

COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

PIPERAZINE

SUMMARY REPORT (2)

1. Piperazine (diethylenediamine), as its dihydrochloride and citrate salts, is used in veterinary medicine as an anthelmintic in pigs and poultry including laying hens because of its activity against nematodes especially *Ascaris* species.

Single or repeated doses of piperazine or its salts (piperazine dihydrochloride and citrate) are given as water soluble powder or as solutions mostly mixed into water or animal feed. The posology depends on animal species, and also on the salt used. The amount of anthelmintic base in active piperazine salts is 35% for citrate and 50 to 53% for dihydrochloride. Doses expressed in terms of the amount of piperazine base are: for swine 110 mg/kg bw and for poultry 32 mg/kg bw. To poultry the substance is given in each of 2 successive feedings or in drinking water for 2 days (can be repeated 7 or 14 days later).

Piperazine and its salts are also used as anthelmintic in humans.

Currently, piperazine is included in Annex III of Council Regulation (EEC) No 2377/90 as follows:

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Piperazine	Piperazine	Porcine	400 µg/kg 800 µg/kg 2000 µg/kg 1000 µg/kg	Muscle Skin + Fat Liver Kidney	Provisional MRLs expire on 1.7.2001
		Chicken	2000 µg/kg	Eggs	

and in Annex II of Council Regulation (EEC) No 2377/90 in accordance with the following table:

Pharmacologically active substance(s)	Animal species	Other provisions
Piperazine dihydrochloride	Chicken	All tissues except eggs

Additional data were provided in response to the list of questions, further to the establishment of provisional MRLs for piperazine.

2. Piperazine and its salts, as an γ -aminobutyric acid (GABA)-like substance, induce a reversible flaccid paralysis in the nematode parasites. This is provoked by a hyperpolarisation of the cell membrane followed by suppression of spontaneous spike potentials. The paralysed nematodes are expelled from the gut lumen by normal peristaltic actions.

In mammalian species piperazine can produce electroencephalogram alterations. A dose-dependent contraction of isolated smooth muscle is produced which appears to be mediated by muscarinic cholinergic receptors, since the effects are blocked by atropine. In rabbits, the most sensitive animal species, it can provoke dose-dependent electroencephalogram alterations at repeated daily oral doses of 50 mg/kg bw and above. Piperazine exerts effects on smooth, cardiac (decrease of muscle action) and skeletal muscle (potentiation) after intravenous administration of doses between 15 and 300 mg/kg bw. High intravenous doses (100 to 500 mg/kg bw) produce cholinergic effects, inhibition of muscle contractions and respiratory arrest in the cat, another sensitive animal species.

3. Following oral administration in humans, piperazine has shown to be absorbed rapidly and to be excreted predominantly and quickly in the urine. During the first 24 hours after administration nearly 40% of the dose was excreted. The shape of the curve for the elimination of piperazine in urine indicates at least two phases in excretion. For the fastest and dominating phase the half-life is in the order of a couple of hours. It was shown that already 30 minutes after administration there was partial metabolism in the gastric juice to N-mononitrosopiperazine which was not detected in the blood but was excreted in the urine mainly within the first 6 hours with half of this appearing within 3 hours.
4. Piperazine was rapidly absorbed following oral administration to 4 laying hens (300 mg/kg bw ¹⁴C-piperazine dihydrochloride, equivalent to 154.5 mg/kg bw piperazine base). Peak plasma concentrations of radioactivity were present 1 hour after administration (26.72 µg piperazine base/ml of plasma). The major route of elimination was via the excreta and consistent with the rapid decline in the plasma concentration of radioactivity. A quantitative recovery of radioactivity in excreta was achieved with approximately 85% of the dose recovered 168 hours post administration, 70% already within 24 hours after administration. The major component in excreta was unchanged piperazine (approximately 60% at 24 hours and approximately 50% at 7 days after administration).

