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

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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

PIRLIMYCIN

SUMMARY REPORT (1)

1. Pirlimycin hydrocloride is a lincosaminide antibiotic closely related to lincomycin and clindamycin. Pirlimycin is effective against common mastitis pathogens, mostly Gram-positive bacteria such as *staphylococci* (e.g. *Staphylococcus aureus*) and *streptococci* (*Streptococcus agalactiae*, *S. uberis*, *S. dysgalactiae*). It is intended for treatment of subclinical and clinical staphylococcal and streptococcal mastitis in lactating dairy cows at a dose rate of 50 mg/quarter administered in two treatments at 24-hour interval by intramammary infusion of an aqueous gel.

2. The mechanism of action of pirlimycin is the inhibition of protein synthesis of the bacterial cell by binding to the 50S ribosomal subunit and thereby inhibiting peptidyl transferase.

3. In rats given orally 29 mg/kg bw of ^{14}C-pirlimycin hydrochloride for 5 days, at the end of treatment highest mean concentration was found in liver (18 000 µg/kg), followed by kidney (7 500 µg/kg), muscle (1 500 µg/kg) and fat (1 000 µg/kg). The parent compound accounted for 57 to 76% and pirlimycin sulphoxide for 21 to 42% of liver radioactivity; the levels of the metabolite were higher in males. At the end of treatment, urinary elimination accounted for about 5% of the administered dose, whereas about 60% was eliminated in the faeces.

4. Human serum protein binding determined *in vitro* was approximately 45%.

   Male volunteers were treated with a single oral administration at approximately 1.8 mg of pirlimycin hydrochloride/kg bw) in capsules or as solution. Mean peak plasma concentrations were lower than (capsule) or approximately equal to (solution) the limit of detection of the microbiological assay used (0.156 µg/ml). Urinary excretion in 48 hours averaged 4.4% or the total dose with the capsule and 7.3% with the oral solution.

   In a further study, male adult volunteers (five subjects/treatment) received each oral doses of approximately 0.83, 2.1, 4.15 and 8.3 mg/kg bw of pirlimycin. Plasma peak values for pirlimycin, 4 hours after administration, were lower than 0.01, 0.1, 0.2 and 0.6 µg/ml after the dose of 0.83, 2.1, 4.15 and 8.3 mg/kg bw, respectively. Urinary excretion during 24 hours ranged from 2.8 to 6.9% of the dose for pirlimycin. Faecal excretion (measured by bioassay over 72 hours) averaged 32% (range 19 to 34%) of the administered doses for pirlimycin.

   According to the faecal recovery data, the unabsorbed fraction of pirlimycin remaining in the gastrointestinal tract of humans can be conservatively estimated at 50%.

5. The oral LD_{50} in rats was 2524 mg/kg bw (range 1976 to 4414 mg/kg bw). Necropsy of non-surviving rats revealed congestion of the glandular stomach and of kidney medulla.

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6. In a 30-day oral toxicity study, rats were treated with doses of 50, 160 or 500 mg pirlimycin hydrochloride/kg bw/day. At 500 mg/kg bw, a few myeloid figures and a slight increase in lysosomes were noted in hepatocytes. Serum levels of glutamic oxaloacetic transaminase and of glutamic pyruvic transaminase were slightly elevated in all dose groups. A dose related increase of stomach histological lesions suggestive of focal irritation was observed at 500 and 160 mg/kg bw, whereas at 50 mg/kg bw histological examination was not performed. Stomach lesions were not observed in control animals. Due to the lack of histological examination at the lower dose level, a NOEL could not be established.

   In a 13-week oral toxicity study, rats were treated with doses of 10, 30, 100 or 300 mg pirlimycin hydrochloride/kg bw/day. Haematological changes (higher mean corpuscular haemoglobin concentration) and plasma biochemical changes (such as reduced total protein, globulin, albumin, and creatinine) were present in males at dose levels of 30 mg/kg bw and higher. Lower relative liver weight was also noted in males at dose levels of 30 mg/kg bw and higher. The NOEL was 10 mg/kg bw.

