



## COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

### RAFOXANIDE

#### SUMMARY REPORT (1)

1. Rafoxanide, a halogenated salicylanilide, [3'-chloro-4'-(p-chlorophenoxy)-3,5-diiodosalicylanilide] is used as a fasciolicide in cattle, sheep, goats and horses. Rafoxanide is also active against gastrointestinal nematodes (*Haemonchus*, *Bunostomum*, *Oesophagostomum*, and *Gaigeria* species) and against the sheep nasal bot fly (*Oestrus ovis*). The establishment of MRLs was requested for non-lactating cattle and sheep. The recommended doses range from 5 to 15 mg/kg bw and rafoxanide is generally administered by oral route by gavage and/or by the intraruminal route. However, rafoxanide can also be used at a dose of 3 mg/kg bw by subcutaneous route, but this therapeutic regimen and this administration route are not defended in this submission.
2. Flukes treated *in vivo* by rafoxanide had elevated ratios of [NAD<sup>+</sup>]/[NADH] and [oxaloacetate]/[malate]. Rafoxanide acts by uncoupling oxidative phosphorylation of flukes, including reduced ATP levels, decreased glycogen content and accumulation of succinate.

In one *in vitro* test rafoxanide, at final bath concentrations above 0.2 µg/ml, produced concentration-related inhibitions in acetylcholine-, serotonin- and histamine-induced contractions of the ileum of guinea pigs. These inhibitions reached a level of significance at 20 µg/ml when compared with the vehicle-induced effects for acetylcholine and histamine. A significant inhibition of ileum contractions induced by serotonin was noted for all doses of rafoxanide when compared with vehicle-induced effect, the effect observed at the lowest concentration of rafoxanide being without biological significance.

In a series of *in vivo* pharmacological studies, rafoxanide had no effects up to a single administration of 100 mg/kg bw administered by intraduodenal route in dogs on the cardiovascular and respiratory systems or by oral route in mice on the locomotion activity, on hexobarbital sleeping time and on the intestinal motility (charcoal propulsion test). In rats, a single oral dose of 10 mg/kg bw of rafoxanide did not induce adverse effects on urine output and urinary electrolytes. In another *in vivo* test in rats to study the influence of rafoxanide on blood coagulation, no effects were reported on the prothrombin time test and activated partial thromboplastin time up to 30 and 100 mg/kg bw respectively. However, no conclusion could be given for the effect on whole blood clotting time due to significant effects observed at all the doses tested (10, 30 and 100 mg/kg bw). This finding was explained by the high value of the control group for this parameter when compared to the treated groups.

3. Three hours after a single intravenous administration of 2 mg/kg bw of <sup>14</sup>C-rafoxanide in infested rats, plasma radioactivity accounted for 16.8 to 19.9% of the total dose administered, rafoxanide representing more than 95% of plasma radioactivity. Within 3 hours after injection, 3.9 to 2.7% and 10 to 14% of the total dose were recovered in the bile and in the liver, respectively.

Within 168 hours after a single oral administration of 12 mg/kg of <sup>14</sup>C-rafoxanide in Sprague-Dawley rats, nearly all the radioactivity was excreted in both sexes, the major fraction being excreted via the feces (99%). Only 0.15% of the administered dose was excreted in urine. Most of the faecal excretion occurred during the 0-24 hour period (84.8 and 78.5% of the dose in males and females, respectively). Most of the radioactivity in faeces was associated with the parent compound (65.3 to 68.2% of the radioactivity quantified by HPLC). In faeces, 3 metabolites were quantified by HPLC: a polar compound amounting to 14%, and 2 further metabolites representing 1.7% and 3.2%, respectively, but no attempt was made to identify them.

After a single oral administration of 12 mg/kg of <sup>14</sup>C-rafoxanide in rats, the highest plasma concentration of radioactivity (12 000 and 17 500 µg equivalents/kg, in males and females, respectively) was seen at 4 or 6 hours after dosing. At sacrifice 168 hours after dosing only low levels of radioactivity were measured in liver (195 µg/kg) and in the gastrointestinal tract (15 µg/kg). In liver, the parent compound and polar metabolites were detected but could not be quantified due to the too low concentrations of radioactivity in the pooled samples. In absence of an intravenous study, it is not possible to estimate the oral bioavailability of rafoxanide.