Piperazine was rapidly absorbed following oral administration to 1 male and 1 female pig (300 mg/kg bw ¹⁴C-piperazine dihydrochloride equivalent to 154.5 mg/kg bw piperazine base). Peak plasma concentrations of radioactivity were present 1 hour after administration (22.75 µg piperazine base/ml of plasma). The major route of excretion was via urine, consistent with the rapid decline in the plasma concentrations of radioactivity, piperazine and its metabolites were rapidly excreted. Within 24 hours of dosing 46% of the dose was recovered in urine. The major component in both urine and faeces was unchanged piperazine (in urine respectively 82% and 61% at 24 hours and 168 hours after administration), although some unidentified metabolites were present in both excreta. At 168 hours after administration, a total of 55.7% and 15.9% of the administered radioactivity were recovered respectively in urine and in faeces.

5. Acute toxicity studies have been performed using piperazine and several of its salts. The salts, which are rapidly hydrolysed to generate piperazine, show a correlation of decreased toxicity with decreased solubility. The following LD₅₀ values for mice after oral administration were observed: piperazine phosphate 22 350 mg/kg bw (9500 mg/kg bw expressed as piperazine base), piperazine citrate 13 200 mg/kg bw and 8500 mg/kg bw (5280 mg/kg bw and 3400 mg/kg bw expressed as piperazine base), piperazine dihydrochloride 6200 mg/kg bw (4360 mg/kg bw expressed as piperazine base), piperazine hexahydrate 7000 mg/kg bw (3100 mg/kg bw expressed as piperazine base), piperazine phosphate and hydrochloride 6900 mg/kg bw (2900 mg/kg bw expressed as piperazine base). In mice piperazine base has shown an LD₅₀ of 1900 mg/kg bw and 2730 mg/kg bw in two different studies. The oral LD₅₀ in rat was 2050 mg/kg bw with the piperazine base and 11 200 mg/kg bw with piperazine citrate (4500 mg/kg bw expressed as piperazine base). With piperazine sultosylate, which is a compound with hypolipoproteinemic properties and is not used in veterinary medicine, the LD₅₀ was indicated as greater than 11 000 mg/kg bw (equivalent to 2200 mg/kg bw piperazine base) for both rat and mice.

6. For the repeated dose toxicity it was shown that a 30-day study in the rat with doses of piperazine hexahydrate given by gavage up to 150 mg/kg bw/day (equivalent to 66 mg piperazine base/kg bw/day) did not provoke adverse effects. However, the average lipid content of liver, muscle, heart, kidney, lungs and serum in the treated group was significantly decreased compared to the control group, while the overall weight gain was not different in both groups.

Three groups of rats (each group 6 males and 6 females) were given piperazine sultosylate by gavage at dosages of 0, 150 and 1500 mg/kg bw/day during 6 weeks. No abnormalities were found in the weight curves, blood and urine analyses, organ weights or histopathological examination of the organs of the three groups.

Two groups of 80 rats were subdivided into 4 subgroups each of 10 males and 10 females, receiving piperazine sultosylate by oral route at dosages of 0, 40, 400 and 1200 mg/kg bw/day. Group A received the treatment for 26 weeks and group B for 52 weeks. Enhanced salivation was observed during the first weeks in the rats receiving 1200 mg/kg bw/day. One female died in the 1200 mg/kg bw/day-group of group B at 26 weeks. In group B there was a reduction in the relative weight of the spleen in females at 40 mg/kg bw/day (15.2%) and of the kidneys in females at 1200 mg/kg bw/day (10.8%). In males there was a fall in the relative weight of the prostate at all 3 doses (27.6%, 33.3%, 38.1%) and of the brain at 40 mg/kg bw/day (17.7%) and 400 mg/kg bw/day (14%). However, the effects were not dose related and no clear effect of piperazine sultosylate was established.

