The European Agency for the Evaluation of Medicinal Products
Veterinary Medicines Evaluation Unit

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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

NITROXINIL

SUMMARY REPORT

1. Nitroxinil is a fasciolicide which is effective against *Fasciola hepatica* infestations and has some activity against gastrointestinal nematodes such as *Haemonchus contortus*. It is administered to cattle and sheep by the subcutaneous route at a dose level of 10 mg/kg bw, usually as the water-soluble N-ethylglucamine salt. It is contra-indicated for use in lactating animals. Nitroxinil is also administered to game birds (pheasant and red legged partridge poults), in the drinking water, for the treatment of syngamiasis; the dose level depends on the age of the poults.

2. Nitroxinil is of similar chemical structure to the herbicides ioxynil and bromoxynil. Like them it is an uncoupler of oxidative phosphorylation. Its mode of action has been attributed to this effect. It also adversely affects fluke spermatogenesis resulting in fewer fertile eggs from surviving flukes. Nitroxinil had a Minimum Uncoupling Concentration (MUC) of 27 to 35 µM (93 to 121 µg/ml) in rat liver mitochondria.

3. Nitroxinil was well absorbed after oral administration to rats. Peak plasma concentrations of around 67 µg/ml were achieved 5 hours after dosing with 10 mg/kg bw. Plasma concentrations declined in a mono-exponential manner and the terminal phase half-life in plasma was approximately 22 hours. Excretion was predominantly *via* the urine and consisted of a mixture of metabolites together with some unmetabolised nitroxinil.

4. In cows, sheep and rabbits, nitroxinil was highly bound to plasma protein. The extent of binding (97 to 98%) was constant over the concentration range of 1 to 100 µg/ml. In all species, residues in plasma were higher than the residues in tissues and consisted almost entirely of nitroxinil.

5. The metabolism of nitroxinil was studied in rats after oral administration and in cattle and sheep after subcutaneous administration. In all 3 species, nitroxinil was extensively metabolised and there were no significant species differences in the metabolic pathway. However there were quantitative differences in the percentages of nitroxinil and its metabolites in tissues and excreta. In all 3 species, unmetabolised nitroxinil was the major component of the residues in muscle and fat. Of the extractable radioactivity in rat liver, around 30% consisted of the metabolite 4-cyano-2-nitrophenol and only 8% were unmetabolised nitroxinil. Nitroxinil comprised around 35% of the extractable radioactivity in rat kidney. The chemical structure of some of the metabolites suggested that they would have toxicological properties similar to those of nitroxinil; no information on these aspects was provided.

6. The acute toxicity of the N-ethylglucamine and N-methylglucamine salts of nitroxinil was studied in rats, mice and dogs. The studies were 30 years old and were not well reported. The acute oral LD_{50} of the N-methylglucamine salt of nitroxinil ranged from 200 to 450 mg/kg bw. The acute oral LD_{50} of the N-ethylglucamine salt was 170 mg/kg bw in rats of both sexes but unstated strain. The overt signs of toxicity in these studies were ataxia, sedation, prostration and hyperpnoea. Both salts were less toxic *via* the dermal route. The acute oral toxicity of the N-ethylglucamine moiety was stated to be greater than 5000 mg/kg bw.

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7. Three repeated-dose toxicity studies were carried out in dogs. The studies were carried out during the 1960s and 1970s and had major deficiencies in design and reporting. Deaths occurred at doses of 10 mg/kg bw. The overall NOEL was around 2 mg/kg bw/day, based on reduced body weight gain at doses of 4 mg/kg bw and above.

8. A GLP study was commissioned in which groups of Sprague-Dawley rats were given daily oral doses of 0 (solvent vehicle), 2, 10 or 32 mg/kg bw/day nitroxinil (base) for 13 weeks followed by a 5-week recovery period. The study paid particular attention to the investigation of effects on thyroid function and morphology which had not been examined in the previous inadequate study. In males, T_3 and T_4 values were significantly reduced in a dose-related manner in all treated groups; the difference from the control group increased as treatment progressed. Thyroid-stimulating hormone values were not affected by treatment. Following the recovery period, T_3 and T_4 values were still significantly below the control values in the 10 and 32 mg/kg bw groups. Histopathological changes attributable to treatment were observed in the thyroids of treated males. Minimal or moderate increased height of the follicular epithelium with associated decreased colloid were observed in all treated groups in a dose-related manner. A NOEL for males was therefore not established. No histopathological changes were observed in the thyroids of any rats in the recovery groups. In females, significant reductions in T_3 and T_4 values were observed only during weeks 4 and 8 and the small changes at 2 mg/kg bw were not consistent and were within the range of historical control values. There were no substance-related histopathological changes in females. The NOEL in female rats was therefore 2 mg/kg bw/day.