In sheep, after an oral administration of <sup>14</sup>C-rafoxanide at a dose of 11.25 mg/kg bw, about 0.12% of the administered dose was recovered in urine within 3 days. The major metabolite was 3,5-diiodosalicylic acid, representing 91% of the excreted radioactivity; rafoxanide accounted for only 9%. No information on the percentage of radioactivity excreted via feces was given. The maximum plasma concentration of radioactivity (19750 µg equivalents rafoxanide/kg) occurred between 1.4 to 1.8 days after administration. An apparent radioactive elimination half-life of 8.9±1.2 days was calculated. This persistence of rafoxanide residues was due to the fact that the compound was strongly bound to plasma proteins (greater than 99%). No information on the ratio of the parent compound to total radioactivity in plasma was available.

In cattle, after an oral administration of <sup>14</sup>C-rafoxanide at a recommended therapeutic dose of 11.25 mg/kg bw, less than 0.6% of the administered dose was recovered in the urine within 6 days. Unchanged rafoxanide and 3,5-diiodosalicylic acid were the 2 major substances detected. However, due to low concentrations of these compounds and as the different system of chromatographic analysis led to different separation, no figures can be given. No information on the percentage of radioactivity excreted via faeces was given. The maximum plasma levels (close to 20 000 µg equivalent rafoxanide/kg) occurred at 1.8 days post dose. The apparent radioactive elimination half-life was 3.87 ± 0.59 days. No information on the ratio of the parent compound to total radioactivity in plasma was available.

4. No GLP acute toxicity studies were conducted. From published data, the oral LD<sub>50</sub> values were higher than 2000 mg/kg bw in rats and 232 mg/kg bw in mice. The intraperitoneal LD<sub>50</sub> values were close to 1700 mg/kg bw in rats and to 100 mg/kg bw in mice.
5. In an oral 13-week repeated dose toxicity rats received rafoxanide by gavage at doses of 0, 12, 24 and 48 mg/kg bw/day. Dose-related findings, including lower levels of calcium and cholesterol and higher relative weights of liver, thyroid and adrenals, were reported at all doses. Centrolobular hepatocyte enlargement and thyroid follicular epithelial hypertrophy were seen in males from all dose groups and in females treated with 48 mg/kg. After a 4-week recovery period, the findings in the highest dose group were partially reversible. No NOEL could be derived from this study.

In dogs, 3 to 11 oral administrations of 100 mg/kg bw rafoxanide induced neurotoxic and other effects, including bilateral equatorial cataracts, papilloedema, vacuolation of the optic nerve, optic chiasma, white matter of the brain and of the spinal cord and focal vacuolation of sciatic nerve.

In an oral 13-week repeated dose toxicity study, followed by a 4-week recovery period for the high dose and control groups, dogs received rafoxanide at doses of 0, 0.05, 0.4 and 2.5 mg/kg bw/day. In the high dose group, a significant decrease in body weight gain ( $p \leq 0.01$ ) was noted (2 kg versus 2.9 kg in the control group). At sacrifice after 13 weeks, histological findings of vacuolation were observed in optic nerves and central nervous system (in the optic nerve fibre of 1 female, in spinal cord white matter of another). Submeningeal white matter vacuolation, in either cerebrum, cerebellum, mid-brain or medulla was reported in all dogs, both males and females. At the end of the recovery period, the decrease in body weight reported in the high dose group had disappeared. One male showed focal optic nerve fibre vacuolation. Submeningeal white matter vacuolation, in either cerebrum, cerebellum or mid-brain was reported. These findings were not reported in any control recovery animal. No compound related effects were noted at 0.05 and 0.4 mg/kg bw/day. The NOEL was 0.4 mg/kg bw/day.

