% of organisms; MIC_{90}: minimum concentration required to inhibit the growth of 90% of organisms
Source: Pridmore (2004a)

Step 2: Do residues enter the human colon?

Yes. A number of residue studies using ^{14}C radiolabelling to detect total residues or analytical chemistry methods to detect parent lasalocid sodium have been conducted in chicken, turkey, quail, pheasant and eggs, as described in the residue report (FAO JECFA Monographs 15). In poultry, 90–95% of an oral dose of lasalocid sodium is excreted in faeces. Muscle contains little or no lasalocid sodium–derived residue, regardless of the period between withdrawal of medication and slaughter. However, residues may be present at low levels in offal, fat and skin. Pharmacokinetic studies in mice and rats indicated rapid absorption of lasalocid sodium, with 90–95% of the oral dose excreted in the faeces. Therefore, lasalocid sodium–related residues could enter the colon of a person ingesting tissues from treated animals.

Step 3: Do the residues entering the human colon remain microbiologically active?

Yes. Lasalocid sodium is the main compound entering the colon when the drug is ingested. However, lasalocid sodium residue should have very reduced activity within the colon of the consumer, as it will become substantially bound to faecal material.

To determine the effect of faecal binding on the antibacterial activity of lasalocid sodium, selected lasalocid concentrations of 0, 1, 2, 5, 10, 20, 50 and 100 µg/mL were incubated for up to 8 hours with increasing concentrations of sterilized human faeces (0%, 10%, 20% and 50% weight/volume [w/v] in Mueller Hinton Broth, collected from three individual donors) (Pridmore, 2004b). These human volunteers were healthy and received no antibiotic therapy during the 3 months prior to the faecal sample collection. Lasalocid sodium activity was determined using *Enterococcus faecalis* ATCC 29212 as an indicator organism, as it is susceptible to lasalocid sodium. The antibacterial activity of the supernatant obtained from centrifugation of the incubation mixture was assessed for the presence or absence of indicator organism growth before and after 24 and 48 hours of incubation. Control experiments indicated that without prior drug exposure to faecal suspensions, a lasalocid sodium concentration of 1 µg/mL consistently inhibited *E. faecalis* growth at each sampling point. Following drug exposure to 10% faeces at the various time intervals, the initial lasalocid sodium concentration required in the test system to inhibit *E. faecalis* growth after interaction with faeces increased to greater than 100 µg/mL. These data suggest that greater than 99% of the initial lasalocid sodium concentration was bound to faeces. All three faecal samples had maximal (> 99%) binding of lasalocid sodium at 10%, 20% and 50% concentrations. The degree of lasalocid sodium binding was rapid and remained constant throughout the 8-hour incubation period, indicating that the binding was irreversible. Based on this in vitro study (Table 5), it was determined that the binding of lasalocid sodium residues to 10%, 20% and 50% w/v concentration faecal material would be likely to exceed 99% in comparison with the activity in the absence of faeces. However, as no additional lasalocid sodium faecal binding interaction studies were conducted to validate and confirm the microbiological assay results (e.g. an HPLC/mass spectrometry chemical assay to directly detect lasalocid sodium remaining in the supernatant or bound to the faecal pellet), a conservative conclusion from the lack of lasalocid sodium activity under the test study protocol conditions would be that it is reduced by greater than 90% by contact with faecal material in the colon. In addition, lasalocid sodium is poorly absorbed, with 90–95% of an oral dose excreted in the faeces of experimental animals. Therefore, a value of 10% or 0.1 availability would be appropriate to use in calculating the microbiological ADI.

Table 5. Growth of* Enterococcus faecalis *ATCC 29212 in supernatants derived from lasalocid sodium/faeces mixtures at the 30-minute faecal binding period

		Lasalocid concentration (µg/mL)							
		0	1	2	5	10	20	50	100
Concentration of faeces (%)	0	+	–	–	–	–	–	–	–
	10	+	+	+	+	+	+	+	+
	25	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+

Key: + = growth after 24 hours
– = no visible growth after 48 hours
Note: The data from the other time intervals tested are identical to the results found at the 30-minute faecal binding period.
Source: Pridmore (2004b)

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern (i.e. disruption of the colonization barrier or resistance development)?

Lasalocid sodium does not appear to select for true acquired resistance in bacteria and is not used in human medicine. Results from the in vitro studies determining the potential for antimicrobial resistance to lasalocid sodium indicate that the development of resistance and cross-resistance to a number of commonly used antimicrobials in veterinary and human medicine is unlikely (Devriese et al., 1997; Wheadon, 2002, 2003). Thus, the only potential adverse effect on human intestinal microbiota would be disruption of the colonization barrier as the end-point of concern for determining the microbiological ADI. Interestingly, the majority of lasalocid sodium residues in the colon are bound to faeces and biologically inactive. Therefore, lasalocid sodium residues are not likely to disrupt the colonization barrier of the human intestinal microflora following the consumption of edible products from poultry. However, as there is potential for trace levels of lasalocid sodium to occur in the gastrointestinal tract, a microbiological ADI for lasalocid sodium residues was determined.

Step 5: Derivation of a microbiological ADI using the VICH GL36 approach

The formula for calculating the microbiological ADI is as follows:

$$\text{Upper bound of the ADI } (\mu g/kg \text{ bw}) = \frac{MIC_{calc} \times \text{Mass of colon content}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

The equation terms are derived as described below.

MIC_{calc}: In accordance with Appendix C of VICH GL36, calculation of the estimated no-observed-adverse-effect concentration (NOAEC, or MIC_{calc}) for colonization barrier disruption uses MIC values from the lower 90% confidence

limit of the mean MIC_{50} for the most relevant and sensitive human colonic bacterial genera. The strains needed to determine the MIC_{calc} were chosen according to these guidelines, which state that an intrinsically resistant bacterial genus should not be included. Based on the genera with a MIC_{50} – *Eubacterium* (0.125 µg/mL), *Bacteroides* (32 µg/mL), *Bifidobacterium* (0.25 µg/mL), *Fusobacterium* (1 µg/mL), *Peptostreptococcus* (2 µg/mL), *Clostridium* (0.125 µg/mL), *Enterococcus* (0.5 µg/mL) and *Lactobacillus* (0.125 µg/mL) – the MIC_{calc} is 0.228 µg/mL.

Mass of colon content: A value of 220 g is based on the colon content measured from humans.

Fraction of oral dose available to microorganisms: It is recommended that the fraction of an oral dose available for colonic microorganisms be based on in vivo measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine. Human data are encouraged, but, in their absence, non-ruminant animal data are recommended. In the absence of data to the contrary, it should be assumed that metabolites have antimicrobial activity equal to that of the parent compound. The fraction may be lowered if the applicant provides quantitative in vitro or in vivo data to show that the drug is inactivated during transit through the intestine. Lasalocid sodium is poorly absorbed and is excreted in faeces primarily in unchanged form. Lasalocid sodium binds rapidly and extensively to faecal contents; therefore, the value is 0.10.

Body weight: The body weight of an adult human is assumed to be 60 kg.

The upper bound of the microbiological ADI for lasalocid sodium is calculated as indicated below:

$$\text{Upper bound of the ADI} = \frac{0.228\ \mu\text{g/mL} \times 220\ \text{g}}{0.10 \times 60\ \text{kg bw}}$$

$$= 8.4\ \mu\text{g/kg bw}$$

Therefore, a microbiological ADI of 0–8.4 µg/kg bw could be derived from in vitro MIC susceptibility testing and bioavailability studies.

2.3 Observations in humans

No observations in humans were identified.

3. COMMENTS

The Committee considered data on pharmacodynamics, pharmacokinetics, short-term and long-term toxicity, genotoxicity, reproductive and developmental toxicity, carcinogenicity and microbiological safety. In addition to the sponsor's submission, a number of studies were retrieved from the published literature. Although most of the studies submitted to the Committee pre-date GLP implementation, the overall package of data was sufficient to allow the derivation of a robust ADI. Those studies that were not performed to GLP standards are identified in this report.

3.1 Biochemical data

Following oral administration of a single radiolabelled dose of lasalocid sodium to mice, radioactivity was rapidly absorbed and excreted. Peak concentrations of radiolabelled material were seen in whole blood 15 minutes after administration, and levels had declined to background within 24 hours. The half-life of elimination of radioactivity in whole blood was 3 hours. Radioactivity was widely distributed to tissues, with the highest concentrations seen in liver, where they peaked 1 hour after administration. Approximately 95% of radioactivity was excreted in the faeces, and approximately 1% in urine, within 24 hours. A similar pattern was seen following multiple oral administrations, with radioactivity peaking in whole blood 30 minutes after the last dose and declining to background levels by 24 hours. Tissue levels were highest in the liver, where they remained detectable 48 hours after administration. Seventy-seven per cent of radioactivity was excreted in faeces within 4 hours of the last dose, and 95% within 24 hours. Excretion was observed to be more rapid in female mice than in male mice, with radioactivity in faeces peaking between 4 and 8 hours in females and between 8 and 12 hours in males.

The pattern of pharmacokinetic behaviour in rats following a single oral administration of radiolabelled lasalocid sodium was comparable to that seen in mice, with rapid absorption and excretion and a wide distribution of radioactivity in tissues. Whole blood radioactivity peaked at 3 hours, and the half-life of elimination was 4.8 hours. Radioactivity was widely distributed to tissues, with the highest levels seen in the liver, where it peaked at approximately 6 hours after administration. Approximately 85% of the administered dose was excreted in faeces within 24 hours, and approximately 1% was excreted in urine over the same period. Similar results were seen after seven daily oral doses.

In bile duct–cannulated male rats administered a single oral dose of radiolabelled lasalocid, approximately 61% of the dose was absorbed. Approximately 59% of the dose was excreted in bile within 48 hours.

In a comparative metabolism study in pig, dog, rat, mouse, chicken and turkey, the radioactive metabolite profile was similar in the faecal and liver fractions, although the relative proportions varied. The only component identified was lasalocid A, which represented the major component of the TRR in the faeces and liver in all species.

Although other residues were not identified, they were present at low levels and are considered to be minor.

3.2 Toxicological data

Critical studies are summarized in Table 6.

The acute toxicity of lasalocid sodium has been investigated in a number of species. Oral LD_{50} values were 146, 122, 33 and 40 mg/kg bw in the mouse, rat, neonatal rat and rabbit, respectively. The increased sensitivity of the rabbit may be due to the increased sensitivity of this species to effects of antimicrobial drugs on the intestinal microflora.

Table 6. Studies relevant to risk assessment

Species/study type (route)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Mouse				
Two-year study of toxicity and carcinogenicity (dietary)	0, 1.5, 5.25, 18	No relevant findings	18[a]	–
Rat				
Thirty-month toxicity and carcinogenicity study (dietary)	Males: 0, 0.5, 1.8, 6.2 Females: 0, 0.6, 2.2, 8.1	Increased incidence of impaired righting and grasping reflexes in females	2.2	8.1
Multigeneration reproductive toxicity study, including teratogenicity study (dietary)	0, 0.5, 1.75, 6	Parental toxicity: Reduced body weights	1.75	6
		Reproductive toxicity: Decreased numbers of corpora lutea and implantations, decreased implantation efficiency	**0.5**[b]	1.75
		Offspring toxicity: Decreased number of pups surviving to weaning, decreased body weight of pups surviving to weaning	1.75	6
		Embryo and fetal toxicity: Decreased fetal weights, increased incidence of visceral and skeletal variants	1.75	6
Rabbit				
Developmental toxicity study (gavage)	0, 0.5, 1, 2	Maternal toxicity: Decreased body weight gain, decreased feed consumption and altered faecal output	–	0.5[c,d]
		Embryo and fetal toxicity: Decreased litter weights, increased incidence of forelimb flexure and minor skeletal abnormalities/variants	**0.5**[b]	1

Table 6 (continued)

Species/study type (route)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Dog				
Two-year toxicity study (dietary)	0, 0.25, 1, 5	Transient intermittent paralysis of limbs and increased serum AP	1	5

AP: alkaline phosphatase; bw: body weight; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] Highest dose tested.

[b] Pivotal study value (Reno et al., 1980b; Clubb & Sutherland, 2003b).

[c] Lowest dose tested.

[d] Maternal toxicity was likely due to the sensitivity of rabbits to antibacterial effects on the microflora of the gastrointestinal tract. It is not considered appropriate to consider the maternal toxicity in relation to derivation of an ADI.

Lasalocid sodium was not irritating to the skin of rabbits but caused corneal irritation, conjunctival redness and chemosis when applied to the eyes.

Lasalocid sodium did not cause skin sensitization in the guinea-pig maximization test.

In a non-GLP 13-week study in rats, lasalocid sodium was administered in the diet at concentrations adjusted to achieve doses of 0, 2, 5 and 20 mg/kg bw per day. Based on reduced feed consumption, increased liver to body weight ratios and increased haemosiderin in the liver in females, the LOAEL was 5 mg/kg bw per day, and the NOAEL was 2 mg/kg bw per day.

In a non-GLP 13-week study in weanling rats, lasalocid sodium was administered in the diet at concentrations adjusted to achieve doses of 0, 1, 2, 3 and 10 mg/kg bw per day. Based on increased AP levels seen in males at all doses at week 13, the LOAEL was 1 mg/kg bw per day. No NOAEL could be established. It is noted, however, that the low-dose effect on AP seen in this study was not seen in other rat studies.

In a non-GLP 13-week study performed in weanling rats that had been exposed to lasalocid sodium in utero (parents were exposed prior to and during mating, gestation and lactation), the substance was administered in the diet at concentrations adjusted to achieve doses of 0, 1, 2, 3 and 10 mg/kg bw per day. Based on increased haemosiderin seen in the liver of males and (predominantly) females, the LOAEL was 3 mg/kg bw per day, and the NOAEL was 2 mg/kg bw per day.

In a non-GLP 13-week toxicity study in dogs, lasalocid sodium was administered in gelatine capsules at doses of 0, 2, 5 and 10 mg/kg bw per day. Transient muscle weakness involving primarily the hindlimbs was noted in animals at the top dose only. Based on decreased serum chloride levels, increased spleen weights, increased congestion in the spleen and increased hepatocyte vacuolation, the LOAEL was 5 mg/kg bw per day, and the NOAEL was 2 mg/kg bw per day.

In a 2-year toxicity study in dogs, lasalocid sodium was administered in the diet at concentrations of 0, 10, 35 and 180 mg/kg feed (equivalent to 0, 0.25, 1 and 5 mg/kg bw per day, respectively). Based on the transient intermittent paralysis of limbs occurring on a single day and on increased AP levels, the LOAEL was 180 mg/kg feed (equivalent to 5 mg/kg bw per day), and the NOAEL was 35 mg/kg feed (equivalent to 1 mg/kg bw per day).

In a 24-month carcinogenicity study, mice were administered lasalocid sodium in feed at a concentration of 0, 10 (low-dose animals were dosed with 20 mg/kg feed for the first 5 weeks of the study, after which the dose was adjusted downward), 35 (mid-dose animals were dosed with 60 mg/kg feed for the first 5 weeks, after which the dose was adjusted downward) or 120 mg/kg feed (equivalent to 0, 1.5, 5.25 and 18 mg/kg bw per day, respectively, after week 5). Lasalocid sodium did not show evidence of tumorigenic potential. The NOAEL was 120 mg/kg feed (equivalent to 18 mg/kg bw per day), the highest dose tested.

In a 30-month toxicity and carcinogenicity study, rats were administered lasalocid sodium in feed at a concentration of 0, 10, 35 or 120 mg/kg (equal to mean doses of 0, 0.5, 1.8 and 6.2 mg/kg bw per day for males and 0, 0.6, 2.2 and 8.1 mg/kg bw per day for females, respectively). The animals used in this study were weanlings bred from parental animals administered the same doses of lasalocid sodium during breeding, gestation and lactation. Lasalocid sodium did not demonstrate tumorigenic properties in this study. Based on a transient impairment of righting and grasping reflexes seen in females between weeks 31 and 49, the LOAEL was 120 mg/kg feed (equal to 8.1 mg/kg bw per day), and the NOAEL was 35 mg/kg feed (equal to 2.2 mg/kg bw per day).

Lasalocid sodium did not show evidence of genotoxic potential in a range of in vitro tests covering the end-points of gene mutation and chromosomal aberration. Although there was no in vivo test for chromosomal effects, the Committee considered that this was unnecessary in view of the existing genotoxicity and carcinogenicity data.

In a multigeneration reproductive toxicity study incorporating a teratology arm, rats were administered lasalocid sodium in feed at a concentration of 0, 10, 35 or 120 mg/kg (equivalent to 0, 0.5, 1.75 and 6 mg/kg bw per day, respectively). At weaning, F_1 animals were randomly selected to become the parents of the F_2 generation; at weaning of F_2 animals, these were randomly selected to become parents of the F_3 generation. F_0 animals and F_2 animals were mated more than once in order to allow for evaluations of teratology. In the high-dose group (120 mg/kg feed), reduced body weights were seen in parental females. The NOAEL for parental toxicity was 35 mg/kg feed (equivalent to 1.75 mg/kg bw per day). The mean numbers of corpora lutea and implantations per pregnant dam were reduced in both the high-dose (120 mg/kg feed) and mid-dose (35 mg/kg feed) groups, resulting in decreased implantation efficiency in these groups. The high-dose group also showed decreased pregnancy and fertility rates. Based on these effects, the NOAEL for reproductive toxicity was 10 mg/kg feed (equivalent to 0.5 mg/kg bw per day). In the high-dose group (120 mg/kg feed), the number of pups surviving to weaning and the body weights of pups surviving to weaning were reduced. In the high-dose group of the teratogenicity arm, fetal weights were slightly reduced, and the incidence of visceral and skeletal

variants was increased. The NOAEL for offspring and embryo/fetal toxicity was therefore 35 mg/kg feed (equivalent to 1.75 mg/kg bw per day).

In a developmental toxicity study in rabbits, lasalocid sodium was administered by oral gavage over days 6–28 of gestation at a dose of 0, 0.5, 1 or 2 mg/kg bw per day. A NOAEL for maternal effects could not be established, as soft stools and effects on body weight gain and feed consumption were seen at all doses. This is likely the result of the known sensitivity of rabbits to antibacterial effects on the microflora of the gastrointestinal tract, and consequently it is not considered appropriate to consider the maternal toxicity in relation to the derivation of an ADI. The LOAEL for embryo and fetal toxicity was 1 mg/kg bw per day, based on decreased litter weights, increased incidence of forelimb flexure and minor skeletal abnormalities/variants at this dose. Although the Committee acknowledges the possibility that these effects may have been secondary to maternal toxicity, it considers the NOAEL for embryo and fetal toxicity to be 0.5 mg/kg bw per day.

No original studies dedicated specifically to the evaluation of the neurotoxic potential of lasalocid sodium were provided. Literature data indicate that polyether ionophores, including lasalocid, do have neurotoxic potential. In line with this, a number of the repeated-dose studies summarized above did include examination of neurological end-points. Evidence of neurotoxicity, consisting of transient patterns of muscle weakness involving primarily the hindlimbs, was seen in the 13-week and 2-year dog studies. These effects were seen only at the highest dose and resolved spontaneously, despite continued administration of the drug. In addition, in the 30-month rat study, impairment of the righting and grasping reflexes was seen. A clear effect was evident only at the top dose and, as with the effects seen in the dog, resolved spontaneously, despite continued administration of the drug.

No observations in humans were identified.

3.3 Microbiological data

A JECFA decision-tree approach that was adopted by the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and complies with VICH GL36 (VICH, 2004) was used by the Committee to determine the need for, and to establish, if necessary, a microbiological ADI for lasalocid sodium. Studies of microbiological activity against bacterial strains representative of the human colonic flora were evaluated.

The microbiological ADI was derived from in vitro MIC data as described in VICH GL36. The strains needed to determine the MIC_{calc}, which is the minimum inhibitory concentration derived from the lower 90% confidence limit for the mean MIC_{50} of the relevant genera for which the drug is active, were chosen according to these guidelines, which state that an intrinsically resistant bacterial genus should not be included. The genera with a MIC_{50}, including *Eubacterium* (0.125 μg/mL), *Bacteroides* (32 μg/mL), *Bifidobacterium* (0.25 μg/mL), *Fusobacterium* (1 μg/mL), *Peptostreptococcus* (2 μg/mL), *Clostridium* (0.125 μg/mL), *Enterococcus* (0.5 μg/mL) and *Lactobacillus* (0.125 μg/mL), were used to determine the MIC_{calc}.

Lasalocid sodium residues may be present at low levels in meat products consumed by humans; therefore, lasalocid sodium–related residues could enter the colon of a person ingesting edible tissues from treated animals. The Committee used pharmacokinetic studies and faecal binding studies to determine the fraction of the oral dose available to the human intestinal microflora. Lasalocid sodium was poorly absorbed after oral administration in animals and also binds extensively (> 90%) to faecal contents. Therefore, low levels of lasalocid sodium residues entering the human colon will remain biologically active. There is potential for disruption of the colonization barrier in the human gastrointestinal tract, as MIC values for some of the most relevant and predominant genera in the gastrointestinal tract were susceptible to lasalocid sodium. Lasalocid sodium does not appear to select for resistance in bacteria, and carboxylic polyether ionophores are not used in human medicine.

The formula for calculating the microbiological ADI is as follows:

$$\text{Upper bound of the ADI } (\mu g/kg \text{ bw}) = \frac{MIC_{calc} \times \text{Mass of colon content}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

The equation terms are derived as described below.

MIC_{calc}: In accordance with Appendix C of VICH GL36, calculation of the estimated NOAEC (MIC_{calc}) for colonization barrier disruption uses MIC values from the lower 90% confidence limit of the mean MIC_{50} for the most relevant and sensitive human colonic bacterial genera. The strains needed to determine the MIC_{calc} were chosen according to these guidelines, which state that an intrinsically resistant bacterial genus should not be included. Based on the MIC_{50} values for *Eubacterium* (0.125 µg/mL), *Bacteroides* (32 µg/mL), *Bifidobacterium* (0.25 µg/mL), *Fusobacterium* (1 µg/mL), *Peptostreptococcus* (2 µg/mL), *Clostridium* (0.125 µg/mL), *Enterococcus* (0.5 µg/mL) and *Lactobacillus* (0.125 µg/mL), the MIC_{calc} is 0.228 µg/mL.

Mass of colon content: A value of 220 g is based on the colon content measured from humans.

Fraction of oral dose available to microorganisms: It is recommended that the fraction of an oral dose available for colonic microorganisms be based on in vivo measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine. Human data are encouraged, but, in their absence, non-ruminant animal data are recommended. In the absence of data to the contrary, it should be assumed that metabolites have antimicrobial activity equal to that of the parent compound. The fraction may be lowered if the applicant provides quantitative in vitro or in vivo data to show that the drug is inactivated during transit through the intestine. Lasalocid sodium is poorly absorbed and is excreted in faeces of experimental animals, primarily in unchanged form. Lasalocid sodium binds rapidly and extensively (> 90%) to faecal contents; therefore, the fraction of oral dose available to microorganisms would be 0.10.

Body weight: The body weight of an adult human is assumed to be 60 kg.

The upper bound of the microbiological ADI for lasalocid sodium is therefore calculated as follows:

$$\text{Upper bound of the ADI} = \frac{0.228\ \mu g/mL \times 220\ g}{0.10 \times 60\ kg\ bw}$$

$$= 8.4\ \mu g/kg\ bw$$

Therefore, a microbiological ADI of 0–8.4 µg/kg bw could be derived from in vitro MIC susceptibility testing and bioavailability studies.

4. EVALUATION

The Committee considered the toxicological effects of lasalocid sodium to be the most relevant for the purpose of establishing an ADI. A toxicological ADI of 0–5 µg/kg bw was established based on the NOAEL of 0.5 mg/kg bw per day from the developmental toxicity study in rabbits and the multigeneration reproductive toxicity study in rats, with application of an uncertainty factor of 100 for interspecies and intraspecies variability.

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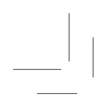
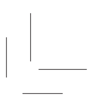
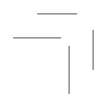
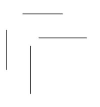

RECOMBINANT BOVINE SOMATOTROPINS (addendum)

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1. EXPLANATION

Somatotropins are proteins secreted by the anterior pituitary gland that stimulate growth, cell regeneration and reproduction in humans and animals. Most anabolic and growth-promoting effects of somatotropins are mediated through insulin-like growth factor-I (IGF-I). Bovine somatotropins produced by recombinant deoxyribonucleic acid (DNA) techniques (rbSTs) are used in lactating dairy cows to increase milk production. Four bovine somatotropin (bST) analogues, somagrebove, sometribove, somavubove and somidobove, were previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its fortieth meeting (Annex 1, reference *104*) and further evaluated at its fiftieth meeting (Annex 1, reference *134*). Although the chemical properties of the recombinant products vary slightly from those of pituitary bST (for chemical structures, see Annex 1, reference *106*), the Committee considered the recombinant products to be biologically and toxicologically similar, as they all act by binding with high affinity to the bST receptor.

The Committee at its fortieth meeting established an acceptable daily intake (ADI) and maximum residue limits (MRLs) "not specified" for these four rbSTs. The term "not specified" was used because of the lack of bioactivity following oral intake of rbSTs and IGF-I and the low concentrations and non-toxic nature of the residues of these compounds. The ADI and MRLs "not specified" were reaffirmed by the Committee at its fiftieth meeting.

Draft Codex standards for rbSTs have been held at the final step (before adoption) for more than a decade. When considering the adoption of these standards, the Codex Alimentarius Commission at its Thirty-fifth Session (FAO/WHO, 2012) requested a re-evaluation of the four analogues of natural bST, somagrebove, sometribove, somavubove and somidobove, by JECFA, noting that the scientific assessment of bST dated back to the 1990s. In particular, the Commission requested that JECFA (i) update the toxicological evaluation, (ii) update the exposure assessment based on any new occurrence data in food, (iii) evaluate potential adverse health effects and (iv) consider the need to revise or maintain the ADI and MRLs for rbSTs. The Commission further requested that JECFA consider new data and information related to other factors pertaining to human health, including (i) the possible increased use of antimicrobials to treat mastitis in cows, (ii) the possibility of increased levels of IGF-I in the milk of cows treated with rbSTs, (iii) the potential effects of rbSTs on the expression of certain viruses in cattle and (iv) the possibility that exposure of human neonates and young children to milk from rbST-treated cows increases health risks (e.g. the development of insulin-dependent diabetes mellitus). JECFA was also asked to consider aspects of antimicrobial resistance associated with the use of rbSTs in relation to human health.

rbSTs are registered in 21 countries in the world, including Bolivia (Plurinational State of), Brazil, Chile, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Jamaica, Lebanon, Mexico, Pakistan, Panama, Peru, Republic of Korea, South Africa, Uruguay, Venezuela (Bolivarian Republic of), the USA and Puerto Rico for use in dairy cows and in Pakistan for use in buffaloes. Sometribove, marketed as Lactotropin, Posilac, Somatech or Lactotropina, is authorized for use at a dosage of 500 mg subcutaneously every 14 days in all cases. A dose of 375 mg is also authorized for use in Mexico. Treatment commences approximately 50–90 days

postpartum until the end of lactation. Somavubove, marketed as Boostin or Hilac, is also registered for use in the Republic of Korea and is exported to Mexico, Brazil, Colombia, Pakistan and South Africa. A zero withdrawal period exists in all cases. bST is administered to cattle either subcutaneously or intramuscularly.

In response to JECFA's call for data, data were submitted to the Committee by a sponsor and two Member countries. Additionally, the Committee undertook a systematic review to address the following questions:

- What are the hormone levels in the milk and/or meat of cattle, goats or sheep treated with rbSTs compared with untreated animals?
- Are the incidences of clinically relevant mastitis different between cattle, sheep and goats treated with rbSTs compared with untreated animals? Are there differences in antimicrobial residue levels in the milk and meat products from treated compared with untreated animals?
- Are retroviral/lentiviral levels and serotype distributions different between cattle, sheep and goats treated with rbSTs compared with untreated animals?
- Are prion levels in meat and milk and prion infectivity different between cows treated with rbSTs compared with untreated animals?
- Is consumption of milk or meat from rbST-treated cattle, sheep or goats associated with increased rates of morbidity and mortality in infants or in the general population compared with the equivalent age groups consuming meat or milk from untreated animals?

Details of the search strategy and databases used are available on the WHO website as supplementary information to the meeting report at http://www.who.int/foodsafety/chem/jecfa/publications/reports/en/index.html.

In addition, PubMed and Web of Knowledge databases were searched for toxicity studies of rbSTs in laboratory animals, bioavailability/bioactivity of oral IGF-I and analytical methods.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

The Committee at its fortieth and fiftieth meetings concluded that human and bovine somatotropins are structurally different and have species-specific receptor binding activity. Furthermore, the total concentration of bSTs detected in tissues and milk of rbST-treated cattle is similar to that from untreated cattle, and rbSTs are denatured by high temperatures (e.g. by cooking and pasteurization) and biodegradation processes in the gut.

2.1.1 Laboratory animals

No new studies on biochemical aspects of rbSTs were submitted with the recent call for data, and none was available in the literature. Since the assessment of rbSTs by the fiftieth meeting, a Health Canada (1999) expert panel

has suggested, based on the detection of anti-rbST antibodies in rats, that some rbSTs administered orally could potentially be absorbed. The study that reported this finding (Richard, Odaglia & Deslex, 1989) was a 90-day study in rats. This study also included a satellite investigation on anti-rbST antibodies in sera of rats administered an rbST by gavage. The fortieth meeting had reviewed the toxicity data from this study; however, the results of the satellite study on the anti-rbST antibodies were not discussed in the toxicological monograph from the fortieth meeting and are summarized below.

(a) Rats

Sometribove was administered daily by gavage at a dose of 0, 0.1, 0.5, 5 or 50 mg/kg body weight (bw) per day or subcutaneously at 1 mg/kg bw per day (positive control) to Charles River CD VAF rats (30 rats of each sex per group) for 13 consecutive weeks. Of these 30 rats of each sex per group, 15 rats were considered part of a satellite study to investigate the development of anti-rbST antibodies. Ten rats of each sex per group from the satellite study were euthanized at week 14, and five rats of each sex per group were maintained without dosing for an additional 14 weeks of recovery. Blood samples were collected from all rats pretreatment and at week 14 (i.e. at the end of the treatment period), at week 7 from 10 rats of each sex per group that were euthanized at week 14 and at week 28 from the remaining 5 rats of each sex per group.

Sera were analysed by radioimmunoprecipitation, and the radioactivity in the pellet was corrected for nonspecific binding. The titre in the test sera was expressed as the percentage of the corrected counts per minute in the precipitate over the total counts per minute tested. Greater than 11% sometribove binding capacity, which was equivalent to the upper 75th percentile plus 1.5 times the interquartile range for negative control sera, was used as a cut-off to classify a sample as antibody positive.

All rats were seronegative at the start of the study. Animals in the negative control and 0.5 mg/kg bw per day groups remained seronegative for sometribove antibodies throughout the experiment. In contrast, 20% of the animals were seropositive on both week 7 (4/20) and week 14 (6/30) in the 5 mg/kg bw per day group. In the 50 mg/kg bw per day group, 15% (3/20) and 30% (9/30) of the animals were seropositive on weeks 7 and 14, respectively. One animal (3%) was seropositive only on week 14 in the lowest-dose group (0.1 mg/kg bw per day). All but one positive control animal administered sometribove subcutaneously were seropositive (Richard, Odaglia & Deslex, 1989). Antibody levels in orally dosed animals were generally lower than those observed in the positive controls. Oral doses of rbST did not increase body weight or feed consumption, although a concomitant marked increase in body weight and feed consumption was recorded in the positive control group from week 2 of the experiment.

The study did not measure rbST in sera and cannot confirm whether intact rbST was absorbed into the systemic circulation. Also, there was no effect on body weight or feed intake, suggesting that a sufficient quantity of bioactive sometribove was not absorbed into the systemic circulation. Consequently, it is not possible to confirm whether the anti-rbST antibody response was a result of absorption of

intact rbST or only an immunologically active peptide fragment (epitope or antigenic determinant) of rbST into the systemic circulation or due to mucosal immunity in the gut. It is known that exposure to ingested foreign proteins could stimulate a mucosal immune response in the gut, and activated antibody-producing cells could enter and produce antibodies in the systemic circulation (McCluskie & Davis, 1999; Valdes-Ramos et al., 2010; Shin et al., 2013). The findings of this study therefore do not confirm the systemic bioavailability of orally administered rbSTs.

Considering the similar levels of total bST detected in milk or tissues of animals treated with rbSTs (see section 2.3), the expected level of human exposure to rbSTs would be much lower than the dose used in anti-rbST antibody–positive rats. Furthermore, because of the structural dissimilarities between human and bovine somatotropins, species-specific receptor binding, destruction of rbSTs by high temperatures (e.g. cooking or pasteurization) and biochemical degradation by gastrointestinal enzymes, small quantities of rbSTs in milk or tissues of treated animals, if present, are not expected to have biological activity when administered orally.

(b) Cattle

In a recent study (Le Breton et al., 2009), the elimination kinetics of an rbST in serum was characterized in a cow in which the concentrations after treatment with a single subcutaneous injection of 500 mg sometribove (Lactotropin, Monsanto, Elanco Animal Health) were measured using liquid chromatography coupled to tandem mass spectrometry in positive electrospray ionization mode. This allowed for the unambiguous identification and quantification of the rbST in serum. Detection of the rbST was possible from 4.5 hours to 4 days after administration, and concentrations up to 10 ng/mL were reported.

No other new biological or pharmacokinetic studies were available.

2.2 Toxicological studies

The Committee at its fortieth meeting evaluated the toxicity of different rbSTs. Acute oral toxicity studies in rats with rbST doses up to 5 g/kg bw, two 2-week oral feeding studies in rats with doses of rbSTs up to 10 mg/kg bw per day and two 4-week oral feeding studies in rats with doses up to 50 mg/kg bw per day caused no effects up to the highest dose tested. Similarly, no treatment-related effects were observed at the highest dose tested in two 90-day oral feeding studies in rats with rbSTs at doses up to 100 mg/kg bw per day and a 90-day oral feeding study in dogs at doses up to 10 mg/kg bw per day.

No new toxicity studies on rbSTs were available since the previous evaluation of rbSTs by the Committee at the fiftieth meeting.

2.2.1 Long-term studies on toxicity and carcinogenicity of recombinant mouse and rat somatotropins

A search of the published literature identified long-term (2-year) carcinogenicity studies in mice and rats for related, but distinct, compounds (i.e. mouse and rat growth hormones) (Farris et al., 2007). These studies did not use

the oral/gavage route for administration of the test articles and did not test rbSTs. The Committee therefore considered these data not directly relevant to the risk assessment of rbSTs, but relevant to understanding the carcinogenic potential of other related somatotropins in respective mammalian species. The study findings are therefore summarized briefly in this monograph.

(a) Mice

In a 2-year study compliant with good laboratory practice (GLP), groups of CD-1 mice 39 days of age and weighing 18.5–27.5 g (females) and 20.2–32.8 g (males) at the beginning of the study were allocated into five groups (50 mice of each sex per group). Mice received daily subcutaneous injections of vehicle (two groups) or recombinant mouse somatotropin (rmST) at 0.1, 0.2 or 0.5 mg/kg bw. Animals were observed daily for mortality and weekly for clinical signs. Body weight measurements and ophthalmic examinations were conducted routinely. Dead mice and those euthanized at the end of the study were necropsied, and 58 tissues per mouse were examined for gross and histopathological lesions.

Daily subcutaneous injection of rmST over 2 years elicited no treatment-related mortality or physical or ocular signs in mice. No effects on body weight were seen in trend analysis in either sex. The final mean body weights were 36.8, 37.5, 37.1 and 38.2 g in females and 46.1, 48.3, 49.3 and 47.6 g in males in the control, 0.1, 0.2 and 0.5 mg/kg bw per day treatment groups, respectively. Examination of the pituitary gland at necropsy did not reveal treatment-related gross changes or changes in pituitary weight. When compared with concurrent or historical controls, there was no significant treatment-related increase in the incidence of tumours in any tissue examined in both males and females (Farris et al., 2007).

