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

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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

CLENBUTEROL

SUMMARY REPORT (2)

1. Clenbuterol (4-amino-3,5-dichloro-α[[(1,1-dimethylethyl)amino]methyl]-3,5-dichlorobenzyl alcohol, CAS-no 37148-27-9) is a direct-acting β_2-sympathomimetic agent, which is usually administered as its hydrochloride salt. It is manufactured as a 50:50 racemic mixture. Most of the pharmacological activity is associated with the *laevo*-form.

   Clenbuterol is used as a bronchodilator for horses by oral and parenteral administration. The recommended treatment schedule is 0.8 µg/kg bw twice daily for up to 10 days. It may be administered by oral, intramuscular or intravenous routes of administration. Clenbuterol is also used as a tocolytic in cattle and horses. The recommended treatment is a single parenteral injection equivalent to 0.8 µg/kg bw in cattle and 0.8 µg/kg bw orally in horses.

   Considering the previous CVMP opinion on clenbuterol and in accordance with the provisions of Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having hormonal or thyrostatic action and of beta-agonists, clenbuterol was entered 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
Clenbuterol hydrochloride	Clenbuterol	Bovine	0.1 µg/kg 0.5 µg/kg 0.5 µg/kg 0.05 µg/kg	Muscle Liver Kidney Milk	Provisional MRLs expire on 1.7.2000 Indication: Solely for tocolysis in parturient cows
		Equidae	0.1 µg/kg 0.5 µg/kg 0.5 µg/kg	Muscle Liver Kidney	Provisional MRLs expire on 1.7.2000 Indications: Tocolysis and the treatment of respiratory ailments

   Further data have now been provided to support the establishment of final MRLs for clenbuterol.

2. Clenbuterol is also used in human medicine for the treatment of chronic obstructive airway diseases. The recommended dosage is 10 to 20 µg, twice a day.

3. Clenbuterol was well absorbed after oral administration to laboratory animals, humans and the target species. In most species peak blood concentrations were achieved 2 to 3 hours after oral dosing. The substance was widely distributed to the tissues and was shown to cross the placenta in pregnant rats, dogs, baboons and cows. In the target species, residues depleted slowest from the liver and kidney; residues in these organs consisted mostly of clenbuterol shortly after administration but the percentage declined with increasing withdrawal time.

7 Westferry Circus, Canary Wharf, London, E14 4HB, UK
Tel. (44-20) 74 18 84 00 Fax (44-20) 74 18 84 47
E-mail: mail@emea.eudra.org http://www.eudra.org/emea.html

4. In all species, excretion was predominantly via the urine as unmetabolised clenbuterol. Following administration of a single oral dose of 843 µg to cows, 81% was excreted in the urine, almost all of this was within 24 hours of treatment. After administration of 3 consecutive oral doses of 442 µg to cows, 76% of the dose was excreted in the urine over a period of 240 hours. Oral or intravenous administration of 0.8 µg/kg bw to horses resulted in approximately 80% of the administered dose excreted in the urine; excretion was essentially complete within 60 hours of treatment; single and multiple dosing produced a similar pattern of excretion. In small species, amounts excreted in faeces were very small. The metabolic pathways were similar in all the species studied though there were quantitative differences in the amounts of metabolites formed.

5. Most of the toxicity studies were carried out with clenbuterol hydrochloride a number of years ago and the design and reporting were not in accordance with modern standards. All of the studies, except for some acute toxicity studies, used the racemic mixture.

6. Clenbuterol hydrochloride was of moderate to high acute toxicity. The acute oral LD_{50} of the racemic mixture ranged from 80 to 180 mg/kg bw. It was more toxic by parenteral routes. The *laevo*-form was more acutely toxic than the *dextro*-form; the acute intravenous LD_{50} values were 23.8 and 50 mg/kg bw respectively, in the mouse.

7. Several repeated-dose studies were carried out in rats using oral and parenteral administration. The duration of these studies ranged from 1 month to 18 months. Increased respiration and heart rate were observed following repeated intravenous dosing at 16 mg/kg bw/day. Myocardial lesions were found in several studies on histopathological examination; the severity of this finding was dose-related. There was also evidence of hepatotoxicity with increased serum glutamic-pyrruvic transaminase and alkaline phosphatase concentrations in several studies; focal necrosis was found in the group which received 16 mg/kg bw/day in the intravenous study. A NOEL of 1 mg/kg bw/day was established in a 1-month study using oral dosing, based on changes in blood chemistry at the next dose level of 10 mg/kg bw/day. However 1 mg/kg bw was not a NOEL in a 6-month study using oral dosing because myocardial lesions were found at this dose level, which was also the lowest dose level used. The effects seen in the 18-month study were not consistent with the results of other studies; bradycardia instead of the expected tachycardia was observed. A NOEL was not established due to a reduction in enzyme activity in both the left and right ventricle walls which was observed on histochemical examination of the myocardium; surprisingly, the effect was greatest at the lowest dose level of 0.1 mg/kg bw/day. Due to the design of the studies and the inconsistent results observed, it was not possible to come to a conclusion regarding an overall NOEL in the rat, from these experiments.