Three groups of beagle dogs (4 male and 4 female in each group) were treated with piperazine dihydrochloride by oral route during 13 weeks at dosage levels (dietary levels) of 92.3 mg/kg feed (equivalent to 3 mg piperazine dihydrochloride/kg bw/day) and 369.2 mg/kg feed (12.3 mg/kg bw/day) for the low and intermediate groups and at 1476.8 mg/kg feed (49.7 mg/kg bw/day) from week 1 through 5 and 3692 mg/kg feed (121.8 mg/kg bw/day) from week 6 through week 13 for the high level group. A fourth group served as control. No compound related signs of systemic toxicity were observed in any of the test animals with regard to appearance and behaviour, body weight changes, ophtalmoscopic finding, organ weights, and gross and microscopic pathology. At 4 weeks and at 13 weeks serum glutamic-oxaloacetic transaminase values fell in the control group whereas they remained constant in treated groups. Since the high dose of 121.8 mg/kg bw/day of piperazine dihydrochloride was not maintained for the entire administration period, the dose of 49.7 mg/kg bw/day of piperazine dihydrochloride (equivalent to 25 mg/kg bw/day of piperazine base) was considered as the NOEL in dogs.

7. In the light of the wide field experience of its use piperazine is not considered to present any significant concern with respect to target animal tolerance. Very young animals (e.g. 4-week old calves) can be treated without ill effects. Adult horses and foals tolerate 6 to 7 times the therapeutic dose of 110 mg/kg bw without any side effects. Four times the therapeutic dose of piperazine adipate in calves (110 mg/kg bw) causes transitory diarrhoea, tympany and anorexia. Forced oral administration of 4 times the therapeutic dose to swine resulted in semi-liquid faeces and impaired appetite but no lasting effects. In domestic animals the therapeutical index is at least a factor 3 (cats) to 6 (horses) when the calculation refers to the base. This factor however has not been established for piperazine sultosylate. The risk for unwanted effects due to overdose is self-limited due to an induced vomiting reflex reducing the amount of product absorbed.

However, in a GLP study 4 pigs received a dose of 300 mg piperazine dihydrochloride/kg bw by oral gavage for three consecutive days. Two male pigs received 1500 mg/kg bw/day (5 times the recommended dosage) and two more male pigs received 3000 mg/kg bw/day (10 times). All pigs were 11 to 13 weeks old. The pigs in the high and the intermediate dosage group died. These animals showed adverse signs within 0.5 hour after the first administration predominantly related to the central nervous system as vomiting, inappetence, abnormal respiration, ataxia, prostration, muscle tremors, convulsions, bright yellow urine and death. All dead animals showed a vacuolar tubular nephropathy. In the 300 mg/kg dosage group animals showed transient discoloration of urine (bright yellow), one male was inappetent, shivering and subdued. The severe unexpected effects were attributed to the administration of a bolus instead of administration by drinking water.

8. Reproduction and teratology studies were undertaken:

A two generation reproduction study in the rat (32 animals per sex and dose) with piperazine dihydrochloride in the diet at the following concentrations: 5000 mg/kg feed (278 to 780 mg/kg bw/day depending on sex and study week), 12 000 mg/kg feed (669 to 1923 mg/kg bw) and 25 000 mg/kg feed (1476 to 4353 mg/kg bw/day), during 17 weeks showed a NOEL of 5000 mg/kg feed equivalent to 278 to 780 mg/kg bw/day.

In rats (n=5) in a first experiment the animals were dosed once daily by gavage from day 6 to 15 of gestation with doses between 250 and 5000 mg/kg bw/day piperazine phosphate. The number of implantation sites and live foetuses, pre- and post-implantation losses and sex ratios were similar for all groups. No evidence of teratogenicity at any dose was shown, although maternal toxicity and foetal weight retardation were observed at the highest dose. Here a NOEL of at least 1000 mg/kg bw/day could be safely accepted. The same experiment was repeated in groups of 24 rats and there again a NOEL of 1000 mg/kg bw/day was confirmed.

