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

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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

ACETYLISOVALERYLTYLOSIN

SUMMARY REPORT (2)

1. Acetylisovaleryltylosin, used as the tartrate salt, is a new macrolide antibiotic which is active against Gram-positive bacteria. It is proposed as an aid in the prevention and treatment of Swine (Mycoplasma) Enzootic Pneumonia (Swine Epidemic Pneumonia) caused by sensitive organisms. The proposed product is a 5% w/w premix which is intended for incorporation into pig feed, for up to 5 days, at a rate of 0.4 to 1 kg product per tonne of finished feed, equivalent to 20 to 50 mg/kg feed acetylisovaleryltylosin activity.

   Acetylisovaleryltylosin was included until 1 July 2001 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
Acetylisovaleryl-tylosin	Sum of acetyl-isovaleryltylosin and 3-O-acetyltylosin	Porcine	100 µg/kg 100 µg/kg 100 µg/kg 100 µg/kg	Muscle Skin + fat Liver Kidney	Provisional MRLs expire on 1.7.2001

   Together with the Annex III recommendation, an additional list of questions was forwarded to the applicant. After assessment of the responses to the list of questions a CVMP Opinion on acetylisovaleryltylosin was adopted stating that the establishment of MRLs as referred to in Article 2 of the aforementioned Council Regulation for acetylisovaleryltylosin in porcine species, could not be recommended in view of the fact that the analytical method available was not fully validated according to Volume VI of the Rules Governing Medicinal Products in the European Union.

   Afterwards a new application has now been submitted to include acetylisovaleryltylosin in Annex I of Council Regulation (EEC) No 2377/90.

2. Acetylisovaleryltylosin has a similar chemical structure to tylosin. The substance is manufactured from tylosin by a bioconversion process. The drug substance contains at least 80% acetylisovaleryltylosin and also contains some impurities derived from substances present in the starting material.

3. A series of *in vivo* and *in vitro* studies indicated that the substance had no important pharmacological effects apart from the antimicrobial activity.

4. The substance was rapidly absorbed after oral administration to rats and pigs. In pigs, peak blood concentrations were attained 2 hours after oral dosing. The substance and a metabolite 3-O-acetyltylosin were widely distributed to the tissues. In both rats and pigs, residues of 3-O-acetyltylosin were higher than residues of the unchanged substance within 2 hours of administration. Highest concentrations were found in the liver, bile and kidneys. The studies were carried out during the middle 1980's and the raw data were no longer available.

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5. *In vitro* metabolism studies confirmed that acetyisovaleryltylosin was rapidly metabolised to 3-O-acetyltylosin.

6. A GLP-compliant study in which Sprague-Dawley rats were given ^{14}C-acetylisovaleryltylosin tartrate in oral doses of 20 mg/kg bw/day for 7 days was performed. The substance was poorly absorbed with peak plasma concentrations of 0.03 to 0.18 µg equivalents/ml achieved 2 to 4 hours after the first dose. There was no evidence of accumulation of radioactivity in plasma after repeated dosing. Most of the administered dose was excreted in the faeces with 88.3% and 85.6% of the administered dose recovered from the faeces of males and females respectively, over the 288 hour collection period. Excretion in the urine over the same time period accounted for 2.8% and 1.2% in males and females.

   The amount of radioactivity remaining in the tissues 3 hours after the last dose was low, with highest concentrations in the liver of 3450 and 2890 µg equivalents/kg in males and females, followed by the spleen (1460 and 1900 µg equivalents/kg in males and females), kidney (1240 and 1190 µg equivalents/kg in males and females) and bone marrow (1060 and 1100 µg equivalents/kg in males and females). By 144 hours after the last dose, concentrations of radioactivity in most tissues were at or below the limit of reliable detection. The substance was extensively metabolised with up to 6 and 9 components in the urine from male and female rats respectively and up to 10 components in faeces. The only substances that were identified in urine and faeces were acetylisovaleryltylosin and the metabolite 3-O-acetyltylosin. Four components were detected in pooled liver and kidney samples. One of these substances was tentatively identified as the metabolite 3-O-acetyltylosin and accounted for 16 to 17% of total residues in liver extracts and 56 to 67% of total residues in kidney extracts.