7. In a preliminary, 30-day oral toxicity study, beagle dogs were dosed orally with doses of 30, 100 and 300 mg/kg bw. Increased serum levels of asparatat aminotransferase and of alanin aminotransferase levels and hepatocytic hydropic degeneration were observed at 100 and 300 mg/kg bw; myeloid bodies were also detected by electron microscopy in the hepatocytes at the same dose levels. No effects were detected at 30 mg/kg bw/day.

   In a 13-week toxicity study, beagle dogs were treated orally with doses of 4, 16, 40 or 160 mg/kg bw/day of pirlimycin hydrochloride in gelatin capsules. Serum asparatat aminotransferase and alanin aminotransferase levels were elevated in the highest dose group. Salivation and vomiting were observed in animals treated with 160 and 40 mg/kg. Histological lesions suggestive of gastric irritation (inflammation, lymphoid hyperplasia) were noted in females at 160 and 40 mg/kg bw. The NOEL was 16 mg/kg bw/day.

8. No effects on fertility or pup survival and growth were observed in a 2-generation reproduction study on rats treated by gavage with doses of 100, 200 or 400 mg pirlimycin hydrochloride/kg bw/day. Salivation, irritability, and reduced weight gain were observed in adult animals at 200 and 400 mg/kg bw. The NOEL for general toxic effects was 100 mg/kg bw/day.

9. In an embryotoxicity/foetotoxicity study, pirlimycin hydrochloride was administered by gavage to pregnant rats at dose levels of 200, 400 or 800 mg/kg bw/day from day 6 to 15 of gestation. Maternal toxicity, as evidenced by soft stools, salivation and reduced weight gain during the dosing period, was observed at 400 and 800 mg/kg bw. The mean number of implantation per litter was slightly decreased at 400 and 800 mg/kg bw, and the resorption rate was slightly increased at the upper dose level; as a consequence, the number of live foetuses per litter was slightly, but not significantly, reduced at 800 mg/kg bw. A significant, dose-related increase in minor skeletal anomalies of the sternebrae was observed at all dose levels. A NOEL for embryotoxicity/foetotoxicity could not be identified.

   In a further study, pregnant mice were treated by gavage with doses of 100, 400 or 1600 mg/kg bw from day 6 to 15 of gestation. At the upper dose level maternal toxicity was evidenced by moderate to severe diarrhoea with a few fatalities. At the same dose level, the only treatment-related effect was a moderate, but statistically significant, reduction in foetal weight. In mice, the NOEL for both maternal toxicity and foetotoxicity was 400 mg/kg bw**.**

   It is concluded that pirlimycin is devoid of any teratogenic potential.

10. Negative results were observed in the following *in vitro* tests both with and without S-9 fraction metabolic activation: the Ames test (two assays in strains TA97, TA98, TA100, TA102, TA1535, TA1537, TA1538, in mammalian cell forward gene mutation assay (HPRT locus) on Chinese hamster ovary (CHO) cells and AS52 cell lines, in the unscheduled DNA synthesis assays (UDS) in primary rat hepatocytes.

Negative results were also found in the micronucleus tests by intraperitoneal route in rats and mice (at 90 to 360 mg/kg bw and 150 to 320 mg/kg bw of free base, respectively) and in a sex-linked recessive lethal assay in *Drosophila melanogaster*.

As pirlimycin hydrochloride gave negative results in a comprehensive battery of *in vitro* and *in vivo* genotoxicity assays, the range of endpoints tested *in vitro* and *in vivo* provide adequate evidence that pirlimycin is devoid of any mutagenic/genotoxic potential.

11. No carcinogenicity studies have been carried out. However, since pirlimycin does not possess any mutagenic or genotoxic potential and is not related to known carcinogenic agents, no further information on carcinogenicity is required.

12. An *in vitro* study on pirlimycin inhibitory activity against intestinal bacteria was performed. Pirlimycin was poorly active or almost inactive against Gram-negatives such as *E.coli*, whereas the lowest MIC_{50} values were obtained against Gram-positive anaerobes.