9. To establish a NOEL for male rats, another 13-week repeated dose study was carried out using doses of 0 (solvent vehicle), 0.25, 0.50, 0.75, 1.00 and 10 mg/kg bw/day. T_3 and T_4 values were significantly reduced in the 1.00 and 10 mg/kg bw groups. T_4 (but not T_3) values were significantly reduced in the 0.75 mg/kg bw group during weeks 4 and 8. The incidence of histopathological changes in the thyroid was significantly increased in the 10 mg/kg bw group. The dose of 0.50 mg/kg bw/day was a NOEL in male rats.

10. Nitroxinil was well tolerated in cattle and sheep at the therapeutic dose level of 10 mg/kg bw/day. Doses of 20 mg/kg bw were tolerated with minimal side effects. Adverse effects such as hyperthermia and hyperpnoea, associated with the uncoupling of oxidative phosphorylation, were observed at higher dose levels. At doses of 40 mg/kg bw and above, these effects could result in the death of the target species.

11. In a 2-generation reproduction study in Sprague-Dawley rats, nitroxinil (base) was administered in diets calculated to provide 0, 1, 4 or 15 mg/kg bw/day. Both parental animals and offspring in the 4 and 15 mg/kg bw groups developed yellow fur. Maternal toxicity (reduced body weight gain and food consumption) was also observed at these dose levels. There were no treatment-related effects on mating, gestation, litter size, pup weight at birth or pup viability. However pup weight gain during lactation was significantly reduced in the 15 mg/kg bw group. Pup development *post partum* (ears/eyes open, startle response, righting reflex) was significantly impaired in the 4 and 15 mg/kg bw groups. The dose of 1 mg/kg bw/day was a NOEL for both maternal toxicity and pup development *post partum*.

12. Three teratology studies were carried out in rabbits. None of the studies was entirely satisfactory and it was not possible to draw any conclusions regarding a NOEL for foetotoxicity because of the inadequate reporting of skeletal variations. An overall NOEL of 4 mg/kg bw/day was established for maternal toxicity. Nitroxinil was not teratogenic in the rabbit at doses of up to 8 mg/kg bw/day.

13. In a GLP teratology study in rats, oral doses of 10 and 50 mg/kg bw/day caused reduced maternal body weight gain. The NOEL for maternal toxicity was 2 mg/kg bw. The incidence of malformations in treated groups was higher than in the controls, in which suprisingly there were no malformations. However, the absence of any statistical significance and the random nature and distribution of the malformations indicated that nitroxinil was not teratogenic in this study. The NOEL for foetotoxicity was 10 mg/kg bw/day, based on increased incidences of delayed ossification and extra ribs at the top dose of 50 mg/kg bw.

14. Nitroxinil was not mutagenic in an *in vitro S.typhimurium* assay for gene mutation at concentrations of 1.28 to 800 µg/plate, with or without metabolic activation, nor in an *in vitro* assay for gene mutation in mammalian cells (mouse lymphoma) at doses of 500 to 5000 µg/ml, with or without metabolic activation. In an *in vitro* cytogenetics assay using cultured human lymphocytes, a clastogenic response (significantly increased structural aberrations) was observed, when tested to its limit of toxicity (1160 µg/ml) in the presence of metabolic activation. No cytogenic effects were observed in the absence of metabolic activation. Nitroxinil did not induce micronuclei in *in vivo* assays in bone marrow in mice or hepatocytes in rats. The mice were exposed to a single intraperitoneal dose of 76.9 mg nitroxinil/kg bw (42% of the LD_{50}) and rats to 2 doses of 62.5 or 125 mg/kg bw, 24 hours before and after partial hepatectomy (the latter dose being the maximum tolerated dose). Overall, it was concluded that nitroxinil was not mutagenic *in vivo.*