(b) Rats

In a GLP-compliant 2-year study, groups of Sprague-Dawley rats 37 days of age and weighing 102–149 g (females) and 129–195 g (males) at the start of the study were allocated into five groups (50 rats of each sex per group). Rats received daily subcutaneous injections of vehicle (two groups) or recombinant rat somatotropin (rrST) at 0.2, 0.4 or 0.8 mg/kg bw. Animals were observed daily for mortality and weekly for clinical signs. Body weight measurements and ophthalmic examinations were conducted routinely. Dead rats and those euthanized at the end of the study were necropsied, and 57 tissues per rat were subjected to gross and histopathological examination.

Daily subcutaneous injection of rrST over 2 years elicited a treatment-related decrease in mortality in female rats, but there was no effect on mortality of male rats. Eighty-two per cent of female rats treated with rrST at 0.4 mg/kg bw per day and 80% of female rats treated with rrST at 0.8 mg/kg bw per day survived to study termination, compared with 62–64% of the control groups. The increased survival was attributed in part to reduction in deaths due to pituitary tumours in females. No treatment-related physical or ocular signs were observed. Female rats treated with rrST had a higher average body weight ($P < 0.001$) at all doses. At the end of the study, mean body weights of female rats were 324, 343, 363 and 381 g in the control,

0.2, 0.4 and 0.8 mg/kg bw per day treatment groups, respectively. In male rats, the body weights in the 0.4 and 0.8 mg/kg bw per day groups were significantly higher than those in the control and 0.2 mg/kg bw per day dose groups. Body weights at the end of the study in male rats were 627, 630, 647 and 650 g at 0, 0.2, 0.4 and 0.8 mg/kg bw per day, respectively. Overall, when compared with concurrent or historical controls, no significant difference in tumour incidence was detected in the different treatment groups. However, after adjustment for multiplicity of statistical tests, the incidence of pituitary adenoma in female rats showed a decreasing trend when the treatment dose was increased (Farris et al., 2007).

2.3 Bovine somatotropin in tissues and milk

Bovine somatotropin is not readily transferred from blood/plasma to milk. At the fortieth meeting of the Committee, it was concluded that studies of rbST residues in milk demonstrate that the proposed use of rbSTs, even at exaggerated doses, will not lead to any detectable concentrations of total bST in milk above those normally present in milk from untreated cows (0.9–1.6 µg/L). Similarly, cows treated with rbSTs have, at most, a 2-fold increase in residues in tissues, to total bST concentrations of 3.1–4.2 µg/kg in muscle and 16–25 µg/kg in liver compared with 2.2–3.7 µg/kg in muscle and 9–13 µg/kg in liver of untreated cows (Annex 1, reference *106*).

The fiftieth Committee meeting evaluated a published study (Choi et al., 1997) in which rbST was administered in two different dosage forms by subcutaneous injection to beef cattle every 2 weeks for 20–24 weeks. Treated cattle were slaughtered 2 weeks after the final dose. Tissue concentrations of total bST ranging from 1.45 ± 0.86 to 4.94 ± 1.47 µg/kg in muscle,4.82 ± 1.95 to 9.33 ± 5.23 µg/kg in fat, 3.56 ± 1.73 to 5.36 ± 1.21 µg/kg in liver and 3.58 ± 1.14 to 4.49 ± 1.83 µg/kg in kidney were reported. Total bST concentrations were measured using a radioimmunoassay procedure. There were no significant differences between treated animals and controls in the concentrations of total bST in muscle, fat, liver or kidney (Annex 1, references *135* and *136*).

A limited number of studies that provide new data on bST residues in tissues (Kweon et al., 2000) and in milk of lactating cows and buffaloes have been published since the fiftieth Committee meeting (Mishra et al., 2005; Mishra, Mahapatra & Shukla, 2006; Vicini et al., 2008). Also, the results of several studies published before the previous meeting were not discussed in the reports of the previous meetings (Torkelson & Miller, 1987; Groenewegen et al., 1990).

Torkelson & Miller (1987) injected eight cows intramuscularly and another eight subcutaneously at 14-day intervals with 500 mg rbST in a sustained release formulation. Ten untreated animals served as controls. Milk and blood samples were collected 2 days prior to injection, on the day of injection and on days 1, 2, 3, 4, 6, 8, 10, 12 and 14 after injection during the fourth treatment cycle. The concentration of total bST in milk was determined by radioimmunoassay. No further information on validation of the assay was provided. The results demonstrated no correlations between total bST concentrations in blood and milk, regardless of the route of administration. Total bST concentrations in most milk samples were below the limit of detection (< 0.3 ng/mL).

Groenewegen et al. (1990) determined the concentrations of total bST in milk of untreated cows (*n* = 3) at 82.3 ± 17 days postpartum and cows (*n* = 3) treated at 78 ± 6 days postpartum. Cows in the treated groups received 10.6 mg rbST (American Cyanamid) daily starting at 28 days postpartum. This formed part of the study in which the bioactivity of milk from cows treated with rbST was examined in hypophysectomized rats. The concentration of total bST in milk was measured by radioimmunoassay, with a level of detection of 0.5 ng/mL and an average recovery of 96%, and was reported as 3.3 and 4.2 ng/mL in milk of control and treated cows, respectively.

Mishra et al. (2005) reported somatotropin concentrations in milk of lactating buffaloes (*n* = 20) treated with rbST (Boostin-250, LG Chemicals India) subcutaneously at 250 mg on three occasions at 14-day intervals, compared with saline-treated controls (*n* = 10). Total somatotropin concentrations were measured in six fortnightly milk samples starting from 15 days pretreatment to 60 days post-injection using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) that utilized (r)bST-specific antibodies. The assay was validated for sensitivity, specificity, precision and recovery. Parallelism was demonstrated between the standard curve using rbST (National Hormone and Peptide Program [NHPP], California, USA) and serially diluted serum, milk and pituitary-derived growth hormone. The sensitivity of the assay was 0.1 ng/mL. The specificity of the assay was determined by western blot using nonspecific proteins such as bovine serum albumin, gelatine and bovine prolactin with rbST. Presence of a single band only on the rbST column indicated that the antibody used in the assay was specific to bST only. The intra-assay and inter-assay variations for serum and milk were 3.36–8.81% and 6.01–14.31%, respectively. Recovery of exogenous bST from serum and milk ranged from 90% to 102% and from 96% to 108%, respectively. Mean total somatotropin concentrations pretreatment and post-treatment in both rbST-treated and control animals at each fortnightly collection are summarized in Table 1. No significant difference in the total somatotropin concentrations was observed between rbST-treated and control animals. These concentrations are similar to those reported for cattle at the fortieth Committee meeting.

Table 1. Fortnightly changes in total somatotropin concentrations in milk in lactating buffaloes treated with rbST (n = 20) compared with saline-treated buffaloes (n = 10)

Treatment group	Somatotropin concentrations (ng/mL)						Overall mean	Significance
	Pretreatment	1	2	3	4	5		
Saline	1.27 ± 0.07	1.10 ± 0.11	1.06 ± 0.10	1.10 ± 0.10	1.18 ± 0.06	1.13 ± 0.05	1.14 ± 0.04	NS
rbST	1.39 ± 0.03	1.16 ± 0.08	1.17 ± 0.07	1.22 ± 0.07	1.19 ± 0.04	1.25 ± 0.06	1.23 ± 0.03	

NS: not significant; rbST: recombinant bovine somatotropin
Source: Adapted from Mishra et al. (2005)

Mishra, Mahapatra & Shukla (2006) performed a study, similar in design to the one previously reported in buffaloes (Mishra et al., 2005), in lactating crossbred (*Bos taurus* × *Bos indicus*) cows (n = 20) treated with rbST (Boostin-250, LG Chemicals India) subcutaneously at 250 mg on three occasions at 14-day intervals, compared with saline-treated control cows (n = 10). No significant difference (P > 0.05) was found in the mean total bST concentrations in milk from rbST-treated cows (1.16 ± 0.08 ng/mL) compared with control cows (1.10 ± 0.34 ng/mL) measured in fortnightly milk samples by indirect sandwich ELISA. No validation information on the assay used was provided in the publication.

In a cross-sectional study, total bST concentrations were determined in retail milk samples (n = 344) collected from stores in 48 contiguous states within the USA where rbST is approved for use (Vicini et al., 2008). Samples were obtained in blocks over a period of 3 weeks from purchased milk labelled as conventional (milk that did not contain any claims about supplementation with rbST or organic practices), rbST-free (milk that has a processor claim that cows were not supplemented with rbST) or organic (milk from farms that were certified as meeting United States Department of Agriculture [USDA] organic standards). A block consisted of a shipping container collected on one day by one sampler and in one city to minimize the effects of shipping conditions. At least two blocks of samples were collected from each state. More samples were collected from states with larger populations or larger quantities of milk production. The freshest (based on expiry date) pasteurized whole milk in plastic or paper containers of any retail brand was preferred. Ultra-high-temperature pasteurized milk was avoided. bST concentrations in milk were measured by electrochemiluminescent immunoassays (ECLIA) using a Sector Imager 6000. Assays were performed at Monsanto. No information on the validation of the assay was provided. The milk samples were also examined for quality (antimicrobials and bacterial counts), nutritional value (fat, protein and solid-not-fat) and additional hormonal composition. There were no significant differences (P > 0.05) in concentration of total bST in milk, regardless of label type. Approximately 82% of milk samples had total bST levels below the limit of quantification (0.033 ng/mL), and 72% were less than the limit of detection (0.010 ng/mL) for the assay.

Another study in which 32 Holstein bulls and steers were randomly assigned to one of four groups, (a) bull group, (b) untreated steer group, (c) steers treated with rbST when they were about 80 kg live weight ($rbST_1$) or (d) steers treated with rbST when they were about 300 kg live weight ($rbST_2$), was reported by Kweon et al. (2000). Treated steers were given rbST every 14 days at 0.03 mg/kg bw per day intramuscularly, alternatively in the rump and shoulder. Concentrations of total bST were measured using an immunoradiometric assay. No details on the validation procedure of the analytical method were provided. The concentrations of total bST in tissue with or without rbST treatment are summarized in Table 2. There were no significant differences between rbST-treated and untreated steers. The tissue concentrations of total bST reported in this study in both control and rbST-treated animals are slightly higher than those reported at the fortieth and fiftieth Committee meetings.

Table 2. Concentrations of total bST in tissues of rbST-treated and untreated steers

Tissues	Total bST in tissues of untreated steers (ng/mL)	Total bST in tissues of treated steers (ng/mL)		
		$rbST_1$	$rbST_2$	SEM
Injection site	5.80	7.23	8.83	0.89
Muscle	6.18	6.85	7.63	0.91
Kidney	15.93	17.75	23.05	1.77
Liver	19.83	18.05	20.10	1.15

bST: bovine somatotropin; $rbST_1$: steers treated with recombinant bovine somatotropin when they were about 80 kg live weight; $rbST_2$: steers treated with recombinant bovine somatotropin when they were about 300 kg live weight; SEM: standard error of the mean
Source: Adapted from Kweon et al. (2000)

2.4 Insulin-like growth factor-I in tissues and milk

2.4.1 IGF-I concentrations in milk

The fortieth Committee meeting cited an average concentration of IGF-I in milk of 3.7 ng/mL for untreated cows. An average concentration of 5.9 ng/mL was reported in cows treated with rbST; although this average concentration was significantly higher than that in milk from untreated cows, most of the concentrations were less than 10 ng/mL and within the normal physiological range observed in the milk of lactating cows. IGF-II concentrations in cow's milk were not affected by rbST treatment.

At the fiftieth Committee meeting, it was noted that the IGF-I content in normal bovine milk was highly variable, depending on the state of lactation, nutritional status and age. Over an entire lactation, IGF-I concentrations in milk ranged between 1 and 30 ng/mL, with the highest concentrations in colostrum and a constant decline thereafter. Multiparous animals were reported to have higher concentrations of IGF-I in milk compared with primiparous cows. Bulk milk from cows not given rbST had IGF-I concentrations of 1–9 ng/mL. In milk from rbST-treated cows, the concentrations of IGF-I ranged from 1 to 13 ng/mL in most studies.

Since the fiftieth Committee meeting, there have been limited additional data published on IGF-I residues in milk from untreated lactating cows (Daxenberger, Sauerwein & Breier, 1998; Liebe & Schams, 1998; Taylor et al., 2004) and from lactating cows treated with rbSTs (Daxenberger, Sauerwein & Breier, 1998; Pauletti et al., 2005; Collier et al., 2008). Additionally, concentrations of IGF-I in retail milk in the USA based on the label (e.g. rbST-free, organic or conventional; Vicini et al., 2008) were reported. Changes in IGF-I concentrations in milk from lactating buffaloes and goats following treatment have also been reported (Faulkner, 1999; Prasad & Singh, 2010; Castigliego et al., 2011). A summary of all new studies is provided in Table 3.

Table 3. Summary of the normal variation of IGF-I concentration in cow's milk and the effect of rbST treatment on IGF-I concentrations in milk

(a) Naturally occurring IGF-I

Study	No. of samples	IGF-I concentrations	Assay method
Daxenberger, Sauerwein & Breier (1998)	5 777	Range 1–83 ng/mL; median 4.4 ng/mL; 90th percentile 9.5 ng/mL; 95th percentile 12.5 ng/mL	Non-extraction radioimmunoassay following defattening
Liebe & Schams (1998)	12 in barned study 12 with clinical mastitis 22 with subclinical mastitis	Healthy quarters: 8.3 ± 1.7, 8.5 ± 2.1, 14.1 ± 1.7 and 15.1 ± 1.8 ng/mL in loose housing and 10.7 ± 2.1 and 6.6 ± 1.5 ng/mL in tied portion of barned study Clinical mastitis: 35.5 ± 23.5 vs 21.2 ± 6.8 ng/mL in healthy quarters Subclinical mastitis: 36.9 ± 31.3 vs 17.7 ± 11.3 ng/mL in healthy quarters	Extraction radioimmunoassay in skimmed milk
Taylor et al. (2004)	50 multiparous	> 16 ng/mL 1st week of calving; 6–9 ng/mL 2–20 weeks postpartum	Ethanol–acetone–acetic acid radioimmunoassay in whole milk

(b) rbST treatment studies

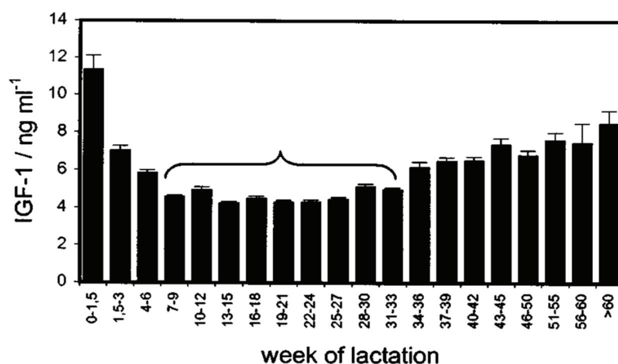
Study	Treatment	No. of animals	IGF-I concentrations		Assay method
			No rbST	rbST	
Cows					
Daxenberger, Sauerwein & Breier (1998)	1 × 500 mg sometribove (Posilac, Monsanto)	34 (33 for data analysis)	~4 ng/mL	Increase of 2.3 ng/mL for lactation 1; 1.6 ng/mL for lactation 2–6; and 1.9 ng/mL (48%) for all lactation	Non-extraction radioimmunoassay following defattening

Table 3 (continued)

Study	Treatment	No. of animals	IGF-I concentrations		Assay method
			No rbST	rbST	
Collier et al. (2008)	25 mg/day sometribove (winter)	6 per group	3.7 ng/mL	4.8 ng/mL	Radioimmunoassay
	25 mg/day sometribove (summer)	6 per group	3.4 ng/mL	3.8 ng/mL	
Pauletti et al. (2005)	3 × 500 mg (Boostin) at 14-day intervals from day 35 prepartum until parturition	21 per group	Day 1 postpartum (colostrum): 674 ± 270 ng/mL Day 7 no significant differences from treated animals	Day 1 postpartum (colostrum): 875 ± 335 ng/mLDay 7 postpartum: 12.9 ng/mL	Immune radiometric assay
Buffaloes					
Castigliego et al. (2011)	5 × 500 mg (Boostin) sc at 14-day intervals	8 per group	1.5–3.0 ng/mL	4.5–7.0 ng/mL	Sandwich ELISA
Prasad & Singh (2010)	5 mg rbST (Boostin) iv daily for 5 days	10	29.7 ± 4.5 to 38.1 ± 3.4 ng/mL	42.0 ± 5.2 ng/mL (highest concentration measured on day 1 after treatment)	Double-antibody radioimmunoassay
Goats					
Faulkner (1999)	2 × 3 mg sc of ovine somatotropin	5	~5 ng/mL	Maximum of ~15 ng/mL	Double-antibody radioimmunoassay
Retail milk survey					
Vicini et al. (2008)	Conventional; rbST-free and organic labelled milk		"rbST free" 3.0 ± 0.1 ng/mL; "organic" 2.7 ± 0.1 ng/mL	"Conventional" 3.1 ± 0.1 ng/mL	ECLIA

ECLIA: electrochemiluminescent immunoassay; ELISA: enzyme-linked immunosorbent assay; IGF-I: insulin-like growth factor-I; iv: intravenously; rbST: recombinant bovine somatotropin; sc: subcutaneously

Fig. 1. Mean IGF-I concentrations (± SEM) in milk from cows not treated with rbST during the entire lactation



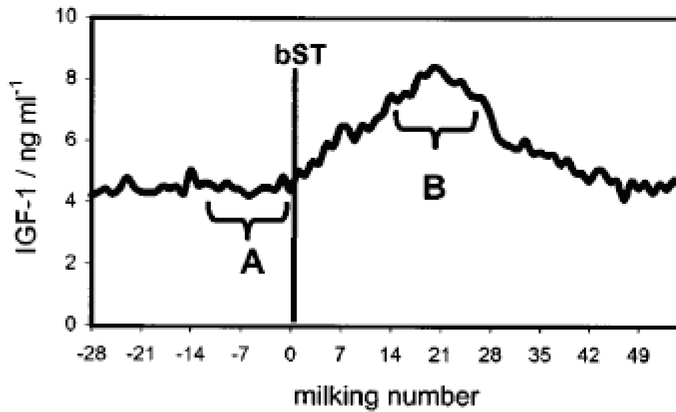
IGF-I: insulin-like growth factor-I (given as IGF-1 in figure); rbST: recombinant bovine somatotropin; SEM: standard error of the mean

Source: Reproduced by permission of the publisher, the Royal Society of Chemistry, from Daxenberger A, Sauerwein H, Breier BH (1998). Increased milk levels of insulin-like growth factor 1 (IGF-1) for the identification of bovine somatotropin (bST) treated cows. Analyst. 123:2429–35 (http://pubs.rsc.org/en/content/articlelanding/1998/an/a804923h/unauth#!divAbstract).

Daxenberger, Sauerwein & Breier (1998) determined naturally occurring IGF-I concentrations in 5777 random milk samples from dairy cows (not treated with rbST) collected over a 1-year period covering all regions of Bavaria. In samples from lactation weeks 7 through 33, the effect of somatic cell count, protein content and parity was quantified and corrected to obtain a normal distribution of the corrected logarithmic IGF-I concentrations. IGF-I concentrations in the milk were measured using a validated non-extraction radioimmunoassay following defattening. The method involved competitive displacement of IGF-I from IGF binding proteins by IGF-II and had an intra-assay variation of 5.1% and an inter-assay variation of 13.4%. IGF-I concentrations in milk from untreated animals ranged from 1 to 83 ng/mL. The distribution of the IGF-I was skewed to the right, with a median concentration of 4.4 ng/mL and 90th and 95th percentiles of 9.5 and 12.5 ng/mL, respectively. There was no detectable effect of region, season, the quantity of milk produced or the milk's fat content on IGF-I concentrations. Stage of lactation strongly influenced the concentration of IGF-I in milk (Fig. 1).

IGF-I concentrations in milk varied 2- to 3-fold across lactation, with the average concentration being the highest in the first 1.5 weeks of lactation, at approximately 11.5 ng/mL, then falling rapidly before levelling out between weeks 7 and 33 at approximately 5 ng/mL before rising steadily again to reach a concentration of approximately 8 ng/mL in late lactation. Somatic cell count in milk and milk protein percentage had small but positive correlations with IGF-I concentrations in milk. The number of lactations (first, second or third to sixth) and breed also had some influence on the IGF-I concentration in milk.

Fig. 2. Mean IGF-I concentrations in milk after rbST treatment. Statistical analysis was based on (A) the control period and (B) the main effect period.



IGF-I: insulin-like growth factor-I (given as IGF-1 in figure); rbST: recombinant bovine somatotropin (given as bST in figure)
Source: Reproduced by permission of the publisher, the Royal Society of Chemistry, from Daxenberger A, Sauerwein H, Breier BH (1998). Increased milk levels of insulin-like growth factor 1 (IGF-1) for the identification of bovine somatotropin (bST) treated cows. Analyst. 123:2429–35 (http://pubs.rsc.org/en/content/articlelanding/1998/an/a804923h/unauth#!divAbstract).

High variability in IGF-I concentrations was observed in cows after six lactations. Samples of Holstein-Friesian cows showed slightly higher IGF-I concentrations compared with other breeds. The study by Daxenberger, Sauerwein & Breier (1998) also included an animal phase in which 34 Brown Swiss cows were given a single treatment of rbST (Posilac, Monsanto) according to the label instructions (500 mg). Milk samples were taken twice daily from these animals for 2 weeks during the pretreatment period and for 4 weeks in the post-treatment period. Statistical analysis was performed on the changes in IGF-I concentration in milk derived from 33 animals from days 7 to 13 after treatment (period B) compared with the 7 days before treatment (period A) (Fig. 2). The IGF-I concentration in milk pretreatment was close to 4 ng/mL, which increased significantly after treatment, with the maximum concentration (approximately 8 ng/mL) detected 10 days after treatment. The mean increase in IGF-I compared with that of the contemporary control was 2.3 ng/mL for lactation 1, 1.6 ng/mL for lactation 2–6 and 1.9 ng/mL (48%) for all lactations combined.

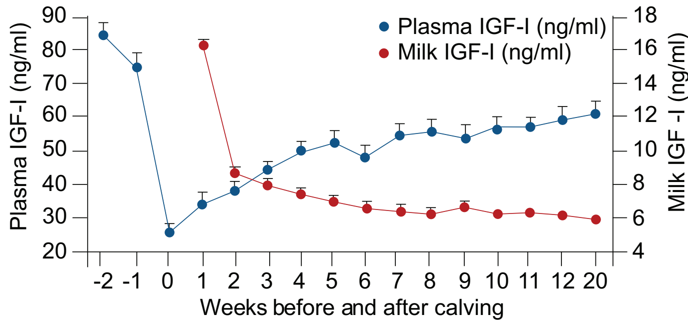
Liebe & Schams (1998) studied the interrelationship between the concentrations of IGF-I, basic fibroblast growth factor and somatic cell count in normal milk and the presence of these growth factors in the milk from cows with clinical and subclinical mastitis. Twelve Brown Swiss cows in their fourth lactation and in their 1st to 10th months of lactation were used. The study was performed in two periods, with four and eight cows in periods 1 and 2, respectively. Cows with chronically elevated somatic cell count in at least one quarter due to a history of mastitis or trauma were selected from their loose housing and moved to a

separate stanchion barn for a period of 5 days and then transferred back to the original loose environment. The periods of 5 days before and after relocation were referred to as control. Four milk samples from each quarter were taken daily at the morning milking. In addition, quarter milk samples (n = 48) from 12 cows affected by clinical mastitis and quarter milk samples (n = 88) from 22 cows (German Fleckvieh) affected by subclinical mastitis obtained from four small Bavarian farms were investigated. IGF-I concentrations were measured in skimmed milk samples by using an extraction radioimmunoassay technique with 3.8% intra-assay and 16% inter-assay coefficients of variation. The concentrations of IGF-I in milk in the relocation portion of the study in the controls were 15.1 ± 1.8 and 14.1 ± 1.7 ng/mL before and after being barned in the first period of the study and 8.3 ± 1.7 and 8.5 ± 2.1 ng/mL in the second period; concentrations of IGF-I were 10.7 ± 2.1 and 6.6 ± 1.5 ng/mL during the time barned during the first and second study periods, respectively. The concentrations of IGF-I in milk from quarters with clinical (35.5 ± 23.5 ng/mL) and subclinical (36.9 ± 31.3 ng/mL) mastitis were almost twice the concentrations detected in corresponding healthy quarters (21.2 ± 6.8 ng/mL and 17.7 ± 11.3 ng/mL, respectively).

Taylor et al. (2004) reported the concentrations of IGF-I in blood from Holstein-Friesian cows not treated with rbST and the influence of stage of lactation from 142 primiparous and 177 multiparous (mean lactation number of 3, range 2–8) cows. Blood samples were collected from 1 week before to at least 12 weeks after calving in the multiparous cows and before calving and 3, 5 and 8 weeks after calving in the primiparous cows. The concentrations of IGF-I in milk were measured in 50 of the multiparous cows. Whole milk samples were collected weekly after calving until week 12 and at week 20 and frozen until assayed for IGF-I. The concentrations of IGF-I in plasma and milk were determined by radioimmunoassay after ethanol–acetone–acetic acid extraction of IGF-I binding proteins. The inter-assay and intra-assay coefficients of variation were 11.2% and 6.7%, respectively. Concentrations of IGF-I in plasma were significantly ($P < 0.001$) higher in the primiparous cows (about 130 and 100 ng/mL) than in the multiparous cows (85 and 60 ng/mL) before and after calving, respectively. IGF-I concentrations in milk in the 1st week after calving were above 16 ng/mL, decreased rapidly in subsequent weeks and thereafter fluctuated between 6 and 9 ng/mL until 20 weeks post-calving (Fig. 3). There was no direct correlation between concentrations of IGF-I in blood plasma and milk.

Collier et al. (2008) investigated the effect of rbST on IGF-I concentrations in milk from lactating cows separately in summer and winter. Summer and winter each consisted of six treatment periods: (1) season farm management of all cows for the first 30 days; (2) 7 days' adjustment to conditions in the climate chambers; (3) exposure of one half of the animals to thermoneutral conditions and exposure of the other half to appropriate cold or hot conditions for 10 days; (4) cold or hot adjustment for 4 days; (5) reversed temperature exposure from period 3 for 10 days; and (6) 5 days post-treatment in a switchover design. Winter conditions were 5 °C and climate chambers for cold set at –5 to +5 °C and for thermoneutral conditions at 15–22 °C. Summer conditions were 18–35 °C and climate chambers set at 24–35 °C for hot conditions and at 15–22 °C for thermoneutral conditions.

Fig. 3. IGF-I concentrations in plasma and milk from 50 multiparous Holstein-Friesian cows



IGF-I: insulin-like growth factor-I

Source: Reproduced by permission of the publisher, BMJ Publishing Group Ltd, from Taylor VJ, Cheng Z, Pushpakumara PGA, Beever DE, Wathes DC (2004). Relationships between the plasma concentrations of insulin-like growth factor-I in dairy cows and their fertility and milk yield. Vet Rec. 155:583–8.

Cows were given daily injections of rbST (sometribove, USAN; 25 mg/day; six cows each study) or saline (control; six cows each study). During on-farm periods, blood and milk (morning and afternoon) samples were collected once weekly. During climate chamber periods, blood samples were collected every 2 days, and milk samples (morning and afternoon) were collected daily. Plasma and milk concentrations of IGF-I and IGF-II were determined by radioimmunoassay. IGF-I and IGF-II concentrations in plasma were increased in cows treated with rbST. Milk yields in experimental cows were higher in winter (31.3 kg/day) than in summer (27.0 kg/day), but the response to rbST in milk production was numerically greater in summer than in winter (7.5 kg/day versus 5.0 kg/day). A pronounced seasonal pattern in basal and rbST-stimulated IGF-I concentrations, but not IGF-II concentrations, was detected in plasma. Higher basal and rbST-stimulated IGF-I concentrations in plasma occurred in summer, despite large decreases in feed intake and energy balance. IGF-I and IGF-II concentrations in milk were not affected by rbST treatment or season (Table 4). Although IGF-I and IGF-II concentrations in milk were unaffected by rbST treatment, total IGF output increased due to increased milk yield. It was concluded that the observed seasonal patterns in plasma IGF-I concentrations (winter: 3.7 ng/mL versus 4.8 ng/mL; and summer: 3.4 ng/mL versus 3.8 ng/mL, in control and treated groups, respectively) may be indicative of seasonal differences in the coupling of the somatotropin–IGF axis. The studies failed to detect an uncoupling of the somatotropin–IGF-I axis in summer, despite an induced negative energy balance during thermal stress.

Pauletti et al. (2005) studied the changes in IGF-I concentrations in colostrum in 42 pregnant multiparous Holstein cows randomly assigned to equally sized groups treated with either 500 mg of rbST (Boostin, Cooper) or vitamin E, used as control. The treatments were initiated 35 days prepartum and repeated

Table 4. The effect of treatment and season on milk yield, IGF-I and IGF-II concentrations in milk and total milk IGF-I and IGF-II output

	Milk yield (kg/day)	Milk IGF-I		Milk IGF-II	
		Concentration (ng/mL)	Output (µg/day)	Concentration (ng/mL)	Output (mg/day)
Treatment					
Control	26.0	3.91	101.6	45.7	1.2
rbST	32.3**	4.26	137.6**	51.2	1.7*
Season					
Winter	31.3***	4.67	146.2***	48.2	1.5
Summer	27.0	3.51**	94.8	48.7	1.3

IGF-I: insulin-like growth factor-I: IGF-II: insulin-like growth factor-II; rbST: recombinant bovine somatotropin; *: rbST different from control, $P < 0.05$; **: rbST different from control, $P < 0.01$ ***: winter different from summer, $P < 0.01$

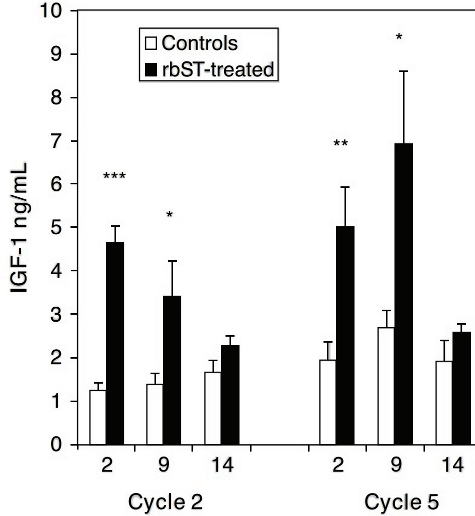
Source: Reprinted from *Domestic Animal Endocrinology*, **35**, Collier, R.J., et al., Effects of recombinant bovine somatotropin (rbST) and season on plasma and milk insulin-like growth factors I (IGF-I) and II (IGF-II) in lactating dairy cows, pp. 16–23 (2008), with permission from Elsevier.

each 14 days until parturition. Colostrum and mammary secretions were collected daily for 7 days postpartum. IGF-I concentrations in serum, colostrum and milk were measured using an immunoradiometric assay. The mean IGF-I concentration in colostrum of rbST-treated cows was significantly ($P < 0.05$) higher than that of the control cows (874.5 ± 335.0 ng/mL versus 674.2 ± 269.5 ng/mL) on day 1 after calving. No significant differences ($P > 0.05$) in IGF-I concentrations in milk were subsequently observed between the two treatment groups; by day 7 postpartum, IGF-I concentrations in milk had decreased to 12.9 ng/mL. At days 6 and 8, concentrations of IGF-I in milk in the control group were higher than those in rbST-treated cows, but not significantly.

In the cross-sectional study on retail milk samples (Vicini et al., 2008), described above, the mean concentrations of IGF-I in conventionally labelled milk and milk labelled as rbST-free and organic were 3.1 ± 0.1, 3.0 ± 0.1 and 2.7 ± 0.1 ng/mL, respectively. The mean IGF-I concentration was not different ($P > 0.05$) between conventional and rbST-free labelled milk, but was significantly lower ($P < 0.05$) in organic labelled milk. IGF-I concentrations in milk were measured by ECLIA using a Sector Imager 6000. Assays were performed at Monsanto. No information on the validation of the assay was provided.

Castigliego et al. (2011) determined hormone variations in serum and milk as potential indicators of treatment with an rbST in buffaloes. Eight lactating Italian buffaloes (*Bubalus bubalis*) were treated 5 times with a slow-release formulation of an rbST (Boostin® LG Life Sciences) at 500 mg subcutaneously every 2 weeks over a period of 10 weeks. An additional eight buffaloes were administered physiological saline and used as controls. Blood samples were collected on the day before treatment and on days 2, 5, 9 and 14 following each treatment. Milk samples were collected at the end of the mechanized morning milking on the day prior to the second and fifth treatment cycles and

Fig. 4. IGF-I variation in buffalo milk after rbST treatment. Comparisons between treated buffaloes (n = 8) and the controls (n = 8) are reported for days 2, 9 and 14 of the cycles of injection 2 and 5. Data are reported as means ± SEM; * P < 0.05; ** P < 0.01; *** P < 0.001.



IGF-I: insulin-like growth factor-I (given as IGF-1 in figure); rbST: recombinant bovine somatotropin; SEM: standard error of the mean

Source: Reproduced with permission from the publisher, Cambridge University Press, from Castigliego L, Li XN, Armani A, Grifoni G, Boselli C, Rosati R et al. (2011). Hormone variations in serum and milk of buffaloes (*Bubalus bubalis*) as potential indicators of treatment with recombinant bovine somatotropin. J Dairy Res. 78:412–20.

on days 2, 9 and 14 following these two treatments. Concentrations of total somatotropin in serum and concentrations of IGF-I in milk were measured using a sandwich ELISA validated for each compound and matrix. Total somatotropin concentrations in serum increased on day 2 after rbST treatment. The average total somatotropin concentrations were approximately 20 times higher in treated relative to control buffaloes and were significantly different ($P < 0.001$) in all five treatment cycles. IGF-I concentrations in serum increased rapidly after rbST treatment and persisted at least until day 9, with significant differences ($P < 0.001$) in treated and control animals. The IGF-I concentrations in milk were significantly ($P < 0.05$ to < 0.001) higher in treated animals compared with the control animals at each day after treatment on each treatment cycle (Fig. 4). The IGF-I concentration in milk increased after treatment but returned to a concentration similar to that of controls by 14 days post-treatment. The highest IGF-I concentrations reported in milk from treated buffaloes were 4.5–7 ng/mL, compared with 1.5–3 ng/mL in untreated controls.

Prasad & Singh (2010) determined the influence of short-term treatment of rbST on plasma growth hormone, IGF-I, prolactin and milk production of Murrah buffaloes in early lactation. Ten Murrah buffaloes in early production were each infused with 5 mg of an intravenous solution of rbST (rbST, Monsanto; NHPP–National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], lot M010-001) per day for 5 consecutive days (days 21–25 postpartum). A mean IGF-I concentration in milk of 34.8 ± 3.5 ng/mL (29.7 ± 4.5 to 38.1 ± 3.4 ng/mL) was observed before treatment. IGF-I concentrations were low at the start of the treatment on days 1, 2 and 3 but increased on day 4 onwards, reaching a maximum of 42.0 ± 5.2 ng/mL on day 1 after the last treatment and declining thereafter. No significant changes ($P > 0.05$) in IGF-I concentration in milk were observed in pooled data of all three phases (before, during and after treatment) of the study.

Faulkner (1999) studied the changes in concentrations of glucose and IGF-I in plasma and milk in response to ovine somatotropin in five British Saanen goats in their third to fifth lactations. Lactating goats were treated with 3 mg ovine somatotropin subcutaneously on the 3rd and 4th days of the study. The concentrations of IGF-I were determined in milk after defattening using a double-antibody radioimmunoassay. Prior to determination of total IGF-I in fat-free milk, samples were extracted for 48 hours at pH 3.7 in glycylglycine to remove or inactivate binding proteins. The concentration of total IGF-I in milk increased significantly ($P < 0.04$) immediately after ovine somatotropin treatment (30-minute sample) from pretreatment concentrations of about 5 ng/mL, reaching a peak of about 15 ng/mL, and preceded that in plasma by approximately 48 hours. This would indicate that the increased concentrations of IGF-I in milk are due to increased local production within the environment of the mammary gland or as a result of an efficient extraction of IGF-I from the circulation.

Although the species most commonly used for milk production is cattle, references to administration in goat (Faulkner, 1999) and buffaloes (Prasad & Singh, 2010; Castigliego et al., 2011) showed that even using different dosages, the resulting effects and concentrations of rbST and IGF-I are constant, regardless of the species.