    At standard inoculum density (10^8 cfu/ml), the following MIC values were obtained: 0.50 µg/ml for *Lactobacillus*, 0.25 µg/ml for *Bacteroides* and *Eubacterium*, 0.06 µg/ml for *Fusobacterium*, *Peptococcus*/*Peptostreptococcus* and 0.03 µg/ml for *Bifidobacterium*.

    The same spectrum of activity was observed at 10^{10} cfu/ml inoculum density, which is closer to human gut situation, *Bifidobacterium* was the most sensitive organism with a MIC_{50} of 0.12 µg/ml.

    Therefore, the MIC_{50} for *Bifidobacterium*, as it was the most sensitive microorganism, at high inoculum density was selected to derive the microbiological ADI.

13. The *in vitro* activity of pirlimycin hydrochloride and its main metabolite, pirlimycin sulphoxide, was investigated. The lowest MIC_{50} for pirlimycin hydrochloride was observed in *Bifidobacterium* spp. and *Eubacterium* spp. (0.06 µg/ml). Pirlimycin sulphoxide was much less active, with a lowest MIC_{50} of 1.0 µg/ml in *Eubacterium* spp.

    Therefore, in these conditions of assay the parent compound showed a 16-fold higher antimicrobial activity as compared to pirlimycin sulphoxide.

14. Healthy male volunteers (five subjects/treatment) received each oral doses of approximately 0, 0.83, 2.1, 4.15 and 8.3 mg/kg bw of pirlimycin. One and/or six days after dosing *Clostridium difficile* was found in stools of 2, 4, 5 and 3 of the subjects treated with pirlimycin at 50, 125, 250 or 500 mg, respectively and in 0, 0, 1 and 0 in the control subjects. Although *C. difficile* is a relevant parameter to assess effects on human gut flora, the data are too limited to reach any definitive conclusion about a level of pirlimycin without effect on human gut flora *in vivo*.

15. The effects of milk concentrations of pirlimycin of 40 to 2400 µg/l on the decrease of pH after incubation were examined with 5 dairy starter cultures (1 yoghurt, 3 Italian cheese and 1 buttermilk/sour cream starters). It was calculated that adverse effects on production processes based on these starters could be expected at average concentrations of 140 to 590 µg/l; The lowest of the 5% prediction limits for the different starters for a doubling of the time needed to attain a pH of 4.6, used as an estimate for clotting time, was 110 µg/l. Considering also that complete raw data of the study were not provided, a clear no-effect concentration on microorganisms relevant to dairy industries was not established.

16. No specific studies on immunotoxicity were provided. However, in repeated dose toxicity studies pirlimycin did not cause any significant effect on white blood cells or organs relevant to immune function.

    Lymphoid hyperplasia concomitant with focal irritation was observed in the stomach of female dogs treated orally for 13 weeks with dose levels of at least 40 mg/kg bw. The NOEL for such effect was 16 mg/kg bw.

17. In humans, mild headache was observed in one out of 10 subjects after single oral administration of 125 mg pirlimycin hydrochloride (approximately 1.8 mg/kg bw). No gastrointestinal disturbances were observed.

    Healthy male volunteers (five subjects/treatment) received each oral doses of approximately 0, 0.83, 2.1, 4.15 and 8.3 mg/kg bw of pirlimycin. The treatment did not elicit any clinical (including gastrointestinal) disturbances. Eosinophils, serum inorganic phosphorus and urine specific gravity showed, in pirlimycin-treated subjects, statistically significant increases.

18. A toxicological ADI can be determined on the basis of the NOEL of 10 mg/kg bw observed in the 13-week rat study, with lower relative liver weight and haematological and serum biochemical changes occurring at dose levels of 30 mg/kg bw and higher. Since the data are of adequate quality, and there is no evidence of effects on reproduction, teratogenicity or mutagenicity/genotoxicity, a safety factor of 100 is adequate to derive the ADI value. Based on this data, a toxicological ADI of 0.100 mg/kg bw (6 mg in a 60 kg individual) was established.

    However, a lower ADI value can be derived for the effects on human gut flora *in vitro*.

