The European Agency for the Evaluation of Medicinal Products
Veterinary Medicines and Information Technology

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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

OXOLINIC ACID

SUMMARY REPORT (2)

1. Oxolinic acid is a synthetic quinolone antibiotic. Oxolinic acid is authorised in veterinary medicine for use in fin fish, calves, pigs and poultry. It is administered by the oral route, in the feed, the drinking water or as a bolus. The recommended doses are for fin fish: 12 mg/kg bw/day for up to 7 days; for pigs and poultry: 20 mg/kg bw/day for up to 5 days and for calves: 20 mg/kg bw/day for up to 10 days.

   Currently oxolinic acid is included 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
Oxolinic acid	Oxolinic acid	Bovine	100 µg/kg 50 µg/kg 150 µg/kg 150 µg/kg	Muscle Fat Liver Kidney	Provisional MRLs expire on 1.1.2001
		Porcine	100 µg/kg 50 µg/kg 150 µg/kg 150 µg/kg	Muscle Skin + fat Liver Kidney	
		Chicken	100 µg/kg 50 µg/kg 150 µg/kg 150 µg/kg 50 µg/kg	Muscle Skin + fat Liver Kidney Eggs	
		Fin fish	300 µg/kg	Muscle and skin in natural proportions	

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

2. Oxolinic acid acts by inhibiting bacterial DNA-gyrase (also known as type II topoisomerase) activity. The comparable human enzyme is much less susceptible to inhibition by quinolone antibiotics. In humans, therapeutic dose levels of oxolinic acid have been reported to induce psychopharmacological effects such as nervous excitation, stereotyped behaviour and insomnia. Oxolinic acid inhibits cytochrome P4501A2 activity resulting in reduced metabolism of co-administered xenobiotics.

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3. The absorption of oxolinic acid after oral administration was shown to be variable and depended on species, formulation, meal influence and disease status. Micronisation enhanced the bioavailability in fin fish, poultry and mammals; C_{max} values doubled when the particle size was reduced from 6.4 µm to 1.0 µm diameter. In humans, mean peak blood concentrations of 1853 µg equivalents/ml were attained 4 hours after oral administration of 1g ^{14}C-oxolinic acid in gelatin capsules. Autoradiography experiments in rodents, quails and eels indicated that the substance was widely distributed to the tissues; highest concentrations were found in the kidneys, bile and liver. In rodents, oxolinic acid crossed the placenta. Excretion was rapid; an oral dose of 20 mg ^{14}C-oxolinic/kg bw acid was almost completely eliminated in mice within 48 hours. In all species, excretion was via both the urine and faeces with less than 0.5% in expired air. In humans, 43% of an oral dose of 1g ^{14}C-oxolinic acid was recovered in the urine and faeces in 24 hours and 67% of the dose in 48 hours. In rats, 27% of an oral dose of 10 mg/kg bw ^{14}C-oxolinic acid was recovered in the urine within 24 hours and 41% in the faeces; the corresponding values for rabbits given the same dose were 49% in the urine and 37% in the faeces.

4. In humans, oxolinic acid was metabolised to at least 8 urinary metabolites. At least 2 metabolites had microbiological activity. The radioactivity of urine collected 0 to 6 hours after an oral dose of 1g ^{14}C-oxolinic acid consisted of 43% oxolinic acid glucuronide, 1.4% of a labile complex of oxolinic acid, 37.5% of glucuronides derived from oxolinic acid metabolites and 18% non-glucuronide metabolites.

5. Oxolinic acid was of low acute toxicity to adult animals. Acute oral LD_{50} values in adult mice ranged from 1890 to more than 6000 mg/kg bw. It was more acutely toxic to neonatal animals with acute oral LD_{50} values in the range from 128 mg/kg bw (mice) to 573 mg/kg bw (rat).

6. The report of a 6-month study in rats was provided. The study was carried out by a discredited laboratory and there was no independent audit. Consequently the findings could not be accepted as valid. In a more recent, but non-GLP study, Sprague-Dawley rats were given daily oral doses of 125, 250 or 500 mg/kg bw/day of oxolinic acid for 13 weeks. The NOEL was 125 mg/kg bw/day. Overt signs of toxicity (hyperactivity, piloerection) and reduced bodyweight gain were observed at higher dose levels. No individual animal data were provided and only 15 organs/tissues were examined at the terminal histopathology.