7. Acute oral LD_{50} values of 758 and 819 mg/kg bw in male and female mice respectively and greater than 3016 mg/kg bw in rats were obtained. The substance was of low toxicity when administered subcutaneously. It was more toxic when administered intravenously with acute LD_{50} values of 121 and 134 mg/kg bw in male and female mice and 58 and 48 mg/kg bw in male and female rats, respectively. The metabolite 3-O-acetyltylosin and 3 by-products of the manufacture of the substance were also of low acute oral toxicity (values greater than 1000 mg/kg bw).

8. A repeated-dose toxicity study was carried out in which Sprague-Dawley rats (10 animals per sex and group) were fed diets containing 0, 400, 2000, 10 000 or 50 000 mg/kg feed for 4 weeks. The dose level of 50 000 mg/kg feed was toxic causing the deaths of several animals. Body weight gain and food consumption were significantly reduced at 50 000 mg/kg feed and slightly reduced in the 10 000 mg/kg feed group. Haematological examinations were indicative of mild anaemia at 50 000 mg/kg feed. Pathological changes included effects such as caecal distention which is a common effect caused by feeding large doses of antibiotics to rodents, and changes in organ weight which reflected the reduced nutritional status of the animals. At the lowest dose level of 400 mg/kg feed, the only treatment-related effect was caecal distention in 2 males and 2 females. A LOEL of 400 mg/kg feed was retained for the study.

9. The previous study was repeated using the same dose levels with the treated diets administered for 13 weeks. The effects observed were similar to those seen in the 4-week study. The NOEL was 2000 mg/kg feed, equivalent to 135.9 and 159.1 mg/kg bw/day for male and female rats respectively.

10. Groups of 10 male and 10 female CD-1 mice were fed diets containing 0, 250, 2500 or 25 000 mg/kg feed of acetylisovaleryltylosin tartrate for 13 weeks. For males and females, the achieved test substance intakes were 39 to 55 and 47 to 64 mg/kg bw/day for the low dose group, 407 to 554 and 543 to 691 mg/kg bw/day for the mid-dose group and 4188 to 5919 and 5046 to 6673 mg/kg bw/day for the top dose group, respectively. Overt signs of toxicity were observed in both sexes administered 2500 and 25 000 mg/kg feed. In males, there was a dose-related reduction in body weight gain in all treated groups. In females, body weight gains were significantly reduced in the groups administered 2500 and 25 000 mg/kg feed. Food consumption was significantly reduced in both sexes at the top dose. There were some minor changes in haemoglobin and haematocrit values, which were observed at all dose levels.

Based on the small decrease in body weight gain in males and the minor changes in haematology values, the lowest dose level of 250 mg/kg feed, equivalent to approximately 46 and 55 mg/kg bw/day in males and females respectively, was retained as a LOEL.

11. Up to 5 times the indicated dose in pigs (the target species) was tolerated with no significant adverse effects.

12. In a 2-generation reproduction study, parental groups of 28 male and 28 female Sprague-Dawley rats were fed diets containing 0, 400, 2000 or 10 000 mg/kg feed of acetylisovaleryltylosin tartrate for 10 weeks prior to mating. Treatment continued throughout mating, gestation and lactation. In both generations receiving 2000 and 10 000 mg/kg feed, there was an initial reduction in body weight gain of males. In females, the body weight gain of the F1 parents administered 10 000 mg/kg bw was reduced during gestation. For the F1 litters, there was a significant dose-related reduction in litter and pup weights in the 2000 and 10 000 mg/kg feed groups. For the F2 litters, pup weights were significantly reduced in the 10 000 mg/kg feed groups. The NOEL was 400 mg/kg feed, corresponding to approximately 22 to 65 and 32 to 67 mg/kg bw/day in males and females respectively.

13. A teratogenicity study was carried out in which mated female Sprague-Dawley derived rats were given daily oral doses of 0, 125, 500 or 2000 mg/kg bw/day of the test substance from day 7 to 17 of pregnancy. Maternal toxicity was evident at 500 and 2000 mg/kg bw with a dose-related increase in salivation and water intake and reduced food consumption during the treatment period. There was no evidence of foetotoxicity or teratogenicity at any dose level.