The Committee considered all new information on the normal variation in IGF-I concentrations in cow's milk and the effect of rbST treatment on IGF-I concentrations in milk, as summarized in Table 3, and noted that the conclusions made at the fortieth and fiftieth Committee meetings are not substantially changed. No new information provided by the sponsor or sourced from the literature was obtained from studies performed according to GLP. Analytical methods used for bST and IGF-I in the various biological matrices are all immunologically based and measure mostly total content. Nevertheless, the available data examined corroborate the Committee's previous conclusions that IGF-I concentrations in cow's milk are highly variable and are influenced by parity, stage of lactation, season, udder health and somatic cell counts of the milk. Treatment of cows with rbST increases the mean IGF-I concentration in milk, but such increases are within the normal physiological variations observed in lactating cows. The wide range of IGF-I concentrations and different conclusions about the increase after bST application might be due to different analytical methods used, including potential interference caused by IGF binding proteins.

2.4.2 IGF-I concentrations in tissues

The fortieth Committee meeting reported that IGF-I concentrations in biopsied muscle and liver of rbST-treated cows increased at most 2-fold when compared with those of untreated cattle. The concentrations of IGF-I in muscle and liver ranged from 91 to 312 μg/kg and from 72 to 162 μg/kg, respectively, in rbST-treated cattle, compared with 68–272 μg/kg and 70–77 μg/kg, respectively, in untreated cattle. It was suggested that the elevated IGF-I concentrations in muscle could have been attributed to wound healing and not to rbST treatment. At the fiftieth Committee meeting, no significant differences were found between treated cows and untreated controls in the concentrations of IGF-I in muscle, fat, liver or kidney. Concentrations of IGF-I measured by radioimmunoassay varied from 34.9 ± 15.2 to 131.8 ± 24.6 μg/kg in muscle, from 203.6 ± 52.6 to 339.1 ± 229.2 μg/kg in fat, from 294.4 ± 88.4 to 389.6 ± 132.3 μg/kg in liver and from 821.1 ± 124.0 to 997.0 ± 140.2 μg/kg in kidneys. Previous assessments of the Committee summarized that the residues of rbST or IGF-I in various tissues of rbST-treated cows did not significantly differ from those of controls or that the slight increase in tissue residues is unlikely to be of concern for human health. A literature search did not identify new information on tissue IGF-I concentrations in rbST-treated animals.

2.5 Analytical methods

The analytical methods used to determine bST and IGF-I in milk and tissues evaluated at the fortieth and fiftieth meetings of the Committee were exclusively immunoassay procedures and could not distinguish between natural bST and rbST.

Methods for assaying IGF-I were considered by the present Committee. Although incomplete removal of IGF binding proteins or variation of standard source and extraction methods might influence reported values, these factors were not perceived to materially alter the conclusions that were taken. While some studies reported higher concentrations of IGF-I in milk, the Committee considered these studies to reflect differences in extraction procedures.

Some of the new methods that have been developed for detection of rbST/bST are summarized in Table 5. Most of the methods (e.g. immunoassays) do not differentiate between native bST and rbSTs. However, a few mass spectrometry methods allow the unambiguous identification of endogenous and recombinant forms (Pinel, André & Le Bizec, 2004; Bailly-Chouriberry et al., 2008). These methods were developed to identify non-compliant use of rbSTs in countries where they are not authorized.

The Committee noted that a recent review by Dervilly-Pinel et al. (2014) described the state of the art in the detection of rbSTs in food-producing animals.

2.6 Bioavailability and bioactivity of IGF-I

The fortieth Committee meeting concluded that many of the physiological effects of rbSTs are mediated by bovine IGF-I, which is structurally identical to human IGF-I and is likely to have similar effects in humans. The Committee meeting further concluded that IGF-I had no bioactivity when administered orally to normal and hypophysectomized rats at doses up to 2 mg/kg bw per day.

Table 5. Summary of recent bST analytical methods

Method	Species and tissues	Sensitivity	Reference
ECLIA	Bovine milk	< 5 pg/mL	McGrath et al. (2008)
ELISA	Bovine milk	0.05 ng/mL	Castigliego et al. (2007)
ELISA	Buffalo serum and milk	0.1 ng/mL	Mishra et al. (2005); Mishra, Goswani & Shukla (2007)
ELISA	Shrimp feed	10 µg/g	Munro & Boon (2010)
LC-MS/MS	Goat plasma	10 ng/mL	Le Breton et al. (2008)
LC-MS/MS	Bovine serum	10 ng/mL	Le Breton et al. (2009)
LC-MS/MS	Bovine milk	$CC\alpha \leq 1.24$ ng/mL $CC\beta \leq 1.92$ ng/mL	Le Breton et al. (2010a)
LC-MS/MS	Bovine blood	$CC\alpha \leq 2.5$ ng/mL $CC\beta \leq 6.8$ ng/mL	Le Breton et al. (2010b)
LC-MS/MS	Trout serum	0.5 µg/mL	Rochereau-Roulet et al. (2013)

bST: bovine somatotropin; CCα: decision limit; CCβ: detection capability; ECLIA: electrochemiluminescent immunoassay; ELISA: enzyme-linked immunosorbent assay; LC-MS/MS: liquid chromatography–tandem mass spectrometry

The fiftieth Committee meeting reported that IGF-I is found in abundance in a variety of body fluids (Table 6).

The fiftieth Committee meeting indicated that for quantitative risk assessment, the slight increases in IGF-I concentrations in milk from rbST-treated cows have to be compared with the physiological variations of IGF-I during lactation as well as with the concentrations in human breast milk, in the secretions of the gastrointestinal tract and in serum. It estimated that the incremental human exposure to IGF-I through consumption of 1.5 L/day of rbST-treated cow's milk represented 0.79% of the IGF-I secreted daily in the gastrointestinal tract and less than 0.09% of the daily production (10^7 ng/day) of IGF-I in adults. Whereas the fortieth meeting of the Committee considered IGF-I to be completely and rapidly degraded in the gastrointestinal tract, the fiftieth Committee meeting considered that some milk-borne IGF-I may escape digestion by gastrointestinal enzymes and be bioavailable, leading to some absorption. Nonetheless, the fiftieth meeting of the Committee concluded that even if IGF-I in milk were absorbed, the additional amount would be negligible and unlikely to have an adverse impact in humans. Limited additional data available on the bioavailability or bioactivity of IGF-I since then, and summarized below, do not substantially change the previous conclusions of the Committee.

Consistent with previous reports of the Committee, new in vitro digestion studies (Rao et al., 1998; Shen & Xu, 2000; Fellah et al., 2001; Anderle et al., 2002; Nabil et al., 2011) suggest that IGF-I is degraded by intestinal enzymes, but in vivo

Table 6. IGF-I concentrations in milk and body fluids of humans

Fluid	IGF-I concentration (ng/mL)
Cow's milk (bulk milk)	
Untreated	1–9
Treated with rbSTs	1–13
Human milk	
Milk	5–10
Colostrum	8–28
Human plasma	
Children	17–250
Adolescents	182–780
Adults	123–460
Human gastrointestinal secretions	
Saliva	6.8
Gastric juice	26
Pancreatic juice	27
Bile	6.8
Jejunal chyme	180
Daily production by adult humans	10^7 ng/day

IGF-I: insulin-like growth factor-I; rbSTs: recombinant bovine somatotropins
Source: Adapted from Annex 1, reference *135*

IGF-I degradation by gastrointestinal enzymes could be delayed by the components in milk/colostrum (Shen & Xu, 2000). Also, analytical methods used could influence the outcome of such measurements. For example, degradation of IGF-I measured by trichloroacetic acid precipitation often overestimated the amount of intact IGF-I when compared with the data from receptor binding assays (Rao et al., 1998; Shen & Xu, 2000).

New data from in vivo studies in laboratory animals (Philipps et al., 2000, 2002) demonstrate that a fraction of orally administered IGF-I is absorbed from the intestines. Suckling rats 10–12 days of age were administered ^{125}I-labelled recombinant human (rh) IGF-I (4×10^6 counts per minute) by gavage in milk, and the radioactivity in portal and cardiac blood was examined at 5, 10, 20 and 30 minutes post-treatment (Philipps et al., 2000). Purified radioactive samples were tested by gel chromatography and receptor binding assays. Radioactivity was detected in both portal and cardiac blood (maximum levels detected at 20–30 minutes post-treatment), but it was lower in the latter. The radioactivity present in the cardiac blood co-migrating at the position of native IGF-I was highest at 5 minutes post-treatment, but decreased significantly thereafter. However, a statistically non-significant numerical increase in radioactivity was observed in the portal blood from 5 to 30 minutes post-treatment. It was estimated that approximately 17–26% of the dose administered, as measured by radioactivity, reached the portal blood, but only a fraction of that reached the systemic circulation. Also, the radioactive

peak found in hepatic blood from IGF-I-fed animals was receptor active, although its binding in the competitive assay was weaker when compared with native IGF-I binding. Owing to extremely low concentrations, the authors could not perform adequate competitive binding studies on purified radioactive material from cardiac blood. This study, while demonstrating that almost a quarter of IGF-I administered in milk is absorbed from the intestine, could not definitively determine what proportion of IGF-I absorbed into the portal circulation enters the systemic circulation. In a subsequent study, the intestinal transport of IGF-I in suckling rats was shown to be non-saturable up to 1 μg/mL of IGF-I, a concentration 200-fold in excess of that in colostrum (Philipps et al., 2002).

Kim et al. (2006) demonstrated that weanling mice (n = 35) administered a single oral dose (1 μg/g) of IGF-I in phosphate-buffered saline (PBS) had a transient higher concentration of serum IGF-I between 4 and 8 hours after treatment, with the highest concentration at 4 hours, when compared with PBS-treated controls (n = 35). Serum concentrations of IGF-I and IGF-II did not differ in weanling mice (n = 20) administered five separate doses of IGF-I at 1 μg/g repeated every 3 days compared with PBS-treated controls (n = 20) at days 7 and 13 post-treatment. Although the authors concluded that increased serum concentrations of IGF-I in treated rats are evidence of its oral bioavailability, the experimental design cannot rule out whether such an increase was modulated by the local action of IGF-I in intestinal mucosa or due to its systemic availability. The dose of IGF-I administered to the mice, which is more than 150 times the amount that a person will consume per day in milk from rbST-treated cows (9 μg per 1.5 L of milk, as concluded at the fiftieth Committee meeting), may further have contributed to the systemic absorption.

Also, there is some evidence in the literature that orally administered IGF-I might have some local activity in the gut (e.g. increase in the weight of small intestine, increased enzyme activities) of laboratory animals (Burrin, 1997; Houle et al., 2000; Alexander & Carey, 2001; Burrin et al., 2001; Kim et al., 2006).

Le Breton et al. (2010a) conducted a study on the effects of industrial processes on milk stability together with the detection of rbSTs. The study was conducted on commercial ultra-high-temperature milk as well as on raw milk, condensed milk and milk powder. The milk treatments analysed were defatting, heating, freezing, pasteurization and spray-drying. The results concluded that the processes that did not involve heating allowed a recovery of the hormone up to 90%, whereas heating, pasteurization and spray-drying induced a significant loss. Regarding the concentration of IGF-I, it is known that higher temperatures, such as those associated with infant formula preparation, will denature it.

Studies in humans suggest that low nutrition level, including malnutrition, starvation, semi-starvation, fasting and caloric restrictions, lowers the IGF-I concentration in plasma (Livingstone, 2013). IGF-I concentrations in plasma are also affected by various physiological or pathological stages in humans (Livingstone, 2013). Several studies have indicated that IGF-I concentrations in human serum could be associated with nutritional status and milk intake. Milk consumption is particularly shown to be associated with an increase in concentrations of IGF-I in plasma in both the young and adults. In an intervention study, when men aged

55–85 years were instructed to drink three servings of nonfat or 1% milk per day as part of their normal diet, IGF-I concentrations in serum increased significantly (10%) in the intervention group by the end of the 12-week intervention period compared with concentrations in those who maintained their normal diet (Heaney et al., 1999). In another intervention study in Mongolia, after a month of drinking whole milk, 10- to 11-year-old school children had higher mean levels of IGF-I, ratios of IGF-I to IGF binding protein 3 (IGFBP-3) and 75th percentiles of growth hormone levels in plasma. A similar, albeit smaller and non-significant, increase in IGF-I, IGF-I/IGFBP-3 and growth hormone levels in plasma was also observed after a week of drinking low-fat milk by girls aged 6–8 years in Boston, Massachusetts, USA (Rich-Edwards et al., 2007). A Danish intervention study demonstrated that IGF-I concentrations in serum and serum IGF-I/IGFBP-3 ratio in 8-year-old boys ($n = 12$) increased from baseline after daily consumption of 1.5 L of milk for 7 days. However, in boys ($n = 12$) supplemented with similar levels of protein from 250 g of low-fat meat, these changes were not observed, suggesting that consumption of milk, but not animal protein alone, is associated with the increase in IGF-I level in plasma (Hoppe et al., 2004). A case–control study in the USA also suggested that low-fat milk intake, but not red meat, poultry and fish intake, was positively associated with IGF-I level in serum and IGF-I/IGFBP-3 ratio (Ma et al., 2001). A European prospective investigational study (Crowe et al., 2009) associated dairy protein and calcium intake with increased IGF-I concentrations in serum. A mean increase in IGF-I concentration in plasma of 13.8 ng/mL (95% confidence interval 6.1–21.5) in intervention groups consuming cow's milk when compared with the controls was reported in a meta-analysis of published literature (Qin, He & Xu, 2009). Evidence therefore points to the fact that drinking milk is associated with an increase in IGF-I levels in plasma, which, however, could be modulated by the existing nutritional or health status of a person. The effect of nutrition or foods, especially milk, on IGF-I level in plasma is, however, short lived (i.e. with no long-term effect). In a British long-term study (Carnegie [Boyd Orr] Survey) involving 728 subjects followed up for 65 years, IGF-I level in adulthood was negatively correlated with childhood family diets (based on 7-day household food inventories) high in milk (Martin et al., 2007).

Although the studies reviewed above demonstrated that consumption of milk could increase the IGF-I concentrations in blood, whether such increases were due to absorption of IGF-I from milk into the systemic circulation or stimulation of endogenous IGF-I production was not investigated.

Studies on the absorption of orally consumed IGF-I in humans were also available. In one study, the effect of enteral IGF-I supplementation on feeding tolerance, growth and gut permeability in premature infants during the 1st month of life in a prospective, double-blind, randomized study was examined (Corpeleijn et al., 2008). The study was conducted according to European good clinical practice regulations. Neonates received either standard infant formula ($n = 32$) or standard formula supplemented with IGF-I, extracted from bovine whey, at 100 µg/L ($n = 28$) during the first 28 days of life. Enteral IGF-I supplementation had no statistical effects ($P > 0.05$) on concentrations of IGF-I, IGFBP-1 and IGFBP-3 or growth hormone in serum compared with the control group throughout the study. No statistical difference in the primary

end-points of days to full enteral feeding, days to regain birth weight or rate of weight gain as well as a range of clinical and anthropometric measures was observed. The results of a lactulose/mannitol excretion test as a secondary end-point, performed at 7-day intervals as a measure of intestinal permeability, indicated no statistically significant differences ($P > 0.05$) between the two groups on day 1, 7, 21 or 28. On day 14, the ratio was significantly reduced ($P = 0.022$), indicating reduced gut permeability in the IGF-I-treated group. There were no differences in intestinal maturation expressed as lactase activity at the same time points. This study, where the controls were supplemented with similar formula with lower levels of IGF-I, provided no evidence of oral absorption of IGF-I at a dose roughly 1–2 times the concentration found in human colostrum (Table 6) and at about 20 times that of milk from contemporary rbST-untreated cows.

The second study specifically examined the effect of bovine colostrum supplementation on IGF-I concentrations in serum in one portion of the study and the oral absorption of IGF-I in a second portion in adult athletes (Mero et al., 2002). In the first portion of the study, adult male and female athletes were randomly assigned in a double-blind design to either a colostrum-treated group ($n = 19$) or a placebo-treated control group ($n = 11$). The colostrum-treated group received an oral bovine colostrum supplement (20 g) that contained a total of 74 μg IGF-I, and the control group received maltodextrin (20 g), daily during a 2-week training period. A significant increase (17%; $P < 0.01$) in IGF-I concentrations in serum was observed in the colostrum-treated group compared with the placebo-treated group. The concentration of circulating IGF-I steadily increased (0.38 nmol/L per day) over the 14-day treatment period, which was ascribed to either direct absorption of IGF-I from the colostrum supplementation or enhanced stimulation of human IGF-I synthesis. In the second portion of the study, the absorption of ^{123}I-labelled rhIGF-I orally administered to six male (mean age 29.1 years) and six female (mean age 23.9 years) athletes was examined. The study involved the preparation of ^{123}I-labelled IGF-I, validation of the biological activity of the radiolabelled IGF-I by receptor binding assays and blood sampling ($n = 7$) of subjects over the test day following oral administration of the ^{123}I-labelled rhIGF-I. IGF-I concentrations in serum measured using a two-site immuno-enzymometric assay showed no significant differences during the first 180 minutes after ^{123}I-labelled rhIGF-I treatment. At 7 hours after treatment, following a standard lunch, the concentrations were significantly increased (17%; $P < 0.01$) compared with the pretreatment concentration (20 nmol/L). Gel filtration of serum samples demonstrated radiolabel in low molecular weight substances, but no radioactivity at the elution positions of free IGF-I or the IGF-I binding proteins. The results provided no evidence for the absorption of orally consumed IGF-I in adult athletes; alternatively, the absorbed IGF-I was subject to an extensive first-pass effect.

Four separate randomized controlled studies investigated whether supplementing bovine colostrum with IGF-I (2 mg/kg) would increase the concentrations of IGF-I in plasma from human volunteers who were active athletes or participating in endurance training (Buckley et al., 2002; Coombes et al., 2002; Kuipers et al., 2002; Buckley, Brinkworth & Abbott, 2003). Volunteers were supplemented with 60 g of bovine colostrum or 60 g of concentrated whey protein for 4 or 8 weeks. In all four studies, IGF-I concentrations in plasma from

the intervention group did not differ either pretreatment or during or at the end of the supplementation when compared with whey protein–fed controls. Data reviewed in section 2.4 above and those reviewed by the fiftieth meeting of the Committee (Annex 1, reference *135*) suggest that the mean IGF-I concentrations in milk from rbST-treated and control cows are approximately 6 ng/mL and 4 ng/mL, respectively. A person consuming 1.5 L of milk from rbST-treated cows would therefore be exposed to 9000 ng of IGF-I per day, and the incremental increased exposure coming from the rbST use would be only 3000 ng/day. In contrast, in the trials reviewed above, study participants were supplemented with 120 000 ng of IGF-I per day. However, the IGF-I concentrations in their plasma did not differ from those of whey protein–fed controls. These findings suggest that the circulating IGF-I concentrations in humans would increase by ingestion of milk (or its components), but would not be affected by the amount of IGF-I ingested in food.

2.7 Milk nutritional composition

The Committee at its fortieth and fiftieth meetings examined the effects of rbST on milk composition and concluded that nutritional components and further processing characteristics of milk are not altered by rbST treatment. Furthermore, the composition of milk from treated cows is well within the normal variation observed during the course of a lactation.

The composition of milk from cows treated with rbST and the composition of milk from untreated controls that are available from recent publications are compared in Table 7. In concurrence with the conclusions of the previous meetings, these data demonstrate that there is no impact of rbSTs on the nutritional qualities of milk.

Table 7. Milk yield and protein, fat and lactose contents among rbST-treated and control animals

Species	Group treatment	Milk yield (kg/day or L/day)	Protein (%)	Fat (%)	Lactose (%)	Reference
Cattle	Control	23.5	3.65	4.29	9.00	Kim & Kim (2012)
	rbST	27.7	3.30	3.84	8.89	
Cattle	Control	20.7	3.16	3.50	4.51	Campos et al. (2011)
	rbST	22.6	3.16	3.52	4.39	
Cattle	Control	15.6	3.27	3.67	–	Macrina, Tozer & Kensinger (2011)
	rbST	17.9	3.28	3.65	–	
Cattle	Control	41.9	2.86	3.65	–	Rivera et al. (2010)
	rbST	45.4	2.81	3.30	–	
Cattle	Control	36.1	2.90	3.82	–	Liboni et al. (2008)
	rbST	37.6	2.83	3.78	–	
Cattle	Control	12.9	3.45	3.94	4.90	Chaiyabutr et al. (2007, 2008)
	rbST	14.6	3.51	4.24	4.62	

Table 7 (continued)

Species	Group treatment	Milk yield (kg/day or L/day)	Protein (%)	Fat (%)	Lactose (%)	Reference
Cattle	Control	33.5	3.08	3.53	–	Al-Seaf, Keown & van Vleck (2007a, 2007b)
	rbST	36.8	3.06	3.55	–	
Cows	Control	22.3[a]	3.0	3.6	4.8	Annen et al. (2007)
	rbST	22.4[a]	3.1	3.5	4.9	
Cattle	Control	38.8	2.84	3.61	–	Blevins, Shirley & Stevenson (2006)
	rbST	39.6	2.78	3.54	–	
Cattle	Control	32.5	3.11	3.57	4.75	Rose, Weekes & Rowlinson (2005)
	rbST	36.6	3.03	4.33	4.79	
Cattle	Control	13.11	3.27	3.60	4.52	Maksiri, Chanpongsang & Chaiyabutr (2005)
	rbST	16.02	3.16	4.70	4.79	
Cattle	Control	16.2	3.22	3.65	–	Fike et al. (2002)
	rbST	17.7	3.23	3.80	–	
Cattle	Control	25.9	3.13	3.55	5.00	Capuco et al. (2001)
	rbST	29.3	2.84	3.80	4.98	
Cattle	Control	40.2	2.92	3.12	–	Moallem, Folman & Sklan (2000)
	rbST	45.4	2.94	3.19	–	
Cattle	Control	29.0	3.05	3.13	4.89	Tarazon Herrera et al. (1999)
	rbST	32.6	3.05	3.31	4.95	
Cattle	Control	30.5	3.3	4.2	4.8	Miller et al. (1999)
	rbST	25.2	3.3.	4.2	4.7	
Cattle	Control	28.8	3.15	3.64	–	Bauman et al. (1999)
	rbST	33.0	3.17	3.57	–	
Buffaloes	Control	7.17	3.78	4.69	4.75	Feckinghaus (2009)
	rbST	8.59	3.78	4.85	4.90	
Buffaloes	Control	5.67	4.75	6.96	–	Jorge, Gomes & Halt (2002)
	rbST	7.53	4.58	6.82	–	
Goats	Control	0.960	3.14	4.64	3.58	Qudus et al. (2013)
	rbST	1.473	3.28	4.76	3.92	
Goats	Control	8.9	3.31	4.39	4.34	Moraes e Amorim et al. (2006)
	rbST	9.0	3.30	4.44	4.47	
Sheep	Control	1.23	4.89	6.14	–	Andrade et al. (2008)
	rbST	2.51	4.88	5.92	–	

Table 7 (continued)

Species	Group treatment	Milk yield (kg/day or L/day)	Protein (%)	Fat (%)	Lactose (%)	Reference
Sheep	Control	0.683	4.6	3.6	4.8	Sallam, Nasser & Yousef (2005)
	rbST	0.868	4.8	3.8	4.8	

rbST: recombinant bovine somatotropin
[a] Half udder milk yield.

2.8 Possible effects of rbSTs on the expression of certain viruses and prions in cattle

The fiftieth meeting of the Committee evaluated whether the immunomodulatory effect of bST would affect expression of retroviruses or prion proteins in treated animals and concluded that (a) available studies provided no evidence that rbSTs affect the expression of retroviruses in cattle and (b) the possibility of a link between rbST treatment and bovine spongiform encephalopathy (BSE) was highly speculative, and there was no evidence for a direct link between rbST treatment and BSE.

The literature search as described above for publications from 1998 to August 2013 retrieved 126 unique articles that included the term “virus” OR “lentivirus” OR “retrovirus” OR “prion”. None of these articles, however, investigated the effects of rbSTs on the expression of viruses or prions in cattle or other ruminants. No new information on the role of rbSTs in the expression of retroviruses or prion proteins in ruminants was available from the literature.

2.9 Possible increased health risks to human neonates and young children

2.9.1 Diabetes

The published literature does not associate milk or dairy consumption with type 2 diabetes (Aune et al., 2013; Gao et al., 2013). However, the literature is inconsistent on an association between milk or dairy consumption and risk for development of type 1 diabetes. Some, but not all, published studies have indicated that in children genetically predisposed to type 1 diabetes, cow’s milk feeding in early infancy, when an infant’s gastrointestinal tract is not fully developed, could stimulate the production of antibodies that can cross-react with pancreatic islet β-cell surface antigens (Knip, Virtanen & Akerblom, 2010; Norris, 2010). These autoantibodies may be a risk factor for activation of autoreactive T cells and type 1 diabetes (Skyler, 2007). Stimulation of aberrant immune response in infancy, however, is not limited to milk components alone, as infants genetically predisposed to type 1 diabetes also have a generalized aberrant immune response to several other proteins, including those from cereals, fruits, berries, bacteria and viruses (Harrison & Honeyman, 1999; Vaarala, 2005, 2012; Simpson & Norris, 2008; Atkinson, 2012; Eringsmark Regnell & Lernmark, 2013; Pugliese, 2013).

Studies reviewed by the fiftieth meeting of the Committee as well as those published in the scientific literature since then (see Table 7) suggest that the composition of milk from rbST-treated cows does not differ from that of untreated

controls. The only exception is a transient increase in the mean concentration of IGF-I in the milk from rbST-treated cows, which, however, falls within the normal physiological range observed in untreated animals (see section 2.4).

Data primarily from knockout mice, but also from human studies, suggest that IGF-I is unlikely to have an adverse impact on the pathogenesis of diabetes in humans. When IGF-I was locally expressed in pancreatic islet β-cells, transgenic mice treated with streptozotocin had milder type 1 diabetes, and all transgenic mice survived, in contrast to control mice, which developed severe diabetes and died (George et al., 2002). Similarly, transgenic CD-1 mice expressing IGF-I in β-cells were also able to counteract the effect of autoimmune destruction of β-cells (Casellas et al., 2006). Results from other studies (Agudo et al., 2008; Robertson et al., 2008) also support that IGF-I produced locally in the islet of Langerhans promotes β-cell replication, reduces apoptosis and has antidiabetic effects by improving islet cell survival and/or providing insulin-like effects. Locally expressed IGF-I, however, did not cause the growth or mass increase of the islet itself. The parenteral administration of IGF-I or IGF-I/IGFBP-3 combinations reduced the severity of insulitis and reduced the onset of type 1 diabetes in non-obese diabetic transgenic mice (Chen et al., 2004).

In general, circulating levels of IGF-I are lower in patients with diabetes (Capoluongo et al., 2006), and case reports in humans have demonstrated that patients with severely insulin-resistant type 1 diabetes could become insulin sensitive for a prolonged period after weekly intravenous bolus infusion of IGF-I at 500 μg/kg bw (Usala et al., 1994). A clinical trial (Thrailkill et al., 1999) evaluated the efficacy of rhIGF-I in patients with type 1 diabetes in a randomized double-blind study. Treatment with rhIGF-I and insulin improved glycaemic control and significantly reduced the glycosylated haemoglobin level and daily insulin requirements. Other studies in humans have also demonstrated beneficial effects of IGF-I in the treatment of type 1 (Carroll et al., 2000) or type 2 diabetes (Moses et al., 1996; Murphy, 2006).

Available evidence suggests that IGF-I is unlikely to have an adverse impact on the pathogenesis of type 1 or type 2 diabetes in humans. As the milk composition did not materially differ between cows treated with rbSTs and untreated cows, the milk from rbST-treated cows would not pose an additional risk for the development of diabetes.

2.9.2 Cancer

The Committee considered the potential cancer risk to humans associated with the consumption of milk from rbST-treated cows. rbSTs are not absorbed from the gastrointestinal tract, have species-specific receptor binding and are not bioactive in humans. Also, the orthologue (e.g. mouse and rat) somatotropins did not cause cancer in mice and rats, respectively, when administered subcutaneously (see section 2.2.1). Therefore, the carcinogenicity risk of rbSTs themselves was considered negligible.

The normal physiological range of IGF-I in human plasma is very wide, ranging from 17 to 250 ng/mL in children, from 182 to 780 ng/mL in adolescents and from 123 to 460 ng/mL in adults (see Table 6). Several prospective and

case–control epidemiological studies have shown that circulating IGF-I levels are higher, although within the normal physiological range, in some cancer patients (Clayton et al., 2011). Moreover, these findings were inconsistent between studies and between different types of cancer. No significant difference was noted in the concentrations of IGF-II or IGF binding proteins in blood between cancer patients and their controls (Clayton et al., 2011). Most of the observations on higher levels of circulating IGF-I in cancer patients were made in epidemiological studies in which the impact of reverse causation cannot be ruled out. Additionally, a recent review on possible carcinogenic hazard to consumers from IGF-I in the diet concluded that the available database is insufficient to link dietary IGF-I directly with breast cancer (Committee on Carcinogenicity, 2012).

Literature reviewed on the bioavailability of IGF-I (section 2.6) suggested that milk consumption could increase the concentrations of IGF-I in human serum. However, evidence was lacking that the increase was due to absorption of IGF-I in milk. The endogenous IGF-I production in humans will therefore be influenced by whether a person consumes milk at all, irrespective of whether the milk comes from rbST-treated or untreated cows. Further, when compared with the overall daily IGF-I production in human adults of 10 mg (see Table 6), the putative contribution of milk-borne IGF-I is considered negligible. For example, a person consuming 1.5 L of milk from rbST-treated cows on average will be exposed to 9000 ng of IGF-I per day, which is equal to 0.09% of the daily production of IGF-I in an adult.

2.10 Increased use of antimicrobial agents to treat mastitis in cows treated with rbSTs

The effect of rbST treatment on mastitis incidence and somatic cell count in milk from treated cows was not reviewed by the Committee at its fortieth meeting, as these effects were considered outside the Committee's terms of reference. At its fiftieth meeting, the Committee reviewed published information and the results of a post-approval monitoring programme for sometribove (Posilac) in the USA on the influence of rbSTs on mastitis and animal health. The Committee concluded that the effects of rbSTs on the incidence of mastitis and general health as well as the resulting days of treatment per animal with any medication are an issue of animal health and outside the terms of reference of the Committee. However, the Committee did consider the results of the post-approval monitoring programme on the percentage of milk discarded due to non-compliant (violative) drug residue as a consequence of antimicrobial use after the market availability of Posilac. It was concluded, based on the results of the programme, that the use of rbSTs will not result in a higher risk to human health due to the use of antimicrobial agents to treat mastitis and that the increased potential for drug residue in milk could be managed by practices currently in use by the dairy industry and by following label directions for use.

The present Committee updated the assessment performed at the fiftieth meeting of the Committee. While acknowledging the issue of mastitis per se to be one of animal health and outside the terms of reference of the Committee, the

Committee performed a systematic review of the literature concerning the effects of rbSTs on mastitis incidence and somatic cell counts, with particular reference to antimicrobial residues in milk. The literature search, as described above, for publications from 1998 to August 2013 retrieved 29 unique articles that included the term “somatic cell count(s)” OR “antibiotic” OR “mastitis”. Some studies were located that evaluated the effects of rbSTs as a treatment for mastitis or that evaluated the effects of rbSTs on animal health parameters other than mastitis. These studies were excluded as irrelevant. An additional four relevant papers identified from review articles by De Vliegher et al. (2012) and Pezeshki et al. (2010) were also included in the review (Table 8).

The meta-analysis publication by Dohoo et al. (2003) was a reanalysis of data already published prior to approval of Posilac (1989–1994) and included 53 randomized clinical trials that Monsanto had provided to Health Canada (Health Canada, 1998). These represented the experimental data considered in previous evaluations by the fortieth and fiftieth Committee meetings. This study reported a 25% increase in incidence of mastitis in rbST-treated herds versus non-treated herds. In contrast, a systematic review by the present Committee of clinical (Brozos et al., 1998; Judge et al., 1999; Collier et al., 2001; Vallimont et al., 2001; Gulay et al., 2003, 2007; VanBaale et al., 2005) and epidemiological studies (Ruegg, Fabellar & Hintz, 1998) published since then (see Table 8) found no effect of rbST on mastitis incidence, possibly due to insufficient power to detect differences in mastitis incidence and exclusive use of multiparous animals as test subjects. It was noted that many of the studies listed in Table 8 and reviewed by the Committee did not follow the label recommended use directions.

Regarding the incidence of subclinical mastitis, assessed as increased somatic cell count scores in milk, the vast majority of studies reported no effect of rbST treatment on somatic cell count values (Ruegg, Fabellar & Hintz, 1998; Chiofalo et al., 1999; Vallimont et al., 2001; Dohoo et al., 2003; Gulay et al., 2003, 2007; VanBaale et al., 2005; Schneider et al., 2012; USDA, 2012), although a few studies reported small, transient increases (Brozos et al., 1998; Bauman et al., 1999; Boutinaud et al., 2003).