15. In a carcinogenicity study, groups of Carworth CFY rats were fed diets containing 0, 20, 80 or 320 mg nitroxinil (as the N-ethylglucamine salt)/kg for 2 years (estimated intakes 0.7, 2.8 or 11.0 and 0.8, 3.4 or 14.0 mg/kg bw/day for males and females, respectively). Survival was not affected by treatment and exceeded 50% in all groups at termination. However the limited histopathological examinations were confined to tissues from 10 rats/group and 20 from the 320 mg/kg group together with gross lesions from the remaining rats. Body weight gain was reduced in females throughout the experiment and was reduced in males during weeks 10 to 45. There were some minor changes in calcium concentrations in treated groups but thyroid hormone concentrations were not monitored. Pituitary weights were increased in treated groups but thyroids were not weighed. The incidence of pituitary adenomas in males given 320 mg/kg was significantly increased compared to the controls. Although the incidence of mammary tumours in females was significantly increased in the 80 mg/kg group, there was no dose response and no increase in mammary tumours in the top dose group. There was an increased incidence of thyroid carcinomas in the 320 mg/kg males. The increases in pituitary adenomas and thyroid carcinomas in the high dose males were probably treatment-related and not of spontaneous origin. The negative *in vivo* mutagenicity studies in the rat and mouse indicate that these tumourigenic effects are due to a non-genotoxic aetiology. Nitroxinil contains iodine and its effect on thyroid hormones and morphology was clearly illustrated in the 13-week study. Any increase in thyroid (and pituitary) tumour incidence may therefore be explained by its goitrogenic effect.

16. Nitroxinil is goitrogenic in rats, particularly males. Chronic exposure at a dietary dose of 11 mg/kg bw resulted in increases in pituitary adenomas and thyroid carcinomas in males. No tumourigenic effects were observed in males at doses of 0.7 to 2.8 mg/kg bw, or in females at doses of up to 14 mg/kg bw. In 90-day oral repeated dose studies in the rat, a NOEL of 0.5 mg/kg bw was established for effects on T_3 and T_4 levels and thyroid morphology in males. An ADI of 0.005 mg/kg bw (0.3 mg for a 60 kg adult) can be established from this NOEL, using a safety factor of 100. This ADI would give a safety margin of over 500-fold for the tumourigenic effects observed in the carcinogenicity study. This safety margin is considered adequate, as despite some methodological shortcomings (no thyroid hormone measurements, no thyroid weights and limited histopathology) increased tumour incidence was observed in male rats only, a clear threshold was evident and appeared to be due to a non-genotoxic goitrogenic mechanism.

17. The composition of the residues in edible tissues was studied in one calf and 3 sheep following a 5-day withholding period between treatment and slaughter. At this time point, nitroxinil was the major component of the residues in calf kidney, muscle and fat, accounting for approximately 56%, 69% and 78% of the residues, respectively, when determined by HPLC. The 4-cyano-2-nitrophenol was the major component of the residues in calf liver with unmetabolised nitroxinil comprising only 2% of the residues. In the sheep, nitroxinil was the major component of the residues in kidney, muscle (including injection site muscle) and fat accounting for 45 to 56%, 90 to 100% and 64 to 100% of the extractable residues respectively, at the 5-day withdrawal point. In sheep liver, most of the residues were present as 3-iodo-4-hydroxy-5-amino-benzamide and not more than 4% was unmetabolised nitroxinil.

18. In cattle, the ratio of marker to total residue in the relevant tissues were assessed by comparing nitroxinil concentrations determined by HPLC in a non-radiolabelled study (4 animals per time point) with those determined in a radiometric ^{14}C-Nitroxinil study (1 animal per time point). The percentage of marker residue to total residue values were reported to be 2% for liver, 56% for kidney, 69% for muscle and 78% for fat at 5 days after treatment; 4% for liver, 34% for kidney, 100% for muscle and 100% for fat at 30 days after treatment; and 6% for liver, 19% for kidney, 100% for muscle and 90% for fat at 45 days after treatment.

    In sheep (n=3), the tissue marker to total residue ratios were assessed in a single study by comparing the nitroxinil content of tissue when determined by HPLC with those determined by LSC. The percentage marker to total residue values for liver, kidney, muscle and fat were reported to have been 1, 53, 97 and 81 at 5 days after treatment.

19. GLP residue depletion studies were carried out in cattle and sheep using an analytical method based on HPLC. These experiments, in which the first time point studied was 30 days after withdrawal of the treatment, confirmed that residues of nitroxinil were most persistent in the kidney and injection site. In cattle, residues in kidney declined from a mean of 252 µg/kg at 30 days after withdrawal of treatment, to a mean of 107 µg/kg at 45 days after treatment, and below the limit of quantification (90 µg/kg) at 60 days. Residues at the injection site were variable and declined from less than 90 to 504 µg/kg at 30 days after withdrawal of treatment to less than 90 to 207 µg/kg at 45 days after withdrawal of treatment. At 60 days, all residues at the injection site were below 90 µg/kg. In non-injection site muscle, nitroxinil concentrations declined from a mean of 83 µg/kg at 30 days, to 45 µg/kg at 45 days and below 50 µg/kg at 60 and 90 days. In fat, nitroxinil concentrations declined from a mean of 182 µg/kg at 30 days to 84 µg/kg at 45 days and to less than 50 µg/kg at days 60 and 90 (limit of quantification: 50 µg/kg). In liver, nitroxinil concentrations declined from a mean of 11 µg/kg at 30 days, to 10 µg/kg at 45 days, to less than 1 to 1 at 60 days and below 1 µg/kg at 90 days (limit of quantification:1 µg/kg).