14. Groups of 30 mated female CD-1 mice were given daily oral doses of 0, 200, 400 or 700 mg/kg bw/day of the test substance by gavage from days 6 to 15 of gestation. Three dams given 700 mg/kg bw/day died. Overt signs of toxicity were observed in approximately 50% of the dams in the 700 mg/kg bw/day group and in 4 dams from the 400 mg/kg bw/day group. Mean foetal litter weight was reduced in the 400 and 700 mg/kg bw/day groups but the reduction was small and not statistically significant. The overall NOEL for the study was 200 mg/kg bw/day.

15. Acetylisovaleryltylosin tartrate gave negative results in poorly reported assays for gene mutation in prokaryotic systems. Negative results were obtained in an *in vitro* assay for gene mutation in the mouse lymphoma L5178Y cell line, in both the presence and absence of metabolic activation. Positive results were obtained in an *in vitro* chromosomal aberration assay in Chinese hamster ovary cells; a stronger positive response was obtained in the presence of metabolic activation. In a second *in vitro* chromosomal aberration assay in Chinese hamster ovary cells, a positive response was again obtained with acetylisovaleryltylosin tartrate in the presence of metabolic activation but negative results were obtained with the metabolite 3-O-acetyltylosin. An *in vivo* micronucleus test in the bone marrow of mice given two oral doses of 200, 400 or 800 mg/kg bw, with an interval of 24 hours between the doses, gave negative results.

16. No carcinogenicity studies were carried out. However the chemical structure of the substance did not possess any alerting features. No pre-neoplastic lesions were observed in the repeated-dose toxicity studies, though these were of short duration (up to 13 weeks). It was concluded that carcinogenicity studies were not required.

17. A toxicological ADI of 220 µg/kg bw was retained by applying a safety factor of 100 to the NOEL of 22 mg/kg bw/day which was established in the 2-generation study.

18. *In vitro* MIC values were determined for 98 separate strains from 10 genera of enteric bacteria of human origin, at 2 different inoculum densities. *Escherichia* and *Proteus* strains were insensitive. A geometric mean MIC_{50} of 0.325 µg/ml was calculated for all sensitive genera (*Bifidobacterium, Eubacterium, Clostridium, Bacteroides, Fusobacterium, Enterococcus, Peptostreptococcus* and *Lactobacillus*), at the higher inoculum density, and subtracting the lower 10% confidence limit of the mean to obviate the need for an arbitrary mathematical component in CF1.

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

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

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

$$\text{ADI} = \frac{\dfrac{0.325 \text{ x } 1}{1} \text{ X } 150}{0.8 \text{ x } 60} = 1.02 \text{ µg/kg bw, i.e.} = 61 \text{ µg/person}$$

The following assumptions were made:

- CF1 = 1 because the geometric mean of all sensitive genera was used,
- CF2 = 1 because the MIC values did not differ significantly when the inoculum density was increased (geometric mean MIC_{50} was 0.266 µg/ml at approximately 10^4 to 10^5 cfu/spot and 0.325 µg/ml at approximately 4.5 x 10^6 cfu/spot),
- 150 g was the weight of the daily faecal bolus,
- the fraction of the oral dose available for microorganisms was considered to be 0.8, because although 70 to 88% of the total radiolabelled oral dose was excreted in the faeces of rats and pigs, only 1 to 7% was identified as parent compound and, in the absence of data to show otherwise, the metabolites were assumed to have the same antimicrobial activity as the parent compound.

19. Eight male and 8 female Landrace pigs were administered daily oral doses of ^{14}C-acetylisovaleryltylosin tartrate on 7 consecutive days, in gelatin capsules, at a target dose of 2.5 mg/kg bw/day. The dose was intended to mimic the dietary administration of 50 mg acetylisovaleryltylosin tartrate per kg feed. Faeces and urine were collected from 2 males and 2 females over 24 hour periods from 24 hours pre-dose up to 5 days following the last dose. The major route of elimination was *via* the faeces. Over the 264-hour collection period, approximately 70% of the total dose was excreted in faeces in males and 74% in females. Excretion in the urine accounted for around 3 to 4% of the total dose. HPLC analysis indicated that 3-O-acetyltylosin accounted for approximately 10% of the total radioactivity in pooled samples of urine and faeces. Acetylisovaleryltylosin accounted for 1 to 2% of the total radioactivity in the urine samples and 2 to 7% of the radioactivity in the faecal samples. Eight unidentified components were also present, each accounting for 3 to 17% of the radioactivity in urine and 6 to 16% of the radioactivity in faeces. The metabolite profiles in urine and faeces were similar following the first dose and the last dose indicating that the metabolism did not change over the 7-day dosing period.