7. A 6-month study in Beagles had been carried out at a discredited laboratory and there was no independent audit. Consequently the results could not be accepted as valid. In a more recent but non-GLP 13-week study, a NOEL of 125 mg/kg bw/day was established. However, the age of the dogs was not reported, no individual animal data were provided and the joints were not examined at termination. The overt signs of toxicity included hyperactivity and stereotyped movements. A 14-day study was carried out using dose levels of 100 and 500 mg/kg bw/day in Beagle dogs which were 3-months old at the start. No NOEL was established. The overt signs of toxicity in this study included hyperactivity, hindlimb stiffness and stilted gait. Pathological examination revealed changes in the articular cartilage of the major synovial joints.

8. A new GLP-compliant study was undertaken to identify a NOEL for arthropathy in juvenile dogs. Groups of 3-month old Beagle dogs (2/sex/dose) received daily oral doses of 0, 2, 10 or 50 mg oxolinic acid/kg bw for 4 weeks. No mortality, treatment-related clinical signs, or effects of bodyweight or food consumption were observed. No treatment-related macroscopic or microscopic effects on the articular cartilage was observed at any dose-level up to 50 mg/kg bw.

9. In a single-generation reproductive toxicity study in rats, 3 litters were bred. The dose levels were 20, 40 and 80 mg/kg bw/day. Pup mortality was increased in all treated groups during the first week after birth. Pup survival at weaning was significantly reduced in all treated groups. These effects were exacerbated following the second and third mattings. Pregnancy rates were very low in all groups including the controls. Another study was carried out to try and clarify these effects. The study included a cross-over design in which pups from treated groups were exchanged with those from untreated mothers. Increased pup mortality was again observed. No NOEL was established. It was not possible to draw any conclusions regarding possible effects on male or female fertility from these studies.

10. In a GLP-compliant study, to investigate effects on fertility and early embryonic development, groups of 24 male and 24 female rats per dose were fed diets containing the equivalent 0, 2, 10 or 50 mg oxolinic acid/kg bw. Males were treated for 64 days before mating. At termination the males were necropsied and reproductive organs and sperm samples were examined microscopically. Females were treated for 15 days before mating and then divided into groups of 12 pregnant females; the first group was treated up to day 7 of gestation and then subjected to Caesarean section on day 15; the second group was allowed to deliver naturally and rear the offspring to weaning. Pup developmental landmarks including righting reflexes were monitored. There was no adverse effects on male or female fertility, sperm numbers or motility, growth or development of the offspring.

11. Teratogenicity studies were carried out in rabbits, mice and rats. Groups of New Zealand White rabbits were given daily oral (gavage) doses of 0, 50 or 200 mg/kg bw/day from days 6 to 18 of gestation. In a second study, New Zealand White rabbits were given daily oral (gavage) doses of 0, 50, 100 or 200 mg/kg bw/day from days 6 to 18 of gestation. In these studies, there was no evidence of maternal toxicity, teratogenicity or foetotoxicity at any dose level. Groups of CD-1 mice were fed diets equivalent to 0, 45.5 or 205 mg/kg bw/day from days 7 to 16 of gestation. Maternal bodyweight was reduced at 205 mg/kg bw but there was no evidence of teratogenicity or foetotoxicity at any dose level, the NOEL for maternotoxicity was 45.5 mg/kg bw.

    Two teratogenicity studies were carried out in rats. In the first study, Carworth CFN rats were fed diets equivalent to 0, 21, 42 or 75 mg/kg bw/day from days 6 to 15 of gestation. Maternal bodyweight gain was reduced at 42 and 75 mg/kg bw and the incidence of resorptions was increased at 75 mg/kg bw but there was no evidence of teratogenicity at any dose level. In the second study, Sprague-Dawley rats were given daily oral (gavage) doses of 0, 50, 100 or 200 mg/kg bw/day from days 6 to 15 of gestation. Maternal bodyweight gain was reduced at 200 mg/kg bw and the incidence of resorptions was significantly increased at this dose but there was no evidence of teratogenicity. In the rat, the NOELs for materno- and foetotoxicity were 21 and 42 mg/kg bw, respectively. Although these studies did not comply with modern guidelines in terms of groups sizes and the individual animal litter data were not available, it was clearly demonstrated that oxolinic acid was not teratogenic.