    In sheep, residues of nitroxinil in the kidney declined from a mean value of 382 µg/kg at 30 days to 208 µg/kg at 45 days, and less than 102 µg/kg at 60 days (limit of quantification: 100 µg/kg). At the same time points, residues at the injection site declined from 161 to 508 µg/kg (day 30), to 102 to 189 µg/kg (day 45) and to less than 90 µg/kg at 60 days (limit of quantification 50 µg/kg). In liver, nitroxinil concentrations declined from a mean of 14 µg/kg at 30 days and below 1 µg/kg at 45, 60 and 90 days (limit of quantification: 1 µg/kg). In fat, nitroxinil concentrations declined from a mean of 155 µg/kg at 30 days, to less than 50 to 99 µg/kg at 45 days and less than 50 µg/kg at days 60 and 90 (limit of quantification: 50 µg/kg). In non-injection site muscle, nitroxinil concentrations declined from a mean of 338 µg/kg at day 30, to 114 µg/kg at day 45 and less than 50 µg/kg on days 60 and 90.

20. Some metabolites of nitroxinil which were present as residues in tissues had a chemical structure similar to that of nitroxinil. Consequently they were likely to possess similar toxicological properties. The metabolites of greatest concern were 4-cyano-2-nitrophenol (the major component of calf liver at the 5-day time point) and 3-iodo-4-hydroxy-aminobenzamide. The contribution of these metabolites have been taken into account when evaluating consumer intake.

21. The *in vitro* metabolism of nitroxinil was similar in the rat, dog, sheep, cow, calf and hen. The *in vivo* metabolism of nitroxinil was the same in the rat, calf and sheep. Plasma concentrations of nitroxinil following oral administration to hens were found to be comparable to those detected in orally treated rats.

    In game birds, there were no data to illustrate the pattern of residues depletion because in the residues study, birds were dosed orally *via* drinking water, only one 30 day time point had been employed and all residues in all tissues were below the limit of quantification (25 µg/kg in skin+fat and 90 µg/kg in muscle and liver) of the HPLC analytical method.

22. Considering that no marker residue could be identified in game birds and that the ratio of residue marker to total residues in the relevant tissues could not be established in these species, no MRLs could be recommended for game birds.

23. The routine analytical method for the determination of residues of nitroxinil in tissues was based on HPLC with UV detection. The limits of quantification were 50 µg/kg for bovine and ovine fat and muscle, 90 µg/kg for bovine kidney and 100 µg/kg for ovine kidney. For the determination of residues in liver, a more sensitive method based on HPLC with electrochemical detection had been developed; the limit of quantification was 1 µg/kg for both bovine and ovine liver. The methods were presented in an ISO 78/2 format, practicable and applicable and of acceptable accuracy and precision. No validated routine analytical method for determining nitroxinil in the relevant tissues of game birds was provided.

Conclusions and recommendation

Considering that:

- an ADI of 0.005 mg/kg bw (0.3 mg for a 60 kg human) was established,
- *in vivo* and *in vitro* studies had indicated that the metabolism of nitroxinil was the same in cattle and sheep,
- nitroxinil is the marker residue relevant to all tissues of the major target species,
- the percentage of marker residue to total residues was 4% for liver, 34% for kidney, 100% for muscle and 100% for fat at 30 days after treatment,
- validated routine analytical methods were presented for determining the marker residue in the relevant tissues of cattle and sheep;

the Committee recommends the inclusion of nitroxinil in Annex I of Council Regulation (EEC) No 2377/90 in accordance with the following table:

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Nitroxinil	Nitroxinil	Bovine, ovine	400 µg/kg 200 µg/kg 20 µg/kg 400 µg/kg	Muscle Fat Liver Kidney	

Based on these MRLs, the theoretical maximum daily intake of total residues was calculated to be 152 µg for bovine and ovine tissues equivalent to 51% of the ADI.