In the rabbit (n=5) a reproduction study oral administration of doses of 100, 250 or 500 mg piperazine phosphate/kg bw per gavage showed dose dependent maternal toxicity culminating at the highest dose in death of does (2), abortion (1) and increases of major abnormalities in pups (23% *versus* 1.7% in controls). The main abnormalities observed were cleft palate and umbilical hernia, otherwise rarely seen in the strain of rabbits used. It seems thus that piperazine in doses higher than 100 mg/kg bw/day could provoke maternotoxicity, which can contribute to teratological effects as a consequence (at high dose levels).

9. A series of GLP compliant mutagenicity studies in prokaryotic and eukaryotic cells, both *in vitro* and *in vivo* has been completed and showed no evidence of mutagenic effect: a bacterial mutation test with piperazine phosphate either in presence or absence of metabolic activation ; similarly with mouse lymphoma cells with piperazine phosphate up to its limit of solubility (400 µg/ml), *in vitro* studies at levels up to 110 µg piperazine phosphate/ml (with or without metabolic activation) failed to induce incidence of chromosomal aberrations, an *in vivo* micronucleus test in mouse with piperazine phosphate at 5000 mg/kg bw did not show the induction of micronuclei.
10. Taking into account the fact that piperazine is known as a secondary amine that can interact with nitrites to produce nitrosamines, which may be carcinogens, this biochemical risk has been evaluated against data generated in several studies.

In mice, doses of about 625 mg piperazine base/kg bw (6250 mg/kg feed) was applied for 28 weeks together with 0.1% nitrite in the drinking water. After an additional period of 12 weeks on a basal diet without piperazine and nitrite, a 10 fold increase in numbers of lung adenomas *versus* control numbers was observed. Mice receiving piperazine alone during the same time period showed afterwards no increase in tumour incidence.

In a different study an increased frequency of lung adenomas in mice was seen when oral doses corresponding to 1250 mg piperazine/kg bw/day plus 5 mg sodium nitrite/kg bw/day or 138 mg piperazine/kg bw/day plus 200 mg sodium nitrite/kg bw/day were given. Doses of 1250 mg piperazine/kg bw/day plus 1 mg sodium nitrite/kg bw/day did not result in any increased tumour frequency.

In rats receiving 30 mg piperazine/kg bw/day plus 70 mg (0.05%) nitrite daily for 75 weeks there was no increase in tumour incidence.

In three studies, orally administered piperazine to humans was nitrosated to N-mononitroso-piperazine to an extent of respectively 0.1, 1 and 5%. N-Mononitrosopiperazine is considered as a carcinogen in experimental animals however there is still considerable debate whether N-mononitrosopiperazine should be considered as being of carcinogenic risk to man under realistic conditions of exposure. It has been postulated that the carcinogenic action of N-mononitroso-piperazine is due to a disproportion of high doses of N-mononitrosopiperazine to 1,4-dinitrosopiperazine. N-Mononitrosopiperazine is water-soluble whilst 1,4-dinitrosopiperazine is lipophilic. 1,4-Dinitrosopiperazine passes cell membranes and reaches reaction sites (e.g. enzymes) which are important for a carcinogenic activity. It therefore seems probable that

1,4-dinitrosopiperazine, not N-mononitrosopiperazine, is the carcinogenic nitrosamine of piperazine. 1,4-Dinitrosopiperazine could not be detected in the analysis of human gastric juice, blood or urine after ingestion of piperazine. Calculations to quantify the possible carcinogenic risk of piperazines to humans (in the assumption that N-mononitrosopiperazine is carcinogenic in humans) with the help of various mathematical models (e.g. from the US Environmental Protection Agency Carcinogen Assessment Group) and with due attention to those reservations that exist in this context, indicate that if there is any risk at all it seems to be extremely small.

