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

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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

CLAVULANIC ACID

SUMMARY REPORT (2)

1. Clavulanic acid (3 (2-hydroxyethylidene)7-oxo-4-oxo-l-azabicyclo-(3.2.0) heptane-2carboxylic acid), CAS No 58001-44-8, is a beta-lactam compound structurally related to the penicillins. It is a fermentation product of *Streptomyces clavigulerus*.

   In veterinary medicine, clavulanic acid is always used in combination with amoxicillin. The formulations contain a ratio of 1:4 clavulanic acid (usually as potassium clavulanate) to amoxicillin trihydrate and are intended for use in cattle, pigs and sheep as intramuscular injectable suspensions (1.75 mg/kg bw once daily for 5 days), in lactating cows for intramammary infusion (50 mg/quarter, 0.4 mg/kg bw, twice daily, i.e. 0.8 mg/kg bw/day, for 3 days) and for oral treatment in preruminant calves (2.5 mg/kg bw, twice daily, i.e. 5 mg/kg bw/day, for 3 days).

   Clavulanic acid has been widely used in human and veterinary medicine for several years in combination with amoxicillin.

   Currently, clavulanic 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
Clavulanic acid	Clavulanic acid	Bovine, ovine, porcine	200 µg/kg 200 µg/kg 200 µg/kg 200 µg/kg	Muscle Fat Liver Kidney	Provisional MRLs expire on 1.7.2001
		Bovine, ovine	200 µg/kg	Milk	

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

2. Clavulanic acid is a specific and irreversible inhibitor of a wide range of bacterial beta-lactamases. Addition of clavulanic acid to amoxicillin formulation enhances the effectiveness of this antibiotic against bacteria that are ordinarily resistant to amoxicillin. Activity of clavulanic acid is linked to an intact beta-lactam structure.

3. No pharmacological alterations were elicited in the rat by oral single doses of 80 mg/kg bw or less on body temperature and on digestive, excretory and neurological/neuromuscular functions. Marked alterations of heart rate, blood pressure and electrocardiogram were observed in dogs after a single intravenous injection of 125 mg/kg bw of potassium clavulanate and higher.

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4. Pharmacokinetic studies were carried out *in vivo* with ^{14}C-potassium clavulanate in rats and dogs following oral administration, and also using biotransformation assays *in vitro* on liver homogenates of rat, dog and calf.

   Following oral administration significant amounts of the radiolabelled dose were absorbed. Urine was the main excretion route accounting for 42% and 52% of the administered dose in rat and dog respectively. Elimination of radioactivity also occurred via expired air and represented 17% of the dose in both species.

   Blood pharmacokinetics were determined in dairy cows, calves, pigs, sheep following parenteral treatment. In dairy cows, half-life was approximately 2 hours, in calves and pigs approximately 1 hour and in sheep 0.76 hour. In ruminants, plasma maximum concentrations following oral treatment normally occur within a few hours post dosing.

   The main identifiable compounds in the urine of rats and dogs were the parent compound (16 to 23% and 14 to 38% of the urinary recovery in the rat and the dog, respectively) and the metabolite 1-amino-4-hydroxybutan-2-one (21 to 35% and 10 to 20% of the urinary recovery in the rat and the dog, respectively). The latter was identified as the main metabolite also *in vitro*. This was also the main metabolite in calf liver homogenates assayed *in vitro.*

   Pharmacokinetic studies were carried out *in vivo* with ^{14}C-potassium clavulanate in humans following oral administration. Urine was the main excretion route accounting for 73% of the administered dose. Most of the radioactivity in the urine was excreted in the first 24 hours after treatment (68% of the dose). Elimination of radioactivity also occurred via expired air with 17% of the dose and the amount recovered from human faeces was only 8% of the dose. Clavulanic acid was the major radioactive component present in urine following oral administration (23% of the dose). The two major metabolites were 2,5-dihydro-4-(2-hydroxyethyl)-5-oxo-1H-pyrrole-3-carboxylic acid (15.6% of the dose) and 1-amino-4-hydroxybutan-2-one (8.8% of the dose). Clavulanic acid and 1-amino-4-hydroxybutan-2-one were the major components in plasma following oral administration. Terminal half-life of clavulanic acid in plasma was 0.8 hours. The metabolite did not retain the beta-lactam structure and the antimicrobial activity was only referred to the parent compound.