19. For the assessment of the microbiological risk, use was made of the formula that was recommended by the CVMP:

$$\text{ADI} = \text{(µg/kg bw)} = \frac{\frac{\text{geometric mean } MIC_{50} \text{ x CF2}}{\text{CF1}} \text{ (µg/ml)} \times \text{daily faecal bolus (150 ml)}}{\text{fraction of an oral dose available for microorganisms} \times \text{weight of human (60 kg)}}$$

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

$$\text{ADI} = \frac{\frac{0.12 \times 10}{1} \times 150}{0.50 \times 60} = 6 \text{ µg/kg bw i.e. } 360 \text{ µg/person}$$

    The following assumptions were made:

    - 0.12 µg/ml is the MIC_{50} in the most sensitive microorganism (*Bifidobacterium*);
    - CF_1 = 1 justified by the use of the most sensitive relevant species and the evidence of a lack of emerging resistance (neither common nor rapid in over 10 millions strains surveyed) to lincosaminides;
    - CF_2 = 10 to take into account the differences between *in vitro* and *in vivo* findings; the *in vivo* data show that a CF2 = 1 would be too conservative. Pirlimycin is stable at low pH;
    - 0.5 was the fraction of an oral dose available for microorganisms;
    - 150 ml was the weight of the daily faecal bolus.

20. After intramammary infusion in dairy cows of 200 mg ^{14}C-pirlimycin hydrochloride/quarter in 4 quarters two times at 24-hour interval plasma pharmacokinetic parameters estimated following non-compartmental analysis showed a two-phase kinetic. Mean values were: AUC_{0-120}: 2270 to 7110 µg.hr/l; $t_{1/2}$ of absorption phase: 2.89 hours; C_{max}^1: 83 µg/l; $t_{1/2}$ of terminal phase: 37 hours; C_{max}^2: 131 µg/l; K_{el} : 0.0224 .

    Approximately 50% of the intramammary administered dose reached the systemic circulation.

Pirlimycin comprised 80.6% of the total residue excreted in urine, pirlimycin sulphoxide 8.0% and two polar unknown compounds 3.8% and 6.7%. In faeces 44.6% of the total residue was pirlimycin, 1.5% pirlimycin sulphoxide, 32% and 18% the two polar unknown compounds.

21. Milk residue depletion kinetics were described in four studies. According to the study, the milk samples were analysed for residues of pirlimycin either by liquid scintillation counting, and/or by HPLC method (limit of detection: 40 µg/l; limit of quantification: 100 µg/l) and/or by microbiological method based on the activity against *M. luteus* and sensitive to 20 µg /l.

    In a first GLP study, 12 dairy cattle in mid-lactation were administered ^{14}C-pirlimycin hydrochloride by intramammary infusion at a dose rate of 200 mg/quarter into four quarters, twice, at 24 hour interval. The mean concentrations of total pirlimycin, parent pirlimycin and microbiological active residues were in the same magnitude : 43 900 µg equivalents/l, 40 700 µg equivalents/l and 41 500 µg/l respectively 12 hours after the end of the treatment. At 96 hours after the end of the administration, the mean concentrations were : 140 µg/l for total radioactivity, 110 µg for the parent compound and 150 µg/l for the microbiologically active residues.

    In another GLP study, 23 dairy cattle in mid-lactation were administered ^{14}C-pirlimycin hydrochloride by intramammary infusion at the recommended dose of 50 mg/quarter of pirlimycin hydrochloride twice at 24-hour interval. In milk, the mean concentrations of total radioactivity were 18 400, 2 030, 420, 170, 110 and 80 µg equivalent/l at 12, 24, 36, 48, 60 and 72 hours after the end of the treatment, respectively. The concentrations of the microbiologically active residues ranged from 17 000 µg/l (12 hours after dosing) to 70 µg/l (72 hours after dosing).

    In 2 non-radiometric studies, cows, mastitic in one quarter (n=26) and non mastitic (n=20), were treated in all four quarters at the recommended dose of 50 mg/quarter of pirlimycin hydrochloride twice at 24-hour interval. For both studies, the mean concentrations of microbiologically active residues in milk were in the magnitude 850, 230, 110 and 70 µg microbiological active compound/l, at 24, 36, 48 and 60 hours after the end of the administration.