6. Published data on tolerance showed that rafoxanide administered orally at doses up to 16.5 mg/kg bw in sheep and cattle did not induce signs of systemic toxicity. However, in the case of overdosage (approximate oral doses estimated to 150-450 mg/kg bw in sheep and subcutaneous doses of 45 to 60 mg/kg in cattle), blindness and mydriasis were recorded. The anurosis observed in sheep was caused by spongy lesions in the cerebral cortex, demyelination, oedema, congestion and haemorrhage of the optic nerves.
7. In a 2-generation study carried out in rats, rafoxanide was administered orally at doses of 0, 0.75, 3 and 12 mg/kg bw/day. An increase of pup deaths throughout the lactation period was reported: 48% in the F0 litter compared with 62% in the F1 litter. The high dose induced a reduction in pregnancy rate in the F1 generation (54% pregnant versus 83% in the control group). In F1 males receiving 12 mg/kg bw/day, there was a marked statistically significant lower mean sperm count, sperm motility and lower percentage of morphologically normal sperm in comparison to controls. In the F1 generation, a statistically significant increase in the incidence of ophthalmoscopic lesions, such as opacification of the lens nucleus and persisted pupillary membrane were observed at the two highest doses, 3 and 12 mg/kg bw/day. No significant compound related effects were observed at 0.75 mg/kg bw/day. The NOEL was 0.75 mg/kg bw/day.
8. In a teratogenicity study carried out in rats, rafoxanide was administered orally by gavage to pregnant Sprague-Dawley females, from day 6 to day 16 of gestation at doses of 0, 5, 12 and 30 mg/kg bw/day. In the highest dose group, a reduction of the body weight gain in dams, a lower mean litter foetal weight and a slight increased incidence of early embryonic deaths were reported. At 30 mg/kg bw/day, a significant increase in the incidence of minor skeletal abnormalities, kinked ribs, patchy ossification, particularly of the skull bones and irregular ossification of ribs were noted. No significant effects of biological significance were reported for the two lowest dosages. The NOEL for this study was 12 mg/kg bw/day.

In another teratogenicity study carried out in rabbits, rafoxanide was administered orally to pregnant females, from day 6 to day 18 of gestation at doses of 0, 0.5, 2 and 5 mg/kg bw/day. In the 5 mg/kg bw dose group, the mean body weight gain was lower than the control one (44% versus 100%). The mean foetal weight was significantly decreased. Approximately 55% of the foetuses showed central ocular opacities and this difference was statistically significant when compared to the control. There was a non significant increased incidence of foetuses with reduced ossification. In the 2 mg/kg bw dose group, the weight gain was lower than that of the control group (63% versus 100%) and no significant difference when compared to the control group was reported for the ocular opacities of foetuses. In the 0.5 mg/kg bw dose group, no significant differences when compared to the control group were reported. The NOEL was 0.5 mg/kg bw/day.

9. Rafoxanide was devoid of mutagenic activity in three *in vitro* tests: *Salmonella*-microsomal assay, test for gene mutation in Chinese hamster ovary (CHO) cells at the HPRT locus and L5178Y mouse lymphoma cells at the TK locus. However, it gave positive results in the presence of metabolic activation in the *in vitro* human lymphocytes chromosome aberration test at the highest concentration (250 µg/ml), inducing a reduced mitotic index of 51%. *In vitro* in a chromosome aberration test in CHO cells, rafoxanide was clastogenic in the presence of metabolic activation at toxic concentrations higher or equal to 15 µg/ml (mitotic index lower than 50%) and sporadic increases in chromosome damage was reported at 10 µg/ml. At lower concentrations rafoxanide was not clastogenic in presence of metabolic activation. In absence of metabolic activation increase of chromosomal aberrations were observed at non toxic doses without dose relationship.

Rafoxanide was not mutagenic in 2 *in vivo* tests: in the bone marrow micronucleus test in mice after oral administration, and in a test for unscheduled DNA synthesis (UDS) in rat hepatocytes.

Since the bacterial test and 2 independent *in vivo* mammalian tests were negative, although clastogenic effects were reported at toxic concentrations, it was concluded from the overall data provided that rafoxanide was not mutagenic.