12. Most of the mutagenicity assays were satisfactorily conducted and were in accordance with GLP. These studies included *in vitro* bacterial assays for gene mutation, an *in vitro* assay for gene mutation in mammalian cells (HPRT locus in V79 cells), an *in vitro* metaphase analysis in human lymphocytes, an *in vitro* unscheduled DNA synthesis (UDS) assay in rat hepatocytes (which was not independently replicated) and an *in vivo* micronucleus test. All these assays gave negative results. Although some published papers claimed positive results in assays in prokaryotic organisms, full details of the methods and results were not available. In a published study, high doses of oxolinic acid resulted in DNA damage, as shown by increased levels of single strand DNA in rats granuloma cells from the skin pouch. Little data are available regarding this assay and the sensitivity is not known-therefore the study is difficult to assess and no firm conclusions can be drawn. It was concluded that oxolinic acid was not mutagenic.

13. Oxolinic acid was not carcinogenic in a published carcinogenicity assay in which ICR mice were fed diets containing 0, 50, 150 or 500 mg/kg feed oxolinic acid (equivalent to 0, 4.9/5.3, 15.2/15.7, or 59.7/67.9 mg/kg bw/day for males/females, respectively) for up to 78 weeks. In another study, Wistar rats were fed diets containing 30, 100, 300 or 1000 mg/kg feed oxolinic acid (equivalent to 0, 1.1/1.3, 3.6/4.4, 10.9/13.2 or 37.6/49.1 mg/kg bw/day for males/females, respectively) for 104 weeks. Oxolinic acid induced a significant increase in Leydig cell tumours and Leydig cell hyperplasia in male rats given the top dose level. Further published work suggested that a non-genotoxic mechanism might be involved in the induction of these tumours.

In a 104-week study in which Wistar rats were fed diets containing the equivalent of 0, 4.2, 43 or 145 mg/kg bw/day of oxolinic acid, serum luteinising hormone (LH) concentrations were significantly increased at 43 and 145 mg/kg bw but not at 4.2 mg/kg bw. There was no effect on testosterone concentrations at any dose level. Further studies showed that the increase in LH concentrations was reversible on cessation of dosing and that serum concentrations were significantly reduced by administration of a dopamine-antagonist. It was concluded that oxolinic acid induced chronic stimulation of LH release by a dopaminergic-mediated effect on the hypothalamo-pituitary axis; the prolonged elevated serum LH concentrations stimulated testicular Leydig cells leading to hyperplasia and subsequent tumour development. The NOEL, based on effect on LH, was 4.2 mg/kg bw/day.

14. Rainbow trout were given intraperitoneal doses of 0.1, 1 or 10 mg/kg bw oxolinic acid on days 6, 4 and 2 prior to immunisation with Yersinia ruckeri O-antigen. Oxolinic acid did not cause immunosuppression in either the nonspecific defence or specific immune system compartments. There was no evidence to suggest that oxolinic acid could affect the immune system in the repeated dose studies conducted in mammalian species.

15. Oxolinic acid has been used in human medicine in several countries in the past. Its use in human medicine has largely been replaced by the fluoroquinolone antibiotics. The usual dose is 750 mg, administered orally, twice a day (equivalent to approximately 25 mg/kg bw/day). It is contra-indicated in children, during pregnancy and lactation because of the risk of arthropathy. Adverse effects have been reported in 10 to 45% of patients. Central nervous system effects (such as insomnia, restlessness, dizziness) were the most commonly reported. A NOEL for the central nervous system effects in humans could not be determined from the data available and the mechanism for the induction the central nervous system effects had not been established with certainty. To induce overt the central nervous system effects in rats and dogs, doses higher than 50 mg/kg bw/day had to be administered. It was concluded that there would be no risk of the central nervous system effects at the residue concentrations likely to be encountered by consumers.