20. In the same study, the pigs were slaughtered (in groups of 2 males and 2 females) at 12 hours, 1, 3 and 5 days after the last dose. Total residues in tissues were determined by combustion and liquid scintillation counting. Total residues were highest in liver and depleted from mean values of 483 µg equivalents/kg at 12 hours to 234 and 90 µg equivalents/kg at 1 and 3 days. Mean total residues in kidney depleted from 308 µg equivalents/kg at 12 hours to 202 and 107 µg equivalents/kg at 1 and 3 days respectively. Mean total residues in muscle depleted from 29 µg equivalents/kg at 12 hours to 11 µg equivalents/kg at 1 day. Mean total residues in composite fat samples were 187 µg equivalents/kg at 12 hours, 64 µg equivalents/kg at 1 day and 43 µg equivalents/kg at 3 days. Mean total residues in samples of skin with fat were 110 µg equivalents/kg at 12 hours, 120 µg equivalents/kg at 1 day and 47 µg equivalents/kg at 3 days. Samples of liver and kidney from one male and one female animal killed 12 hours after the last dose and 1 female killed 1 day after the last dose were extracted with methanol and re-analysed by liquid scintillation counting. The samples were then analysed by HPLC-radioprofiling. In the pigs killed 12 hours after dosing, 3-O-acetyltylosin accounted for approximately 6 to 8% and

9 to 10% and acetylisovaleryltylosin accounted for approximately 6 to 9% and 7 to 16% of the total residues in liver and kidney respectively. In the pig killed 1 day after dosing, 3-O-acetyltylosin accounted for approximately 5% and 13%, and acetylisovaleryltylosin accounted for approximately 5% and 6% of the total residues in liver and kidney respectively. Up to 8 unidentified components were found in the liver and kidney samples, each accounting for 1 to 32% of the total residues.

21. In the same study, all samples of fat, kidney, liver, muscle and skin with fat were analysed for residues of acetylisovaleryltylosin and 3-O-acetyltylosin using the proposed routine analytical method based on HPLC with mass spectrometric detection. In all samples, residues of both analytes were below the limit of quantification (below 50 µg/kg in each case).

22. The proposed routine analytical method was based on HPLC with mass spectrometric detection and was described in the ISO 78/2 format. The method was validated for porcine tissues in accordance with Volume VI of the Rules Governing Medicinal Products in the European Community. The limits of quantification were 25 µg/kg in all relevant tissues. The limits of detection were less than 1 µg/kg for all tissues. The extraction procedure involved the use of chloroform.

Conclusions and recommendation

Having considered:

- an ADI of 1.02 µg/kg bw (i.e. 61 µg/person) was established for acetylisovaleryltylosin,
- in pigs, the sum of acetylisovaleryltylosin and 3-O-acetyltylosin was identified as the marker residue and considering that the marker residue represents 15, 25, 100 and 100% of the total residues in liver, kidney, muscle and skin+fat respectively between 12 and 24 hours after treatment,
- the total residue in all tissues was rapidly depleted so that at 12 hours, 24 and 72 hours after treatment the amount of residues likely to be ingested by consumers represents 134, 66 and 30% of the ADI respectively,
- marker residue concentrations were below the limit of quantification in all tissues by 12 hours after treatment therefore MRLs were set at a value corresponding to twice the limit of quantification,
- a validated routine analytical method for the determination of the marker residue in edible tissues of pigs is available;

the Committee for Veterinary Medicinal Products recommends the inclusion of acetylisovaleryltylosin 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
Acetylisovaleryl-tylosin	Sum of acetyl-isovaleryltylosin and 3-O-acetyltylosin	Porcine	50 µg/kg 50 µg/kg 50 µg/kg 50 µg/kg	Muscle Skin + fat Liver Kidney	

Based on these MRLs values, the daily intake will represent about 100% of the ADI.