    The ratio of pirlimycin to total residues and microbiologically active residues was estimated at 100%.

22. The depletion of pirlimycin in edible tissues was followed in two radiometric studies and one non-radiometric study.

    Two radiometric studies were carried out in cattle. ^{14}C-pirlimycin hydrochloride was administered by intramammary infusion at a dose regimen of two treatments at 24 hour interval and a dose rate of 200 mg (n=12) or 50 mg/quarter (n=23) into four quarters.

    After administration of 200 mg^{14}C-pirlimycin/quarter, the concentrations of total residues were 7 130, 780, 50 and 20 µg pirlimycin equivalents/kg in liver, kidney, muscle and fat respectively at 6 days after the end of the treatment. Then they declined to reach 3 570, 260, 20 and 10 µg pirlimycin equivalents/kg in liver, kidney, muscle and fat respectively at 14 days after the end of the treatment and 500, 10 µg pirlimycin equivalents/kg in liver and kidney at 28 days after the end of the treatment and were below 2 µg/kg for muscle and fat at this sampling time.The concentrations in udder were 130, 30 and lower than 2 µg/kg on days 6, 14 and 28.

    After administration of 50 mg ^{14}C-pirlimycin/quarter, the mean concentrations of total were 2 180, 300, 20, and 10 µg pirlimycin equivalents/kg in liver, kidney, muscle and fat respectively at 6 days after the end of the treatment. Then, they declined to reach 990, 60, 10 and 10 µg pirlimycin equivalent/kg in liver, kidney, muscle and fat respectively at 14 days after the end of the treatment and 890 and 40 µg pirlimycin equivalent/kg in liver and kidney respectively at 18 days after the end of the treatment and were below 5 µg/kg for muscle and fat for this sampling time. In udder, significant amounts of pirlimycin could be measured 330, 160 and 40 µg equivalents/kg in udder at 6, 14 and 18 days after the end of treatment, respectively.

In a non-radiometric study after intramammary infusion into all 4 quarters of two doses of 50 mg/quarter of pirlimycin hydrochloride by at 24 hours intervals to 20 dairy cows significant amounts of pirlimycin could be measured at 2 days after the end of the treatment: 1 470 µg/kg in liver, 460 µg/kg in kidney, 20 µg/kg in muscle and lower than 25 µg/kg in fat. At 7 days, pirlimycin could only be measured in liver (240 µg/kg) and in kidney (60 µg/kg). At later sampling times (14, 21, 28 days), pirlimycin concentrations were below the limit of quantification.

In liver, pirlimycin represented 24.9%, of the total residues radioactivity pirlimycin sulphoxide 63.9% and pirlimycin sulfone 8.1%. In kidneys, pirlimycin represented 43.1%, pirlimycin sulphoxide 46.1%, and pirlimycin sulfone 7.2%.

23. Three specific depletion studies of pirlimycin amounts in liver were conducted. The concentrations of pirlimycin in the liver determined by the *M. luteus* microbiological method (limit of detection: 20 µg/kg; limit of quantification: 30 µg/kg) or the HPLC/TSP/MS method (limit of quantification and limit of detection: 25 and 20 µg/kg)

    After administration of 50 mg/quarter of pirlimycin hydrochloride into all 4 quarters twice of 31 cows at 24 hour interval, the concentrations of microbiologically active residues in the liver were: 1 650 µg/kg, 600 µg/kg, 280 µg/kg, 240 µg/kg and 160 µg/kg at 2, 6, 10, 14 and 21 days after the end of the treatment respectively.

    After administration of 50 mg/quarter of pirlimycin hydrochloride administered twice at 24-hour interval in two quarters of 24 animals, the mean concentrations of microbiologically active residues in liver at 14, 21 and 30 days were 50, 30 and 30 µg/kg and those of the parent compound 90, 40 and 50 µg/kg respectively.