The Committee at its fiftieth meeting compared the non-compliant antimicrobial drug residues in bulk tank milk in the USA 2 years before approval of rbST (1992–1993) and 2 years after approval of rbST (1994–1995) as part of a post-approval monitoring programme. Results of the same programme were available for the years 1996–2012 (NMDRD, 2013) for the present Committee to review. The National Milk Drug Residue Database (NMDRD) is a voluntary industry reporting programme, whereas mandatory reporting is required by state regulatory agencies under the National Conference on Interstate Milk Shipments (NCIMS). Data are reported on the extent of the national testing activities, the analytical methods used, the kind and extent of the animal drug residues identified and the amount of contaminated milk that was removed from the human food supply. The system includes all of the milk supply, of which approximately 95% is regulated through the NCIMS by state regulatory agencies. The trend in milk tankers positive for antimicrobial residues in the USA since 1995 is presented in Fig. 5. As noted

Table 8. Studies investigating rbST use and mastitis or milk somatic cell counts in dairy animals

Study	Study design	Test animal	No. per group	Treatment	Results
Bauman et al. (1999)	Epidemiological	Dairy herds of the north-eastern USA during years 1994–1998	Herd nos per group: 164–176	Herds that used Posilac during specified time period vs herds that did not use Posilac	Significant increase in SCC in rbST-treated herds vs control ($P < 0.01$)
Boutinaud et al. (2003)	Prospective clinical	Saanen goats (INRA Experimental Farm, Brouessy, France) in week 32 of lactation	3	5 mg rbST/day sc for 23 days vs control. Each goat milked 3×/day on right udder half and 1×/day on left udder half	Increased SCC with rbST from treatment days 5 to 17, after which no difference
Brozos et al. (1998)	Prospective clinical	Polytocous Chios ewes (Institute of Reproduction and AI, Ionia, Thessaloniki)	11	160 mg rbST sc every 14 days during lactation days 5–182 vs control (no injection)	Increase in mean SCC after lactation day 105; no significant differences in percentages of bacteriologically positive milk samples, distribution of bacterial isolates or prevalence of subclinical mastitis
Campos et al. (2011)	Prospective clinical	Dairy cows	12–14	500 mg rbST every 14 days, starting on 63rd day of lactation; 500 mg rbST every 12 days, from the 63rd day of lactation, treatment continued until 280 days in milk; control	No effects on SCC or mastitis incidence
Chadio et al. (2000)	Prospective clinical, switch-back design with three 28-day periods	Multiparous crossbred alpine goats in lactation week 8	4	160 mg sustained release rbST sc every 14 days vs control	No significant difference in SCC
Chiofalo et al. (1999)	Prospective clinical	Multiparous Comisana lactating ewes	40	120 mg rbST sc every 21 days (total two treatments) vs control	No effect on SCC

Table 8 (continued)

Study	Study design	Test animal	No. per group	Treatment	Results
Collier et al. (2001)	Prospective clinical	Commercial dairy herds (Holstein or Jersey cows) in the north-eastern, south-eastern, upper Midwest and western USA	Primiparous: 209–210; multiparous: 352–355	500 mg sometribove (zinc-oil formulation sc/14 days) or control (oil excipient sc), lactation week 9 or 10 to dry-off or lactation day 400	No effects on percentages of cows with mastitis, average mastitis cases/100 cow-days, mastitis case duration, use of mastitis therapies, mastitis ORs for primiparous or multiparous cows and numbers of cows culled for mastitis
De Souza Paula & da Silva (2011)	Prospective clinical	Dairy cows in Santa Rosa, Brazil	12	rbST, 2 applications, 14 days apart vs saline control	Increased SCC values with rbST treatment
Dohoo et al. (2003)	Meta-analysis of prospective clinical trial data	Dairy cows	Unstated	Unstated	Significant increase in incidence rates and RRs (~25%) for clinical mastitis in rbST-treated cows; no significant effect on incidence rate or RR for subclinical mastitis (as increase in SCC)
Feckinghaus (2009)	Prospective clinical	Lactating Murrah water buffaloes	14	Single application 500 mg rbST vs no injection	No effect on SCC on 1st, 3rd, 5th, 7th, 10th and 14th days after application
Fitzgerald et al. (2007)	Prospective clinical	Healthy primiparous Holstein cows (2nd gestation, 1st dry period; University of Arizona) with SCC scores of < 300 000	4	Control vs 500 mg rbST/14 days during the 60-day dry period through lactation day 30, with half-udder treatments of either 2×/day milking or 4×/day milking	No significant differences in SCC during the 1st 30 days postpartum

Table 8 (continued)

Study	Study design	Test animal	No. per group	Treatment	Results
Gulay et al. (2003)	Prospective clinical	Multiparous Holstein cows (University of Florida), 4 weeks prior to calving	95–98	Control vs biweekly 142.9 mg rbST sc 21 ± 3 days prior to calving through postpartum day 42; all cows received Posilac beginning 100 ± 4 days postpartum	No significant differences in SCC, incidences of health problems (types unspecified) or culling rates
Gulay et al. (2004)	Prospective clinical	Multiparous Holstein cows (University of Florida) were assigned to treatment groups in a 2 × 3 × 2 factorial arrangement 8–9 weeks prior to calving	42	Control vs biweekly injections 0.4 mL (142.9 mg) Posilac per cow from 21 ± 3 days prior to calving through 42 ± 2 days postpartum; all cows treated with rbST after 56 ± 2 days postpartum	Decreased SCC in treated cows through 42 ± 2 days postpartum
Gulay et al. (2007)	Prospective clinical, also data from a retrospective study analysed separately	Holstein cows in the University of Florida Dairy Research herd	162–166 (prospective) 109 (retrospective cohort)	142.9 mg rbST/cow sc 2-week intervals, 19–24 days before calving until 39–45 days postpartum vs control	Decreased incidences of mastitis and total disease in rbST-treated vs controls
Judge et al. (1999)	Prospective clinical	Commercial dairy herds in Michigan Dairy Herd Improvement Association	261–277	500 mg rbST every 14 days between lactation days 63 and 301 vs control	No effect of rbST on incidence of mastitis
Kim, Chang & Kim (2002)	Prospective clinical	Holstein dairy cows	9	Group I: rbST alone; Groups II, III and IV: rbST treatment + retinyl palmitate and cholecalciferol; an untreated control group	No significant effect on mastitis incidence, but there was decreased SCC in rbST + retinyl palmitate and cholecalciferol–treated groups
Kim & Kim (2012)	Prospective clinical	Lactating Holstein dairy cows in Kyunggi Province, Republic of Korea	25	Boostin-250 and vehicle (control), administered weekly; Boostin-S and Posilac every 14 days	No effect on incidence of clinical mastitis and SCC

Table 8 (continued)

Study	Study design	Test animal	No. per group	Treatment	Results
Liboni et al. (2008)	Prospective clinical	Multiparous Holstein cows (University of Florida)	25–27	Group I: no rbST; Group II: postpartum rbST; Group III: prepartum rbST; Group IV: prepartum and postpartum rbST; prepartum rbST every 2 weeks beginning 21 days before calving; postpartum rbST during the first 63 days of lactation every 2 weeks; all cows received rbST after 63 days in lactation	No changes in SCC between treatment groups
Lucci et al. (1998)	Prospective clinical	Crossbred Holstein first-lactation pregnant heifers	9	rbST 500 mg dose, Groups (A) control; (B) bST each 28 days; (C) bST each 21 days; (D) bST each 14 days for 112 days	No effect on SCC
Masoero et al. (1998)	Prospective clinical	Italian Friesian lactating cows	25 per trial × 2 trials, 1. winter–spring, 2. autumn–winter	rbST (500 mg, every 2 weeks for 10 times) vs control	No effect on SCC
Moraes e Amorim et al. (2006)	Prospective clinical	Toggenburg goats at farm in Água Limpa, Brazil	12	250 mg rbST, every 14 days (four injections) vs saline (control)	Decreased SCC in treated goats
Mukherjee (2007)	Prospective clinical	Lactating buffaloes	30	Boostin (250 mg/2 weeks); no control group	Increase in SCC on days 4, 18 and 32, bacterial plate count $< 0.40 \times 10^3$ cfu/mL
Posada et al. (2008)	Prospective clinical	Holstein-Freisian cows, 1–4 parity, 60–180 days in milk (Antioquia, Colombia)	10	Group 1, rbST (500 mg) + vitamin E + lecithin, group 2, rbST (500 mg), and control group without treatment; nine injections every 2 weeks	No significant effect on mastitis incidence (measured by California Mastitis Test, and analysed for proportion affected by confidence intervals)

Table 8 (continued)

Study	Study design	Test animal	No. per group	Treatment	Results
Requena et al. (2010)	Prospective clinical	Lactating Manchega dairy ewes (Polytechnic University of Valencia, Spain)	18	Control vs 40, 80 or 120 mg of bST every 14 days from 2 to 20 weeks of lactation	No effect on SCC
Ruegg, Fabellar & Hintz (1998)	Epidemiological	32 dairy herds in Indiana, Michigan and Ohio surveyed August 1994 –August 1995	Herd nos per group: 13–19	rbST used for ≥ 25% cow- days vs control	No effects on culling density, or rate or incidences of SCC-related or mastitis-related culling
Schneider et al. (2012)	Prospective clinical	Holstein heifers, southern Brazil, 35 days prior to expected calving date	15–16	500 mg rbST/cow sc 35, 21 and (if relevant) 7 days before calving vs control	Significantly decreased SCC with rbST
Vallimont et al. (2001)	Prospective clinical	Multiparous Holstein dairy cows	13–15	500 mg sustained release Posilac, sc 28 and 14 days prior to calving vs control	No effects on mastitis incidence or SCC
VanBaale et al. (2005)	Prospective clinical	Multiparous Holstein cows at Arizona commercial dairy	60	rbST, 60–66 to 305 days in milk vs control (cows in both groups were milked 6×/day during the first 21 days in lactation, and 3×/day thereafter)	Increased SCC in cows treated with rbST
Studies available in abstract form only					
Bayram et al. (2006)	Prospective clinical	Anatolian buffaloes in mid- and late lactation	10	500 mg rbST sc every 14 days vs control	No effect on SCC
Hassan et al. (2007)	Prospective clinical	Buffaloes in 2nd–3rd lactations, 70–80 days postpartum	6	Control vs biweekly low (250 mg/head) and high (500 mg/head) doses of rbST for 90 days	Significantly ($P < 0.01$) increased SCC

cfu: colony-forming units; OR: odds ratio; rbST: recombinant bovine somatotropin; RR: risk ratio; sc: subcutaneously; SCC: somatic cell count

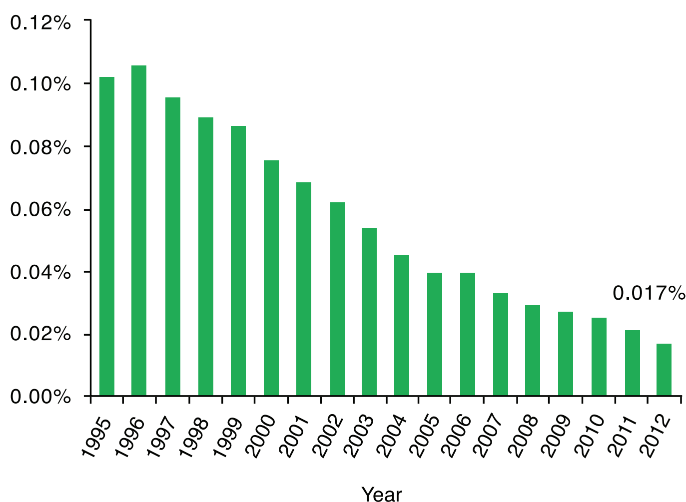
at the fiftieth Committee meeting, the USA switched to a more sensitive test for antimicrobial residues in 1995, corresponding to the highest level of residue non-compliance reported. The bulk milk tankers positive for antimicrobial residues increased slightly between 1995 and 1996. Since 1996, the percentage of bulk milk tankers positive for antimicrobial residues has steadily declined to 0.017% in 2012, compared with 0.10% in 1995 (Fig. 5). These results provide no evidence of increased human risk for exposure to antimicrobial drug residues associated with the use of rbSTs in the dairy industry in the USA over the last 19 years.

Several factors could influence the observed decline in non-compliant drug residues, including adherence to good veterinary practice and improved animal husbandry practices. Moreover, the available data did not provide individual animal–level data to correlate with the use of rbSTs. Nonetheless, the available evidence suggests that in the USA, the approval of rbSTs was not associated with an increased incidence of non-compliant antimicrobial residues in bulk milk. However, no relevant monitoring data were available from other jurisdictions where rbSTs are authorized for use.

A survey of retail milk in the USA (Vicini et al., 2008), which tested 334 retail milk samples labelled as conventional, rbST-free or organic milk from stores in 48 contiguous states within the USA, did not detect any antimicrobial residues.

The use of antimicrobial agents is an important tool in the management of clinical mastitis. However, the Committee could not analyse the potential association between the use of rbSTs and the use of antimicrobial agents. This was due to the unavailability of data on the use of antimicrobial agents to treat mastitis in

Fig. 5. Percentage of bulk tankers positive for antimicrobial residues from 1995 to 2012



Source: NMDRD (2013)

farms using rbSTs when compared with farms not using rbSTs. The results of the systematic literature review of the studies published since the last Committee meeting and the antimicrobial residue monitoring data from the USA, however, provided an indirect indication that when antimicrobial agents are used in accordance with the label directions, human exposure to antimicrobial residues is unlikely to increase due to potential increased use of antimicrobial agents to treat mastitis in rbST-treated cows.

An excerpt from the USDA's Animal and Plant Health Inspection Service's Centers for Epidemiology and Animal Health fact sheet on bulk tank milk somatic cell counts (BTSCC) was also provided (Bauman & Collier, 2013). BTSCC refers to the number of white blood cells (primarily macrophages and leukocytes), secretory cells and squamous cells per millilitre of raw milk. The average BTSCC in milk in the USA was stable between 1998 and 2003 and has declined steadily since 2003. BTSCC declined from 319 000 cells/mL in 2003 to 233 000 cells/mL in 2009 (27% decline). An average BTSCC of 224 000 cells/mL in 2010 and 206 000 cells/mL in 2011 indicates that the pattern of decline continues. Operations with increased BTSCC are more likely to have milk that is non-compliant with antimicrobial residues (van Schaik, Lotem & Schukken, 2002). A continuous decrease in somatic cell count in milk in the USA is an additional indirect support for the lack of evidence linking the use of rbSTs with an increased risk for antimicrobial residues in milk.

Studies from the USDA's National Animal Health Monitoring System (NAHMS) reported that 10.1% of cows in the USA in 1996, 22.3% in 2002 and 17.1% in 2007 were treated with rbSTs (USDA, 2007). During those years, the percentages of cows with mastitis increased slightly, from 13.4% (1996) to 14.7% (2002) to 16.5% (2007). Although the slight increase in prevalence of mastitis from 1996 to 2002 could be linked with a more than doubling in the percentage of cows given rbSTs, mastitis prevalence continued a trend upwards in 2007, despite a 5% decrease in the percentage of cows administered rbSTs. The increase in mastitis prevalence was more closely related to the increased annual milk yield per cow of 1–3% per year since 1991 (USDA, 2007).

Ruzante et al. (2010) analysed data collected during the NAHMS Dairy 2007 study (USDA, 2007) from dairy farms in the USA to study factors associated with the presence of *Salmonella* in environmental samples in dairies in the USA. Environmental samples to test for *Salmonella* were collected from a subset of 260 dairy operations used in the overall study. The association of the presence of *Salmonella* in environmental samples with the use of rbSTs was examined as one of the factors. A higher presence of *Salmonella* in the environment was observed with the use of rbSTs. The biological significance of this finding is unclear, and the study was not designed to capture any related factors, such as management practices.

In its systematic review of the literature, the Committee did not find specific studies that investigated the associations between the use of rbSTs and the development of antimicrobial resistance in mastitis pathogens. Controlled studies have not determined whether the use of rbSTs may increase this risk or, for that matter, help to decrease it. Although bovine mastitis is considered the single most important reason for antimicrobial use in lactating dairy cows (Erskine et al., 2004) and although antimicrobial resistance in mastitis pathogens is a cause for concern (Oliver, Murinda & Jayarao, 2011;

Oliver & Murinda, 2012), in the absence of properly designed studies, whether the use of rbSTs in cows or farms increases antimicrobial resistance remains speculative. It is concluded that there is a lack of evidence that the use of rbSTs in dairy herds contributes to antimicrobial resistance in dairy herds.

Available new information therefore does not change the conclusion of the fiftieth Committee meeting in regards to the risk to human health due to the use of antimicrobial agents to treat mastitis.

3. COMMENTS

3.1 Biochemical data

The Committee at its fortieth and fiftieth meetings concluded that human and bovine somatotropins are structurally different and have species-specific receptor binding activity. Furthermore, the total concentration of bST detected in tissues and milk of rbST-treated cattle is similar to that from untreated cattle, and bST is denatured by high temperatures (e.g. by cooking or pasteurization) and biodegradation processes in the gut. No new biochemical data on rbSTs were available since the previous evaluation of the compound by the Committee at the fiftieth meeting. The Committee evaluated a part of a study submitted to previous JECFA meetings, but not specifically discussed in the respective monographs. This study investigated the serum level of anti-rbST antibodies as a surrogate measure for oral absorption/bioavailability in rats administered an rbST by gavage for 90 days. The results indicated increased levels of circulating anti-rbST antibodies in 20% and 30% of rats treated with the rbST at 5 and 50 mg/kg bw per day, respectively, and in one animal (3%) treated with the rbST at 0.1 mg/kg bw per day. The experimental design, however, did not allow an assessment as to whether the antibody response was a result of absorption of intact rbST or only an immunologically active peptide fragment (epitope or antigenic determinant) of the rbST into the systemic circulation or due to mucosal immunity in the gut. Also, there were no systemic effects on growth or feed intake in orally treated rats. These data, together with the data evaluated at previous meetings of the Committee, confirm the absence of the biological activity of rbSTs following oral intake.

3.2 Toxicological data

The Committee at its fortieth meeting evaluated the toxicity of different rbSTs. Acute oral toxicity studies in rats with rbST doses up to 5 g/kg bw, two 2-week oral feeding studies in rats with rbST doses up to 10 mg/kg bw per day and two 4-week oral feeding studies in rats with rbST doses up to 50 mg/kg bw per day caused no effects up to the highest dose tested. Similarly, no treatment-related effects were observed in two 90-day oral feeding studies in rats at rbST doses up to 100 mg/kg bw per day and a 90-day oral feeding study in dogs at rbST doses up to 10 mg/kg bw per day, the highest doses tested. No new toxicity studies on rbSTs were available since the previous evaluation of rbSTs by the Committee at the fiftieth meeting.

The present Committee evaluated long-term carcinogenicity studies in rats and mice using related, but distinct, compounds (i.e. rrST and rmST). Daily subcutaneous administration of rrST and rmST to groups of rats and mice, respectively, for 2 years did not show any carcinogenic effects. Although the Committee considered these data not directly relevant to the risk assessment of rbSTs, these observations do illustrate that other somatotropins are not potential carcinogens.

3.3 Concentrations of rbSTs and IGF-I in milk and tissues

Previous meetings of the Committee have concluded that owing to the structural dissimilarity between bovine and human somatotropins and species-specific receptor binding, rbSTs are not biologically active in humans. Also, similar concentrations of total bST are detected in milk and tissues of rbST-treated and untreated cows. Very few new publications investigating the concentrations of bST in milk and tissues following treatment with rbSTs were available in the literature since the fiftieth meeting of the Committee. Available information supports the conclusions of the previous Committee that there is no significant change in the concentrations of total bST detected in milk and tissues of rbST-treated cows when compared with untreated controls.

Available new information supports previous conclusions that the IGF-I concentration in milk varies widely in lactating cows and is influenced by parity, stage of lactation, nutritional status, season and somatic cell counts (an indication of udder health) of the milk. IGF-I concentrations measured in colostrum are substantially higher than concentrations in milk produced subsequently. Treatment of cows with rbSTs transiently increased the mean IGF-I concentration in milk by up to 50%, but such increases were within the physiological variations observed in untreated cows.

A new cross-sectional study of retail milk in the USA suggests that the IGF-I concentrations in retail milk labelled as conventional, which includes milk from both rbST-treated and untreated cows (3.1 ± 0.1 ng/mL), were not different from concentrations in milk labelled to be from rbST-free cows (3.0 ± 0.1 ng/mL). However, the percentage of conventional milk that comes from cows treated with rbSTs is not known.

The fiftieth meeting of the Committee considered that some milk-borne IGF-I may escape degradation by gastrointestinal tract enzymes and get absorbed from the gastrointestinal tract. In vitro digestion studies indicated that IGF-I is rapidly degraded by gastrointestinal tract enzymes. However, subsequent studies in experimental animals showed that the rate of degradation could be reduced by the components in milk/colostrum. In vivo studies in laboratory animals suggested that up to 25% of IGF-I fed with milk could be absorbed from the gastrointestinal tract, although only a fraction of it would reach the systemic circulation. Studies in infants showed that feeding a formula supplemented with a 20-fold higher concentration of IGF-I did not increase the IGF-I concentrations in serum compared with feeding a standard formula. Randomized trials in active adult athletes did not detect any difference in IGF-I concentrations in plasma from an intervention group fed up to 120 000 ng IGF-I per person per day from bovine colostrum for up to 8 weeks when compared with controls fed whey protein during pretreatment, treatment or post-treatment periods.

The literature suggests that the concentration of IGF-I in serum in humans is influenced by a number of factors, including age, physiological stage and nutritional status. Consumption of milk per se was associated with increased blood IGF-I concentrations in humans. There is evidence that orally administered IGF-I has some local bioactivity in the gastrointestinal tract. However, given the large quantity of IGF-I secreted in the digestive tract of humans, the small additional quantity of IGF-I in milk from cows treated with rbSTs is unlikely to make a biologically relevant contribution to the effects of endogenous IGF-I. The endogenous IGF-I production in humans will be more influenced by the consumption of milk per se, irrespective of whether it is from rbST-treated or untreated cows.

The present Committee concluded that some milk-borne IGF-I may not be degraded by gastrointestinal enzymes. However, even if some of the IGF-I in milk were absorbed, the incremental human exposure would be negligible when compared with total daily human production of IGF-I of 10 mg/day, as reported by the Committee at the fiftieth meeting. This is consistent with the previous conclusion of the Committee.

3.4 Expression of retroviruses and prion proteins

The fiftieth meeting of the Committee concluded that the available studies provided no evidence that rbSTs affect the expression of retroviruses in cattle. The Committee also concluded that the possibility of a link between rbST treatment and BSE was highly speculative, as there was no evidence for a direct link. No new information on the role of rbSTs in the expression of retroviruses or prion proteins in ruminants was available from the literature.

3.5 Risk of type 1 diabetes in genetically susceptible infants

There is evidence that in infants genetically susceptible to type 1 diabetes, exposure to cow's milk early in infancy, when an infant's gastrointestinal tract is not fully developed, may stimulate the production of antibodies that can cross-react with pancreatic islet β-cell surface antigens. This may be a risk factor for the development of type 1 diabetes. Stimulation of aberrant immune response in infancy, however, is not limited to milk components alone, as infants genetically predisposed to type 1 diabetes also have a generalized aberrant immune response to several other proteins (e.g. cereals, fruits, bacteria, viruses).

Animal and human studies suggest that IGF-I is unlikely to have an adverse impact on the pathogenesis of diabetes in humans. The composition of milk from cows treated with rbSTs did not differ materially from that of untreated cows, and therefore consumption of milk from rbST-treated cows would not pose an additional risk for the development of diabetes.

3.6 Risk of cancer

The Committee also considered the potential cancer risk in humans associated with the consumption of milk from rbST-treated cows. The Committee concluded that any carcinogenic risk from rbSTs themselves was negligible, because they are not absorbed from the gastrointestinal tract, they are not bioactive

in humans and the respective orthologues did not cause cancer in rats or mice when administered subcutaneously.

As stated above, the IGF-I exposure from consumption of milk from cows treated with rbSTs represented a small fraction of the physiological amounts produced in humans, and endogenous IGF-I production in humans will be influenced more by the consumption of milk per se than by whether the milk is from rbST-treated or untreated cows. Circulating IGF-I concentrations at the higher end of the normal physiological range were observed in some cancer patients, although these were inconsistent between studies and between different types of cancers. Moreover, these observations came from epidemiological studies in which the impact of reverse causation cannot be excluded.

3.7 Risk to human health from use of antimicrobial agents

The fiftieth Committee meeting concluded that the use of rbSTs would not result in a higher risk to human health due to the use of antimicrobial agents to treat mastitis and that increased potential for drug residues in milk could be managed by practices currently in use within the dairy industry and by following the directions for use.

The potential risk to human health due to the potential for increased use of antimicrobial agents to treat mastitis or increased incidence of non-compliant residues in milk of cows treated with rbSTs was also considered by the present Committee. A meta-analysis published in 1998 observed that cows treated with rbSTs had a higher incidence (up to 25%) of mastitis compared with untreated cows. A systematic review of the literature published since the fiftieth meeting of the Committee did not find any significant difference in the incidence of mastitis between rbST-treated and untreated cows. However, the Committee did not have data to determine the use of antimicrobial agents to treat mastitis on farms using rbSTs.

The fiftieth meeting of the Committee had assessed the data from a post-approval monitoring programme established in the USA to monitor the effects on animal health, including mastitis and non-compliant drug residues in milk. Additional monitoring data for 1996–2012 from the same programme were assessed for the long-term trend in antimicrobial residues in bulk milk. Since 1996, there has been a consistent decrease in the number of bulk milk samples positive for non-compliant antimicrobial residues, with only 0.017% of samples testing positive in 2012, compared with 0.1% in 1996. Several factors could influence the observed results, including adherence to good veterinary practice and improved animal husbandry practices. Moreover, the available data did not provide individual animal-level data to correlate with the use of rbSTs. Nonetheless, the Committee considered that the available evidence suggested that in the USA, the approval of rbSTs did not lead to an increased incidence of non-compliant antimicrobial residues in bulk milk. The Committee found no relevant monitoring data from other jurisdictions where rbSTs are authorized for use.

Although the Committee was aware of the concern regarding potential antimicrobial resistance, its systematic review of the literature did not find specific studies correlating the use of rbSTs with the development of antimicrobial resistance in mastitis pathogens.

Based on the data reviewed, the Committee concluded that there was no evidence to suggest that the use of rbSTs would result in a higher risk to human health due to the possible increased use of antimicrobial agents to treat mastitis or the increased potential for non-compliant antimicrobial residues in milk.

4. EVALUATION

Based on the above assessment, the Committee's responses to the issues raised by the Codex Alimentarius Commission are as follows:

(i) update the toxicological evaluation

No new toxicological studies were available. Owing to structural differences between bovine and human somatotropins, species-specific receptor binding of somatotropins and lack of bioactivity of rbSTs following oral intake, the Committee concluded that if any rbST residues are present in milk or tissues, they would pose a negligible risk to human health.

(ii) update the exposure assessment based on any new occurrence data in food

The Committee concluded that similar concentrations of total bST were present in milk and tissues of rbST-treated and untreated cows.

(iii) consider new data and information related to the possibility of increased levels of IGF-I in the milk of cows treated with rbSTs

There is a transient increase in IGF-I concentrations in milk of rbST-treated cows, which fall within the normal physiological range. IGF-I is substantially, if not completely, degraded in the gut and is unlikely to be absorbed from the gut and be bioavailable at biologically relevant exposures. Therefore, the contribution of exogenous IGF-I resulting from the ingestion of milk from rbST-treated cows is extremely low in comparison with endogenous production.

(iv) evaluate potential adverse health effects, including the possibility that exposure of human neonates and young children to milk from rbST-treated cows increases health risks (e.g. the development of insulin-dependent diabetes mellitus)

Exogenous IGF-I from milk makes no significant contribution to circulating levels of IGF-I in humans, and there are no significant differences in the composition of milk from rbST-treated cows when compared with the milk from untreated cows. The Committee concluded that there was no additional risk for the development of type 1 diabetes due to the consumption of milk from rbST-treated cows. The Committee also concluded that the literature did not support a link between exposure to IGF-I in milk from rbST-treated cows and an increased risk of cancer.

(v) consider new data and information related to the potential effects of rbSTs on the expression of certain viruses in cattle

There was no new information on the link between rbST use and either potential stimulation of retrovirus expression or prion protein expression in cattle.

The present Committee considers that the position expressed by the previous Committee remains valid.

(vi) consider new data and information related to the possible increased use of antimicrobials to treat mastitis in cows and aspects of antimicrobial resistance associated with the use of rbSTs in relation to human health

The Committee concluded that there was no evidence to suggest that the use of rbSTs would result in a higher risk to human health due to the possible increased use of antimicrobial agents to treat mastitis or the increased potential for non-compliant antimicrobial residues in milk. The Committee did not find specific studies linking the use of rbSTs with the development of antimicrobial resistance. The present Committee considers that the position expressed by the previous Committee remains valid.

(vii) consider the need to revise or maintain the ADI and MRLs for rbSTs

The Committee reaffirmed its previous decision on ADIs and MRLs "not specified" for somagrebove, sometribove, somavubove and somidobove.

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ZILPATEROL HYDROCHLORIDE

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1. EXPLANATION

Zilpaterol hydrochloride (zilpaterol HCl, Chemical Abstracts Service No. 119520-06-8) is a β_2-adrenoceptor agonist used for promoting body weight gain, feed efficiency and carcass muscle ratio in cattle fed in confinement before slaughter. There are four enantiomers of zilpaterol HCl: (6*R*,7*R*), (6*R*,7*S*), (6*S*,7*R*) and (6*S*,7*S*). The product in use, which has the code name RU 42173, is racemic *trans* zilpaterol HCl, a mixture of the (6*R*,7*R*) and (6*S*,7*S*) enantiomers; it will be referred to as zilpaterol HCl in this report.

Zilpaterol HCl exhibits the characteristics of a β_2-adrenergic agonist, inducing relaxation of the muscles of the bronchi, an increase in heart rate and a decrease in diastolic blood pressure (Fichelle & Plassard, 1984; Plassard et al., 1985; Filloux, 1994; Corbier & Petit, 1999a). Zilpaterol HCl also produces the biochemical effects of stimulation of the β-adrenergic receptors, including increased lipolysis and reduced lipogenesis in adipose cells and increased protein synthesis and reduced proteolysis in striated muscle fibres (Kern et al., 2009).

The only use of zilpaterol HCl is as a veterinary drug in cattle. The recommended dose added to cattle feed is 7.5 mg/kg (on a 90% dry matter basis) of the total daily ration during the last 20–40 days of the feeding period before slaughter. This level in the feed is equivalent to approximately 0.15 mg/kg body weight (bw) per day or 60–90 mg/animal per day. Where use is authorized, a withdrawal period ranging from 2 to 4 days is applied.

Zilpaterol HCl has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated zilpaterol HCl at the current meeting at the request of the Twentieth Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2012). The Committee was asked to establish an acceptable daily intake (ADI) and recommend maximum residue limits (MRLs) for zilpaterol HCl in cattle tissue.

The Committee considered the results of studies on blood protein binding in vitro, pharmacokinetics, pharmacological effects in vitro and in vivo, acute, short-term and long-term toxicity, genotoxicity, reproductive and developmental toxicity, and relay pharmacology, as well as observations in humans. The majority of the studies were performed in accordance with good laboratory practice (GLP).

The structure of zilpaterol HCl is shown in Fig. 1.

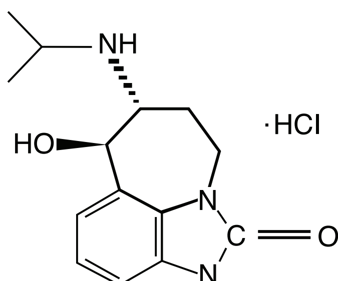
2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 *Blood protein binding in vitro*

The binding of zilpaterol HCl to blood proteins was investigated with blood serum collected from rats, dogs and humans. Blood serum from fasted male Sprague-Dawley rats (pooled sample from 30 rats weighing about 200 g) or from fasted male Beagle dogs (pooled sample from four dogs weighing about 10.7 kg) was loaded with [^{14}C]zilpaterol HCl (radiopurity > 97%) at final concentrations of

Fig. 1. Structure of zilpaterol HCl



$C_{14}H_{19}N_2O_2$·HCl (molecular weight: 297.783 g/mol)

Synonyms: RU 42173 corresponds to racemic *trans* zilpaterol HCl, a mixture of the (6*R*,7*R*) and (6*S*,7*S*) enantiomers

0.005, 0.05, 0.5, 10 and 100 µg/mL. After dialysis to the equilibrium point, the binding percentage was calculated from the concentrations measured by counting the radioactivity (liquid scintillation counting). The average binding to serum proteins of rats and dogs was 14% and 15%, respectively, at 37 °C (Tremblay, Biechler & Cousty, 1990a, 1990b).

The characteristics of the binding of [^{14}C]zilpaterol HCl (radiopurity 95%; tested concentrations 7.4–29.8 µg/mL) to the various serum proteins were assessed in vitro using human blood elements. Pooled human serum from healthy volunteers was used, and the protein binding was assessed by equilibrium dialysis using either the serum or isolated proteins (human serum albumin, α_1-glycoprotein, lipoproteins – very low density lipoprotein, low-density lipoprotein or high-density lipoprotein – and γ-globulins). Binding to erythrocytes was also determined using either erythrocytes in buffer or whole blood. In human serum, the binding percentage was constant at 14.9 ± 0.8%, and binding was non-saturable, with a binding coefficient of 0.158 ± 0.005. The test item did not bind to α_1-glycoprotein, lipoproteins or γ-globulins. The non-saturable binding to human serum albumin in phosphate buffer (pH 7.4) was 14.1 ± 1.5%, with a binding coefficient of 0.152 ± 0.002. Binding of [^{14}C]zilpaterol HCl to erythrocytes was the same, in the range 7.4–29.8 µg/mL, regardless of the concentration tested, with a total erythrocyte fraction of 55.1 ± 3.3% and a binding constant of 1.57 ± 0.02. In the presence of plasma, the total erythrocyte fraction was 55.2 ± 3.6%, demonstrating that zilpaterol HCl is not retained by the plasma compartment. The results show that zilpaterol HCl binds to human serum protein with only a weak affinity and in a non-saturable way, and its affinity for erythrocytes is moderate (Bree, N’Guyen & Tillement, 1987).

2.1.2 Absorption, distribution, metabolism and excretion

(a) Rats

In a non-GLP study, the absolute oral bioavailability of zilpaterol HCl was determined in 12 male Sprague-Dawley rats weighing about 202 g and administered a single dose of [^{14}C]zilpaterol HCl (radiopurity > 98%). Each of six animals per

group was administered a dose of [^{14}C]zilpaterol HCl in saline at 1 mg/kg bw by either the intravenous or oral (gavage) route. Animals were fasted 21 hours before and 6 hours after dosing. Combined urine samples were collected from two rats per cage from 0 to 24 hours post-dosing, and then unchanged zilpaterol was determined by thin-layer chromatography following solid/liquid-phase microcolumn extraction. The amount of unchanged parent compound eliminated in urine over 24 hours was 27.7 ± 0.6% of the dose after intravenous dosing and 27.5 ± 0.6% of the dose after oral administration. Therefore, the absolute oral bioavailability calculated on the basis of urinary excretion was 99.3% (Tremblay et al., 1990a).

A GLP-compliant study was conducted to compare the pharmacokinetics of zilpaterol HCl in plasma after a single administration or administration for 2 weeks by the oral route. Zilpaterol HCl was administered at two different dose levels (0.055 and 1.10 mg/kg bw) by admixture in the diet or by gavage to Sprague-Dawley rats about 8 weeks of age and with an average body weight of 272 g (males) or 213 g (females). Two groups, each with 15 animals of each sex per dose, received the test item via dietary admixture, and two other groups, each with 20 animals of each sex per dose, received the test item by gavage (in water), at the respective dose levels. The purity of the test item was 100.6%. Blood samples were collected at various times at the beginning and end of the single or 2-week administration period. Animals were observed at least once daily for clinical signs or mortality/morbidity, and feed consumption and body weight were recorded twice per week. Plasma free zilpaterol was assayed using a validated radioimmunoassay with a limit of quantification of 0.02 ng/mL. No deaths or clinical signs were observed at any time throughout the study, and treatment had no impact on either feed consumption or body weight. Area under the plasma concentration–time curve over 24 hours ($AUC_{24\ h}$) and peak concentrations in plasma (C_{max}) were generally 2–4 times higher in females than in males, but were 8-fold higher in females than in males in the low-dose group administered the compound in the diet for 2 days. Treatment via single gavage resulted in shorter time to reach C_{max} (T_{max}) (0.25–0.75 hour versus 3–23 hours) and higher $AUC_{24\ h}$ (1.5–4 times) and C_{max} (3–13 times) compared with the dietary admixture. Repeated administration for 2 weeks had no change in these pharmacokinetic parameters, other than a reduction in T_{max} (3–7 hours) in animals receiving the compound in their diet. The mean C_{max} values were approximately proportional to the dose in males. In females, a higher variation was observed for C_{max}, and the C_{max}/dose ratio was lower at 1.10 mg/kg bw per day than at 0.055 mg/kg bw per day. The mean $AUC_{24\ h}$/dose ratio was higher (approximately 1.8–3.9 times) via gavage than via dietary admixture (Sauvez, 1995).

In a non-GLP experiment, groups of 10 fasted Sprague-Dawley rats (five animals of each sex per group) weighing about 203 g were administered [^{14}C]zilpaterol HCl dissolved in saline at 1 mg/kg bw once orally by gavage (radiopurity 93.6–98.9%). The organs of the treated animals were collected 0.5 and 24 hours after dosing to measure the distribution of radioactivity as a function of time. Concentrations of radioactive material in all tissues were substantially lower at 24 hours than at 0.5 hour, demonstrating that the radioactivity was in the elimination phase at 24 hours, and there was no tissue retention of radioactivity. Tissue/plasma concentration ratios (males: skin, eyes, sex organs, erythrocytes, adrenals and liver; females: subcutaneous fat and ovaries) at 24 hours increased

significantly compared with those at 0.5 hour, reflecting incomplete distribution at 0.5 hour for these organs. There was no sex difference except for tissue/plasma concentration ratio in subcutaneous fat at 0.5 hour, which was higher in females than in males, and tissue/plasma concentration ratios in muscle and kidneys at 24 hours, which were higher in males than in females (Tremblay et al., 1989).

Sprague-Dawley (Crl CS (SD) BR) rats about 6 weeks of age at study initiation (six animals of each sex per group) were administered zilpaterol HCl (purity 99.2%) dissolved in water orally by gavage at a dose of either 0.05 or 1 mg/kg bw per day for 13 consecutive weeks. Toxicokinetic evaluation was performed on days 1 and 92. Toxicokinetic analyses demonstrated the test item in all sampled animals, proving intestinal absorption of the compound. A mean apparent T_{max} of 1 hour was found in both treated groups. Mean C_{max} values were 1.14 and 3.72 ng/mL in males (days 1 and 92, respectively) and 2.73 and 4.48 ng/mL in females (days 1 and 92, respectively) treated with 0.05 mg/kg bw per day. These values were 28.9 and 39.6 ng/mL in males and 32.3 and 85.4 ng/mL in females given 1 mg/kg bw per day, respectively. For both tested doses, the half-life ranged from 2.4 to 5.5 hours, and zilpaterol levels were below the limit of quantification at 24 hours after dose administration. Total AUC values on days 1 and 92 were, respectively, 6.21 and 10.36 ng·h/mL in males and 9.75 and 14.59 ng·h/mL in females treated with 0.05 mg/kg bw per day. In the high-dose group, these values were, respectively, 93 and 187 ng·h/mL in males and 296 and 433 ng·h/mL in females, reflecting a higher systemic exposure in females than in males, together with a slight accumulation of the test item, with C_{max} being 1.5–3.5 times higher on day 92 than on day 1, and T_{max} being 1.5–2 times greater. Total AUC values were approximately proportional to dose in male rats. In females, the AUC value was higher in the high-dose group than would be expected from a strictly linear relationship (Sauvez, 1994b).