8. Several repeated dose studies were carried out in the dog using oral and parenteral routes of administration. Cardiotoxicity was the most notable finding in these studies. In a 3-month study using oral doses of 0, 0.4, 4 and 20 mg/kg bw/day, a dose-related tachycardia was apparent after the first dose and no NOEL was established. In other studies, myocardial necrosis was apparent at doses of 2.5 mg/kg bw/day and above. There was no overall NOEL in the dog due to the tachycardia at 0.4 mg/kg bw/day, the lowest dose level tested.

9. In a 1-month repeated dose study in the mouse, increased liver weights were observed following oral dosing at 12.5 mg/kg bw/day and above. The NOEL was 2.5 mg/kg bw/day. However the study was not to modern standards and clinical chemistry was not monitored.

10. Repeated dose studies were carried out in rodents and primates using inhalational administration. These studies suffered from methodological problems; there was doubt over the achieved drug intakes and cardiotoxicity was not always adequately assessed. Consequently no reliance can be placed on the results of these studies.

11. In the target species there were no significant adverse effects except for an increase in heart rate and a fall in diastolic blood pressure.

12. In a study to investigate perinatal toxicity, groups of pregnant female rats were given daily oral doses of 0, 1, 7 or 50 mg clenbuterol hydrochloride/kg bw/day from day 15 of gestation up to day 21 post-partum. Maternal food consumption was significantly reduced in all treated groups. The numbers of pups born dead were increased in all treated groups in a dose-related manner. Post-natal mortality increased in all treated groups. By day 7 of lactation, all pups in the 50 mg/kg bw/day group had died. A NOEL could not be established.

13. In a reproductive toxicity study, groups of rats were given daily oral doses of 0, 1, 7 or 50 mg/kg bw/day of clenbuterol hydrochloride. Treatment of the males began 10 weeks prior to mating. Treatment of the females began 2 weeks prior to treatment and continued throughout gestation and lactation. On day 14 of gestation 50% of the females were killed and the uterine contents examined. The remainder were allowed to deliver naturally and rear the offspring to weaning. To investigate the high pup mortality in the perinatal study, the litters of 6 control dams were exchanged with the litters from dams given 50 mg/kg bw. There was no substance-related effect on fertility, corpora lutea, implantations or resorption rates. There was evidence of maternal toxicity (reduced body weight gain) at 50 mg/kg bw. Pup weights at birth were significantly reduced in all treated groups and there was a decrease in the numbers of viable pups in the 7 and 50 mg/kg bw groups. All pups in the 50 mg/kg bw group died during the first day of lactation; it made no difference whether the pups were suckled by their own mothers or by mothers from the untreated control group. The majority of the pups from the control group who were suckled by dams given 50 mg/kg bw survived to weaning. A NOEL was not established.

14. The previous study was repeated using lower dose levels of 0, 1.5, 7.5 or 15 µg/kg bw/day. There was no effect on fertility, gestation length, numbers of corpora lutea, implantation rate, incidence of resorptions, teratogenicity or foetotoxicity at any dose level. Pup survival and body weight gain were unaffected by treatment. Performance in behavioural tests was unaffected by treatment. The NOEL was therefore more than 15 µg/kg bw/day.

15. Two teratology studies were carried out in the rabbit using oral administration. The first study used oral doses of 0, 0.03, 0.1 and 0.3 mg/kg bw/day but was badly conducted with some animals already pregnant at the start and others dying of bronchopneumonia. It was stated that there were no effects on the incidence of resorptions, foetal weights or the incidence of malformations. However, very few details were provided and therefore it was not possible to draw conclusions regarding NOELs. In the second study, groups of rabbits were given daily oral doses of 0, 0.01, 1 or 50 mg/kg bw/day on days 8 to 16 of gestation. Maternal toxicity (reduced body weight gain and food consumption) was observed at 50 mg/kg bw. The incidence of resorptions was significantly increased at 50 mg/kg bw with a corresponding reduction in the numbers of viable foetuses. Mean litter and foetal weights were also reduced at 50 mg/kg bw/day. The substance was teratogenic at 50 mg/kg bw with a significant increase in the numbers of malformed foetuses. The malformations included cleft palate and synostosis. The second study was not entirely satisfactory since the numbers of dams which became pregnant in each group was less than that required in the OECD guidelines. A third study was carried out using the inhalational route of exposure and calculated doses of 0 (sham), 0 (propellant control), 46, 146 and 300 µg/kg bw/day. There was no substance-related effect on any parameter. However, there was a high incidence of malformed foetuses in both the sham and propellant control groups which may have resulted from the stress due to the exposure procedure. No conclusions can be drawn because of the small numbers of foetuses available for examination and the high incidences of malformations in the control groups. Although no individual study was entirely satisfactory, overall it may be concluded that clenbuterol was teratogenic in the rabbit at maternally toxic dose levels and that an oral dose of 1 mg/kg bw/day was a NOEL for maternal toxicity, foetotoxicity and teratogenicity.