5. Clavulanic acid has a low oral acute toxicity in both adult rats and mice, with an LD_{50} greater than 2000 mg/kg bw. However, the single dose toxicity was higher in a study on pre-weaning rats, with gastrointestinal signs and deaths occurring even at the lowest dose level tested (125 mg/kg bw).

   Several toxicity studies were performed on laboratory species both with potassium clavulanate and with 1:2 clavulanic acid/amoxicillin. In general, clavulanic acid/amoxicillin appeared slightly more toxic than potassium clavulanate. In the repeated dose toxicity studies, gastrointestinal irritation in rats and dogs treated with clavulanic acid/amoxicillin was observed even at the lowest dose levels tested (30 and 15 mg/kg bw/day, respectively); renal tubular vacuolation in dogs was the most sensitive systemic effect (NOEL 15 mg active substances/kg bw/day i.e. 5 mg/kg bw/day for clavulanic acid). These studies using combination products were not used to conclude on overall NOELs since interference from amoxicillin regarding toxicological effects of clavulanic acid could not be ruled out.

6. A 28-day and a 90-day oral toxicity study with potassium clavulanate were performed both in the rat and in the dog. Clinical, biochemical and haematological effects, reduced bodyweight gain, gastrointestinal irritation and liver toxicity were observed.

   In the rat the most sensitive indicators of potassium clavulanate effects were decreased urine output, increased urine osmolarity and increased white blood cell count; in the dog clinical signs (emesis, salivation) and hydropic changes of hepatocytes were observed. In both species NOEL in the 90-day study was 20 mg/kg bw/day. Caecal enlargement was observed in the rat at lower dose levels (NOEL 10 mg/kg bw/day in the 90-day study).

7. The following oral reproductive and developmental toxicity studies on clavulanic acid are available, one generation in rat; teratogenic effects in rat; teratogenic effects in mouse (2 studies) and peri- and postnatal development in rat. The studies were adequately designed. Overall, a moderate reduction of female fertility and/or growth and survival of the foetuses were seen at dose levels eliciting slight systemic or maternal toxicity. Although a statistically non-significant, but dose-related, reduction in the mean number of *corpora lutea* per dam was detected in both the rat F0-generation and the mouse F1-generation at the lowest dose level in the testing programme, 10 mg/kg bw/day, this dose level could be retained as a NOEL.

8. Clavulanic acid was tested in an adequate set of in *vitro* and *in vivo* genotoxicity studies.

   Results indicating mutagenic effects were obtained in one forward mutation assay in mouse lymphoma cells. Significant increases in mutation rate were obtained in the absence and to the lesser extent in the presence of metabolic activation. However, effective concentrations were high (4000 µg/ml and 8000 µg/ml with and without metabolic activation, respectively) and cytotoxicity was concurrently present.

   Absence of genotoxic effects was observed in the following *in vitro* and *in vivo* tests, gene conversion in *Saccharomyces cerevisiae*, *Samonella*-microsomal assay, with and without metabolic activation, dominant lethal in the mouse at oral dose levels up to 4500 mg/kg bw and the micronucleus test in the mouse at oral dose levels of up to 9000 mg/kg bw. The overall data indicate that clavulanic acid is not a genotoxic agent.

9. No carcinogenicity studies were performed. In view of the absence of genotoxic effects of clavulanic acid such data were not required.

10. Clavulanic acid did not induce skin sensitisation in guinea pig when tested by the Magnusson-Kligman method.

    The subcutaneous administration of clavulanic acid/amoxicillin (more than or equal to 25.5 mg clavulanic acid) slightly reduced antibody titres in the rabbit.

11. Combination products using clavulanic acid/amoxicillin have a history of widespread use in human therapy. As in animals, clavulanic acid is never administered on its own, but always in combination with amoxicillin. Therefore it is not easily possible to separate effects of clavulanic acid from those of amoxicillin. Oral tablets which contain for instance 250 to 875 mg amoxicillin and of 125 mg clavulanic acid are used in human patients. Hypersensitivity reactions and adverse, mostly gastrointestinal, effects have been reported to occur with a rate and severity comparable to that of other beta-lactams. Data on direct administration of clavulanic acid were obtained from a study where male human volunteers received a nominal oral dose of 125 mg of clavulanic acid which is the oral dose usually used in human antibiotic therapy. A substantial number of clinical-pharmacological parameters have been monitored and there were no changes in pharmacological response as concerns haemodynamics (e.g. pulse, blood pressure), haematology, clinical chemistry or urinalysis.