In a study performed in compliance with GLP, six adult Wistar rats weighing 200–250 g (three of each sex) were administered a single dose of [^{14}C]zilpaterol HCl (radiochemical purity > 98%) at 0.2 mg/kg bw by the oral route in a semi-synthetic diet. The metabolite profile in urine and faeces was determined using high-performance liquid chromatography (HPLC) with radiometric detection, and structural determination of metabolites was performed by mass spectrometry. In 8 days, rats eliminated an average of 48.9% of the dose in the urine and 42.3% in the faeces, with no sex-related differences, although males eliminated 37% of the dose in urine over the first 24 hours, compared with 20% for females. The metabolite profile in the urine collected during the first 2 days after dosing showed the parent compound and five metabolite peaks. In urine samples from days 1 and 2, respectively, unchanged zilpaterol represented 60.3% and 46.7% of the total radioactivity. The other metabolites in urine samples collected on days 1 and 2, respectively, were deisopropyl zilpaterol, 19.5% and 14.2%, acetylated deisopropyl zilpaterol, 4.4% and 4.2%, the glucuronide conjugate of hydroxy-zilpaterol, 9.3% and 22.3%, and two unidentified metabolites (B and C), each accounting for less than 2.3% of the radioactivity. In the liver and carcass, residue levels were less than 0.1% and 1% of the dose, respectively (Tulliez, 2000a).

In a GLP study, six adult Sprague-Dawley rats (three of each sex) weighing 200–250 g were administered a single dose of [^{14}C]zilpaterol HCl (radiochemical purity > 98%) at 0.2 mg/kg bw by the oral route in a semi-synthetic diet. The metabolite

profile of zilpaterol HCl in urine and faeces was established by HPLC with online radiometric detection, and structural determination of metabolites was performed by mass spectrometry. Over 8 days, male rats eliminated 55.6% and 40.9% of the administered dose in urine and faeces, respectively. In females, the values were 46.0% and 46.5%, respectively. Parent compound and six metabolites were found in urine, unchanged zilpaterol being the predominant component; the other metabolites were deisopropyl zilpaterol, acetylated deisopropyl zilpaterol, hydroxy-zilpaterol, a glucuronide conjugate of hydroxy-zilpaterol and two unidentified peaks. No single metabolite accounted for more than 8.4% of the radioactivity present in the urine. In faeces, parent compound accounted for less than 10% of radioactivity. The major component present was hydroxy-zilpaterol, which accounted for 60–80% of the radioactivity detected. In the liver, residue levels were less than 0.1% of the administered dose after 8 days (Tulliez, 2000b).

In a non-GLP study aiming to determine the metabolism of zilpaterol HCl, 10 adult Wistar rats (five of each sex) were administered a single oral dose of [^{14}C]zilpaterol HCl (radiochemical purity 98.5%) at 0.2 mg/kg bw by gavage. One male and one female were terminated at each of 12 hours and 48 hours for determination of radioactive concentration in tissues. The remaining six animals were kept in metabolism cages for measurement of elimination rates in urine and faeces (in one male and one female, for which daily collections of urine and faces were made), as well as for measurement of the radioactivity concentration in tissues (three males and three females), 8 days after dosing. About 91% of the dose was eliminated in urine (49%) and faeces (42%) over 8 days, with the greatest elimination occurring during the first 48 hours (63% of the dose). The highest residue concentrations were about 35 and 6 μg/kg in liver and kidney (mean of males and females), respectively, from rats killed 12 hours after dosing. Radioactivity was below the limit of detection in perirenal fat and muscle on day 8. Less than 1% of the radioactivity administered remained in the carcass after 8 days. In the liver collected 12 hours after dosing, bound radioactivity represented about 85% of the total hepatic radioactivity. Four metabolites and unchanged zilpaterol were found in urine samples by radio-HPLC. Metabolite identities were determined in a separate study by mass spectrometry. The main metabolites in urine were deisopropyl zilpaterol, hydroxy-zilpaterol and unchanged zilpaterol, accounting, respectively, for 1.9%, 8.8% and 81.3% of the radioactivity present. Unchanged zilpaterol (44.2% of the radioactivity present), deisopropyl zilpaterol (7.4%) and hydroxylated zilpaterol (40.4%) were the only compounds detected in faecal samples (Zalko, 1993).

An intraspecies (rat) comparison of metabolites, based on the above-described studies, is provided in Table 1.

Six groups of 36 Wistar rats (3 males and 3 females per dose group) were fed for 3 weeks with feed supplemented with zilpaterol HCl at 0, 1 or 10 mg/kg. Animals were then killed on day 28, and microsomes were prepared from the livers. The microsomes were used to assess hepatic microsomal metabolism of zilpaterol HCl and testosterone. Livers from further groups of three male Wistar rats treated with various liver mono-oxygenase enzyme inducers (dexamethasone, phenobarbital, 3-methylcholanthrene and clofibrate) or with corn oil were also

Table 1. Metabolites in urine collected for 24 hours after single oral dosing of rats

<table>
<tr><th rowspan="2">Metabolite</th><th colspan="3">Mean (males and females) percentages of the extractable radioactivity</th></tr>
<tr><th colspan="2">Rat (Wistar)</th><th>Rat (Sprague-Dawley)[a]</th></tr>
<tr><td>(A) Acetylated deisopropyl zilpaterol</td><td>Not detected[b]</td><td>4.35[c]</td><td>Not detected</td></tr>
<tr><td>(B) Unidentified</td><td>Not detected[b]</td><td>2.24[c]</td><td>Not detected</td></tr>
<tr><td>(C) Hydroxy-zilpaterol glucuronide</td><td>Not detected[b]</td><td>9.26[c]</td><td>Not detected</td></tr>
<tr><td>(D) Unidentified</td><td>Not detected[b]</td><td>6.50[c]</td><td>Not detected</td></tr>
<tr><td>(E1) Deisopropyl zilpaterol</td><td>1.9[b]</td><td rowspan="2">19.48[c,d]</td><td>4.3</td></tr>
<tr><td>(E2) Hydroxy-zilpaterol</td><td>8.8[b]</td><td>1.6</td></tr>
<tr><td>(F) Zilpaterol</td><td>81.3[b]</td><td>60.28[c]</td><td>89.6</td></tr>
</table>

[a] Data from Tulliez (2000b).
[b] Data from Zalko (1993).
[c] Data from Tulliez (2000a).
[d] Metabolites E1 and E2 were not resolved.

used for preparation of microsomes. Following incubation of zilpaterol HCl with the hepatic microsomal fraction, unchanged zilpaterol, deisopropyl zilpaterol and hydroxy-zilpaterol were identified. The metabolites were shown to be the products largely, if not entirely, of cytochromes P450 (CYP); deisopropyl zilpaterol seemed to be formed preferentially by members of the CYP1A subfamily, whereas hydroxy-zilpaterol was formed less specifically, possibly involving members of the CYP2B subfamily. Treatment with zilpaterol HCl for 3 weeks had no effect on the hepatic microsomal metabolism of this compound in either males or females. Similarly, hepatic microsomal metabolism of testosterone remained unaffected by 3 weeks of treatment with zilpaterol HCl, and there was no effect on total microsomal P450 content (Zalko, 1993).

(b) Dogs

In a non-GLP study, the absolute oral bioavailability of zilpaterol HCl was determined in four fasted male Beagle dogs with an average body weight of 10.0 kg. [^{14}C]Zilpaterol HCl (radiopurity > 98%) dissolved in saline was administered by either the intravenous or the oral (gavage) route at a dose of 1 mg/kg bw. Urine samples were then collected from 0 to 48 hours post-dosing. Unchanged zilpaterol was determined by coincidence of the ultraviolet and radioactive traces by HPLC or thin-layer chromatography and then quantified by liquid scintillation counting. The amount of unchanged parent compound eliminated in urine in 48 hours after intravenous dosing was 22.8 ± 2.1% of the administered dose. After oral administration, the amount eliminated in urine was 23.9 ± 2.4%. Hence, the absolute oral bioavailability calculated on the basis of urinary excretion was 100% (Tremblay et al., 1990b).

The absorption, elimination and bioavailability of zilpaterol HCl in rats and dogs are presented in Table 2.

Table 2. Estimated bioavailability[a] and elimination after a single oral dose administered to rats and dogs

Parameters	Rat	Dog
Dose (mg/kg bw)	0.055[b], 0.2[c,d], 1[e], 1.10[b]	1[f]
Absolute oral bioavailability	99.3%[e]	100%[f]
C_{max} (ng_{eq}/mL)	2.35–115[b]	No data
T_{max} (h)	0.25–0.75[b]	No data
% of the dose eliminated in 8 days: urine	48.9%[c], 49%[d]	No data
% of the dose eliminated in 8 days: faeces	42.3%[c], 42%[d]	No data

C_{max}: peak plasma concentration; eq: equivalent; T_{max}: time to reach C_{max}
[a] Relative to an intravenous dose.
[b] Sprague-Dawley rat (data from Sauvez, 1995).
[c] Wistar rat (data from Tulliez, 2000a).
[d] Wistar rat (data from Zalko, 1993).
[e] Sprague-Dawley rat (data from Tremblay et al., 1990a).
[f] Beagle dog (data from Tremblay et al., 1990b).

(c) Humans

In a clinical study, nine healthy male volunteers aged 28–55 years and weighing 56–76 kg were orally administered zilpaterol HCl (1 mg/mL in 2 mL ampoule, added into drinking-water) at single doses of 0.25, 0.5, 1.0 or 2.0 mg/adult to assess tolerance of zilpaterol HCl. Adults of each group were fasted and then received either the test item (increasing doses) or placebo, according to a single-blind protocol using a four-way cross-over design. Four subjects received 0.25 mg and five subjects received 0.5 mg; then, after a 7-day wash-out period, the next higher doses were administered. Blood samples were collected at 15 minutes, then at 1, 2, 3, 4, 5, 6, 8 and 24 hours after dosing. Plasma samples were assayed for zilpaterol using a radioimmunoassay method. The T_{max} was 1 hour, and the half-life was 4–5 hours at all doses. C_{max} and AUC values increased linearly with dose. The pharmacokinetic parameters are presented in Table 3 (Sutton & Budhram, 1987; Tremblay & Mouren, 1988).

Table 3. Plasma pharmacokinetic parameters of zilpaterol HCl in human healthy volunteers after a single oral dose

Parameters	Dose of zilpaterol HCl administered			
	0.25 mg/adult	0.5 mg/adult	1.0 mg/adult	2.0 mg/adult
C_{max} (ng/mL)	0.98 ± 0.16	2.28 ± 0.23	4.47 ± 0.33	8.56 ± 0.89
$AUC_{0-\infty}$ (ng·h/mL)	6.93 ± 0.20	12.3 ± 1.6	26.1 ± 1.7	51.7 ± 5.6
T_{max} (h)	1	1	1	1
$t_{½}$ (h)	4.81 ± 0.97	3.69 ± 0.34	4.15 ± 0.37	4.14 ± 0.26
Regression between C_{max} and the dose	C_{max} = 4.26 × dose + 0.11			

AUC: area under the plasma concentration–time curve; C_{max}: peak plasma concentration; $t_{½}$: half-life; T_{max}: time to reach C_{max}
Source: Sutton & Budhram (1987); Tremblay & Mouren (1988)

2.1.3 Effects on enzymes and other biochemical parameters

Many in vitro, ex vivo and in vivo studies have clearly demonstrated the β_2-agonist effect of zilpaterol HCl. Those effects are manifested as contraction of the musculature of the cardiovascular system, as well as relaxation of the smooth muscles of the vasculature and the bronchi. The pharmacological properties and characterization of zilpaterol HCl and its main metabolite, deisopropyl zilpaterol (free base or hydrochloride form), were assessed in a series of in vitro, ex vivo and in vivo assays.

In a GLP study, the affinity of zilpaterol HCl and its main metabolite, deisopropyl zilpaterol (as free base or hydrochloride form), was determined for rat cortex β_1-adrenoceptor and rat lung β_2-adrenoceptor using in vitro receptor binding assays. The tested concentrations ranged from 10^{-10} to 10^{-4} mol/L. The reference agonist compounds were alprenolol and propranolol, respectively, for β_1- and β_2-adrenoceptors. Ligands were [^{3}H]CGP-26505 (0.6 nmol/L) for β_1-adrenoceptor and [^{125}I]iodocyanopindolol (0.05 nmol/L) for β_2-adrenoceptor. The purity of the test items was 100.6%, 100.3% and 97.5%, respectively, for zilpaterol HCl, deisopropyl zilpaterol HCl and deisopropyl zilpaterol free base. Zilpaterol HCl and its main metabolite displayed low affinity for β_1-adrenoceptors (median inhibitory concentration [IC_{50}]: 2.1–2.8 × 10^{-5} mol/L, compared with the IC_{50} of alprenolol: 2.0 × 10^{-8} mol/L). Regarding the β_2-adrenoceptor, the affinity of the parent compound (IC_{50}: 6.9 × 10^{-6} mol/L) was about 1.5-fold higher than that of its main metabolite (Table 4) (Filloux, 1994).

The agonist activity of zilpaterol HCl, deisopropyl zilpaterol HCl and deisopropyl zilpaterol free base on β-adrenoceptor-coupled adenylyl cyclase was investigated using guinea-pig lung membranes, by measuring the amount of ^{32}P-labelled cyclic adenosine monophosphate synthesized. Tested concentrations ranged from 10^{-7} to 10^{-3} mol/L for each compound, and the positive control was isoproterenol. Antagonist activity was similarly determined, against isoproterenol-induced adenylyl cyclase activation (concentration 10^{-6} mol/L). Propranolol was used as control. The results demonstrated that zilpaterol and deisopropyl zilpaterol are only partial agonists at the β-adrenoceptor in terms of adenylyl cyclase activation (7–41% of basal level compared with 90–95% induced by isoproterenol; 8–22% basal activity remaining when used as an antagonist compared with 0–2% with propranolol) (Filloux, 1994).

Table 4. Affinity of zilpaterol HCl and deisopropyl zilpaterol (as free base or hydrochloride form) for rat cortex β_1-adrenoceptors and lung β_2-adrenoceptors

	IC_{50} (mol/L)			
	Zilpaterol HCl	Deisopropyl zilpaterol HCl	Deisopropyl zilpaterol free base	Reference agonist
β_1-adrenoceptor	2.8 × 10^{-5}	1.8 × 10^{-5}	2.1 × 10^{-5}	2.0 × 10^{-8} (alprenolol)
β_2-adrenoceptor	6.9 × 10^{-6}	1.0 × 10^{-5}	1.0 × 10^{-5}	1.7 × 10^{-9} (propranolol)

Source: Filloux (1994)

In a GLP study, the binding of zilpaterol HCl, deisopropyl zilpaterol HCl and deisopropyl zilpaterol free base to rat adrenoceptors (α_1 and α_2) and dopaminergic receptors (D_1 and D_2) was investigated. The affinity of the compounds for all of the receptors was very low ($IC_{50} \geq 50$ μmol/L), suggesting that there would be no pharmacological effects of the compounds via these receptors (Arbogast, Gilis & Church, 1995a, 1995b).

In a non-GLP study, the β_2-adrenergic activity of zilpaterol HCl was investigated ex vivo using isolated guinea-pig trachea. In this assay, widely used for β_2-adrenoceptor agonist activity testing, trachea were pretreated with propranolol at concentrations ranging from 10^{-9} to 10^{-7} mol/L for 30 minutes, then contracted with carbachol at a concentration of 3×10^{-7} mol/L. After contractions reached a plateau, zilpaterol HCl was tested by cumulative addition. The agonistic effects of zilpaterol HCl were inhibited competitively by propranolol, when the pA_2 (a measure of the affinity of the antagonist for its receptor) of propranonol determined with zilpaterol HCl was 9.07. The pA_2 of propranolol determined with zilpaterol HCl was comparable to that determined in the laboratory under the same conditions with isoprenaline. The pA_2 of propranolol determined with isoprenaline was 9.27. The results demonstrate the β_2-adrenergic agonist activity of zilpaterol HCl (Advenier, 1987).

In a set of three in vivo non-GLP studies, anaesthetized, pithed Sprague-Dawley rats (males, 12–15 weeks old, 6–11 per group) were administered zilpaterol HCl or its main metabolite, deisopropyl zilpaterol (free base or hydrochloride form), intravenously (avoiding any potential low intestinal resorption and potential first-pass effect) at cumulative doses of 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 (deisopropyl zilpaterol + antagonists only) mg/kg bw. Compounds were administered alone or a few minutes after administration of well known and well characterized adrenoceptor antagonists, propranolol (β-adrenoceptor antagonist) at 1 mg/kg bw, atenolol (β_1-adrenoceptor antagonist) at 2.5 mg/kg bw or 5 mg/kg bw, ICI 118,551 (β_2-adrenoceptor antagonist) at 1 mg/kg bw, phentolamine (α-adrenoceptor antagonist) at 1 mg/kg bw or domperidone (peripheral dopamine receptor antagonist, studies with zilpaterol HCl only) at 0.1 mg/kg bw. Zilpaterol HCl induced a dose-dependent decrease in diastolic blood pressure from 0.01 mg/kg bw, with a maximum effect observed at a dose of 0.1 mg/kg bw. Tachycardia was observed from 0.3 mg/kg bw, with a maximum effect at a dose of 3 mg/kg bw. Both the vascular and cardiac effects induced by zilpaterol HCl could be abolished with propranolol. The vascular effects were blocked by ICI 118,551, but not by atenolol. Atenolol had little or no effect on the blood pressure response to zilpaterol HCl, but antagonized the effects on heart rate at high doses. Phentolamine and domperidone had no effect on either the vascular or cardiac response to zilpaterol HCl. Deisopropyl zilpaterol (as either its free base or hydrochloride form) dose-dependently decreased the blood pressure from 0.1 mg/kg bw, with a steady-state maximum effect observed at 0.3 mg/kg bw. In contrast, deisopropyl zilpaterol had no effect on the heart rate. The vascular effects of deisopropyl zilpaterol were antagonized by propranolol and by ICI 118,551, but not by atenolol or by phentolamine.

The in vivo results demonstrate that deisopropyl zilpaterol displays 10-fold lower β_2-agonist activity on the cardiovascular system, compared with the parent compound. Moreover, the use of the specific β_2-adrenoceptor antagonist

Table 5. In vivo activity of zilpaterol HCl and deisopropyl zilpaterol (as the free base or hydrochloride form) in pithed rat

Parameter	Compound	EDx (mg/kg bw)[a]	Dose at which effect reaches maximum (mg/kg bw) (effect size)
Decreased diastolic blood pressure	Zilpaterol HCl	0.01	0.1 (−20.6 ± 1.9 mmHg[b])
	Deisopropyl zilpaterol HCl	0.12	1 (−20.0 ± 0.8 mmHg)
	Deisopropyl zilpaterol free base	0.12	1 (−23.0 ± 1.8 mmHg)
Increased heart rate	Zilpaterol HCl	0.3	3 (+87.2 ± 4.5 bpm)
	Deisopropyl zilpaterol HCl	No effect at 10 mg/kg bw	None observed
	Deisopropyl zilpaterol free base	No effect at 1 mg/kg bw	10 (+22.8 ± 13.1 bpm)

bpm: beats per minute

[a] EDx: $ED_{DBP}10$ = dose that will decrease diastolic blood pressure (DBP) by 10 mmHg (~20% of the initial basal values); $ED_{HR}50$ = dose that will decrease heart rate (HR) by 50 bpm (~20% of the initial basal values).

[b] 1 mmHg = 133.3 Pa.

Source: Corbier & Petit (1999a, 1999b, 1999c)

ICI 118,551 demonstrated that effects on the vascular system are β_2-adrenoceptor mediated, whereas activity on the heart is associated with the β_1-adrenoceptor (Corbier & Petit, 1999a, 1999b, 1999c).

The in vivo effects are summarized in Table 5.

In conclusion, the main pharmacological activity of zilpaterol HCl is mediated by the β_2-adrenoceptor. Its main metabolite, deisopropyl zilpaterol, is also a β_2-specific agonist, but with a potency in vivo about an order of magnitude less than that of the parent compound. Zilpaterol HCl binds very poorly, if at all, to α_1- or α_2-adrenoceptors or dopaminergic receptors (D_1 and D_2), with high inhibitory constants, reflecting its lack of significant pharmacological effect via these receptors.

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Single-dose toxicity studies

The results of single-dose toxicity studies performed with zilpaterol HCl, its main metabolite, deisopropyl zilpaterol (free base form), and the final product (premix ZILMAX 4.8%) are summarized in Table 6. Most of the studies were GLP compliant, and the purity of zilpaterol HCl was greater than 98%, whereas that of deisopropyl zilpaterol was greater than 97.5%. When ZILMAX was tested, the batches were compliant with the release specifications. The results show rather low toxicity of zilpaterol HCl.

Table 6. Single-dose toxicity of zilpaterol HCl, deisopropyl zilpaterol and zilpaterol HCl 4.8% premix

Species (strain)	Sex (no. of animals)	Route	Test article	Dose (mg/kg bw)	LD_{50} (mg/kg bw)[a]	Reference
Mouse (Swiss CD-1)	M (10) F (10)	Oral	Zilpaterol HCl	0, 200, 350, 500, 650	M: 430 (360–490) F: 580 (495–800)	Fournex, Collas & Audegond (1985a)
Mouse (Swiss OF1)	M (5) F (5)	Oral	Zilpaterol HCl	0, 385, 500, 650, 845, 1 100	M: 430 (170–530) F: 680 (520–785)	Catez et al. (1994b)
Mouse (Swiss CD-1)	M (10) F (10)	Intraperitoneal	Zilpaterol HCl	M: 0, 145, 170, 195 F: 0, 110, 145, 195, 265	M: 155 (150–165) F: 170 (150–190)	Fournex, Collas & Audegond (1985d)
Mouse (Swiss OF1)	M (5) F (5)	Oral	Deisopropyl zilpaterol	0, 650, 845, 965, 1 100	M: 1 065 F: 965–1 100 M & F: 1 030 (975–1 100)	Catez et al. (1994a)
Mouse (Swiss OF1)	M (5) F (5)	Oral	Zilpaterol HCl 4.8% premix	2 000	M & F: > 2 000	Catez et al. (1996a)
Rat (SD)	M (10) F (10)	Oral	Zilpaterol HCl	0, 312, 625, 1 250, 2 500, 5 000	M: 1 325 (880–2 020) F: 890 (600–1 275)	Fournex, Collas & Audegond (1985b)
Rat (SD)	M (10) F (10)	Intraperitoneal	Zilpaterol HCl	M: 0, 150, 250, 350, 450 F: 0, 150, 200, 250	M: 280 (225–330) F: 225 (205–240)	Fournex, Collas & Audegond (1985c)
Rat (SD)	M (5) F (5)	Inhalation	Zilpaterol HCl	5.04 mg/L of air	M & F: LC_{50} (4 h) > 5.04 mg/L	Coombs (1998)
Rat (SD)	M (5) F (5)	Dermal	Zilpaterol HCl	2 000	M & F: > 2 000	Manciaux (1998)
Rat (SD)	M (5) F (5)	Oral	Zilpaterol HCl 4.8% premix	2 000	M & F: > 2 000	Catez et al. (1996b)

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

[a] 95% confidence intervals provided in parentheses.

In Swiss CD-1 mice, a single oral administration of zilpaterol HCl at 200, 350, 500 or 650 mg/kg bw in 0.5% methyl cellulose via oral gavage induced apathy, hypomotility, hunched back, breathing or respiratory difficulties, slight locomotor difficulties and piloerection. The clinical signs persisted on the day following dosing. Mortality occurred within 0.25–4 hours of dosing. Animals found dead exhibited moderate congestion of the lungs, stomach and intestines. No abnormalities were observed in animals necropsied on day 14. The median lethal dose (LD_{50}) values were 430 (95% confidence interval [CI]: 360–490) and 580 (95% CI: 495–800) mg/kg bw for males and females, respectively (Fournex, Collas & Audegond, 1985a).

In Swiss OF1 mice, clinical signs of toxicity were observed during the 36- to 48-hour period following treatment with a single oral dose of zilpaterol HCl at 385, 500, 650, 850 or 1100 mg/kg bw, administered by gavage in 0.5% methyl cellulose. The clinical signs included dyspnoea, moderate to marked difficulties in walking, hypomotility and prostration. In addition, tremors and convulsions preceding death were recorded in the first 30 minutes following gavage. Mortality occurred within 0.25–3 hours of dosing. Animals found dead exhibited moderate congestion of the lungs, stomach and intestines. The LD_{50} values were 430 (95% CI: 170–530) and 630 (95% CI: 520–785) mg/kg bw for males and females, respectively (Catez et al., 1994b).

In Swiss CD-1 mice, a single intraperitoneal injection of zilpaterol HCl at 145, 170 or 195 mg/kg bw (males) or 110, 145, 195 or 265 mg/kg bw (females) in 0.5% methyl cellulose induced hypotonia or apathy, hypomotility, hunched back, respiratory difficulties, slight locomotor difficulties and tremor during the 24-hour period following treatment. Mortality occurred immediately after injection in animals receiving the highest dose. The LD_{50} values were 155 (95% CI: 150–165) and 170 (95% CI: 150–190) mg/kg bw for males and females, respectively (Fournex, Collas & Audegond, 1985d).

Deisopropyl zilpaterol, a major metabolite of zilpaterol, administered orally once to Swiss OF1 mice by gavage in 0.5% methyl cellulose at 650, 845, 965 or 1100 mg/kg bw caused hypomotility, moderate to marked difficulties in walking, dyspnoea (from 845 mg/kg bw), apathy or lethargy (1100 mg/kg bw) during the 24-hour period following treatment. Mortality occurred within 3 hours after administration of 1100 mg/kg bw in females and on day 2 for other animals. No abnormalities were observed in animals necropsied on day 14 except for congested appearance of the lung (1100 mg/kg bw). The LD_{50}s were 1065 (confidence interval could not be calculated) and 965–1100 (confidence interval could not be calculated) mg/kg bw for males and females, respectively, with a median for males and females combined of 1030 (95% CI: 975–1100) mg/kg bw, indicating that the main metabolite, deisopropyl zilpaterol, displays an acute toxicity of about half that of the parent compound (Catez et al., 1994a).

A single oral dose of zilpaterol HCl administered to Sprague-Dawley rats by gavage in 0.5% methyl cellulose at 312, 625, 1250, 2500 or 5000 mg/kg bw induced hypomotility, hunched back, breathing or respiratory difficulties, slight locomotor difficulties and piloerection during the 24 hours following treatment. Mortality occurred within 0.75–5 hours of dosing. Animals found dead exhibited moderate

congestion of the lungs, profuse saliva around the snout and a large quantity of water in the stomach. No abnormalities were observed in animals necropsied on day 14. The LD_{50} values were 1325 (95% CI: 880–2020) and 890 (95% CI: 600–1275) mg/kg bw for males and females, respectively (Fournex, Collas & Audegond, 1985b).

Clinical signs observed after a single intraperitoneal injection of zilpaterol HCl in 0.5% methyl cellulose to Sprague-Dawley rats at 150, 250, 350 or 450 mg/kg bw (males) or 150, 200 or 250 mg/kg bw (females) included hypotonia, hunched back and slight locomotor difficulties during the 24 hours following injection. Mortality occurred within 0.3–2 hours of dosing. Animals found dead exhibited moderate congestion of the lungs and liver. No abnormalities were observed in animals necropsied on day 14. The LD_{50} values were 280 (95% CI: 225–330) and 225 (95% CI: 205–240) mg/kg bw for males and females, respectively (Fournex, Collas & Audegond, 1985c).

Sprague-Dawley rats were exposed to zilpaterol HCl by inhalation at an aerosol concentration of 5.04 ± 0.2 mg/L via nose only for 4 hours. The mass median aerodynamic diameter was 3.8 µm, and the percentage of breathable particles (< 7 µm) was 73%. There were no unscheduled deaths during the study. During exposure, clinical signs included exaggerated respiratory movements, gasping and wet fur. Immediately following exposure, clinical signs included exaggerated respiratory movements, partial closing of the eyes, whole body tremors and wet fur. In males, staggering, gasping and lethargy were reported. Matted fur and brown staining were observed in some of the animals for 1–5 days following treatment. Body weights and feed consumption were reduced in males only in the day following treatment. At necropsy, lung weight to body weight ratios were comparable between treated and control animals. One animal exhibited a slightly congested lung, and the majority exhibited discoloration of the tail. The 4-hour median lethal concentration (LC_{50}) for zilpaterol HCl was greater than 5.04 mg/L of air (Coombs, 1998).

The shaved dorsal area of Sprague-Dawley rats was treated with zilpaterol HCl at a dose of 2000 mg/kg bw. The test site was covered by a semi-occlusive dressing for 24 hours, after which the site was washed with water. Ocular secretion was observed in one female from day 9 to day 15. No other clinical signs and no cutaneous reactions were noted during the study. Body weight gain of the animals was reduced when compared with historical control animals (there were no concurrent controls). Three males lost weight between days 1 and 5, and two females lost weight between days 5 and 8. Macroscopic examination of the main organs of the animals revealed no apparent abnormalities. The dermal LD_{50} of zilpaterol HCl was greater than 2000 mg/kg bw in rats (Manciaux, 1998).

Zilpaterol HCl 4.8% premix was administered once to Swiss OF1 mice and Sprague-Dawley rats orally by gavage. Because of the granularity of the preparation, it had to be ground before administration and was delivered as an aqueous suspension in 0.5% methyl cellulose of 50 mg/mL in four batches of 10 mL/kg bw (mice) or 100 mg/mL in two batches of 10 mL/kg bw (rats) over 2 hours. No deaths and no clinical signs were observed. The LD_{50} was greater than 2000 mg/kg bw for both mice and rats (Catez et al., 1996a, 1996b).

(b) Dermal irritation

In a GLP-compliant study, the skin irritation potential of zilpaterol HCl was tested in three adult male hybrid New Zealand White rabbits using 0.5 g of pure material administered as a thin layer to 6 cm^2 of shaved dorsal skin. The material was covered with a semi-occlusive dressing for 4 hours, after which the site was washed with water. No signs of cutaneous irritation were observed over 72 hours. There were no clinical signs noted or abnormal changes in body weight. Zilpaterol HCl was non-irritating to the skin (Catez et al., 1996c).

(c) Ocular irritation

In a GLP-compliant study, the ocular irritation potential of zilpaterol HCl was tested in three adult male hybrid New Zealand White rabbits using 0.1 g of pure material administered once to the right eye via the base of the conjunctival sac. Unrinsed cornea showed moderate opacity in 2/3 animals and slight opacity in 1/3 animals by 24 hours, with a recovery in 3/3 animals by 96 hours. The area involved was slight in 3/3 rabbits at 24 hours and 1/3 rabbits at 48 hours and had disappeared at 72 hours. The iris presented slight congestion at 1 hour in 1/3 animals. No reaction was observed at 24–72 hours. Conjunctiva showed slight redness and discharge at 1 hour and 24 hours after dosing in 3/3 animals. Zilpaterol HCl is slightly irritating to the eye (Catez et al., 1996d).

(d) Dermal sensitization

A GLP-compliant study was performed in the guinea-pig, using the maximization test of Magnusson and Kligman, to assess the potential of zilpaterol HCl to induce delayed-contact hypersensitivity. Male and female guinea-pigs (5 animals of each sex for the control group, 10 animals of each sex for the treated group) were used. On day 1, they were intradermally injected with Freund's complete adjuvant containing zilpaterol HCl (10% weight per weight [w/w]) or sterile isotonic saline solution. The purity of zilpaterol HCl was 100.1%. On day 7, lauryl sulfate was topically applied on the same site to induce skin irritation. The day after, the test item (10% w/w in saline) or control vehicle (saline) was topically applied under occlusion for 48 hours. Twelve days later, animals were challenged by topical application of zilpaterol HCl (10% w/w in saline) on the right flank. The left flank served as control and received the vehicle only. Application sites were maintained under occlusive condition for 24 hours, and skin reactions were evaluated and scored about 24 and 48 hours later. Animals were then terminated.

For the whole study duration, there were no clinical signs or deaths observed. There was no well defined cutaneous reaction observed after the challenge test, and zilpaterol HCl displayed no cutaneous reaction attributable to sensitization potential. Zilpaterol HCl was not a skin sensitizer in guinea-pigs (de Jouffrey, 1996).

2.2.2 Short-term studies of toxicity

A comprehensive set of short-term toxicity studies was performed with zilpaterol HCl in laboratory species, including rodents (rats and mice), Beagle dogs, Cynomolgus monkeys, Yucatan microswine and cattle (the target species). The

pivotal studies were performed in compliance with GLP, with appropriate standards for protocols and conduct.

(a) Mice

Zilpaterol HCl (purity 100.1%) was administered to Swiss CD-1 mice (10 animals of each sex per group) by the oral route (dietary admixture) at 0, 0.2, 2, 4 or 40 mg/kg feed (equal to 0, 46, 460, 895 and 8867 μg/kg bw per day for males and 0, 47, 483, 986 and 9601 μg/kg bw per day for females, respectively) for 4 weeks. In addition, two satellite groups of 32 animals of each sex treated orally with zilpaterol HCl via feed containing either 0.2 or 40 mg/kg were included for toxicokinetic purposes. The concentration of the test substance in the diet was modified weekly to maintain the nominal dose levels. Animals were observed daily for clinical signs and mortality. Feed consumption, body weights and achieved doses were recorded weekly. Haematology and blood biochemistry analyses were performed just before necropsy. Levels of zilpaterol in plasma collected from satellite groups were analysed on days 2–3 and 24–25 during administration. At the end of the treatment period, animals were killed and subjected to complete macroscopic examination. Organs were weighed and preserved for any further microscopic examinations.

No mortality or clinical signs of toxicity were observed at any time throughout the study. Mean feed consumption was slightly, although not statistically significantly, increased (by 8–9%) in females in the 4 and 40 mg/kg feed groups over the last 3 weeks of treatment. Body weight gain in females was increased slightly, but statistically significantly, on weeks 2 and 3 of treatment at the highest dose, but returned to the control range by week 4 of treatment. Toxicokinetics revealed a T_{max} of about 23 hours in both males and females. Plasma levels of the test substance were 2.3- to 3.3-fold higher (0.2 mg/kg feed per day) and 1.3-fold higher (40 mg/kg feed) in males than in females. Mean $AUC_{0–24\ h}$ values were 2.68–3.54 ng·h/mL and 1.07–1.16 ng·h/mL, respectively, in males and females of the 0.2 mg/kg feed group. In the 40 mg/kg feed dose group, $AUC_{0–24\ h}$ values were 674–765 ng·h/mL and 518–601 ng·h/mL in males and females, respectively. The ratio AUC/dose was similar at both doses in males, but was lower in females in the low-dose group. Treatment duration had no impact on toxicokinetic parameters. There was no effect of the treatment on haematology parameters or blood biochemistry. At necropsy, there were no macroscopic findings, and organ weights were unaffected.

There were no treatment-related findings. The no-observed-adverse-effect level (NOAEL) was 4 mg/kg feed (equal to 986 μg/kg bw per day), based on the transient increase in body weight observed in females at 40 mg/kg feed (equal to 9601 μg/kg bw per day) (Sauvez, 1996).

(b) Rats

Sprague-Dawley rats (five animals of each sex per group) about 6 weeks of age with weights ranging from 175 g to 204 g for males and from 154 g to 174 g for females were administered zilpaterol HCl daily by gavage in oral solution in

distilled water for 10 days at a dose of 0 or 50 mg/kg bw per day in a preliminary short-term toxicity study. Animals were observed daily for clinical signs or mortality. Feed consumption and body weight were monitored throughout the study. Haematology, blood biochemistry and urine analysis parameters were also monitored. At study termination, organs were collected, weighed and subjected to histopathological examination.