11. In humans serious adverse effects are rare and generally evidence of overdose or impaired excretion. They fall into three broad categories i.e. gastrointestinal, neurological and allergic. Nausea, vomiting, diarrhoea, abdominal pain, headache, skin rashes, and urticaria occasionally occur. Severe neurotoxicity and electroencephalogram abnormalities have been reported with symptoms including somnolence, dizziness, nystagmus, muscular incoordination and weakness, ataxia, myoclonic contractions, tremor, convulsions, and loss of reflexes. Piperazine is a respiratory sensitizer. In addition hypersensitivity reactions such as erythema multiforme and angioedema have occurred in some humans. In 23 years of clinical reporting only 233 neurological events have been recorded in humans. These were nearly all related to children younger than 6 years and with overdoses above the limit of 3 g so that these cases cannot be accepted as pharmacological effects with therapeutic doses.
12. A toxicological ADI of 0.25 mg piperazine base/kg bw (i.e. 15 mg piperazine base/person) was established for piperazine based on the NOEL of 25 mg piperazine base/kg bw/day derived from the 13-week oral repeated dose toxicity study in dogs, using piperazine dihydrochloride, after applying a safety factor of 100.
13. Twelve laying hens were given a single oral dose of radiolabelled piperazine dihydrochloride (300 mg/kg bw, equivalent to 154.5 mg piperazine base/kg bw). Groups of 3 animals were killed 12, 24, 48 and 96 hours after administration. The residues were measured using radiochromatography (TLC and HPLC with fluorescence detection, with a limit of quantification of 25 µg piperazine base/kg). Twelve hours after administration the tissue concentration of radioactivity was highest in liver (14 140 µg/kg), whilst muscle, skin and fat contained 4564, 2797 and 624 µg piperazine equivalents/kg, respectively. The corresponding values for piperazine base were 2210 µg equivalents/kg in muscle, 1421 µg equivalents/kg in skin, 161 µg equivalents/kg in fat and 3271 µg equivalents/kg in liver.

At 96 hours after treatment the total residues amounted to 307 µg equivalents/kg in muscle, 457 µg equivalents/kg in skin, 356 µg equivalents/kg in fat and 1346 µg equivalents/kg in liver. The corresponding values for piperazine base residues in skin and liver were 28 and 35 µg/kg, while the values for muscle and fat were below the limit of quantification of 25 µg/kg.

At 12 hour after administration, the total intake of laying hens' edible tissues is 3035 µg, which represents 20% of the ADI.

14. Four laying hens were given a single oral dose of radiolabelled piperazine dihydrochloride (300 mg/kg bw, equivalent to 154.5 mg piperazine base/kg) in the pharmacokinetic part of a study investigating pharmacokinetics and residue depletion, where eggs were collected up to 168 hours after treatment and further 6 birds in the residue depletion part, where eggs were collected until 96 hours after treatment, at 24 hours intervals. The highest concentration of radioactivity in eggs was present at 48 hours after administration (mean total radioactivity of 8 240 µg equivalents/kg corresponding to 7970 µg/kg of piperazine base) declining to a mean value of 2483 µg equivalents piperazine base/kg at 96 hours after administration. The percentage of parent piperazine in the total residue content in eggs was 94% and 77% at respectively 48 and 96 hours after administration.

As a worst case scenario, considering the highest concentration of total piperazine residues in eggs (10 300 µg equivalents/kg at 48 hours) and the intake from other edible tissue at 12 hours after administration, the total intake would be 4065 µg, which represent 27% of the ADI.

15. Six male and 6 female pigs were given a single oral dose of radiolabelled piperazine dihydrochloride (300 mg/kg bw, equivalent to 154.5 mg piperazine base/kg bw). Groups of 3 animals were killed 12, 24, 48 and 96 hours after dosing. The concentrations of total radioactive residues declined from 21 670 µg equivalents/kg in muscle, 12 830 µg equivalents/kg in skin and fat, 71 850 µg equivalents/kg in liver and 125 899 µg equivalents/kg in kidney at 12 hours after administration to 7527, 7793, 44 230 and 63 000 µg equivalents/kg in the respective tissues after 24 hours. After 48 hours the respective values were 2626, 3324, 27 410 and 19 580 µg equivalents/kg, which declined to 1824, 2671, 12 890 and 6270 µg equivalents/kg in muscle, skin and fat, liver and kidney, respectively, after 96 hours. The residues were also measured using radio-chromatography (TLC and HPLC with fluorescence detection, with a limit of quantification of 25 µg/kg). The amounts of piperazine determined in porcine muscle, skin and fat, liver and kidney were 3296 µg/kg, 2646 µg/kg, 9049 µg/kg and 42 720 µg/kg at 24 hours, 387 µg/kg, 473 µg/kg, 3459 µg/kg and 6556 µg/kg at 48 hours and 49 µg/kg, 88 µg/kg, 591 µg/kg and 901 µg/kg at 96 hours respectively.