10. In view of the results of the mutagenicity studies, no carcinogenicity studies were provided.
11. In rabbits, no sign of irritation was recorded after skin irritation test and a transient and very slight conjunctival irritation in the eye irritation test. In a Magnusson-Klingman test performed in Guinea-pigs, rafoxanide did not produce evidence of delayed contact hypersensitivity.
12. A toxicological ADI was determined from the NOEL of the most relevant toxicological findings reported after histological examination: vacuolation observed in optic nerves and central nervous system. Having considered the severity of this toxicological finding, a safety factor of 200 was retained. Applying this safety factor to the NOEL 0.4 mg/kg bw/day for this effect in dogs in the 13-week toxicity study, a toxicological ADI of 0.002 mg/kg bw (2 µg/kg, i.e. 120 µg per person) was calculated.
13. In a radiometric study carried out in three groups of two sheep, three days after a single oral administration of 11.25 mg <sup>14</sup>C-rafoxanide/kg bw, the following mean levels of radioactivity were measured: 773 µg equivalent/kg in muscle, 1325 µg/kg in fat, 1690 µg/kg in liver and 2440 µg/kg in kidney. Levels declined slowly to reach 117.5 µg/kg in muscle, 160 µg/kg in fat, approximately 350 µg/kg in liver and kidney, 30 days after administration.

The mean ratio of rafoxanide to total residues was determined using the analytical method proposed as routine analytical method based on liquid chromatography and tandem mass spectrometry (LC-MS/MS). The concentrations of the parent compound were 1049, 1987, 1248 and 1915 µg/kg in muscle, fat, liver and muscle after 3 days. Significant amounts of rafoxanide were still measured in edible tissues 30 days after treatment: approximately 150 µg/kg in muscle and fat, 176.5 µg/kg in liver and 320 µg/kg in kidney. After 60 days the concentrations of rafoxanide were lower than 25 µg/kg in muscle, fat and liver and close to 50 µg/kg in kidney.

At 30 days, rafoxanide represented 100%, 88%, 50% and 87% of the total radioactivity in muscle, fat, liver and kidney respectively.

In a non-radiometric depletion study carried out according to the current requirements, 28 days after a single oral administration of rafoxanide at a dose of 11.25 mg/kg bw to 3 groups of 4 sheep aged 8-months, mean concentrations of rafoxanide in edible tissues were 220, 329, 424 and 324 µg/kg in muscle, fat, liver and kidney, respectively. At 42-days withdrawal, rafoxanide concentrations remained high: 120 µg/kg in muscle, 270 µg/kg in fat, 189 µg/kg in liver and 145 µg/kg in kidney. Sixty days post-dose, rafoxanide could be still quantified, around 29 µg/kg in muscle, 30 µg/kg in fat and liver and 46 µg/kg in kidney.

14. In a radiometric study carried out in 3 groups of 2 calves, 3 days after single oral administration of 11.25 mg <sup>14</sup>C-rafoxanide/kg bw, the following mean levels of radioactivity were measured: 973 µg equivalents rafoxanide/kg in muscle, 2303 µg/kg in fat, 2990 µg/kg in liver and 2880 µg/kg in kidney. Levels declined slowly to reach 20 µg/kg in muscle, 90 µg/kg in fat, approximately 200 µg/kg in liver and 40 µg/kg in kidney, 30 days after the administration. Sixty days post dose, the levels were very low: 25 µg/kg in muscle, 100 µg/kg in fat and below the limit of quantification of 50 µg/kg in liver and kidney. The concentrations of the parent compound were simultaneously measured. They were 458, 1691, 695 and 1682 µg/kg in muscle, fat, liver and muscle, 3 days post dosing. Thirty days post dosing, the levels of rafoxanide were close or below the limit of quantification in all edible tissues (lower than 5 µg/kg).

In a further radiometric study carried out in 4 calves, 14 days after a single oral administration of 11.25 mg of <sup>14</sup>C-rafoxanide/kg bw, the levels of radioactivity ranged from below the limit of quantification (18 µg equivalents rafoxanide/kg) to 24 µg equivalents rafoxanide/kg in muscle, and from below 32 to 46 µg equivalents rafoxanide/kg in fat. In liver and kidney, significant amounts of radioactivity were measured, the corresponding mean concentrations being 140.5 and 63 µg equivalents rafoxanide/kg, respectively. At this time point, the levels of rafoxanide ranged from below the limit of quantification (5 µg/kg) to 15 µg/kg in muscle and were close to 15 µg/kg in fat, 18 µg/kg in kidney. In liver, rafoxanide could not be quantified (less than 5 µg/kg in 2 samples) and could not be detected in the 2 other samples (less than 1 µg/kg).