No mortality or clinical signs of toxicity were observed. Body weight gain following treatment of animals was higher than in control rats. This was more marked and statistically significant in females, although feed consumption was unaffected. Haematology revealed a significant decrease in platelet count (females, by 20%), mainly associated with 2/5 animals. Significantly decreased blood glucose (males and females) and a more marked triglyceride decrease (males only) were observed in treated animals. Alkaline phosphatase was significantly increased by approximately 50% in treated females, although other liver function tests were normal. Blood urea level was significantly increased in female rats, but remained within the normal physiological range. At necropsy, macroscopic examination revealed no abnormal findings. Absolute organ weights were not affected by treatment, although a number of relative organ weights were lower in treated animals, particularly in females. This was associated with the higher body weight of treated animals when compared with the control group. Histopathological examination revealed no abnormal findings. The results of this study were used to set the doses for the 30-day toxicity study in rats (Collas & Fournex, 1984).

Sprague-Dawley (Crl CS (SD) BR) rats about 8 weeks of age at study initiation (four animals of each sex per group) were administered zilpaterol HCl (purity 100.6%) orally via dietary admixture at a dose of 0 or 1 mg/kg bw per day for 15 consecutive days. Achieved doses were 0.9 and 1.1 mg/kg bw per day for females and males, respectively. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weights were monitored twice a week; thereafter, achieved doses were calculated. At the end of the treatment period, animals were sacrificed. No macroscopic examination was performed.

No mortality or clinical signs of toxicity were observed throughout the study duration. Feed consumption was lower in treated animals; on a weekly basis, the mean difference (compared with untreated animals) ranged from −6% to −10%. There was no effect of the treatment on mean body weight. Zilpaterol HCl administered at a dose of 1 mg/kg bw per day (dietary admixture) induced slightly decreased feed consumption (Sauvez, 1994c).

Sprague-Dawley rats about 6 weeks of age at study initiation (10 animals of each sex per group) were administered zilpaterol HCl (purity > 98%) daily by oral gavage in distilled water at a dose of 0, 1, 10 or 100 mg/kg bw per day for 30 consecutive days. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and water consumption were monitored, as well as body weight. Haematology, blood biochemistry and urine analysis parameters were also

monitored, together with ophthalmological examinations and electrocardiograms (ECGs). At termination, following necropsy, organs were collected, weighed and subjected to histopathological examination.

All animals survived, and no clinical signs were observed. Body weight gain of males treated with 10 and 100 mg/kg bw per day and of females receiving 100 mg/kg bw per day was significantly increased throughout the study, although feed consumption remained unaffected. Water consumption was slightly increased in females in the high-dose group on week 1 of treatment. There was no treatment-related effect on blood pressure, and the ECG revealed no major differences. A fall in heart rate was observed in a dose-dependent manner in all treated females (−10%, −11% and −20% at 1, 10 and 100 mg/kg bw per day, respectively). In males, heart rate was reduced (−7%) only in the high-dose group, and the difference was not statistically significant (the raw data on heart rate were not provided in the study report). Haematology revealed a slight, statistically significant increase in total leukocyte count (males, 100 mg/kg bw per day), a significant, dose-dependent, slight decrease in activated partial thromboplastin time in males only (all treated groups) and a minor, dose-dependent, slightly decreased prothrombin time in females (10 and 100 mg/kg bw per day). Some significant variations were observed for blood biochemical parameters (sodium, calcium, phosphorus, chloride, cholesterol, glucose and creatinine kinase), but these remained within normal limits, were not consistent between sexes and were not dose dependent. Proteinograms were normal for females. Males showed significant variations in some proteins, but there was no dose dependency. Males treated with 100 mg/kg bw per day showed significant decreases in α_2- and β_1-globulins. Liver function tests were normal in males. In females, liver function tests were also unaffected, other than a 2-fold increase in lactate dehydrogenase in the high-dose group. However, this increase in lactate dehydrogenase is attributed to haemolysed samples rather than the treatment with zilpaterol HCl. Blood urea level was slightly, although significantly, raised (by approximately 50%) in females treated with 100 mg/kg bw per day, but remained within the normal physiological range. Urine analysis revealed no abnormal findings, and the test for occult blood in faeces was negative.

There were no ocular lesions or any macroscopic findings at necropsy. Absolute weight of heart was increased in males (10 and 100 mg/kg bw per day, with no dose–response relationship) and females (all dose groups, with no relationship with dose at 1 mg/kg bw). Ovarian weight in the high-dose group was significantly higher than in controls. These differences were generally not observed for relative organ weights (other than for heart in low- and mid-dose females), but a statistically significant, non-dose-dependent decrease in liver weight was observed in all treated males. Relative brain weight was significantly decreased in high-dose males and females. Microscopic examination revealed no treatment-induced lesions. The few abnormalities observed were naturally occurring in rats.

The lowest-observed-adverse-effect level (LOAEL) was 1 mg/kg bw per day, the lowest dose tested, based on decreased heart rate in females and decreased activated partial thromboplastin time in males at all dose levels (Fournex et al., 1985).

Sprague-Dawley (Crl CD (SD) BR) rats about 6 weeks of age at study initiation (20 animals of each sex per group) were administered zilpaterol HCl (purity > 98%) in water orally via gavage at a dose of 0, 1, 10 or 100 mg/kg bw per day for 90 days. At completion of the treatment period, eight animals of each sex per group were kept without treatment for a 32-day reversibility period. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weights were monitored weekly. Ophthalmological examination, ECG and blood pressure recording (1–3 hours after dosing) were performed before the first treatment, on weeks 4 and 12, and then at the end of the reversibility period. Haematology, blood biochemistry analysis and urine analysis were performed on weeks 5, 13 and 18 (end of reversibility period). At the end of the treatment and reversibility periods, animals were terminated and subjected to complete macroscopic examination. Selected organs were weighed, and microscopic examinations were performed on several organs and on all organs displaying macroscopic lesions.

One male (1 mg/kg bw per day) and one female (100 mg/kg bw per day) were found dead on days 79 and 78, respectively. No clinical signs other than hypersalivation (female) were observed prior to these deaths. One female of the control group died under anaesthesia (blood sampling, day 32), and another female of the high-dose group was found dead on day 77, previously displaying piloerection, emaciation, pallor of the extremities, dyspnoea and decreased activity. The causes of these deaths were not established with certainty. In surviving animals, dose- and treatment-related hypersalivation was observed in 9/20 males and 12/20 females from the 10 mg/kg bw per day dose group and in all animals from the high-dose group. Salivation was first observed between day 29 and day 37 in most affected animals receiving 10 mg/kg bw per day and between day 2 and day 13 in most high-dose animals. There was no other clinical sign associated with the test item. A dose-dependent, reversible, slightly increased body weight gain (≤ 10% in the high-dose group) was observed in all treated animals. This was statistically significant at 10 and 100 mg/kg bw per day in females and at 100 mg/kg bw per day in males. Feed consumption was significantly increased in males at 10 and 100 mg/kg bw per day. Feed efficiency remained comparable between treated and control animals. No ophthalmic or blood pressure abnormalities related to treatment were observed. A slight dose-dependent reduction of heart rate, associated with increased PQ and QαT conduction times, which was statistically significant at 12 weeks in the high-dose group only (–14% in males, –7% in females), was observed. Values remained, however, within commonly accepted limits for rats. Haematology revealed no variation of any toxicological significance. Blood urea levels were significantly increased in mid- and high-dose males and in all treated groups of females at week 5 and in all treated groups of males and females at week 13. Plasma creatinine levels were significantly increased at 5 weeks in mid- and high-dose males and females and at 13 weeks in high-dose males and in mid-dose females. Although plasma creatinine levels were increased in high-dose females, changes were not significant. Urine analysis revealed significant increases in urine volume in males at 5 and 13 weeks and significant increases in pH, specific gravity and urine volume at 5 and 13 weeks. Some of these changes were apparent in all dose groups. They were due to variations in a few animals per dose group.

However, no significant differences were observed in any of these parameters following the period of reversibility. There was no treatment-related abnormality on either macroscopic or microscopic examinations. Absolute and relative weights of the kidneys were unaffected by treatment, other than an increase in absolute weights in high-dose females. Relative weights of some organs were slightly, but significantly, decreased in high-dose males (thyroid, thymus, brain) and in mid- and high-dose females (liver, uterus, heart). All variations regressed at the end of the reversibility period.

The LOAEL was 1 mg/kg bw per day, the lowest dose tested, based on increased blood urea in females at all doses (Moysan, 1989).

In a follow-up study, Sprague-Dawley (Crl CD (SD) BR) rats about 6 weeks of age at study initiation (six animals of each sex per group) were administered zilpaterol HCl (purity 99.2%) in water orally by gavage at a dose of 0, 0.05, 0.5 or 1 mg/kg bw per day for 4 consecutive weeks. The doses were based on the results from the previous 90-day study. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weight were monitored weekly. ECG and blood pressure were monitored before and at the end of the treatment period. Blood biochemistry was performed about 24 hours after the last treatment. At termination, macroscopic examination was performed on all animals, with recording of any gross observations. Organs were weighed, and microscopy of the spleen was performed.

All animals survived the treatment period, and no clinical signs were observed. Feed consumption was slightly higher in all treated animals (from +5% to +15%), although this was not statistically significant and was less than 10%, other than in high-dose females. Body weights were increased in all dose groups, being statistically significant from week 2 in females from the high-dose group. There was no dose dependency in males or in females in the low- and mid-dose groups. The difference in final body weight was less than or equal to 10%, other than in high-dose females (+13%). There was no effect of the treatment on ECG, heart rate or blood pressure recorded at the end of the treatment period. Biochemical analysis revealed a decreased blood glucose level in males and females from the 0.5 and 1 mg/kg bw per day dose groups, although this was significant only in females, and values remained within the background range. Blood urea and creatinine levels were unaffected by treatment. Absolute heart weight was significantly increased (+17%) in females from the high-dose group, and testes weight was statistically decreased in males from the 1 mg/kg bw per day dose group. This was not treatment related. Males from the 0.5 and 1 mg/kg bw per day dose groups displayed significant decreases in adrenal weight (0.5 mg/kg bw per day: relative weight, 1/6 animals; 1 mg/kg bw per day: relative plus absolute weights, 2/6 animals). Absolute weight of testes was also significantly decreased in males from the high-dose group, mainly associated with one male that had small testes with soft consistency. These changes were not considered to be compound related. Macroscopic examination of the spleen revealed an irregular surface (one male, intermediate-dose group) and several raised greyish/whitish foci or areas (0.5 mg/kg bw: two males and one

female; 1 mg/kg bw: four males and one female), correlated with minimal to marked inflammatory cell infiltration. This was considered not to be compound related, as it is observed spontaneously in this strain of rats.

The NOAEL was 0.5 mg/kg bw per day, based on the significant increase in body weight of females at 1 mg/kg bw per day (Sauvez, 1993a).

Sprague-Dawley (Crl CS (SD) BR) rats about 6 weeks of age at study initiation (20 animals of each sex per group) were administered zilpaterol HCl (purity 99.2%) dissolved in water orally by gavage at a dose of 0, 0.05, 0.5 or 1 mg/kg bw per day for 13 consecutive weeks. These doses were based on the results from the preliminary 4-week and 90-day studies. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weights were monitored weekly, and feed efficiency was estimated. Ophthalmological examinations were performed before the first treatment and on week 13. Systolic blood pressure and ECG were performed on 10 animals of each sex per group before treatment and then at 1–3 hours after dosing on weeks 3, 4, 8 and 13. Haematology, blood biochemistry analysis and urine analysis were performed on week 13 for 10 animals of each sex per group. At the end of the treatment period, animals were terminated and subjected to complete macroscopic examination. Organs were weighed (adrenals, heart, kidneys, liver, ovaries/testes, spleen and thymus), and microscopic examination was performed on several organs and on all organs displaying macroscopic lesions.

No mortality or clinical signs of toxicity were observed at any time throughout the study. A treatment-related increase in feed consumption and body weight was observed in the intermediate- and high-dose groups. Lower mean heart rates were observed during week 8 in females given 0.05 or 0.5 mg/kg bw per day and during week 3 in females given 1 mg/kg bw per day. This was associated with a longer PQ interval, which was also noted in males treated with 0.5 or 1 mg/kg bw per day. Lower systemic blood pressure, sometimes statistically significant, was observed in males at 0.5 and 1 mg/kg bw per day in week 3 or 4. These observations were considered treatment related.

There was no effect of treatment on ophthalmology, haematology, blood biochemistry or urine analysis. Slightly dose-related, statistically significant, higher absolute and relative heart weights were observed in females at 0.5 mg/kg bw per day and in males and females at 1 mg/kg bw per day. There were no major macroscopic or microscopic findings.

The LOAEL was 0.05 mg/kg bw per day, the lowest dose tested, based on decreased mean heart rate observed at all doses in females (Attia, 1994; Sauvez, 1994b).

(c) Dogs

One male and one female Beagle dog 9 months of age were given an oral dose of zilpaterol HCl (purity > 98%) at 100 mg/kg bw per day in capsules (gavage) for 7 consecutive days. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weights were monitored. Haematology

and blood biochemistry were analysed before the first dosing and again at the end of the study. At termination, following necropsy, organs were subjected to macroscopic examination and then to histopathological examination.

Neither animal died. Frequent vomiting, reduced motor activity, apathy, lacrimation, vasodilatation of the abdomen and ears, as well as diarrhoea were observed. Between days 0 and 4, weight loss in the male, associated with partial loss of appetite, was observed, with recovery thereafter. Reductions of erythrocyte number (moderate) and haemoglobin/haematocrit values (marked) were observed in both animals. Blood biochemistry revealed decreases in sodium, chloride and total proteins and increases in transaminases and alkaline phosphatase (male and female). Increases in cholesterol and ornithine carbamyl transferase (male) and in phospholipids and phosphorus (female) were also observed. Macroscopic examination revealed putty-coloured liver of heterogeneous appearance with clear lobular structure, associated at the microscopic level with excess glycogen and lipids in the perilobular hepatocytes.

Overall, numerous clinical signs associated with the pharmacological activity of the test item, together with hepatocellular injury, were observed at the only dose used, 100 mg/kg bw per day (Fournex et al., 1984).

Beagle dogs (three animals of each sex per group) about 8 months of age at study initiation were administered zilpaterol HCl (purity 99.5%) orally in capsules daily at a dose of 0, 0.5, 5 or 50 mg/kg bw per day for 30 consecutive days. These doses were based on the results from the preliminary 7-day study. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and water consumption were monitored daily, and body weights were monitored on a weekly basis. Ophthalmological examinations were performed before the first treatment and on week 13. Systolic blood pressure and ECG were recorded on several occasions. Haematology, blood biochemistry, urine analysis and faecal examination (occult blood) were performed at the end of the treatment period. Intestinal absorption of the test item was determined by blood-level analysis performed after the first and last administrations. At the end of the treatment period, animals were terminated and subjected to complete macroscopic examination. Organs were then subjected to microscopic examination.

One male (0.5 mg/kg bw per day) and one female (5 mg/kg bw per day) died during the experimental phase of the study. The deaths were not related to the test item. A dose-dependent peripheral vasodilatation was observed in all treated animals, occurring about 30 minutes after dosing, persisting for some hours to the whole day in the 50 mg/kg bw per day dose group. Irregular vomiting was observed in two males at 5 mg/kg bw per day and in all animals at 50 mg/kg bw per day. Motor activity was reduced in the 5 and 50 mg/kg bw per day dose groups. Body weight change was unaffected by the treatment in surviving animals. Feed consumption and water consumption were unaffected in the 0.5 and 5 mg/kg bw per day dose groups. In the 50 mg/kg bw per day group, one female had transient and partial loss of appetite, and two females had slightly increased water intake. Blood pressure was reduced in all treated animals 1 hour after dosing and returned to normal values 24 hours after dosing. Increased heart rate was observed in all

treated animals 1 hour after dosing and then returned to a normal beat 24 hours after administration.

Reduced red cell count, haemoglobin level and haematocrit value were observed in one male and one female, as well as a fall in platelet count in one male and two females, at 50 mg/kg bw per day. Blood biochemistry revealed no abnormal findings. Urine analysis revealed glycosuria, which was not correlated with any increase in blood glucose, in one male at 50 mg/kg bw per day and a high bilirubin level, which was not correlated with high blood bilirubin level, in two males at 50 mg/kg bw per day. No occult blood was found in faecal samples.

Ophthalmic examinations revealed no abnormal findings, and there were no macroscopic lesions observed at necropsy. No treatment-related change was observed in organ weights. Abnormal spermatogenesis, not related to the test item, was observed in two, two and one male of the 0.5, 5 and 50 mg/kg bw per day groups, respectively.

Peripheral vasodilatation, rise in heart rate and fall in blood pressure related to the treatment of zilpaterol HCl were observed at all doses. Hence, the LOAEL was 0.5 mg/kg bw per day, the lowest dose tested, based on the results observed (Fournex et al., 1986).

(d) Swine

One male and one female Yucatan microswine 7–8 months of age were given increasing oral doses (via gavage) of zilpaterol HCl (purity 99.2%) in distilled water in a dose range–finding study. The doses were 0, 0.05, 0.5, 1, 5 or 10 mg/kg bw per day over periods of 4 consecutive days, with a 3-day withdrawal period, to assess both the potential toxic and pharmacological effects of zilpaterol HCl. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weights were monitored throughout the study. ECG and blood pressure were recorded before the first administration of each new dose and 1–3 hours after the last administration of each new dose. Haematology and blood biochemistry were monitored on a weekly basis. On completion of the last treatment period, animals were killed and subjected to complete macroscopic examination. Selected organs were weighed and subjected to microscopic examination.

No animal died, and there were no clinical signs of toxicity. Feed consumption and body weight were unaffected by treatment. Although there was no impact of the test item on blood pressure, a slightly increased heart rate associated with a decreased QT interval was observed at the end of the treatment periods (0.5, 1, 5 and 10 mg/kg bw per day in male and 10 mg/kg bw per day in female). The only effects observed were increased heart rate together with a decreased QT interval, which is related to the pharmacological effects of zilpaterol HCl (Sauvez, 1993c).

Groups of two male and two female Yucatan microswine weighing about 12 kg were given a daily oral dose of zilpaterol HCl (purity 99.2%) dissolved in water at 0, 5, 10, 50 or 5000 μg/kg bw per day for 4 weeks. The choice of the doses was based on the previous dose range–finding toxicity study (Sauvez, 1993c). Animals

were observed daily for clinical signs of toxicity and mortality. Feed consumption was monitored, as well as body weight. Haematology, blood biochemistry and urine analysis were also monitored, together with ophthalmological examinations and ECG. At termination, following necropsy, selected organs were collected, weighed and subjected to histopathological examination.

No mortality or clinical signs were observed. Treatment had no impact on body weight, and feed consumption was similar between treated and control animals. Treatment had no effect on either ECG or blood pressure, and ophthalmological examination revealed no differences between control and treated animals. Urine analysis revealed no qualitative or quantitative treatment-related abnormality. Haematology and blood biochemistry revealed no findings of importance. At necropsy, there were no macroscopic or microscopic findings.

A NOAEL of 5000 µg/kg bw per day was identified, the highest dose tested (Sauvez, 1994a).

Yucatan microswine (four animals of each sex per group) about 4–7 months of age at study initiation were administered zilpaterol HCl (purity 100.6%) dissolved in water orally by gavage at a dose of 0, 1, 50, 1000 or 10 000 µg/kg bw per day for 13 consecutive weeks. These doses were based on the results from the previous 4-week toxicity study. Plasma levels of the test item were determined on the day of first dosing, then once on week 5 and at the end of the treatment period. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weight were monitored throughout the study. ECG examination and blood pressure recording were performed twice on both weeks 1 and 2, then once on weeks 4, 8 and 12. Ophthalmological examinations were performed before the first treatment and on week 13. Haematology, blood biochemistry analysis and urine analysis were performed on week 13. At the end of the treatment period, animals were terminated and subjected to complete macroscopic examination. Selected organs were weighed, and microscopic examinations were performed on several organs and on all organs displaying macroscopic lesions.

No clinical signs of toxicity were observed during the study. One male and one female of the 1 µg/kg bw per day dose group were found dead on days 70 and 20, respectively. Deaths were associated with severe bronchopneumonia and abscesses and were not related to test item administration. There was no treatment-related effect on feed consumption. Body weight was slightly increased, although statistically non-significantly, in males from the 1000 and 10 000 µg/kg bw per day dose groups. ECG and blood pressure, ophthalmological examination, haematology, blood biochemistry and urine analysis revealed no effect of the treatment. Macroscopic and microscopic examinations as well as organ weights revealed no effect of treatment.

The NOAEL was 10 000 µg/kg bw per day, the highest dose tested (Sauvez, 1994d).

(e) Monkeys

In a study aiming to evaluate the potential toxic and pharmacological effects of zilpaterol HCl, one male and one female Cynomolgus monkey weighing about 3 kg were given increasing oral (gavage) doses of zilpaterol HCl (purity 99.2%) in distilled water in a dose range–finding study. The doses were 0.05, 0.5, 1, 5 and 10 mg/kg bw per day over a period of 4 consecutive days, with a 3-day withdrawal period. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weights were monitored throughout the study. ECG and blood pressure were recorded before the first administration of each new dose and 1–3 hours after the last administration of each new dose. Haematology and blood biochemistry were monitored on a weekly basis. On completion of the last treatment period, animals were terminated and subjected to complete macroscopic examination. Selected organs were weighed and subjected to microscopic examination.

No animal died, and there were no clinical signs of toxicity. Feed consumption and body weight were unaffected by treatment. Although there was no impact of the test item on blood pressure, a slight to moderate increase in heart rate associated with a decrease in QT interval was observed at the end of the treatment period (0.05, 0.5, 1, 5 and 10 mg/kg bw per day). There was no dose–response relationship in increased heart rate with a decreased QT interval, but association with the test item cannot be excluded (Sauvez, 1993b).

Groups of two male and two female Cynomolgus monkeys weighing about 2.5–2.9 kg were given a dose of zilpaterol HCl (purity 99.2%) dissolved in water orally (by gavage) at 0, 5, 10, 50 or 5000 µg/kg bw per day for 4 weeks. The choice of doses was based on the previous dose range–finding toxicity study. Animals were observed daily for clinical signs of toxicity and mortality, and feed consumption was monitored daily. Body weights were recorded weekly. ECG and blood pressure were monitored throughout the study 1–3 hours after dosing, and ophthalmological examinations were performed at the end of the dosing period. Haematology, blood biochemistry and urine analysis were also monitored. At termination, following necropsy, a complete macroscopic examination was performed, and selected organs were collected, weighed and subjected to histopathological examination.

No mortality or clinical signs were observed. Treatment had no impact on body weight, and feed consumption was similar between treated and control animals. Treatment had no effect on either ECG or blood pressure at 5 and 10 µg/kg bw per day. Blood pressure was slightly decreased in the two highest dose groups, and a slightly increased heart rate with an associated decrease in QT interval was observed from week 2 of treatment, mainly in males. Ophthalmological examination revealed no difference between control and treated animals. Urine analysis revealed no qualitative or quantitative treatment-related abnormalities. Haematology and blood biochemistry revealed no findings of importance, and all individual data remained within the range of the background values. At necropsy, even if some differences in organ weights were observed, there was no difference of major order that was considered to be treatment related. There were no macroscopic or microscopic findings.

The NOAEL was 10 μg/kg bw per day, based on reduced blood pressure and the increased heart rate with a decreased QT interval at 50 μg/kg bw per day (Sauvez, 1993d).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a GLP-compliant study to evaluate the potential tumorigenic activity of zilpaterol HCl, five groups of 65 nulliparous and non-pregnant female CD-1 mice weighing about 23 g and 8 weeks of age at initiation of dosing were given a dose of zilpaterol HCl (purity 99.4%) at 0, 10, 20, 50 or 250 μg/kg bw per day via oral gavage for 18 consecutive months. Ten satellite animals per group were used for determination of zilpaterol concentration in plasma, with animals sampled 1 month after the first dosing. Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weight were recorded on a weekly basis until week 13, then once every 4 weeks. Haematology was monitored at the end of the administration period. At termination, following necropsy, a complete macroscopic examination was performed, and selected organs were collected and subjected to histopathological examination. All masses and macroscopic lesions were also subjected to microscopic examinations.

The incidence, nature and onset of clinical signs did not show any indication of a relationship with treatment or dose. Distribution of mortality and factors contributing to mortality were similar between control and treated animals, and the survival rate was in the range 66–75%. Plasma analysis for zilpaterol revealed systemic exposure in all treated groups, increasing with dose, except in the 20 μg/kg bw per day dose group, with average plasma concentrations of 0.204 ng/mL at 10 μg/kg bw per day, 0.112 ng/mL at 20 μg/kg bw per day, 0.390 ng/mL at 50 μg/kg bw per day and 2.240 ng/mL at 250 μg/kg bw per day. There was no systemic toxicity or tumorigenicity associated with zilpaterol HCl. Feed consumption, body weight and haematological parameters were similar between control and treated animals. Some haematological findings were observed: significantly increased haemoglobin at 50 and 250 μg/kg bw per day and a significantly increased number of red blood cells and haematocrit in these dose groups. Although these changes did not show strong dose dependency, there was a degree of biological consistency. There were also significantly decreased numbers of platelets, absolute neutrophil counts and monocyte numbers at 50 and 250 μg/kg bw per day.

A NOAEL of 20 μg/kg bw per day was identified, based on changes in haematological parameters at 50 μg/kg bw per day (Auletta, 2003).

(b) Rats

In a GLP-compliant study, five groups of 20 male and 20 female Sprague-Dawley rats weighing about 178 g for males and 157 g for females and 6 weeks of age were given zilpaterol HCl (purity 99.7%) incorporated into the feed at a dose of 0, 25, 50, 125 or 250 μg/kg bw per day for 52 consecutive weeks. Every week, the concentration of zilpaterol HCl was adjusted to achieve the nominal doses. Achieved doses were very close to the theoretical values. An additional 10 males

and 10 females in the control and 250 μg/kg bw per day groups were kept for a further 4-week treatment-free (recovery) period. Two satellite groups of 10 males and 10 females received doses of 25 or 250 μg/kg bw per day for determination of toxicokinetics, and plasma samples collected on day 3 and then in weeks 27 and 52 were assayed for zilpaterol using a validated radioimmunoassay method.

Animals were observed daily for clinical signs of toxicity and mortality. Feed consumption and body weight were recorded on a weekly basis. Thereafter, achieved doses were calculated. Ophthalmic examination, ECG and blood pressure were recorded throughout the study. Haematology and blood biochemistry were also monitored on 10 animals of each sex per group throughout the study. At termination, following necropsy, a complete macroscopic examination was performed, and selected organs were collected, weighed and subjected to histopathological examination. Electron microscopic examinations of the heart were performed on three animals of each sex per group.

No mortality or clinical signs attributed to the test item were observed. Plasma levels of zilpaterol ranged from 0 to 5 ng/mL, with concentrations increasing in a dose-related fashion. Systemic exposure was slightly higher in females. Treatment had no significant impact on body weight, and the feed consumption was similar between treated and control animals, including the treatment-free period. Ophthalmological examinations revealed no impact of the treatment. ECG revealed slight decreases in heart rate, without impact on waveform traces, in males and females at 125 and 250 μg/kg bw per day; these were fully reversible at the end of the recovery period. A slightly increased systolic blood pressure, which was fully reversible, was also observed in the high-dose group. Haematology and blood biochemistry revealed no findings attributable to the test item, and all individual data remained within the range of the background values. At necropsy, macroscopic examination revealed no non-neoplastic or neoplastic findings attributable to the test item, and microscopic examinations revealed no impact of the treatment. Treated animals displayed no difference in either absolute or relative organ weights when compared with control rats.

The NOAEL was 50 μg/kg bw per day, based on decreased heart rate (Richard, 1999a).

In a GLP-compliant carcinogenicity study, five groups of 66 male and 66 female Sprague-Dawley rats weighing about 178 g for males and 157 g for females and 6 weeks of age were given zilpaterol HCl (purity 99.7%) incorporated into the feed at a dose of 0, 25, 50, 125 or 250 μg/kg bw per day for 104 consecutive weeks. Every week, the concentration of zilpaterol HCl was adjusted to achieve the nominal doses. Achieved doses did not deviate from the theoretical ones by more than 0.5%. Ten males and 10 females per dose group were sampled during the last treatment week to assess zilpaterol plasma concentration using a validated radioimmunoassay method. Animals were observed daily for clinical signs of toxicity and mortality; after 6 months of treatment, all animals were palpated every 2 weeks to record the date of appearance of any palpable mass. Feed consumption and body weight were recorded on a weekly basis until week 69, then once every 2 weeks. Thereafter, achieved doses were calculated. Haematology was also

monitored throughout the study. At termination, following necropsy (including all animals found dead or moribund during the course of the experimental phase), a complete macroscopic examination was performed, and selected organs were collected, weighed and subjected to histopathological examination. All masses and macroscopic lesions were also subjected to microscopic examinations.

The incidence, nature and onset of the clinical signs, as well as palpable masses, did not show any indication of a relationship with treatment or dose. Distribution of mortality and factors contributing to mortality were similar between controls and animals in all but the highest-dose group. In the highest-dose group, survival decreased in the second half of the study, being about 10–20% lower than in the control group, reaching statistical significance in the males. In plasma, zilpaterol concentrations ranged from 0 to 9 ng/mL and increased with dose. In females, concentrations were slightly higher than in males. Feed consumption was slightly increased in the females at 250 μg/kg bw per day. Body weights were slightly lower in males and females from the highest-dose group, especially in the second half of the study. The test item had no qualitative or quantitative impact on haematological parameters. Macroscopic changes in decedent and surviving animals were similar in incidence and nature between treated and control rats. Organ weights were similar between treated and control animals, other than a marked increase in the weight of the ovaries in the 125 and 250 μg/kg bw per day dose groups. Histopathologically, this may have a possible link to an increased incidence of follicular cysts and the occurrence of leiomyomas. There were no microscopic non-neoplastic or neoplastic lesions attributable to the test item in males. In 3% (2/63 females examined) of the 125 μg/kg bw per day dose group and 8% (5/64) of the 250 μg/kg bw per day dose group, leiomyomas of the suspensory ligament of the ovary were observed. This is a benign tumour known to be associated with the pharmacological activity of β_2-agonist compounds in rats. The severity of this lesion was not increased with the dose.

The NOAEL in this study was 50 μg/kg bw per day, based on increased weight of ovaries with increased incidence of ovarian cysts and increased incidence of ovarian leiomyomas (Richard, 1999b).

Ovarian leiomyomas are tumours that are well known to be related to the use of β_2-adrenoceptor agonists in rodents. The proliferation of the mesovarian smooth muscle is considered to be an adaptive physiological response to prolonged stimulation of the β-receptors, with muscle relaxation as a consequence, because the induction of this type of neoplastic lesion in rodents was blocked by the concomitant use of propranolol, a β_2-adrenoceptor antagonist (Nelson, Kelly & Weikel, 1972; Jack, Poynter & Spurling, 1983; Gibson et al., 1987).

Little or no relaxant response to β_2-agonists can be demonstrated in the uteri of non-pregnant women (Cantox, 2000). There is no evidence in humans of any increased incidence of smooth muscle tumours such as leiomyomas among users of β_2-agonists (Jack, Poynter & Spurling, 1983; Gibson et al., 1987; Gopinath & Gibson, 1987). Thus, the occurrence of ovarian leiomyomas in rats treated with zilpaterol HCl may represent a species-specific effect, and it is considered unlikely that oral exposure in humans would result in ovarian leiomyomas.

2.2.4 Genotoxicity

A comprehensive range of genotoxicity studies using either zilpaterol HCl or its main metabolite, deisopropyl zilpaterol (free base), has been performed in microorganisms, mammalian cultured cells or in vivo in the mouse. Most of these studies complied with GLP. For zilpaterol HCl, the purity of the test items ranged from 99.4% to 99.7%; for deisopropyl zilpaterol free base, the purity either was 88.7%, with impurities containing 10.3% water, or ranged from 96.5% to 97.5%. When required (lack of intrinsic metabolic activity of the test system), a metabolic activating system (liver S9 fractions, from Aroclor-treated rats) was also used for testing the effect of any metabolic activation on potential genotoxicity. The results for zilpaterol HCl and deisopropyl zilpaterol free base are provided in Tables 7 and 8, respectively.

Based on comprehensive tests of genotoxic activity, mainly performed under GLP-compliant conditions, in microorganisms, in cultured mammalian cells or in vivo in mice, it can be concluded that zilpaterol HCl shows no evidence of any genotoxic potential. This is also true of its main metabolite, deisopropyl zilpaterol.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

(i) Rats

In a preliminary GLP-compliant study, four groups of 10 male and 10 female Sprague-Dawley rats approximately 9 weeks of age with average body weights of 344 g for males and 225 g for females were fed with feed supplemented with zilpaterol HCl at concentrations of 0, 0.9, 3.6 and 14.4 mg/kg. The tested doses corresponded to 0, 0.06, 0.23 and 0.94 mg/kg bw per day for males and 0, 0.10, 0.40 and 1.61 mg/kg bw per day for females, respectively. Administration started 15 days before mating and during mating for males and during mating, pregnancy and lactation for females, lasting until termination. For mating purposes, each male was paired with one female from the same dose group until mating or for a duration not exceeding 7 days. Day 0 of gestation was set when vaginal lavage revealed the presence of spermatozoa. Males and females in the F_0 generation were observed for clinical signs and mortality or morbidity on a daily basis. Feed consumption and body weight were recorded on a weekly basis until termination for males and, for females, on a weekly basis until day 21 postpartum and then on days 64 and 71 of the post-weaning period. Females were allowed to deliver normally; after weaning of the last F_1 litter, F_0 parents were terminated and subjected to complete macroscopic examination. The number of implantation sites was recorded in all F_0 females. Microscopic examination was performed on selected organs and on all macroscopic lesions. Each litter was observed daily for clinical signs and for number and sex of live, dead and cannibalized pups, from day 1 postpartum until days 22–23, and body weights were recorded on days 1, 4, 7, 14 and 21 postpartum. Weights of live pups were recorded on days 1, 4, 7, 14 and 21 postpartum. On day 4, random selection was used to adjust each litter size to four males and four females, and pups not selected were preserved in Bouin's fluid. All surviving pups, killed between day 22 and day 25 postpartum, together with pups that had died,

Table 7. Results of genotoxicity studies performed with zilpaterol HCl

Test system	Test object	Concentrations/ doses	Results	Reference
Microorganisms				
In vitro reverse mutation in bacteria	*Salmonella typhimurium* TA98, TA100	100–5 000 µg/ plate (±S9)	Negative (±S9)	Vannier & Chantot (1984)
	S. typhimurium TA98, TA100, TA1535, TA1537, TA1538	10–4 000 µg/ plate (±S9)	Negative (±S9)	Loquet (1987a)
	S. typhimurium TA98, TA100, TA102, TA1535, TA1537 *Escherichia coli* WP2uvrA	312.5–5 000 µg/ plate (±S9)	Negative (±S9)	Haddouk (1997)
Cultured mammalian cells				
Chromosomal aberration	CHO/*HGPRT* locus assay	500–5 000 µg/ mL (±S9)	Negative (±S9)	Adams (1992)
Unscheduled DNA synthesis	Primary Fischer 344 rat hepatocytes	2.50–5 000 µg/ mL	Negative	McKeon (1991)
Forward mutation	L5178Y/ *tk*$^{+/-}$ mouse lymphoma cell	250–5 000 µg/ mL (±S9) 500–3 000 µg/ mL (±S9)	Negative (±S9)	Mitchell (1998d)
In vivo tests				
Micronucleus test	Swiss Cr1:CD-1 (1CR) BR mouse bone marrow cells	400 mg/kg bw	Negative	Loquet (1987b)
Micronucleus test	ICR mouse bone marrow cells	100–400 mg/kg bw	Negative whatever the sacrifice time (24, 48 or 72 h after administration of the test item)	Murli (1991a, 1991b)
Micronucleus test	Swiss Webster mouse bone marrow cells	31.25–500 mg/kg bw	Negative	Mitchell (1998c)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HGPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

Table 8. Results of genotoxicity studies performed with deisopropyl zilpaterol free base

Test system	Test object	Concentrations/doses	Results	Reference
Microorganisms				
In vitro reverse mutation in bacteria	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537 *Escherichia coli* WP2uvrA	312.5–5 000 μg/plate (–S9) 37.5–5 000 μg/plate (+S9)	Negative (±S9)	Molinier (1993)
Cultured mammalian cells				
Unscheduled DNA synthesis	Wistar rat hepatocytes	0.064–5 000 μg/mL	Negative	Ward (1994)
Forward mutation	L5178Y/ $tk^{+/-}$ mouse lymphoma cells	78–5 000 μg/mL (–S9) 400–1 100 μg/mL (+S9)	Weak potential to induce mutation in the mouse lymphoma assay (+S9)[a]	Riach (1994)
Forward mutation	L5178Y/ $tk^{+/-}$ mouse lymphoma cells	100–2 500 μg/mL (–S9) 250–1 700 μg/mL (+S9)	Negative (±S9)	Mitchell (1998a)
In vivo tests				
Micronucleus test	Swiss OF1 mouse bone marrow cells	1 500 mg/kg bw	Negative	Molinier (1994)
Micronucleus test	Swiss Webster mouse bone marrow cells	125–2 000 mg/kg bw	Negative	Mitchell (1998b)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Non-biologically relevant because observed at only highly toxic concentrations (at 900 μg/mL), and other two experiments show negative results.

were subjected to complete macroscopic examination. Male and female mating index and fertility index were calculated. Gestation, live birth, viability (day 4 postpartum) and lactation (day 21 postpartum) indices were also calculated.