Piperazine base represented at 24 hours 34, 44, 68 and 20% of the total piperazine residues in skin+fat, skeletal muscle, kidney and liver, respectively, and 14%, 15%, 34% and 13% respectively at 48 hours, and dropped at 96 hours to 3, 3, 14 and 5%, respectively. The metabolites were not identified. Piperazine base was retained as marker residue.

At 24 hours the calculated residue intake from porcine tissues (8600 µg/kg) and chicken's eggs (1030 µg/kg) represents 57 % of the ADI.

16. A routine analytical method for the analysis of piperazine in the edible tissues of pigs, except for chicken's eggs based on LC-MS using electrospray has been developed and described according to the ISO 78/2 format. The limits of quantification are 200 µg/kg for muscle, 400 µg/kg for skin+fat, 1000 µg/kg for liver and 500 µg/kg for kidney. However, all raw data were lacking and the validation data (linearity, specificity and limit of detection) did not meet the requirements of Volume VI of the Rules Governing Medicinal Products in the European Community.

Conclusions and recommendation

Having considered that:

- a toxicological ADI of 0.25 mg piperazine base/kg bw (i.e. 15 mg piperazine base/person) was established,
- piperazine base was identified as the marker residue in both chicken's eggs and pig tissues and that the marker residue represents 77% of the total residues in chicken's eggs 96 hours after treatment, and, respectively 15%, 14%, 13% and 35% of the total residues in porcine muscle, skin+fat, liver and kidney at 48 hours after treatment,
- at 12 hours after treatment of chicken with piperazine dihydrochloride the amount of residues likely to be ingested by consumers from edible chicken tissues other than eggs represents 20% of the ADI,
- no information on residues in edible chicken tissues after treatment with other piperazine salts than the dihydrochloride salt were available;
- the routine analytical method for the analysis of piperazine in the edible tissues of pigs and eggs was available but not fully validated,
- the Applicant has committed to address the outstanding issues;

the Committee recommends, in accordance with Article 4 of Council Regulation No 2377/90 as amended, a 2-year extension of the provisional MRLs for piperazine in accordance with the following table:

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Piperazine	Piperazine	Porcine	400 µg/kg 800 µg/kg 2000 µg/kg 1000 µg/kg	Muscle Skin + Fat Liver Kidney	Provisional MRLs expire on 1.7.2003
		Chicken	2000 µg/kg	Eggs	

Based on these MRL values the daily intake from porcine tissues (48 hours after treatment) and chicken's eggs (96 hours after treatment) will represent about 20% of the ADI.

Before the Committee for Veterinary Medicinal Products can consider the inclusion of piperazine for pigs and for chicken's eggs in Annex I of Council Regulation (EEC) No 2377/90, the points included in the list of questions should be addressed.

LIST OF QUESTIONS

1. The applicant should complete the validation of the routine analytical method for all porcine edible tissues mainly with regard to the experimental design (extraction procedure and preparation of the standards), linearity (number of repetitions), specificity (relative intensity of the different ions if existing), limit of detection in accordance with the Volume VI of the Rules Governing Medicinal Products in the European Community. All raw data, including all chromatograms should be provided.
2. The applicant should provide a routine analytical method for the detection of piperazine in chicken's eggs validated in accordance with the Volume VI of the Rules Governing Medicinal Products in the European Community.