12. A toxicological ADI of 0.05 mg/kg bw (i.e 3 mg/person) was established based on the NOEL of 10 mg/kg bw/day observed in the reproductive toxicity studies in rats and mice and applying a safety factor of 200 to take into account the effects on reproductive function induced in rats and mice observed at this dose.

13. MIC data were generated for a range of micro-organisms derived from the human gastro-intestinal tract (approximately 100 strains). MIC_{50} values ranged from *Eubacterium* 128 µg/ml, *Proteus* 32 µg/ml, *Escherichia* 32µg/ml, *Lactobacillus* 32 µg/ml, *Peptostreptococcus* 8 µg/ml, *Enteropcoccus* 512 µg/ml, *Bifidobacterium* 12 µg/ml, *Bacteroides* 8 µg/ml, *Clostridium* 8 µg/ml to *Fusobacterium* 2 µg/ml.

For the assessment of a microbiological ADI use was made of the formula recommended by the CVMP:

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

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

$$\text{ADI} = \frac{\dfrac{8.84 \times 2}{5} \times 150}{0.1 \times 60 \text{ kg}} = 88.4 \text{ µg/kg bw} = 5307 \text{ µg/person}$$

The following assumptions were made:

- CF1 = 5, to account for the fact that both chromosomal and plasmidic resistance was observed;
- CF2 = 2, estimated factor to give allowance for MIC values at conditions more similar to those prevailing *in vivo*; there was an increase of MIC values with increasing bacterial densities and a significant increase after passage of clavulanic acid through a simulated gastro-intestinal tract;
- 0.1 = oral dose available to gut bacteria as determined using oral ^{14}C-clavulanic acid in humans;
- 150 g = weight of the daily faecal bolus;
- 8.84 = 10% lower confidence limit of the geometric mean of MIC_{50} values over the all genera tested.

14. Considering that the toxicological ADI of 0.05 mg/kg bw (i.e. 3 mg/person) is lower than the microbiological ADI of 0.09 mg/kg bw (i.e. 5 mg/person) the toxicological ADI was considered the relevant ADI for assessing the risk for the consumer.

15. The impact of clavulanic acid on the fermentation characteristics of dairy starter cultures was studied using bacterial strains representative of those commonly used in EU dairy industry (e.g. *Lactobacillus delbrueckii* subsp. *bulgaris*, *Streptopcoccus thermophilus*, *Lactobacillus lactis* subsp. *Lactis*; *Lactococcus lactis* subsp *lactis*-mixed starter culture. Based on the pH profiles over time the no effect level for inhibition (i.e. change in pH not greater than 0.3) was 1000 µg/l for the most sensitive organisms.

16. Recent total residue depletion studies in tissues using ^{14}C-labelled clavulanic acid in lactating cows (intramammary), cattle (intramuscular, oral) and pigs (intramuscular) following recommended treatments were provided. These studies were specifically designed to investigate residue depletion at early time points after treatment. The highest total tissue residues were always in kidney followed by liver, muscle and fat.

    In dairy cows (4 animals), total residues at 12, 24, 36 and 48 hours after 3 intramammary doses (45 mg ^{14}C-clavulanic acid) were as follows: 936, 519, 201 and 276 µg equivalents/kg in kidney, 316, 297, 299 and 344 µg equivalents/kg in liver, 58, 41, 16 and 41 µg equivalents/kg in muscle and 51, 15, 6, 14 µg equivalents/kg in fat, respectively.

    In calves (4 animals), following 5 intramuscular doses of 1.7 mg/kg bw ^{14}C-clavulanic acid, mean total residues at 8 and 24 hours after treatment were 5905 and 4664 µg equivalents/kg in kidney, 2478 and 1910 µg equivalents/kg in liver, 619 and 264 µg equivalents/kg in muscle and 283 and 180 µg equivalents/kg in fat, respectively. Mean ^{14}C-total residue concentrations at the injection sites were 16308, 4062, 1955 and 3419 µg equivalents/kg at 8, 24, 32 and 48 hours after treatment, respectively.

Mean ^{14}C-total residues in tissues of 4 calves at 8 and 24 hours following 6 daily oral doses of 3.1 to 3.4 mg/kg bw were 10908 and 12608 µg equivalents/kg in kidney, 5399 and 6769 µg equivalents/kg in liver, 1305 and 1426 µg equivalents/kg in muscle and 3139 and 1808 µg equivalents/kg in fat, repectively.