F_0 parents did not display any clinical signs, and no deaths were observed. Feed consumption was similar between treated and untreated animals, but body weight was significantly increased in all treated males, mainly during the 1st week of treatment. During the premating period, body weight only of females from the high-dose group was increased. This difference lasted during the pregnancy and lactation periods and was attributed to the pharmacological activity of the test item. There was no effect of zilpaterol HCl on mating and fertility (males and females) or on gestation

index and duration of gestation. The mean number of liveborn pups as well as the viability index (day 4 postpartum) were similar in all groups. On day 21 postpartum, the lactation index was similar in all groups, and no clinical signs were registered in pups. Macroscopic examination of pups dying between day 5 and day 21 postpartum revealed no abnormalities. Macroscopic and microscopic examinations performed on both F_0 and F_1 animals revealed no treatment-related changes.

In this reproductive toxicity study with zilpaterol HCl, the only effect observed was an increase in body weight. The LOAEL for parental toxicity was 0.9 mg/kg feed (equal to 0.06 mg/kg bw per day), based on increased body weight in F_0 males at 3.6 mg/kg feed (equal to 0.23 mg/kg bw per day). The NOAEL for reproductive and offspring toxicity was 14.4 mg/kg feed (equal to 0.94 mg/kg bw per day), the highest dose tested (Richard, 1995).

In a multigeneration study aiming to evaluate potential effects of the test item on male and female reproductive performance and on growth and development of the offspring, four groups of 30 male Sprague-Dawley rats approximately 7 weeks of age with an average body weight of 249 g and 30 female Sprague-Dawley rats about 10 weeks of age with an average body weight of 248 g were fed with feed supplemented with zilpaterol HCl (purity 99.7%) at concentrations of 0, 0.9, 3.6 or 14.4 mg/kg in a GLP-compliant study. F_0 treatment started 71 days before mating for males and until termination (after the weaning of F_1 litters) and 15 days before mating for females and until termination after the weaning of F_1 litters (treatment period included mating for males and mating, pregnancy and lactation for females). For mating purposes, each male was paired with one female from the same dose group until mating or for a duration not exceeding 7 days. After mating, females were allowed to deliver normally and to rear their progeny until weaning. After weaning, 25 males and 25 females were selected to constitute the F_1 generation and were administered the same treatment until the end of the study. Achieved doses were 0, 0.06, 0.23 and 0.94 mg/kg bw per day for F_0 males and 0, 0.10, 0.40 and 1.61 mg/kg bw per day for F_0 females. For the F_1 generation, 0, 0.08, 0.32 and 1.26 mg/kg bw per day were administered to F_1 males and 0, 0.12, 0.45 and 1.77 mg/kg bw per day to F_1 females.

Males and females in the F_0 generation were observed for clinical signs and mortality and morbidity on a daily basis. Feed consumption and body weight were recorded on a weekly basis until termination for males and, for females, on a weekly basis during the premating period, during the gestation period at intervals of days 0–6, days 6–13 and days 13–21, and during lactation at intervals of days 1–7, days 7–14 and days 14–21. Females were allowed to deliver normally. F_1 litters were examined daily (number and sex of live, dead and cannibalized, clinical signs and gross abnormalities). Body weight of live pups was recorded on days 1, 4 (before selection), 7, 14 and 21 postpartum. On day 4 postpartum, the size of the litter was randomly adjusted to four males and four females, the remaining pups being killed. Pups were then observed for developmental steps (pinna unfolding and hair growth on day 5 postpartum, incisor eruption on day 13, eye opening and auricular duct opening on day 17) and for reflexes (surface righting reflex on day 5, cliff avoidance on day 11, air righting reflex on day 17). At weaning, one male and

one female from each group and from 25 surviving litters were randomly selected (day 22 postpartum corresponding to day 1 of the F_1 generation). They were further fed with feed supplemented with the same concentrations of zilpaterol HCl as for the F_0 parents until termination (after weaning of the F_2 generation). F_1 males and females were allowed to mate when they were about 12 weeks old and subjected to the same examination protocol (clinical signs, mortality, body weight and feed consumption) as for the parent F_0 generation. F_1 females were allowed to deliver normally. Litter size and the numbers of live, dead and cannibalized F_2 pups were recorded, as well as clinical signs on a daily basis. F_2 pups were weighed on days 1, 4 (before selection), 7, 14 and 21 postpartum and were also observed for developmental steps and for reflexes, as for the F_1 pups. On day 4 postpartum, the size of the litter was randomly adjusted to four males and four females, the remaining animals being terminated. F_0 and F_1 parents were terminated after weaning of most F_1 and F_2 litters, respectively. After weaning, F_2 pups were also killed. At necropsy, F_0, F_1 and F_2 animals were observed macroscopically, and selected organs were collected, weighed and subjected to microscopic examination, together with all macroscopic lesions (for F_0 and F_1 animals only). The male and female mating and fertility indices, female gestation index as well as live birth and viability indices were calculated, together with preimplantation and postimplantation losses.

There were no clinical signs in treated animals, and the test item had no effect on mating, fertility or gestation parameters (F_0 and F_1 generations). During the lactation period, there was no effect on litter size, pup survival, clinical signs, body weight gain, sex ratio or physical or reflex development (F_1 and F_2 pups). At all tested concentrations, the test item induced higher feed consumption and body weight gain in both F_0 and F_1 parents, associated with the pharmacological activity of zilpaterol HCl. Macroscopic and microscopic examinations revealed no treatment-related effect of toxicological relevance (F_0, F_1 and F_2 animals).

In this multigeneration study, the only effect produced by zilpaterol HCl was induction of a slight increase in feed consumption and body weight gain in F_0 and F_1 parent animals at all dose levels. The LOAEL for parental toxicity was 0.9 mg/kg feed (equal to 0.06 mg/kg bw per day), the lowest dose tested, for effects on feed consumption and body weight gain. The NOAEL for offspring and reproductive toxicity was 14.4 mg/kg feed (equal to 0.94 mg/kg bw per day), the highest dose tested (Richard, 1998).

(b) Developmental toxicity

(i) Mice

A GLP-compliant tolerance study in pregnant mice was split into two trials. In the first one, two groups of 10 mated female Swiss CD-1 mice about 13–14 weeks of age were orally (by gavage) administered zilpaterol HCl (purity 99%) in water at either 0 or 300 mg/kg bw per day from days 6 to 18 of pregnancy, day 0 being the day of the presence of the vaginal plug. In the second trial, and based on the results from the first one, two groups of 12 mated females were

administered orally (by gavage) zilpaterol HCl at 0 or 450 mg/kg bw per day from days 6 to 18 of pregnancy. General condition and behaviour of dams were recorded on a daily basis. Body weights were recorded on days 0, 6, 10, 14 and 18, prior to termination. Litter parameters were recorded, and live fetuses were subjected to external examinations.

There was no effect of zilpaterol HCl on general condition or behaviour of the dams or on body weight. Four females died due to dosing incidents, three in the 450 mg/kg bw per day dose group and one in the control group. Pregnancy rates were 100%, and no litter parameters were affected by the test item. No external malformation attributable to the test item was recorded.

The NOAEL for maternal and embryo/fetal toxicity was 450 mg/kg bw per day, the highest dose tested (Fournex & Vannier, 1990).

(ii) Rats

Four groups of 10 female Sprague-Dawley rats weighing 200–225 g were allowed to mate with male Sprague-Dawley rats. The day when vaginal smears revealed the presence of spermatozoa was set as day 0 of pregnancy. Pregnant females were then orally administered zilpaterol HCl (purity 99%) to achieve a dose of 0, 200, 400 or 600 mg/kg bw per day, from day 6 to day 15 of gestation. They were observed for clinical signs, mortality and abortion signs, and body weights of the dams were recorded on days 0, 6, 10, 15, 18 and 20 of gestation. On gestation day 20, animals were killed, and gravid uterus weight, number of corpora lutea, number of early and late resorptions, number of live and dead fetuses as well as number of implantations were recorded. Viable fetuses were observed for external malformations and anomalies. Preimplantation and postimplantation losses were further calculated.

Ten minutes after treatment, some dams from the treated groups showed signs of hypersalivation, with the number of animals affected increasing with the dose. There were no deaths or abortions due to treatment, and the fertility index was similar between treated and untreated females. Females from the 200 mg/kg bw per day dose group showed lower body weight gain than controls. This was considered incidental, because it was not observed in the two highest dose groups. At necropsy, no adverse effects associated with the test item were observed. Slightly increased postimplantation losses were noted in females from the high-dose group, reflecting slight embryotoxicity. One fetus from the 200 mg/kg bw per day dose group and two fetuses from the 600 mg/kg bw per day dose group showed malformations, including amelia of forelimb and right hindlimb; omphalocele, microphthalmia and absence of mandible; and ectrodactyly and cleft lip, respectively.

The LOAEL for maternal toxicity was 200 mg/kg bw per day, based on increased occurrence of salivation at all doses. The NOAEL for embryo/fetal toxicity was 400 mg/kg bw per day, based on the increase in postimplantation loss at 600 mg/kg bw per day, but further studies (see below) were required to clarify whether the compound causes teratogenicity (Bussi, Milone & Fumero, 1987a).

In a GLP-compliant study performed to evaluate the potential embryo and fetal toxicity and teratogenic effects of zilpaterol HCl, 100 mated female Sprague-Dawley rats 12 weeks of age and weighing about 250 g were randomly allocated to four groups of 25 animals. Zilpaterol HCl (purity 99%) was orally administered (by gavage) at a dose of 0, 50, 150 or 450 mg/kg bw per day from day 6 to day 15 of pregnancy (inclusive) in distilled water, day 0 being the day of detection of spermatozoa in the vaginal smear. Dams were observed daily for clinical signs, including abortion, morbidity and mortality. Body weights were recorded periodically throughout the pregnancy. On day 20 of pregnancy, dams were terminated. Fetuses were delivered by caesarean section, and dams were subjected to macroscopic examinations. Litter parameters were recorded. Half of the live fetuses were submitted to soft tissue examination, and the remainder to skeletal examination.

Signs of hypersalivation were observed in all treated dams during the last 2 days of treatment, with dose-related incidence, and one dam died because of a dosing incident. No abortions were observed, and the fertility index was unaffected by zilpaterol HCl. Body weight gain as well as final absolute body weight (excluding gravid uterus) were slightly higher in all treated groups, when compared with the control, being significantly higher in the 50 and 150 mg/kg bw per day dose groups. This was associated with the pharmacological activity of the test item. No embryotoxic effect of zilpaterol HCl was observed, and litter data were similar between groups, sometimes slightly decreased without statistical significance in the treated groups. Fetuses with external malformations were found in all treatment groups without treatment-related incidence. Increased numbers of fetuses with skeletal anomalies were observed in all treated groups, being statistically significant in the high-dose group. Significantly increased numbers of skeletal variants were also found in the treated groups. Visceral variants were also present in all treated groups, with higher frequency in the high-dose group, and were related only to the urinary tract.

The LOAEL for maternal toxicity was 50 mg/kg bw per day, based on the increased occurrence of salivation at all doses. The NOAEL for embryo and fetal toxicity was 150 mg/kg bw per day, based on increased external anomalies at 450 mg/kg bw per day (Bussi, Conz & Fumero, 1988b).

In a GLP-compliant preliminary study aiming to assess the effect of zilpaterol HCl on pregnancy and in utero development in rats, 40 (10 animals per group) female Sprague-Dawley rats about 8–10 weeks old with weights ranging from 170 to 225 g were treated with zilpaterol HCl in distilled water at 0, 400, 600 or 800 mg/kg bw per day from day 6 to day 15 of pregnancy. Dams were then terminated on day 20 and subjected to macroscopic examination. Litter values were determined, and fetuses were examined for gross abnormalities. Treated dams presented signs of hypersalivation, together with a dose-related increase in water consumption. Transient changes in feed intake were observed in treated animals, and body weight gain was transiently lowered in the high-dose group. Litter and fetal weights were reduced in the high-dose group only. The dose of 800 mg/kg bw per day was considered to be the maximum tolerated dose.

In the definitive test, 100 pregnant female Sprague-Dawley rats 8–10 weeks of age and weighing about 161–235 g were allocated to four groups of 25 animals. Zilpaterol HCl was orally administered at a dose of 30, 150 or 750 mg/kg bw per day from day 6 to day 15 of pregnancy, one group receiving the vehicle alone (control group). Dams were observed daily for clinical signs and weighed about every 2 days. Feed and water intakes were determined concomitantly. On day 20 of pregnancy, dams were terminated and subjected to macroscopic examination, and litter values were determined. Fetuses were examined for visceral and skeletal abnormalities. In the 150 and 750 mg/kg bw per day dose groups, some females showed signs of hypersalivation, with the saliva sometimes being stained. The incidence was dose related. From day 8, water intake was increased in treated females, and feed consumption was decreased from day 3 to day 6 at 30 and 150 mg/kg bw per day and from day 6 to day 10 at the high dose. This was associated with reduced body weight gain during the same periods. Thereafter, feed consumption was slightly increased in all treated animals. At necropsy, staining of fur and dirty tails were observed in dams from the high-dose group only. There was no other finding. Litters from the high-dose group only displayed slightly reduced weights, together with a significantly reduced average mean fetal weight. Postimplantation losses were higher in the high-dose group, but not statistically significantly. Sex ratio as well as litter size remained unaffected by the test item. Fetal examination showed significant dose-related increases in skeletal changes in fetuses from all treated dams, including wavy ribs, sternebral anomalies, reduced longbones/digits as well as misshapen scapulas. In fetuses from dams treated with 750 mg/kg bw per day, there was a possible suggestion of visceral changes (two fetuses displaying intraventricular septal defects).

The LOAEL for both maternal toxicity and embryo/fetal toxicity was 30 mg/kg bw per day, the lowest dose tested, based on the results observed (Hughes & Myers, 1990).

In a pivotal, GLP-compliant study aimed at assessing the potential effect of zilpaterol HCl on embryonic and fetal development after oral gavage, mated female Sprague-Dawley rats 12 weeks of age and weighing about 210–230 g were randomly allocated to five groups with 28 animals per group. Zilpaterol HCl (purity 99.2%) was orally administered via gavage in water at a dose of 0, 0.2, 2, 10 or 50 mg/kg bw per day from day 6 to day 15 of pregnancy. Dams were observed daily for clinical signs, morbidity and mortality. Feed and water consumption as well as body weights were recorded periodically throughout the pregnancy. On day 20 of pregnancy, dams were terminated. Fetuses were delivered by caesarean section, and dams were subjected to macroscopic examinations. Litter parameters were recorded. Half of the live fetuses were submitted to soft tissue examination, the remainder to skeletal examination.

Hypersalivation related to the test item was observed in dams from the 2, 10 and 50 mg/kg bw per day dose groups, with a higher incidence in the highest-dose group. No treatment-related deaths or abortions were observed, and there were no findings at necropsy. Feed consumption was higher at 2 mg/kg bw per day during the last treatment days. In the 10 and 50 mg/kg bw per day dose groups,

feed consumption was lower on days 6–9 and then increased thereafter, which was attributed to zilpaterol HCl. Treatment-related increased water consumption was observed in dams from the highest-dose group. Increased body weight was observed in the 2, 10 and 50 mg/kg bw per day dose groups. Feed consumption was increased at 2 mg/kg bw per day; at 10 and 50 mg/kg bw per day, feed consumption decreased transiently and then increased. Zilpaterol HCl displayed no effect on litter data, including mean numbers of corpora lutea, implantation sites and live fetuses, mean fetal weight and postimplantation loss. The test item had no impact on the incidence of external malformations or soft tissue anomalies/malformations when compared with the control group. In the 50 mg/kg bw per day dose group, however, an increased incidence of delayed ossification was observed. This concerned mainly the 5th and 6th sternebrae and the skull. Wavy ribs were also evident. These findings were associated with maternal toxicity of the test item. These results demonstrated slight (2 and 10 mg/kg bw per day) to moderate (50 mg/kg bw per day) maternal toxicity, mainly characterized by increased growth, related to the pharmacological activity of zilpaterol HCl. As a consequence, fetal development was impacted in the highest-dose group only, without embryotoxicity.

The NOAEL for maternal toxicity was 0.2 mg/kg bw per day, based on the change in body weights and the increased occurrence of hypersalivation at 2 mg/kg bw per day. The NOAEL for embyo and fetal toxicity was 10 mg/kg bw per day, based on delayed ossification at 50 mg/kg bw per day (Savary, 1992).

(iii) Rabbits

In a preliminary dose range–finding developmental toxicity study, 32 mated female New Zealand White rabbits weighing about 3.10–3.20 kg were randomly allocated to four groups of eight animals each. Zilpaterol HCl in distilled water was orally administered via gavage at 0, 30, 100 or 300 mg/kg bw per day from day 6 to day 18 of pregnancy, day 0 being the day with a positive vaginal smear. Does were observed daily for clinical signs, morbidity, mortality and possible abortion. Body weights were recorded periodically throughout the pregnancy. On day 29 of pregnancy, animals were terminated and subjected to macroscopic examination. Fetuses were delivered by caesarean section and observed for external malformations. Litter parameters were determined.

No dams displayed clinical signs, and there were no abortions or drug-related mortality. The fertility index was similar between treated and untreated females. Treated does showed a lower body weight increase compared with untreated dams, being more marked in the high-dose group. Necropsy of the animals revealed no findings. Early resorptions were observed in all treated groups, being significantly higher from 100 mg/kg bw per day. In the high-dose group, there were two dams with 100% resorption. There was no dam with dead fetuses. The number of does with only viable fetuses was significantly decreased in the 100 and 300 mg/kg bw per day dose groups. Zilpaterol HCl had no effect on the number of implantations. Early resorption frequency and number of viable fetuses versus number of implantations were significantly higher and lower, respectively, in dams from the high-dose group. External examination of the fetuses revealed no substance-related effect.

The LOAEL for maternal toxicity and embryo/fetal toxicity was 30 mg/kg bw per day, based on effects observed at all doses (Bussi, Milone & Fumero, 1987b).

In a GLP-compliant study aiming to assess the potential effect of zilpaterol HCl on embryonic and fetal development after oral gavage, 80 mated female New Zealand White rabbits weighing about 3.40–3.70 kg were randomly allocated to four groups of 20 animals, to ensure at least 12 gravid does per group. Zilpaterol HCl (purity 99%) in distilled water was orally administered via gavage at a dose of 0, 20, 60 or 180 mg/kg bw per day from day 6 to day 18 of pregnancy, day 0 being the day with a positive vaginal smear. Does were observed daily for clinical signs, morbidity, mortality and possible abortion. Body weights were recorded periodically throughout the pregnancy. On day 29 of pregnancy, animals were terminated and subjected to macroscopic examination. Fetuses were delivered by caesarean section and observed for external malformation. Litter parameters were determined. Fetuses were eviscerated for macroscopic examination of internal organs. One third of the fetuses were decapitated for further cranial examination. The rest of the carcass as well as the carcass from all other fetuses were used for skeletal examination. Observations were classified as malformations, anomalies or variants.

No dams displayed clinical signs. Some cases of death, not substance related, were recorded in all groups. One doe from the control group and another from the 20 mg/kg bw per day dose group were found dead on the day of abortion on days 22 and 25, respectively. One doe from the high-dose group aborted on day 24 and survived. No pathological change was recorded in any of these three does. There was no difference between groups regarding the fertility index. Body weights as well as final body weights (excluding gravid uterus) were slightly higher in treated does, the increase being statistically significant in the 60 mg/kg bw per day dose group. Body weight gain was higher, although not statistically significantly, in all treated groups. This was associated with the pharmacological activity of the test item. At necropsy, there were no macroscopic findings. The frequency of does with either early or late resorptions was similar between groups. In each treated group, there was one doe with 100% postimplantation loss. There was a significantly lower number of corpora lutea compared with number of implantations in treated animals. This was, however, not associated with the test item, because treatment started after the implantation phase. The frequency of early resorption was higher in all treated groups, being statistically significant in the 20 and 60 mg/kg bw per day dose groups. The observed increase in postimplantation losses in the high-dose group was associated with the doe that aborted and survived, although the does that aborted in the control and 20 mg/kg bw per day dose groups died. There was no external malformation observed in fetuses, but one fetus from the 20 mg/kg bw per day dose group presented skeletal malformation (sutural bones near to the fontanella bregmatica, lack of central parietal ossification, complete ossification of frontal sutura, partial ossification of sutura between frontals and parietals, marked ossification of lumbar processes). This was considered incidental. There was no visceral malformation. There was no visceral anomaly, but the number and ratio of skeletal anomalies were higher in all treated groups, reaching statistical significance in the low- and high-dose groups. Skeletal variants were similar in all groups.

The LOAEL for maternal and embryo/fetal toxicity was 20 mg/kg bw per day, based on increased body weight in does and increased ratio of skeletal anomalies at all doses (Bussi, Conz & Fumero, 1988a).

2.2.6 Special studies

(a) Relay pharmacology

As demonstrated by short- and long-term toxicological studies, zilpaterol HCl is a compound of low intrinsic toxicity. The observed effects, mainly on the cardiovascular system, are associated with its β_2-adrenoceptor agonist activity. Thus, a relay pharmacology study was performed in dogs.

A relay pharmacology study was performed to investigate the possible pharmacological effects on blood pressure and heart rate in dogs ingesting meat (muscle, liver) from steers treated orally with zilpaterol HCl. Beagle dogs (13–26 months old and weighing 10–16 kg, groups of one male and one female implanted with transmitters) were fed daily for 5 consecutive days with 200 g of muscle or liver from untreated steers or from steers treated for 50 consecutive days with zilpaterol HCl at 0.2 mg/kg bw per day and slaughtered immediately or 7 days after the end of treatment. A daily complementary ration of 100 g cereal flakes was also provided to all animals. A positive control group of dogs was treated orally with a capsule containing zilpaterol HCl (purity 99.2%) at a dose of 3 μg/kg bw per day, together with muscle or liver from untreated steers and cereal flakes ration.

The ingested dose of zilpaterol HCl when dogs were fed with meat from treated steers was calculated from zilpaterol concentrations determined using a radioimmunoassay method. The impact of the feed on both heart rate and blood pressure was calculated from respective AUC values (global and daily). When dogs were fed with 200 g liver from steers slaughtered immediately after 50 days of daily oral zilpaterol HCl doses of 0.2 mg/kg bw, the ingested doses ranged from 1742 to 1985 ng/kg bw per day. The doses ranged from 252.5 to 275.6 ng/kg bw per day when dogs were fed with 200 g muscle. The corresponding doses when dogs were fed with liver and muscle from steers slaughtered 7 days after treatment was withdrawn were 11.3 ng/kg bw per day with liver and 1.5–1.7 ng/kg bw per day with muscle.

Whatever the feeding regimen (liver or muscle from treated steers slaughtered at the end or 7 days after the end of the treatment), there was no effect on either systolic/diastolic blood pressure or heart rate of dogs. In the positive control group treated with zilpaterol HCl at 3 μg/kg bw per day, a slight increase in the global AUC and daily AUC was observed for heart rate, but not for blood pressure. Thus, ingestion of muscle and liver from zilpaterol-treated steers (50 days at 0.2 mg/kg bw per day) does not induce changes in blood pressure and heart rate in dogs (Vacheron et al., 1995).

2.3 Observations in humans

Four studies were conducted in which human volunteers, either healthy or asthmatic, were administered zilpaterol HCl. The study protocols were approved by ethics committees and carried out according to the guidelines in the Declaration of Helsinki (revised Tokyo version).

Table 10. NOAELs and LOAELs derived from the ascending dose tolerance study in healthy volunteers

End-point	NOAEL (mg/head)	LOAEL (mg/head)
Cardiovascular	–	0.25
- Systolic blood pressure	0.50	1.00 (16.3 mmHg[a])
- Diastolic blood pressure	0.25	0.50 (5 mmHg)
- Stroke volume	0.25	0.50 (25.5 mL)
- Heart rate	–	0.25 (5.75 bpm)
Bronchodilatation	0.25	0.50
Tremor	0.25	0.50
Metabolism (blood glucose)	–	0.25 (2 mmol/L)

bpm: beats per minute; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] 1 mmHg = 133.3 Pa.

Source: Sutton & Budhram (1987)

In the first single-blind and placebo-controlled study, the tolerance of zilpaterol HCl was assessed in eight healthy male volunteers aged 28–55 years given ascending single oral doses of 0, 0.25, 0.50, 1.00 and 2.00 mg zilpaterol HCl dissolved in aqueous vehicle in a four-way cross-over design. The volunteers fasted from midnight on the evening before the study, although water was allowed. Taking individual body weights into consideration, the corresponding doses were 0, 3.6, 7.1, 14.3 and 28.6 μg/kg bw. ECG, arterial blood pressure, cardiac output, stroke volume and ejection velocity were recorded. Blood biochemistry, airway calibre and haematology factors were also recorded.

Heart rate was slightly increased (6 beats per minute [bpm]) at a dose of 0.25 mg and significantly increased at higher doses (44 bpm at 2.00 mg). Systolic blood pressure was significantly increased from 1.00 mg zilpaterol HCl, and airway calibre was significantly increased from 0.50 mg. Blood glucose level was increased in a dose-dependent manner at all doses, from 2 mmol/L at 0.25 mg to 4.81 mmol/L at 2.00 mg, although haematology remained unaffected by the treatments. The occurrence of tremors was evaluated (subjective and inspective), and increased frequency was observed only at a dose of 0.50 mg and above.

The LOAEL was 0.25 mg/person (equal to 3.6 μg/kg bw), based on increased heart rate and blood glucose level observed at all doses (Sutton & Budhram, 1987) (Table 10).

In a second double-blind, comparative placebo-controlled study, 13 healthy male volunteers were administered placebo (*n* = 4) or zilpaterol HCl (*n* = 9) at a dose of 0.25 mg/person, corresponding to 3.68 μg/kg bw, 3 times/day for 7 consecutive days (i.e. a daily dose of 0.75 mg/person or about 11.04 μg/kg bw). One of the nine volunteers was removed from the trial prior to completing the dosing regimen, due to adverse pharmacological effects. This

subject was replaced in the study. Zilpaterol HCl was swallowed, and then blood pressure, heart rate, clinical chemistry parameters and bronchodilatation were measured. Heart rate increased significantly on average by 13 bpm after the first and second doses of the day, then by 6 bpm after the third dose of the day. There was also a significant bronchodilating effect of the test item, and a trend for tremors was also observed.

The LOAEL was 0.25 mg/person (equal to 3.68 μg/kg bw), based on cardiovascular effects, bronchodilatation and tremors (Strauch et al., 1989).

A double-blind, randomized, placebo-controlled cross-over trial was performed in 12 adult asthmatic volunteers given a single oral dose of 0 or 0.25 mg zilpaterol HCl (equal to 0 or 3.85 μg/kg bw). The volunteers fasted before dosing. The aim was to assess both the bronchodilating activity (measured by the forced expiratory volume in 1 second [FEV_1]) and tolerance (heart rate, blood pressure, tremors – clinical examination). Zilpaterol HCl significantly increased bronchodilatation (measured by FEV_1) up to 4 hours after dosing. Heart rate was slightly but significantly increased up to 1.5 hours post-dosing, with no major palpitation or ECG changes. Diastolic blood pressure was slightly but significantly decreased at 0.5 and 1 hour after dosing. Short-lasting finger tremors were seen in 2/12 patients after treatment. Another patient experienced tremulousness without, however, objective tremor of the fingers. A fourth subject who had previously displayed mild tremor following zilpaterol HCl administration showed no tremor increase after drug intake.

A LOAEL of 0.25 mg/person (equal to 3.85 μg/kg bw) for cardiovascular effects, bronchodilatation and tremor was derived from the results of this study (Vivet, 1988).

A double-blind, randomized, placebo-controlled four-way cross-over trial was performed in 11 asthmatic volunteers receiving three single oral doses of 0, 0.05, 0.10 or 0.25 mg zilpaterol HCl (equal to 0, 0.76, 1.52 and 3.79 μg/kg bw, respectively). The volunteers fasted before dosing. Several parameters were assessed during this study, such as bronchodilatation, blood pressure, heart rate, tremor occurrence, blood glucose and potassium. Statistically significant increased bronchodilatation, as measured by FEV_1, was observed after doses of 0.10 or 0.25 mg, from 30 minutes to 4 hours post-dosing. However, at 0.10 mg/person, bronchodilatation was much less pronounced and of shorter duration. The dose of 0.25 mg was associated with a small and asymptomatic increase in heart rate, from 1 to 6 hours after dosing. Blood pressure remained unaffected at all doses. Mild tremor was seen when evaluated clinically by a physician (two patients at 0.05 and 0.10 mg/person and eight patients at 0.25 mg/person). When the tremor was assessed by drawing a line between two parallel sinusoids, no significant differences were observed at any time between the three doses of zilpaterol HCl and the placebo.

The LOAEL was 0.05 mg/person (equal to 0.76 μg/kg bw), based on the increased incidence of tremor (Vivet, 1989) (Table 11).

Table 11. NOAELs and LOAELs derived from the study of bronchodilating activity of zilpaterol HCl in adult asthmatic volunteers

End-point	NOAEL (mg/person)	LOAEL (mg/person)
Cardiovascular	0.10	0.25
- Heart rate	0.10	0.25
- Blood pressure	0.25	–
Bronchodilatation	0.05	0.10
Tremor	–	0.05
Metabolism (blood glucose and potassium)	0.25	–

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
Source: Vivet (1989)

Table 12. NOAELs and LOAELs derived from studies performed in adult healthy and asthmatic volunteers

End-point	NOAEL (mg/person)	LOAEL (mg/person)
Cardiovascular	0.10	0.25
- Systolic blood pressure	0.25	0.50
- Diastolic blood pressure	0.25	0.50
- Stroke volume	0.25	0.50
- Heart rate	0.10	0.25
Bronchodilatation	0.05	0.10
Tremor	–	0.05
Metabolism (blood glucose)	0.25	–

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
Source: Sutton & Budhram (1987); Vivet (1988, 1989); Strauch et al. (1989)

In all studies performed in human volunteers (16 healthy and 23 asthmatic), the observed effects after an oral dose were slight and transitory and typically related to the β_2-adrenergic agonist activity of zilpaterol HCl. Considering all the human data, the LOAEL for zilpaterol was 0.05 mg/person (equal to 0.76 μg/kg bw), the lowest dose tested. An overall NOAEL could not be determined (Table 12).

3. COMMENTS

The Committee considered the results of studies on blood protein binding in vitro, pharmacokinetics, pharmacological effects in vitro and in vivo, acute, short-term and long-term toxicity, genotoxicity, reproductive and developmental toxicity and relay pharmacology, as well as observations in humans. The majority of the studies were performed in accordance with GLP.

3.1 Biochemical data

In rats, dogs and humans, the binding of zilpaterol HCl to serum albumin was low (14–15%). In human red blood cells, the bound fraction was 55%, irrespective of the concentration and the presence of plasma. Zilpaterol HCl was rapidly absorbed via the oral route, with almost 100% bioavailability in rats and dogs. In rats, peak blood concentrations were reached within 1 hour of a single oral gavage dose and by 3–23 hours when given in the diet. Total AUC correlated approximately with dose. The half-life in plasma ranged from 2.4 to 5.5 hours. After repeated dosing, the pharmacokinetic profile in rats was similar to that following a single dose. Systemic exposure was slightly higher in females than in males. In a study in human male volunteers in which zilpaterol HCl was given as a single dose in drinking-water, the time to reach the peak concentration was 1 hour, and the half-life in plasma was 4–5 hours, independent of the dose.

In studies in rats using radiolabelled zilpaterol HCl, approximately 50–55% of the dose was eliminated in the urine and 40–42% in the faeces over 8 days. In urine, unchanged zilpaterol predominated, accounting for at least half of the radioactivity, with the metabolites deisopropyl zilpaterol, acetylated deisopropyl zilpaterol, hydroxy-zilpaterol, a glucuronide conjugate of hydroxy-zilpaterol and two unidentified metabolites each accounting for between 2% and 20% of the radioactivity. Deisopropyl zilpaterol was the main metabolite present. In faeces, parent compound accounted for 10–45% of the radioactivity present and hydroxy-zilpaterol for 60–80%, with a small fraction present as deisopropyl zilpaterol. After 8 days, residues in liver and carcass were less than 0.1% and less than 1%, respectively, of the administered dose; radioactivity could not be detected in fat or muscle.

In studies using rat hepatic microsomal fractions, unchanged zilpaterol was present, together with deisopropyl zilpaterol and hydroxy-zilpaterol. The metabolites were shown to be largely the products of cytochromes P450; deisopropyl zilpaterol was formed preferentially by members of the CYP1A subfamily, whereas hydroxy-zilpaterol was formed less specifically, possibly involving members of the CYP2B subfamily. Zilpaterol HCl did not induce drug-metabolizing enzymes.

Many in vitro, ex vivo and in vivo studies have clearly demonstrated the β_2-agonist effect of zilpaterol HCl and its main metabolite, deisopropyl zilpaterol (as free base or hydrochloride form). These effects are manifested as contraction of cardiac muscle and relaxation of the smooth muscles of the vasculature and the bronchi. There is little or no affinity of zilpaterol or its main metabolite for α_1- or α_2-adrenoceptors or for dopaminergic receptors D_1 and D_2. Studies on guinea-pig lung membranes have demonstrated that zilpaterol HCl and deisopropyl zilpaterol are only partial agonists at the β_2-adrenoceptor in terms of adenylyl cyclase activation.

In rat studies, zilpaterol HCl given intravenously to anaesthetized, pithed animals induced a dose-dependent decrease in diastolic blood pressure from 0.01 mg/kg bw, with a maximum effect observed at 0.1 mg/kg bw, and an increase in heart rate from 0.3 mg/kg bw. Deisopropyl zilpaterol (in either its free base or hydrochloride form) caused a dose-dependent decrease in diastolic blood pressure from 0.1 mg/kg bw, with a steady-state maximum effect observed at 0.3 mg/kg bw, but had no effect on the heart rate. These data indicate that deisopropyl zilpaterol

has 10-fold lower β_2-agonist activity on the cardiovascular system, compared with the parent compound. Use of specific antagonists for β_1- and β_2-adrenoceptors demonstrated that the effect of the compounds on blood pressure was mediated by β_2-adrenoceptors, whereas the activity of zilpaterol HCl on the heart was associated with the β_1-adrenoceptor.

3.2 Toxicological data

A comprehensive set of toxicological studies was performed, mainly in compliance with GLP, in both rodent (mice, rat) and non-rodent species (dog, Cynomolgus monkey and microswine). The purity of the test item was higher than 90% in all studies. The pivotal toxicological studies are summarized in Table 13.