16. In view of the hormone-related increase in Leydig cell tumours observed in male rats exposed to 38 mg/kg bw oxolinic acid for 104 weeks, but not at doses of 1 to 11 mg/kg bw, the toxicological ADI was determined from the NOEL of the most sensitive parameter of hypothalamo-pitiutary-testicular function. Applying a safety factor of 100 to the NOEL of 4.2 mg/kg bw for the effect on serum Luteinising Hormone (LH) in male Wistar rats in a 104-week study a toxicological ADI of 42 µg/kg bw (2520 µg for a 60 kg human) was calculated.

17. *In vitro* minimum inhibitory concentration (MIC) data were provided for a range of microorganisms representing all the genera which were most relevant for the human gut flora. For most genera, *in vitro* MIC values were determined for at least 10 strains. *Escherichia coli* was the most sensitive microorganism with *in vitro* MIC_{50} values of 0.41 and 0.43 µg/ml for 10 strains isolated from healthy human volunteers, when cultured under aerobic and anaerobic conditions respectively. In another study, an *in vitro* MIC_{50} value of 0.38 µg/ml was obtained for 82 *Escherichia coli* isolates from Japanese patients.

Based on the formula adopted by the CVMP, a microbiological ADI was calculated as follows:

$$\text{ADI} = (\mu g/kg\ bw) = \frac{\frac{MIC_{50}\text{ for most sensitive organism} \times CF2}{CF1}\ (\mu g/ml) \times \text{daily faecal bolus (150 ml)}}{\text{fraction of an oral dose available for micro-organisms} \times \text{weight of human (60 kg)}}$$

Based on the above formula, the microbiological ADI can be calculated as follows:

$$\text{ADI} = \frac{\frac{0.4 \times 1}{1} \times 150}{0.4 \times 60} = 2.5\ \mu g/kg\ bw, \quad \text{i.e. } 150\ \mu g/person$$

The following assumptions were made:

- CF1 = 1 because the MIC_{50} value for the most sensitive, relevant strain (*Escherichia coli*) was used; there is no evidence of significant plasmidic resistance to quinolones to date;
- CF2 = 1 because no data were available to correct for extrapolation from the *in vitro* to the *in vivo* situation;
- 150 g was the weight of the daily faecal bolus;
- 0.4 = the fraction of the oral dose available to the micro-organisms at the distal part of the gastro-intestinal tract.

18. The strains of microorganisms used in food processing appeared to be insensitive to oxolinic acid with *in vitro* MIC values for *Lactobacillus spp* of greater than 50 µg/ml. Full details of these studies were not available.

19. Pharmacokinetic and residues depletion data for the target species used unlabelled material and the residues in plasma and tissues were mostly determined using various high pressure liquid chromatography (HPLC) methods. The studies were not in accordance with GLP. In many cases it was not clear whether the residues extracted were oxolinic acid or whether some conjugates were also extracted. Oxolinic acid was quickly absorbed after oral administration in all the target species. Bioavailability after an oral dose of 10 mg/kg bw was approximately 82% in healthy chickens but around 100% in diseased chickens. Oral bioavailability was also high in pigs and calves though the percentage of bioavailability could not be deduced from the data provided for these species.

    Bioavailability was lower in fin fish. In Atlantic salmon, bioavailability after an oral dose of 20 mg/kg bw was approximately 25%. The data on metabolism in the target species were very limited. Oxolinic acid accounted for 62% of the residues in rainbow trout bile, 6 hours after oral dosing with 40 mg/kg bw, and oxolinic acid glucuronide accounted for 38% of the residues. Twenty-four hours after dosing, residues in bile were 29% oxolinic acid, 66% oxolinic acid glucuronide with small amounts of 2 other glucuronides (each comprising 2 to 3%). There was no information concerning the relationship between total and marker residue in the tissues of any of the target species.