Mean ^{14}C-total residues in tissues of 4 pigs which were determined 8 and 24 hours following intramuscular administration of 1.5 to 1.8 mg/kg bw were 3113 and 1937 µg equivalents/kg in kidney, 2158 and 1872 µg equivalents/kg in liver, 390 and 310 µg equivalents/kg in muscle and 462 and 361 µg equivalents/kg in skin + fat, respectively. Mean ^{14}C-total residue concentrations at the injection sites were 1522, 926, 656, and 796 µg equivalents/kg at 8, 24, 32 and 48 hours, respectively.

17. There were several non-radiometric tissue residue depletion studies using commercial amoxicillin/clavulanic acid preparations. Calves (22 animals) were treated orally with a nominal dose of 2.5 to 3.3. mg clavulanic acid/kg bw twice daily for 3 days. Pigs (30 animals) were treated intramusculary with a dose of 1.75 mg clavulanic acid/kg bw once daily for 5 days. Calves (22 animals) were treated intramusculary with a dose of 1.75 mg clavulanic acid/kg bw once daily for 5 days. Cows (20 animals) were treated intramammary twice daily for 3 days with a dose of 1 injector/quarter (containing 50 mg clavulanic acid in combination with amoxicillin and prednisolone) in each quarter of the udder. Clavulanic acid was determined by a validated HPLC method.

    In all these studies, clavulanic acid was only detectable at very early time points mainly in kidney of calves (oral study, 356 µg/kg at 8 hours) and in pigs at intramuscular injection sites (153 µg/kg at 6 hours). In one kidney sample of a dairy cow treated intramammarily a concentration of 258 µg/kg was observed at 48 hours. Any other tissues of cows, calves or pigs investigated showed concentrations of less than the respective limits of quantification (50 to 200 µg/kg) at 12 hours or later after treatment.

18. Clavulanic acid and the tissue metabolite 1-amino-4-hydroxybutan-2-one were also determined by radio-TLC methods in tissues obtained in radiometric studies. The results for clavulanic acid determinations were comparable to those obtained in the non-radiolabelled studies i.e. concentrations were very low even at the early time points investigated. The immediate metabolic and/or degradation product, 1-amino-4-hydroxybutan-2-one was also consistently observed in the edible tissues investigated. This compound was present at almost equal or somewhat greater concentrations than the parent compound. In summary, both the parent compound and 1-amino-4-hydroxybutan-2-one constituted only relatively small fractions of the total residues in most tissues investigated. From the available data the overall ratio of parent compound to total residues in tissues was roughly estimated with 10% for the early time points investigated. In addition to clavulanic acid and 1-amino-4-hydroxybutan-2-one, no other single residue component could be identified. The fact that clavulanic acid and 1-amino-4-hydroxybutan-2-one accounted for relatively small amounts of the total radioactive residue is consistent with the observation of rapid metabolism/degradation of clavulanic acid to highly polar components. There was also indication for the formation of bound radioactivity/residues, possibly by entering of largely degradated metabolites/carbon label into intermediate metabolism.

19. In older tissue residue studies in which tissue residues were investigated at later time points after treatment by means of microbiological assays (e.g. *Klebsiella aerogenes* NCTC 11228) no detectable residues (less than 10 µg/kg) were found in the tissues of young calves treated orally with 8 mg clavulanic acid/kg bw for 3 days and killed after more than 3 days withdrawal, nor in the tissues, including injection sites, of fattening calves, pigs and sheep treated intramuscularly with 1.75 mg/kg bw for 5 days and killed after more than 10, 7 and 14 days after the end of administration, respectively.

20. Total residues in milk of 4 lactating cows were determined at 12 to 48 hours following 3 intramammary administrations of 45 mg ^{14}C-clavulanic acid into each quarter of the udder at 12 hours intervals. Maximum ^{14}C-total residue concentrations in milk were 11367, 937, 192 and 119 µg equivalents/kg at the 1st, 2nd, 3rd and 4th milkings, respectively. Clavulanic acid and the 1-amino-4-hydroxybutan-2-one metabolite could be detected in the 1st to 3rd milking and accounted for 41% to 33 % and 45% to 23% of ^{14}C-total radioactivity, respectively. The results indicated that clavulanic acid is a potential marker residue in milk.