Table 13. Studies relevant to risk assessment

Species/study type (route)	Doses (mg/kg bw per day[a])	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day[a])
Mouse				
Eighteen-month study of toxicity and carcinogenicity (gavage)	0, 0.01, 0.02, 0.05, 0.25	Increased haemoglobin, red blood cell counts, haematocrit; decreased numbers of platelets, absolute neutrophil counts and monocyte numbers	0.02	0.05
Rat				
Thirteen-week study of toxicity (gavage)	0, 0.05, 0.5, 1	Decreased mean heart rate	–	0.05[b]
Two-year study of toxicity and carcinogenicity (dietary)	0, 0.025, 0.05, 0.125, 0.25	Increased weight of ovaries with increased incidence of ovarian cysts; increased incidence of ovarian leiomyomas	0.05	0.125
Two-generation reproductive toxicity study (dietary)	0, 0.06, 0.23, 0.94 (F_0 males); 0, 0.10, 0.40, 1.61 (F_0 females); 0, 0.08, 0.32, 1.26 (F_1 males); 0, 0.12, 0.45, 1.77 (F_1 females)	Parental toxicity: Increased body weight and feed consumption	–	0.06[b]
		Reproductive and offspring toxicity: No effects	0.94[c]	–
Developmental toxicity study (gavage)	0, 0.2, 2, 10, 50	Maternal toxicity: Increased occurrence of hypersalivation, increased body weight gain	0.2	2
		Embryo and fetal toxicity: Delayed ossification	10	50

Table 13 (continued)

Species/study type (route)	Doses (mg/kg bw per day[a])	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day[a])
Rabbit				
Developmental toxicity study (gavage)	0, 20, 60, 180	Maternal toxicity: Increased body weight	–	20[b]
		Embryo and fetal toxicity: Increased incidence of skeletal anomalies	–	20[b]
Dog				
Thirty-day study of toxicity (capsule)	0, 0.5, 5, 50	Increased peripheral vasodilatation, increased heart rate, decreased blood pressure	–	0.5[b]
Microswine				
Thirteen-week study of toxicity (gavage)	0, 0.001, 0.05, 1, 10	No effects	10[c]	–
Cynomolgus monkey				
Four-week study of toxicity (gavage)	0, 0.005, 0.01, 0.05, 5	Increased heart rate with a decreased QT interval	0.01	0.05
Human				
Four-way cross-over design, single-blind and placebo-controlled study (healthy adult)	0, 3.6, 7.1, 14.3, 28.6 μg/kg bw	Increased heart rate and blood glucose level	–	3.6 μg/kg bw[b]
Double-blind, randomized, placebo-controlled four-way cross-over study (asthmatic patient)	0, 0.76, 1.52, 3.79 μg/kg bw	**Increased incidence of tremor**	–	**0.76 μg/kg bw**[b,d]

[a] Except where otherwise noted.
[b] Lowest dose tested.
[c] Highest dose tested.
[d] Pivotal study value (Vivet, 1989).

Zilpaterol HCl and deisopropyl zilpaterol were of relatively low acute toxicity by the oral route. The oral LD_{50} values of zilpaterol HCl were about 1100 mg/kg bw in rats and about 500 mg/kg bw in mice. The main metabolite, deisopropyl zilpaterol free base, had an oral LD_{50} value in mice of about 1000 mg/kg bw, indicating that its acute toxicity is about half that of the parent compound. Zilpaterol HCl

is not irritating to skin and is slightly irritating to eyes. It is not a skin sensitizer in guinea-pigs.

In short-term toxicity studies performed in mice, rats, dogs, microswine and Cynomolgus monkeys, the main effects observed were those classically associated with β-adrenoceptor agonists, such as cardiovascular effects (increased heart rate and decreased diastolic blood pressure). In parallel, increased body weight associated with increased feed consumption was also observed.

In mice, zilpaterol HCl administered orally for 4 weeks at a dose of 0, 0.2, 2, 4 or 40 mg/kg feed (equal to 0, 0.046, 0.46, 0.895 and 8.867 mg/kg bw per day for males and 0, 0.047, 0.483, 0.986 and 9.601 mg/kg bw per day for females, respectively) induced no treatment-related effects except body weight change. There was a small but statistically significant increase in body weight in female mice at the highest dose, but body weight had returned to control values by the end of treatment. The NOAEL was 4 mg/kg feed (equal to 0.986 mg/kg bw per day), based on increased body weight in female mice.

In a 30-day study, rats were given zilpaterol HCl at a dose of 0, 1, 10 or 100 mg/kg bw per day by oral gavage. Body weight was increased at 10 and 100 mg/kg bw per day in males and at 100 mg/kg bw per day in females, with no change in feed consumption. A dose-dependent decrease in heart rate was observed at all doses in female rats. In male rats, total leukocyte count was increased at the highest dose, and activated partial thromboplastin time was slightly decreased at all doses. Decreased prothrombin time was observed at 10 and 100 mg/kg bw per day in females. Microscopic examination revealed no treatment-induced lesions. The LOAEL was 1 mg/kg bw per day, the lowest dose tested, based on decreased heart rate in females and decreased activated partial thromboplastin time in males.

In a 90-day study, rats were given zilpaterol HCl at a dose of 0, 1, 10 or 100 mg/kg bw per day by oral gavage. At 10 and 100 mg/kg bw per day, dose-dependent hypersalivation in both sexes was observed. Statistically significant increases in body weight gain were seen in females at 10 and 100 mg/kg bw per day and in males at 100 mg/kg bw per day, and an increase in feed consumption in males was observed at 10 and 100 mg/kg bw per day. There was a slight increase in blood urea at all doses in both sexes, but it was not accompanied by histopathological changes. Increased plasma creatinine was found at 10 and 100 mg/kg bw per day in males and females on week 5, but levels returned to normal at 10 mg/kg bw per day in males and at 100 mg/kg bw per day in females on week 13. The LOAEL was 1 mg/kg bw per day, the lowest dose tested, based on increased blood urea. In a follow-up study, when zilpaterol HCl was administered to rats at 0, 0.05, 0.5 or 1 mg/kg bw per day for 4 weeks, body weight was significantly increased at the highest dose without significant change in feed consumption in female rats. The NOAEL was 0.5 mg/kg bw per day, based on body weight change in females.

In a further short-term study, when rats were given zilpaterol HCl for 13 weeks at 0, 0.05, 0.5 or 1 mg/kg bw per day by oral gavage, increased body weight was observed in males and females at 0.5 and 1 mg/kg bw per day. Significantly lower mean heart rates were observed at all doses in females and at the highest dose in males on week 3, 4, 8 or 13. This was associated with longer

PQ intervals at all doses in females and at 0.5 or 1 mg/kg bw per day in males. Lower systolic blood pressure was observed at 0.5 or 1 mg/kg bw per day on week 3 or 4 in males. A LOAEL of 0.05 mg/kg bw per day, the lowest dose tested, was derived from the results of this study.

In a 7-day study, dogs were given zilpaterol HCl at 100 mg/kg bw per day in a capsule by oral gavage. Clinical signs included frequent vomiting, reduced motor activity, apathy, vasodilatation and diarrhoea, accompanied by reduced body weight, changes in haematology and blood biochemistry, and morphological changes in the liver.

In a 30-day study, dogs were given zilpaterol HCl at 0, 0.5, 5 or 50 mg/kg bw per day in a capsule by oral gavage. A dose-dependent peripheral vasodilatation was observed at all doses. Reduced blood pressure and increased heart rate were observed at 1 hour, but blood pressure and heart rate had returned to normal values by 24 hours after dosing at all doses. Irregular vomiting and reduced motor activity were found at 5 and 50 mg/kg bw per day. The LOAEL was 0.5 mg/kg bw per day, the lowest dose tested, based on peripheral vasodilatation, increased heart rate and decreased blood pressure.

Microswine (one of each sex per group) were given increasing doses of zilpaterol HCl of 0.05, 0.5, 1, 5 and 10 mg/kg bw per day by oral gavage, each dose being given for 4 days with a 3-day withdrawal period between doses. A slight increase in heart rate associated with a decreased QT interval, without any change in blood pressure, was found from 0.5 mg/kg bw per day in a male and at 10 mg/kg bw per day in a female. In a 4-week toxicity study in which microswine were given zilpaterol HCl at 0, 0.005, 0.01, 0.05 or 5 mg/kg bw per day by oral gavage, the NOAEL was 5 mg/kg bw per day, the highest dose tested, as no treatment-related adverse effects were found. In a 13-week toxicity study in which microswine were given zilpaterol HCl at 0, 0.001, 0.05, 1 or 10 mg/kg bw per day by oral gavage, non-statistically significant increases in body weight were observed only in males at 1 and 10 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day, the highest dose tested.

Cynomolgus monkeys (one of each sex per group) were given increasing doses of zilpaterol HCl of 0.05, 0.5, 1, 5 and 10 mg/kg bw per day by oral gavage, each dose being given for 4 days with a 3-day withdrawal period between doses. A slight increase in heart rate associated with a decreased QT interval, without any change in blood pressure or dose–response relationship, was found at all doses.

In a 4-week toxicity study in which Cynomolgus monkeys were given zilpaterol HCl at 0, 0.005, 0.01, 0.05 or 5 mg/kg bw per day by oral gavage, a reduction in blood pressure, accompanied by an increased heart rate with an associated decrease in QT interval, was observed at 0.05 and 5 mg/kg bw per day. The NOAEL was 0.01 mg/kg bw per day, based on cardiovascular effects.

In a chronic toxicity and carcinogenicity study in female mice, zilpaterol HCl was given orally by gavage at doses of 0, 10, 20, 50 and 250 μg/kg bw per day for 18 months. Significantly increased haemoglobin, associated with significantly increased red blood cell counts and haematocrit, was found at 50 and 250 μg/kg bw per day. There were also significantly decreased numbers of platelets, absolute

neutrophil counts and monocyte numbers at 50 and 250 μg/kg bw per day. There were no neoplastic or preneoplastic findings. The NOAEL was 20 μg/kg bw per day, based on haematological changes.

In a chronic toxicity study in rats, zilpaterol HCl was given in the feed at concentrations adjusted to achieve doses of 0, 25, 50, 125 and 250 μg/kg bw per day for 52 weeks. There were slight decreases in heart rate, without impact on waveform traces, at 125 and 250 μg/kg bw per day and slight increases in systolic blood pressure at the highest dose in both sexes. These effects were fully reversible after a 4-week recovery period. The NOAEL was 50 μg/kg bw per day, based on the decrease in heart rate.

In a chronic toxicity and carcinogenicity study in rats, zilpaterol HCl was given in the feed at concentrations adjusted to achieve doses of 0, 25, 50, 125 and 250 μg/kg bw per day for 104 weeks. Slightly reduced body weight gains were found in males and females at the highest dose. The number of surviving male animals was significantly reduced at the highest dose. A marked increase in the weight of ovaries with increased incidence of ovarian cysts was observed at 125 and 250 μg/kg bw per day. An increased incidence of ovarian leiomyomas of the suspensory ligament was also found in the two highest dose groups. The NOAEL was 50 μg/kg bw per day, based on the increased ovarian weight with increased incidence of cysts and the increased incidence of leiomyomas.

Ovarian leiomyomas are benign tumours known to be related to the use of β_2-adrenoceptor agonists in rodents. The proliferation of the mesovarian smooth muscle is considered to be an adaptive physiological response to prolonged stimulation of the β-receptors, with muscle relaxation as a consequence. The occurrence of this neoplastic lesion in rodents was reported to be blocked by concomitant treatment with propranolol, a β_2-adrenoceptor antagonist. Little or no relaxant response to β_2-agonists can be demonstrated in the uteri of non-pregnant women. There is no evidence in humans of any increased incidence of smooth muscle tumours such as leiomyomas among users of β-agonists. Thus, the occurrence of ovarian leiomyomas in rats treated with zilpaterol HCl may represent a species-specific effect, and it is considered unlikely that oral exposure in humans would result in ovarian leiomyomas.

In an adequate range of tests of genotoxic activity, mainly performed under GLP-compliant conditions, zilpaterol HCl showed no evidence of genotoxic potential in microorganisms, in cultured mammalian cells or in vivo in mice. Like the parent compound, its main metabolite deisopropyl zilpaterol showed no evidence of genotoxicity in an adequate range of tests.

Considering the absence of any evidence of genotoxicity in vitro and in vivo, together with the likelihood that induction of mesovarian leiomyomas observed with zilpaterol HCl is associated with species-specific pharmacological β_2-adrenergic activity, the Committee concluded that zilpaterol HCl is unlikely to pose a carcinogenic risk to humans.

In a one-generation reproductive toxicity study in rats, zilpaterol HCl was given in the feed at a concentration of 0, 0.9, 3.6 or 14.4 mg/kg (equal to 0, 0.06, 0.23 and 0.94 mg/kg bw per day for males and 0, 0.10, 0.40 and 1.61 mg/kg bw per

day for females, respectively) from 15 days prior to and during mating, pregnancy and lactation. There were no effects attributable to the treatment on F_0 reproduction or F_1 litters, apart from significant increases in body weight in all treated males, mainly during the 1st week of treatment, and in females during the entire treatment period at the highest dose. Based on body weight increases, the LOAEL for parental toxicity was 0.9 mg/kg feed (equal to 0.06 mg/kg bw per day in F_0 males), the lowest dose tested. The NOAEL for offspring and reproductive toxicity was 14.4 mg/kg feed (equal to 0.94 mg/kg bw per day), the highest dose tested.

In a two-generation reproductive toxicity study in rats, zilpaterol HCl was given in the feed at a concentration of 0, 0.9, 3.6 or 14.4 mg/kg from 71 days prior to mating and until weaning in F_0 or F_1 males and from 15 days before mating until the end of lactation for F_0 or F_1 females (equal to 0, 0.06, 0.23 and 0.94 mg/kg bw per day for F_0 males; 0, 0.10, 0.40 and 1.61 mg/kg bw per day for F_0 females; 0, 0.08, 0.32 and 1.26 mg/kg bw per day for F_1 males; and 0, 0.12, 0.45 and 1.77 mg/kg bw per day for F_1 females). There were no effects attributable to the treatment on F_0 or F_1 reproduction or on F_1 and F_2 litters, apart from slightly higher feed consumption and body weight gain in both F_0 and F_1 parents at all doses. The LOAEL for parental toxicity was 0.9 mg/kg feed (equal to 0.06 mg/kg bw per day), the lowest dose tested, for effects on feed consumption and body weight gain. The NOAEL for offspring and reproductive toxicity was 14.4 mg/kg feed (equal to 0.94 mg/kg bw per day), the highest dose tested.

In a limited but GLP-compliant developmental toxicity study in mice, zilpaterol HCl was given at a dose of 0, 300 or 450 mg/kg bw per day by oral gavage from days 6 to 18 of pregnancy. There were no effects on general condition or behaviour of the dams or on body weight. No litter parameters were affected, and there were no malformations attributable to zilpaterol HCl. The NOAEL for maternal and embryo/fetal toxicity was 450 mg/kg bw per day, the highest dose tested.

In a series of three developmental toxicity studies in rats, zilpaterol HCl was given at doses ranging from 30 to 750 mg/kg bw per day by oral gavage from days 6 to 15 of gestation. A dose-dependent increase in maternal salivation was seen in all three studies (from 50 mg/kg bw per day). At the two highest doses used (600 and 750 mg/kg bw per day), there was an increase in postimplantation loss and a reduction in fetal weight, respectively. In two of the studies, there were significant increases in the numbers of fetuses with skeletal or visceral anomalies and/or variants, reaching statistical significance at the highest doses (450 and 750 mg/kg bw per day).

In a developmental toxicity study in rats using lower doses, zilpaterol HCl was given at a dose of 0, 0.2, 2, 10 or 50 mg/kg bw per day by oral gavage from days 6 to 15 of pregnancy. Hypersalivation was observed at 2, 10 and 50 mg/kg bw per day, with a more marked incidence in the high-dose group. Maternal body weight gains were increased in a dose-dependent manner from 2 mg/kg bw per day. Feed consumption was increased at 2 mg/kg bw per day; at 10 and 50 mg/kg bw per day, feed consumption decreased transiently and then increased. Water consumption was increased at 50 mg/kg bw per day. The incidences of delayed ossification of some bones (5th and 6th sternebrae, skull) and of wavy ribs were higher at

50 mg/kg bw per day. The NOAEL for maternal toxicity was 0.2 mg/kg bw per day, based on hypersalivation and increased body weight gain. The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on delayed ossification.

In a developmental toxicity study in rabbits, zilpaterol HCl was given at a dose of 0, 20, 60 or 180 mg/kg bw per day by oral gavage from days 6 to 18 of pregnancy. Maternal body weights as well as final body weights (excluding gravid uterus) were slightly higher at all doses, with statistical significance at 60 mg/kg bw per day. The incidence of skeletal anomalies was increased in all treated groups, reaching statistical significance in the low- and high-dose groups. The LOAEL for maternal and embryo/fetal toxicity was 20 mg/kg bw per day, the lowest dose tested.

In a relay pharmacology study in dogs, one male and one female were fed daily for 5 days with either 200 g liver or 200 g muscle from steers treated orally with a zilpaterol HCl dose of 0.2 mg/kg bw per day for 50 days and then slaughtered immediately after the end of the treatment or 7 days later. A positive control group was fed with 200 g muscle from untreated cattle together with zilpaterol HCl (by oral capsule) at 3 μg/kg bw per day. The achieved doses of zilpaterol HCl were 1742–1985 ng/kg bw per day for dogs fed with 200 g liver from steers killed immediately after the 50 days of treatment and 252.5–275.6 ng/kg bw per day for dogs fed with 200 g muscle from steers killed immediately after the 50 days of treatment. The achieved doses of zilpaterol HCl were 11.3 ng/kg bw per day for dogs fed with 200 g liver and 1.5–1.7 ng/kg bw per day for dogs fed with 200 g muscle from steers killed 7 days after the 50 days of treatment. There was no effect of this treatment regimen on either heart rate or blood pressure. In the positive controls treated with 3 μg/kg bw per day, a marginal effect on heart rate, but not on blood pressure, was observed.

3.3 Observations in humans

Studies were conducted in which human volunteers, either healthy or asthmatic, were administered zilpaterol HCl. The study protocols were approved by ethics committees, and the studies were carried out according to the guidelines in the Declaration of Helsinki (revised Tokyo version). The pivotal studies in humans are summarized in Table 13 above.

In a four-way cross-over design, single-blind and placebo-controlled study involving eight healthy male volunteers, ascending single doses of 0, 0.25, 0.50, 1.00 and 2.00 mg/person (equal to 0, 3.6, 7.1, 14.3 and 28.6 μg/kg bw, respectively) were given orally in aqueous vehicle. The volunteers fasted before dosing. The dose of 0.25 mg/person slightly increased heart rate, and higher doses provoked significant increases in heart rate. Airway calibre was significantly increased at 0.50, 1.00 and 2.00 mg/person, and systolic blood pressure was significantly increased at 1.00 and 2.00 mg/person. Haematology remained unaffected, but blood glucose was increased in a dose-dependent manner at all dose levels. Tremor frequency was significantly increased from 0.50 mg/person (equal to 7.1 μg/kg bw). Based on this study, the LOAEL was 0.25 mg/person (equal to 3.6 μg/kg bw), the lowest dose tested, for cardiovascular effects and higher blood glucose levels.

In a double-blind, comparative placebo-controlled study, 13 healthy male volunteers were given placebo or zilpaterol HCl at a dose of 0.25 mg/person (equal to 3.68 μg/kg bw), 3 times a day for 7 consecutive days (i.e. a daily dose of 0.75 mg/person or about 11.04 μg/kg bw). This dosing regimen had significant effects on the cardiovascular system, tremor and bronchodilatation.

A double-blind, randomized, placebo-controlled cross-over trial was performed in 12 adult asthmatic volunteers given a single oral dose of 0 or 0.25 mg zilpaterol HCl per person (equal to 0 or 3.85 μg/kg bw). The volunteers fasted before dosing. Zilpaterol HCl significantly increased bronchodilatation (measured by FEV_1) up to 4 hours after dosing. Heart rate was slightly but significantly increased up to 1.5 hours post-dosing, with no major palpitation or ECG changes. Diastolic blood pressure was slightly but significantly decreased at 0.5 and 1 hour after dosing. Short-lasting finger tremors were seen in 2/12 patients after treatment. The LOAEL was 0.25 mg/person (equal to 3.85 μg/kg bw), the only dose tested, for bronchodilatation, tremors and cardiovascular effects.

In a double-blind, randomized, placebo-controlled, four-way cross-over trial performed in 11 asthmatic volunteers, three single oral zilpaterol HCl doses of 0, 0.05, 0.10 or 0.25 mg/person (equal to 0, 0.76, 1.52 and 3.79 μg/kg bw, respectively) were tested. The volunteers fasted before dosing. Bronchodilatation was observed at 0.10 and 0.25 mg/person, remaining slight and transient, although only the highest dose induced a slight but significant change in heart rate. Blood pressure remained unaffected at all doses. Mild tremor was seen when evaluated clinically by a physician (two patients at 0.05 and 0.10 mg/person and eight patients at 0.25 mg/person). When the tremor was assessed by drawing a line between two parallel sinusoids, no significant differences were observed at any time between the three doses of zilpaterol HCl and the placebo. The LOAEL was 0.05 mg/person (equal to 0.76 μg/kg bw), based on the clinical assessment of tremor.

In all studies performed in human volunteers (16 healthy and 23 asthmatic), the observed effects after an oral dose were slight and transitory and typically related to the β_2-adrenergic agonist activity of zilpaterol HCl. Considering all the human data, the LOAEL for zilpaterol HCl was 0.05 mg/person (equal to 0.76 μg/kg bw). An overall NOAEL could not be identified.

4. EVALUATION

The Committee considered tremors observed in humans, which were consistent with the compound's β_2-adrenergic agonist activity, as the most relevant adverse effect for establishing an ADI for zilpaterol HCl. The LOAEL for tremor was 0.05 mg/person (equal to 0.76 μg/kg bw); the effect was slight at this dose. The Committee established an ADI of 0–0.04 μg/kg bw per day by applying an uncertainty factor of 20, comprising a default uncertainty factor of 10 for human individual variability and an additional uncertainty factor of 2 to account for use of a LOAEL for a slight effect instead of a NOAEL. The Committee noted that the ADI is based on an acute effect. The Committee also noted that the upper bound of the ADI provides a margin of safety of at least 1250 with respect to the NOAEL of 50 μg/kg bw per day for the formation of leiomyomas in rats.

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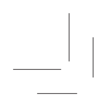
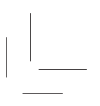
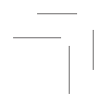
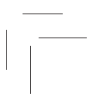
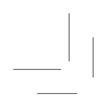
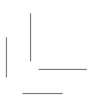
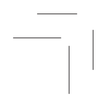
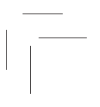





ANNEXES

ANNEX 1

REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

1. *General principles governing the use of food additives* (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. *Procedures for the testing of intentional food additives to establish their safety for use* (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. *Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)* (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. I. *Antimicrobial preservatives and antioxidants*, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. *Specifications for identity and purity of food additives (food colours)* (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. II. *Food colours*, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. *Evaluation of the carcinogenic hazards of food additives* (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. *Evaluation of the toxicity of a number of antimicrobials and antioxidants* (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. *Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents* (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. *Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants* (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. *Specifications for identity and purity and toxicological evaluation of food colours*. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases* (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
12. *Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases*. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.
13. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.
14. *Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents* (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.
15. *Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents*. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.
16. *Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.
17. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics* (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.
18. *Specifications for the identity and purity of some antibiotics*. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.
19. *Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances* (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.
20. *Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances*. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.
21. *Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.
22. *Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents* (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. *Toxicological evaluation of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.
24. *Specifications for the identity and purity of some extraction solvents and certain other substances*. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.
25. *A review of the technological efficacy of some antimicrobial agents*. FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.
26. *Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants* (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.
27. *Toxicological evaluation of some enzymes, modified starches, and certain other substances*. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.
28. *Specifications for the identity and purity of some enzymes and certain other substances*. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.
29. *A review of the technological efficacy of some antioxidants and synergists*. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.
30. *Evaluation of certain food additives and the contaminants mercury, lead, and cadmium* (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.
31. *Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate.* FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.
32. *Toxicological evaluation of certain food additives with a review of general principles and of specifications* (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).
33. *Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents*. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.
34. *Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers*. FAO Food and Nutrition Paper, No. 4, 1978.
35. *Evaluation of certain food additives* (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.
36. *Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.

37. *Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives.* FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.
38. *Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances*. (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.
39. *Toxicological evaluation of some food colours, thickening agents, and certain other substances*. FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.
40. *Specifications for the identity and purity of certain food additives*. FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.
41. *Evaluation of certain food additives* (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.
42. *Toxicological evaluation of certain food additives*. WHO Food Additives Series, No. 10, 1976.
43. *Specifications for the identity and purity of some food additives*. FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.
44. *Evaluation of certain food additives* (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.
45. *Summary of toxicological data of certain food additives*. WHO Food Additives Series, No. 12, 1977.
46. *Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others*. FAO Nutrition Meetings Report Series, No. 57, 1977.
47. *Evaluation of certain food additives and contaminants* (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.
48. *Summary of toxicological data of certain food additives and contaminants*. WHO Food Additives Series, No. 13, 1978.
49. *Specifications for the identity and purity of certain food additives*. FAO Food and Nutrition Paper, No. 7, 1978.
50. *Evaluation of certain food additives* (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.
51. *Toxicological evaluation of certain food additives*. WHO Food Additives Series, No. 14, 1980.
52. *Specifications for identity and purity of food colours, flavouring agents, and other food additives*. FAO Food and Nutrition Paper, No. 12, 1979.
53. *Evaluation of certain food additives* (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.
54. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 15, 1980.

55. *Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives)*. FAO Food and Nutrition Paper, No. 17, 1980.
56. *Evaluation of certain food additives* (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.
57. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 16, 1981.
58. *Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives)*. FAO Food and Nutrition Paper, No. 19, 1981.
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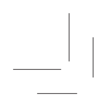
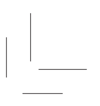
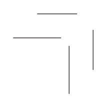
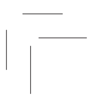
ANNEX 2

ABBREVIATIONS USED IN THE MONOGRAPHS

ADI	acceptable daily intake
AIC	Akaike's Information Criterion
ALAT	alanine aminotransferase
AP	alkaline phosphatase
ASAT	aspartate aminotransferase
ATCC	American Type Culture Collection
AUC	area under the plasma concentration–time curve
BMD	benchmark dose
BMD_{10}	benchmark dose for an extra 10% risk compared with the modelled background incidence
BMDL	lower 95% confidence limit on the benchmark dose
$BMDL_{10}$	lower 95% confidence limit on the benchmark dose for an extra 10% risk compared with the modelled background incidence
BMDS	benchmark dose software
bpm	beats per minute
BSE	bovine spongiform encephalopathy
bST	bovine somatotropin
BTSCC	bulk tank milk somatic cell count
bw	body weight
CCα	decision limit
CCβ	detection capability
cfu	colony-forming units
CHO	Chinese hamster ovary
CI	confidence interval; Colour Index
CLSI	Clinical and Laboratory Standards Institute
C_{max}	peak plasma concentration
CYP	cytochrome P450
DBP	diastolic blood pressure
DIM	days in milk
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DWC	Don Whitley Scientific Ltd culture collection
ECG	electrocardiogram; electrocardiographic
ECLIA	electrochemiluminescent immunoassay
ED	effective dose
ELISA	enzyme-linked immunosorbent assay
eq	equivalent
F	female
F_0	parental generation
F_1	first filial generation
F_2	second filial generation
F_3	third filial generation
FAO	Food and Agriculture Organization of the United Nations

FEV_1	forced expiratory volume in 1 second
GL36	Guideline 36 (VICH)
GLP	good laboratory practice
HGPRT	hypoxanthine–guanine phosphoribosyltransferase
HPLC	high-performance liquid chromatography
HR	heart rate
IC_{50}	median inhibitory concentration
IGF	insulin-like growth factor
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBP-1	insulin-like growth factor binding protein 1
IGFBP-3	insulin-like growth factor binding protein 3
IU	International Unit
iv	intravenously
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC_{50}	median lethal concentration
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LD_{50}	median lethal dose
LOAEL	lowest-observed-adverse-effect level
M	male
MIC	minimum inhibitory concentration
MIC_{50}	minimum concentration required to inhibit the growth of 50% of organisms
MIC_{calc}	minimum inhibitory concentration derived from the lower 90% confidence limit for the mean MIC_{50} of the relevant genera for which the drug is active
MOE	margin of exposure
MRL	maximum residue limit
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAHMS	National Animal Health Monitoring System (USA)
NCCLS	National Committee for Clinical Laboratory Standards (USA)
NCIMS	National Conference on Interstate Milk Shipments (USA)
NCTC	National Collection of Type Cultures (USA)
NHPP	National Hormone and Peptide Program (USA)
NMDA	*N*-methyl-D-aspartate
NMDRD	National Milk Drug Residue Database (USA)
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NR	no results obtained
NS	not significant
OECD	Organisation for Economic Co-operation and Development
OR	odds ratio
pA_2	measure of the affinity of the antagonist for its receptor
PBS	phosphate-buffered saline
rbST	recombinant bovine somatotropin
RCA	reinforced clostridial agar

RCS	reticulum cell sarcoma
rh	recombinant human
rmST	recombinant mouse somatotropin
RNA	ribonucleic acid
RR	risk ratio
rrST	recombinant rat somatotropin
S9	9000 × *g* supernatant fraction from rat liver homogenate
sc	subcutaneously
SCC	somatic cell count
SD	Sprague-Dawley; standard deviation
SEM	standard error of the mean
$t_{½}$	half-life
TJA	tomato juice agar
T_{max}	time to reach C_{max}
TRR	total radioactive residues
USA	United States of America
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
WC	Wilkins-Chalgren agar
WCB	Wilkins-Chalgren agar containing 5% (w/v) defibrinated sheep's blood
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight

ANNEX 3

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Geneva, 5–14 November 2013

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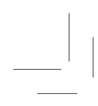
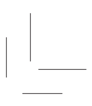
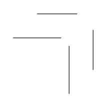
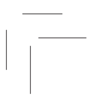

ANNEX 4

RECOMMENDATIONS ON COMPOUNDS ON THE AGENDA AND FURTHER INFORMATION REQUIRED

Derquantel (anthelminthic agent)

Acceptable daily intake: The Committee maintained the ADI of 0–0.3 μg/kg body weight established at its seventy-fifth meeting (WHO TRS No. 969, 2011).

Estimated dietary exposure: There were insufficient data to calculate an estimated daily intake (EDI), and the theoretical maximum daily intake (TMDI) approach was used. Using the model diet and the marker residue to total residue ratio approach with the maximum residue limits (MRLs) recommended, the estimated dietary exposure is 6.8 μg/person, which represents approximately 38% of the upper bound of the ADI.

Residue definition: Derquantel

Recommended maximum residue limits (MRLs)

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Muscle (μg/kg)
Sheep	7.0	0.4	0.8	0.3

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Emamectin benzoate (antiparasitic agent)

Acceptable daily intake: The Committee confirmed the ADI of 0–0.0005 mg/kg body weight established by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2011, based on an overall no-observed-adverse-effect level (NOAEL) of 0.25 mg/kg body weight per day for neurotoxicity from 14- and 53-week studies in dogs, supported by an overall NOAEL of 0.25 mg/kg body weight per day from 1- and 2-year studies in rats. An uncertainty factor of 500 was applied to the NOAEL, which includes an additional uncertainty factor of 5 to account for the steep dose–response curve and irreversible histopathological effects in neural tissues at the lowest-observed-adverse-effect level (LOAEL)

	in dogs, as used by JMPR and confirmed by the current Committee.
Estimated dietary exposure:	The EDI is 11 μg/person per day, which represents approximately 37% of the upper bound of the ADI.
Residue definition:	Emamectin B1a

Recommended maximum residue limits (MRLs)

Species	Muscle (μg/kg)	Fillet[a] (μg/kg)
Salmon	100	100
Trout	100	100

[a] Muscle plus skin in natural proportion.

The Committee extended the MRLs for muscle and fillet in salmon to trout.

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Gentian violet (antibacterial, antifungal and anthelminthic agent)

Acceptable daily intake:	The Committee concluded that it is inappropriate to set an ADI for gentian violet because it is genotoxic and carcinogenic.
Maximum residue limits:	MRLs could not be recommended by the Committee, as it was not considered appropriate to establish an ADI. The Committee also noted that there was limited information on residues.

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Ivermectin (antiparasitic agent)

Acceptable daily intake:	The Committee established an ADI of 0–1 μg/kg body weight at its fortieth meeting (WHO TRS No. 832, 1993).
Estimated dietary exposure:	The fortieth meeting of the Committee (WHO TRS No. 832, 1993) included an estimate of the potential intake from muscle. No further assessment of dietary exposure was undertaken at the current meeting.
Residue definition:	Ivermectin B1a

Recommended maximum residue limits (MRLs)

Species	Muscle (µg/kg)
Cattle	4

Lasalocid sodium (antiparasitic agent)

Acceptable daily intake: The Committee established an ADI of 0–5 µg/kg body weight on the basis of a NOAEL of 0.5 mg/kg body weight per day from a developmental toxicity study in rabbits and a multigeneration reproductive toxicity study in rats, with application of an uncertainty factor of 100 for interspecies and intraspecies variability.

Estimated dietary exposure: An EDI of 80 µg/person per day was calculated, which represents approximately 17% of the upper bound of the ADI.

Residue definition: Lasalocid A

Recommended maximum residue limits (MRLs)

Species	Skin + fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Chicken	600	600	1 200	400
Turkey	600	600	1 200	400
Quail	600	600	1 200	400
Pheasant	600	600	1 200	400

The Committee extended the MRLs in chicken to turkey and quail and extrapolated the MRLs in chicken to pheasant. No information was available for duck, including on approved uses. As the compound is not registered for use in laying hens, according to the sponsor, it is not appropriate to recommend MRLs for eggs.

Monepantel (anthelminthic agent)

Acceptable daily intake: An ADI of 0–20 µg/kg body weight was established by the Committee at its seventy-fifth meeting (WHO TRS No. 969, 2012).

Estimated dietary exposure: Using the model diet and marker residue to total residue ratios of 1.00 for muscle and 0.66 for fat, liver and kidney, and applying a correction factor of 0.94 to account for the mass difference between monepantel sulfone (the marker residue) and monepantel, the EDI is 446 μg/person per day, which represents approximately 37% of the upper bound of the ADI.

Residue definition: Monepantel sulfone, expressed as moneantel

Recommended maximum residue limits (MRLs)

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Muscle (μg/kg)
Sheep	13 000	1 700	7 000	500

These MRLs are consistent with the shortest withdrawal time assigned in Member States with an approved use of monepantel.

Recombinant bovine somatotropins (production aid)

Acceptable daily intake: Based on a systematic review of the literature published since the last evaluation, the Committee reaffirmed its previous decision on ADIs "not specified" for somagrebove, sometribove, somavubove and somidobove, established at the fortieth meeting (WHO TRS No. 832, 1993).

Maximum residue limits: The Committee reaffirmed its previous decision on MRLs "not specified" for somagrebove, sometribove, somavubove and somidobove, established at the fortieth meeting (WHO TRS No. 832, 1993).

Zilpaterol hydrochloride (adrenoceptor agonist, growth promoter)

Acceptable daily intake: The Committee established an ADI of 0–0.04 μg/kg body weight on the basis of a LOAEL of 0.76 μg/kg body weight for tremor in humans.

An uncertainty factor of 20 was applied, comprising a default uncertainty factor of 10 for human individual variability and an additional uncertainty factor of 2 to account for the use of a LOAEL for a slight effect instead of a NOAEL. The Committee noted that the ADI is based on an acute effect. The Committee also noted that the upper bound of the ADI provides a margin of safety of at least 1250 with respect to the NOAEL of 50 μg/kg body weight per day for the formation of leiomyomas in rats.

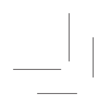
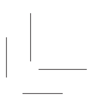
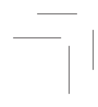
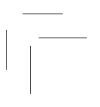
Residue definition: Zilpaterol (in muscle). The Committee was unable to determine a suitable marker residue in other edible tissues.

Maximum residue limits: The Committee concluded that it was not possible to recommend MRLs for zilpaterol.

The following data are needed to establish MRLs:

- results from studies investigating marker residue in liver and kidney;
- results from studies determining marker residue to total residue ratio in liver and kidney;
- results from depletion studies to enable the derivation of MRLs compatible with the ADI.

All such studies should use sufficiently sensitive validated analytical methods capable of measuring zilpaterol and its major metabolites in edible tissues of cattle.

This volume contains monographs prepared at the seventy-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 5 to 14 November 2013.

The toxicological monographs in this volume summarize data on the veterinary drug residues that were evaluated toxicologically by the Committee: gentian violet, lasalocid sodium, recombinant bovine somatotropins and zilpaterol hydrochloride. Annexed to the report is a summary of the Committee's recommendations on these and other drugs discussed at the seventy-eighth meeting, including acceptable daily intakes (ADIs) and proposed maximum residue limits (MRLs).

This volume and others in the WHO Food Additives Series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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