In cattle, the mean ratio of rafoxanide to total residues was determined using the analytical method proposed as routine analytical method based on LC-MS/MS. Three days after administration, rafoxanide represented approximately 50%, 75%, 25% and 60% of the total radioactivity in muscle, fat, liver and kidney. At 14 days after dosing, rafoxanide represented approximately 50%, 50% and 30% of total radioactivity in muscle, fat and kidney, respectively. This ratio was not established in liver, because the concentrations of rafoxanide were below the limit of quantification (5 µg/kg) in all 4 animals. At further slaughtering times (30 and 60 days), the concentrations of parent compound were too low to establish a ratio.

In a non-radiometric depletion study carried out according to the current requirements, 28 days after a single oral administration of 11.25 mg/kg to 2 groups of 4 calves aged 4-months, the mean concentrations of rafoxanide were 6.6, 18.2 and 7.3 µg/kg in muscle, fat and kidney, respectively. In liver, rafoxanide concentrations were lower than the limit of quantification (5 µg/kg). At 42 days post-dose, all concentrations in edible tissues were below the limit of quantification.

15. A liquid chromatographic tandem mass spectrometric (LC-MS/MS) method based on electrospray ionisation was proposed for residue surveillance. Under the LC-MS/MS conditions used, the characteristic ion dissociation of m/z 624→127 was monitored for rafoxanide. This method was presented in ISO 78/2 format and validated in accordance with the requirements of Volume VI of the Rules Governing Medicinal Products in the European Community for all validation parameters except limit of detection and specificity. The limit of quantification was 5 µg/kg for all edible tissues of both target species.

## Conclusions and recommendation

Having considered that:

- a toxicological ADI of 2 µg/kg bw (i.e. 120 µg/person) was established for rafoxanide,
- rafoxanide was identified as marker residue,
- in bovines rafoxanide represented 50%, 50% and 30% of the total residues in muscle, fat and kidney, respectively, 14 days after treatment, and in ovines rafoxanide represented 100%, 88%, 50% and 50% of the total residues in muscle, fat, liver and kidney, respectively, 30 days after treatment;
- the depletion of rafoxanide in bovine and ovine species is quite different, so that the tissue distribution 14 days after treatment was considered for bovines and 42 days after treatment for ovines (as residues around day 30 still exceeded the ADI),
- in bovine liver, the concentrations of rafoxanide were below the limit of quantification in all the animals, so that the MRL for bovine liver was set at twice the limit of quantification;
- an analytical method for monitoring residues is available but not fully validated;

the Committee for Veterinary Medicinal Products recommends the inclusion of rafoxanide in Annex III 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
Rafoxanide	Rafoxanide	Bovine	30 µg/kg 30 µg/kg 10 µg/kg 40 µg/kg	Muscle Fat Liver Kidney	Not for use in animals from which milk is produced for human consumption.
		Ovine	100 µg/kg 250 µg/kg 150 µg/kg 150 µg/kg	Muscle Fat Liver Kidney	Provisional MRLs expire on 1.7.2001

Based on these MRLs values, the daily intake from ovine tissues, which corresponds to the worst case, will represent less than 75% of the ADI.

Before the Committee can consider the inclusion of rafoxanide in Annex I to Council Regulation (EEC) No 2377/90, the points included in the list of questions should be addressed.

## LIST OF QUESTIONS

1. As the routine analytical method for monitoring purposes is based on a LC/MS-MS method, the applicant should provide information on the relative intensity of the different ions (1 precursor and 2 products or 2 precursors and 1 product). The applicant should also determine the limit of detection according to the requirements of Volume VI of The Rules Governing Medicinal Products in the European Community. A revised presentation of the analytical method according to an internationally recognised format (e.g. ISO 78/2) should be provided.