21. In a non-radiolabelled milk residue study, after intramammary treatment of lactating cows (8 animals) with 3 consecutive doses (each 50 mg clavulanic acid in combination with amoxicillin and prednisolone) in each quarter of the udder at 12 hours intervals, clavulanic acid concentrations were measurable in milk up to 36 hours after the last administration (3 milkings). Maximum concentrations ranged from 16693 µg/kg at 12 hours and 2137 µg/kg at 24 hours to 379 µg/kg at 36 hours. From 48 hours onwards, no quantifiable residues were observed (lower than the limit of quantification of 50 µg/l). In a second study where cows were treated with a clavulanic acid/amoxicillin combination without prednisolone maximum clavulanic acid concentrations in milk were in the same range with 11878 µg/kg at 12 hours, 645 µg/kg at 24 hours, 58 µg/kg at 36 hours and lower than the limit of detection of 18 µg/kg at 48 hours. Clavulanic acid residues were determined by a validated HPLC method.

22. Measurable milk residues were also found by microbiological assays in cow's milk following intramammary administration of 125 mg/quarter in all four quarters. Mean residue depleted from 30000 µg/1 at 8 hours to 3500, 860, 70 and 20 µg/1 at 24, 32, 48 and 56 hours, respectively. No detectable residues (less than 4 µg/l) were observed 72 hours after the administration. After a 5-day 1 intramuscular treatment of lactating cows at a dose of 1.75 mg/kg bw/day, residue concentrations of 20 to 40 µg/l were observed after 8 hours; no detectable residues were found after 24 hours.

23. In dairy ewes treated intramuscularly with 5 daily injections of 1.75 mg/kg bw, 20 µg/l were found in milk 8 hours after the end of treatment; no detectable residues were found after 24 hours.

24. A fully validated analytical method based on HPLC with UV detection for the determination of clavulanic acid in milk and tissues is available. The validation was supported by adequate raw data. For milk, the limit of quantification was 50 µg/l. Precision and accuracy (within- and between day examined in the range of 50 to 400 µg/kg) were within the specified acceptance criteria. The validated limits of quantification were 50 µg/kg for muscle and fat or skin + fat in natural proportions, 100 µg/kg for liver and 200 µg/kg for kidney. Acceptable validation (within- and between day) was done in the range from the limit of quantification to 400 and 800 µg/kg, respectively.

25. Inadequate data were provided to assess the residue depletion profiles in sheep following treatment by relevant routes. No validated analytical method for monitoring residues in sheep edible tissues including ewes' milk was available. Therefore, no final MRLs could be proposed for sheep.

26. The radiometric studies carried out in cattle and pigs showed that the total radioactivity in edible tissues declined rapidly within the 24 hours after treatment. Twenty-four after the end of the treatment, the levels of radioactivity quantified in edible tissues of calves following 6 daily oral doses were much higher than the amounts measured in edible tissues of other target species and of calves when the compound was administered by another route of administration. They were in the magnitude of 12000 µg equivalents/kg in kidney, 6500 µg/kg in liver, 1500 µg/kg in muscle and 1800 µg/kg in fat leading to a daily intake susceptible to be ingested representing 91% of the acceptable daily intake. However, although clavulanic acid is rapidly metabolised, it represents 10% of the total residues for the early time points. It was deduced that clavulanic acid concentrations were 1200, 650, 150 and 180 µg/kg in kidney, liver, muscle and fat. This study was retained as the basis to establish the MRLs for cattle and pigs. The depletion studies carried out with the radiolabelled compound gave a more accurate distribution of residues in the edible tissues so that the final MRLs were slightly different from the provisional ones.

Conclusions and recommendations

Having considered:

- that a toxicological ADI of 0.05 mg/kg bw (i.e. 3 mg/person) was established,
- the tissue distribution in calves twenty-four hours after the end of the repeated oral treatment,
- that the parent compound was retained as marker residue which is estimated to constitute approximately 10% of the total residues in bovine and porcine tissues and 33% in bovine milk,
- that insufficient information on residue depletion and validation of the analytical method was available in respect of sheep tissues and milk
- that a validated analytical method for the determination of clavulanic acid in cattle and pigs tissues and in bovine milk is available;

the Committee recommends the inclusion of clavulanic acid for bovine and porcine species in Annex I of Council Regulation (EEC) No 2377/90 as follows:

Pharmacologically Active substance	Marker residue	Animal species	MRL	Target tissue	Other provisions
Clavulanic acid	Clavulanic acid	Bovine	100 µg/kg 100 µg/kg 200 µg/kg 400 µg/kg 200 µg/kg	Muscle Fat Liver Kidney Milk	
		Porcine	100 µg/kg 100 µg/kg 200 µg/kg 400 µg/kg	Muscle Skin+fat Liver Kidney	

Based on these MRLs total residues intake at one day after treatment from food derived from treated animals including milk was estimated to be approximately 55% of the ADI.