    After administration of 50 mg/quarter of pirlimycin hydrochloride administered twice at 24-hour interval in two quarters of 33 animals, the mean concentrations of microbiologically active residues in liver were 430, 80, 60 and 40 µg/kg and those of pirlimycin 490, 70, 40 and 60 µg/kg at 7, 14, 21 and 30 days, respectively.

24. During the development of this application, the interconversion of pirlimycin in special conditions was studied

    After administered by intramammary route into all 4 quarters two doses of 50 mg/quarter of pirlimycin hydrochloride at 24 hours interval, the mean concentration of parent pirlimycin in the liver of the cows slaughtered at 2 and 7 days were 1 470 and 240 µg/kg. For later sampling times (14, 21 and 28 days) , the concentrations in liver were below 25 µg/kg.
    When a incubation phase was introduced during the assay (incubation at 37°C for 24 hours) the mean concentrations of parent pirlimycin in the liver of the cows slaughtered at 2, 7, 14 and 21 days increased to 1 690 µg/kg, 610 µg/kg, 210 and 60 µg/kg, respectively. At 28 days, the concentrations remained below 25 µg/kg. The relative increase in concentrations of parent pirlimycin was greater for samples with low concentrations (7, 14 and 21 days) whereas only minor increases were observed for samples with higher concentrations (2 days).

    A further series of experiments showed that: the increased concentration of parent pirlimycin occurred at the expense of the sulphoxide; the relative degree of the increase of pirlimycin was dependent upon the initial concentration of residue as a function of the slaughter interval following treatment of the dairy cow with pirlimycin; the greatest increase occurred in the first 6 to 16 hour period, but may require 22 to 24 hours to reach a plateau for maximum parent pirlimycin residue measurement. Experiments on frozen liver showed a slight increase in the concentration of parent pirlimycin; an initial freeze-thaw cycle had a slight impact upon the concentration of parent pirlimycin, but subsequent cycles produced no further apparent increase in concentration; the interconversion of pirlimycin sulphoxide to parent pirlimycin was likely to be due to residual activity of reductase enzymes within the liver.

25. The ratio of the marker residue towards total microbiologically active residues was estimated equal to 1 in muscle, fat, liver and kidney. The parent compound was selected as marker residue.

26. Analytical methods for the determination of pirlimycin residue in milk and liver, based on mass spectrometric (MS) detection, were provided in an ISO 78/2 format with supporting validation data. The limits of quantification were 25 µg/kg for liver and 50 µg/kg for milk.
The HPLC/TSP/MS method has been adapted also for kidney, muscle and fat with a limit of quantification of 50 µg/kg, however for these tissues the method is not fully described and validated in accordance with the recommendations of Volume VI of the Rules Governing Medicinal Products in the European Community.

Conclusions and recommendation

Having considered that:

- a microbiological ADI has been established,
- pirlimycin is the marker residue representing 100% of the microbiologically active residues in all edible tissues including milk,
- a post-slaughter interconversion of pirlimycin sulphoxide into the parent compound may occur in liver,
- an HPLC/TSC/MS analytical method is available; however, this method is not fully validated for kidney, muscle and fat;

the Committee recommends the inclusion of pirlimycin 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
Pirlimycin	Pirlimycin	Bovine	100 µg/kg 100 µg/kg 1000 µg/kg 400 µg/kg 100 µg/kg	Muscle Fat Liver Kideny Milk	Provisional MRLs expire on 1.7.2000

Based on these MRLs values, the daily intake will represent about 85% of the microbiological ADI.

Before the Committee can consider the inclusion of pirlimycin 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. The Applicant should provide all the raw data concerning the study on effects on dairy starter cultures and discuss the results in comparison with findings on other lincosaminides.

   If the Applicant is unable to provide the complete raw data, a new GLP-compliant study should be performed.

2. The Applicant should revise the proposed analytical method for liver, taking into account the observed interconversion of pirlimycin sulfoxide to pirlimycin by the inclusion of an incubation step in the analytical procedure.

3. The Applicant should provide a validation report (with supporting chromatograms and raw data) for the analytical method for muscle, kidney and fat; the method should be presented in an internationally recognised format (e.g. ISO 78/2).