20. Broilers were given a single oral dose of 15 mg/kg bw. Groups of 6 birds were slaughtered 1, 3, 6, 8 and 14 days after dosing. Residues of oxolinic acid in tissues were determined using HPLC. The limit of quantification was not given but appeared to be about 15 µg/kg. Mean residues in liver declined from 2 160 µg/kg, one day after dosing, to 490 µg/kg 3 days after dosing and 50 µg/kg 6 days after dosing. Over the same time points, mean residues in kidney declined from 2 380 µg/kg to 910 µg/kg to 160 µg/kg and residues in muscle declined from 1 460 µg/kg to 570 µg/kg to 20 µg/kg. No residues data were provided for skin + fat. In another experiment, broilers were given drinking water containing oxolinic acid for 84 hours. The dose corresponded to 25 mg/kg bw. Groups of 6 birds were slaughtered 1, 2, 4, 5 and 6 days after dosing. Residues of oxolinic acid in tissues were determined using HPLC. The limit of detection was 25 µg/kg. Mean residues in liver declined from 2 570 µg/kg one day after the end of treatment to 108 µg/kg at 2 days. Mean residues in muscle declined from 1 830 µg/kg one day after the end of treatment to 80 µg/kg at 2 days and residues in fat declined from 200 µg/kg to 28 µg/kg. At 4 days, residues in all samples of liver, muscle and fat were below the limit of detection. Mean residues in skin depleted from 814 µg/kg one day after the end of treatment to 120 µg/kg at 2 days and were still detectable at 6 days (124 µg/kg). No kidney samples were analysed.

21. Laying birds were given oral doses equivalent to 15 mg/kg bw/day of oxolinic acid, for 5 days. Peak residues of oxolinic acid in eggs (determined by HPLC; limit of detection 10 µg/kg) were found on the 5th day of dosing: 6 100 µg/kg. One day after the last treatment, mean residues in eggs were 5 610 µg/kg and depleted to 1 240 µg/kg at 3 days, 80 µg/kg at 6 days and were below 10 µg/kg at 9 days.

22. Piglets were given feed containing oxolinic acid at a dose equivalent to 15 mg/kg bw/day for 7 days. The piglets were slaughtered (3 per time point), 24 hours, 3 days and 5 days after the end of treatment. Residues of oxolinic acid in tissues were determined using HPLC. The limit of detection was 25 µg/kg. Mean residues in liver, kidney and muscle were 1 080 µg/kg, 1 350 µg/kg and 1500 µg/kg at 24 hours after the end of treatment. Residues were undetectable in all samples of liver, kidney and muscle at 3 days. Residues in subcutaneous fat depleted from 490 µg/kg at 24 hours to 25 µg/kg at 3 days and were below the limit of detection at 5 days.

23. Groups of carp were given an oral bolus of 5, 10, 20 or 40 mg/kg bw oxolinic acid. The water temperature was 21±1°C. The carp were killed (3 per time-point) at intervals from 6 hours up to 144 hours after dosing. Residues in tissues were determined using a microbiological method with limits of detection of 200 µg/kg for serum and 1 000 µg/kg for tissues. Residues in serum peaked at approximately 15 hours after dosing and were 730, 590, 890 and 2 230 µg/kg for the 5, 10, 20 or 40 mg/kg bw groups respectively. By 72 hours, residues in serum were below 200 µg/kg in the 5 and 10 µg/kg bw groups but were detectable up to 96 hours and 120 hours for the 20 and 40 mg/kg bw groups respectively. Peak residues in hepatopancreas were also observed at approximately 15 hours after dosing and were 1 000, 3 400, 3 400 and 17 000 µg/kg for the 5, 10, 20 or 40 mg/kg bw groups, respectively. Residues in kidney were detectable only in the group treated at 40 mg/kg bw (peak value 2 300 µg/kg). Residues in all muscle samples were below the limit of detection.

24. Rainbow trout were given feed containing oxolinic acid at a rate of 1% bodyweight, corresponding to a dose level of 12 mg/kg bw/day for 7 days. The water temperature was 9 to 10°C. Groups of 10 fish were killed at 24, 48, 96, 144 and 240 hours after the end of treatment. Residues of oxolinic acid in samples of muscle + skin were determined using HPLC (limit of quantification approximately 10 µg/kg). Mean residues depleted from 1 970 µg/kg 24 hours after the end of treatment, to 930 µg/kg at 48 hours, 90 µg/kg at 96 hours and 50 µg/kg at 144 hours. In another study, rainbow trout were given feed containing oxolinic acid at a dose corresponding to 20 mg/kg bw/day for 5 days. The fish were kept at different water temperatures. Residues in tissues were determined using HPLC with a limit of detection of 20 µg/kg. Mean residues in muscle one day after the end of treatment were 1 990 µg/kg in the group kept at 8.5 to 11.5°C and depleted to 540 µg/kg at 3 days, 40 µg/kg at 5 days and 20 µg/kg at 7 days. Mean residues in muscle one day after the end of treatment were 2 090 µg/kg in the group kept at 17.1 to 19.6°C and depleted to 340 µg/kg at 3 days, 70 µg/kg at 5 days and 60 µg/kg at 7 days.

25. Calves were given an oral bolus of 22 mg/kg bw oxolinic acid followed by a second oral bolus of 11 mg/kg bw, 24 hours later. The calves were killed (3 per time point), 3 hours and 9 hours after the second dose. Residues of oxolinic acid in tissues were determined using HLPC. At 3 hours and 9 hours, mean residues in liver were 12 500 µg/kg and 13 300 µg/kg, in kidney were 14 600 µg/kg and 17 500 µg/kg and in muscle were 8 000 µg/kg and 8 600 µg/kg respectively. No fat samples were analysed.

26. The proposed routine analytical methods were presented in the ISO 78/2 format and were based on HPLC with ultra violet-diode array detection. The limits of quantification were 50 µg/kg for bovine, porcine, fin fish and chicken tissues, although no validation data were presented for eggs. Data demonstrating the specificity of the proposed routine analytical methods (i.e. against homologues and analogues of oxolinic acid) were not presented.

Conclusions and recommendation

Having considered that:

- a microbiological ADI of 2.5 µg/kg bw (i.e. 150 µg/person) was established for oxolinic acid,
- like the other quinolones already recommended for inclusion in Annexes I and III of Council Regulation (EEC) No 2377/90, the parent compound, is considered to be an appropriate marker residue for all the target species,
- there was no information on the ratio of marker to total residue in the relevant tissues of any of the target species; taking into account the limited data available on the metabolism of oxolinic acid, and also the evidence that for those quinolones already included in the Annexes to Council Regulation (EEC) No 2377/90, the unmetabolised parent substance generally accounted for the major portion of the residues, a conservative marker to total ratio of 0.5 was used when calculating MRLs for oxolinic acid,
- the proposed routine analytical methods were insufficiently validated for specificity against homologues and analogues of oxolinic acid,
- the applicant has committed to address the oustanding issues;

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

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Oxolinic acid	Oxolinic acid	Bovine	100 µg/kg 50 µg/kg 150 µg/kg 150 µg/kg	Muscle Fat Liver Kidney	Not for use in animals from which milk is produced for human consumption Provisional MRLs expire on 1.1.2003
		Porcine	100 µg/kg 50 µg/kg 150 µg/kg 150 µg/kg	Muscle Skin + fat Liver Kidney	Provisional MRLs expire on 1.1.2003
		Chicken	100 µg/kg 50 µg/kg 150 µg/kg 150 µg/kg 50 µg/kg	Muscle Skin + fat Liver Kidney Eggs	
		Fin fish	300 µg/kg	Muscle and skin in natural proportions	

Based on these MRL values, the maximum daily intake of total residues was estimated to be 118 µg/person, equivalent to 74% of the microbiological ADI of 150 µg/person.

LIST OF OUTSTANDING ISSUES FOLLOWING THE ASSESSMENT OF THE RESPONSE TO THE LIST OF QUESTIONS

1. The Applicant should justify the choice of the ratio of marker to total residues (i.e. residues with antimicrobiological activity) for all edible tissues of the target species and for eggs.

2. The proposed routine analytical method should be validated in accordance with Volume VI of the Rules Governing Medicinal Products in the European Community for chicken eggs.

3. The specificity of the proposed routine analytical methods should be validated in accordance with Volume VI of the Rules Governing Medicinal Products in the European Community (i.e. against homologues and analogues of oxolinic acid) in the edible tissues of all target species.