16. Three teratology studies were carried out in rats using oral administration. In the first study, groups of rats were given daily oral doses of 0, 0.04, 0.2 or 1 mg/kg bw/day on days 6 to 15 of gestation. There was no evidence of maternal toxicity, teratogenicity, or foetotoxicity at any dose level. In the second study the dams were given daily oral doses of 0, 0.01, 1.0, 10 or 100 mg/kg bw/day. Maternal toxicity was observed at 10 and 100 mg/kg bw. The incidence of resorptions was significantly increased in both groups with a corresponding reduction in the numbers of live foetuses. The incidences of malformations and variations were increased at 10 and 100 mg/kg bw. The malformations included hydrocephalus, ansarka, anophthalmia, rib

malformations and splintering of the vertebrae. The NOEL for teratogenicity and foetotoxicity was 1 mg/kg bw/day. The third study used the same dose levels and included a littering phase with 5 dams/dose. Again malformations were increased in a dose-related manner in the 10 and 100 mg/kg bw groups. In the littering phase, pup development was retarded at 100 mg/kg bw; 2 pups with hydrocephalus were found in the 10 mg/kg bw group and 4 with anophthalmia in the 100 mg/kg bw group. As in the previous study, the NOELs for teratogenicity and foetoxicity were 1 mg/kg bw/day. There was also a teratology study in rats using the inhalational route but no conclusions can be drawn because of the lack of information concerning achieved doses.

Overall it may be concluded that clenbuterol was teratogenic in the rat at maternally toxic doses and that an oral dose of 1 mg/kg bw/day was a NOEL for maternal toxicity, foetotoxicity and teratogenicity.

17. Negative results were obtained in 2 *in vitro* bacterial assays for gene mutation though neither assays was to modern standards. An *in vitro* assay for the HGPRT mutation in V79 cells was also negative though this study was also deficient in some aspects. A mouse lymphoma assay was well conducted and gave negative results in the absence of metabolic activation. Small but statistically significant increases in mutant frequency were obtained at the top 2 dose levels of 700 and 800 μg/ml in the presence of metabolic activation but the results were not reproducible.

    In an *in vitro* cytogenetics assay in human lymphocytes, occasional numbers of aberrant cells were observed. These exceeded the concurrent and historical control values but were not reproducible or concentration dependent.

    An *in vivo* micronucleus test gave negative results but the maximum tolerated dose was not achieved and there was no calculation of the polychromatic to normochromatic erythrocyte ratio. There was no evidence that clenbuterol induced chromosomal aberrations in Chinese Hamster bone marrow following subacute (5-day) oral dosing at 50% of the LD_{50} value.

18. In a carcinogenicity study in the mouse, clenbuterol hydrochloride was administered in the drinking water for 2 years. The dose levels were 0, 0.1, 1 and 25 mg/kg bw/day. Survival at termination exceeded 50% in all groups and was in the range 78 to 86% for males and 54 to 60% for females. There was a significant increase in absolute heart rate in males given 1 mg/kg bw and in females given 25 mg/kg bw. Relative heart weights were increased in a dose-related manner in all treated groups. The incidences of non-neoplastic and neoplastic lesions in the treated groups were comparable with the controls. There was no evidence of carcinogenicity.

19. A carcinogenicity study was carried out in 2 strains of rat. Chbb:THOM rats were administered dose levels of 0, 6.25, 12.5 or 25 mg/kg bw/day. Charles River Sprague-Dawley rats were administered 0 or 25 mg/kg bw/day. The test substance was administered in the feed for the first 20 weeks but because of contamination problems, it was administered in the drinking water for the remainder of the study. Survival exceeded 50% in all groups. Treated rats were tense, nervous and aggressive and body weight gain was reduced in a dose-related manner. There was no substance-related increase in the incidence of any tumour type, apart from an increase in mesovarian leiomyomas in the Charles River Sprague-Dawley rats. No mesovarian leiomyomas were found in the Chbb:THOM rats given the same dose level. Several β-agonists have been shown to induce mesovarian leiomyomas in certain strains of rats and there is evidence from a study with salbutamol that co-administration of a β-antagonist such as propranolol abolishes the tumours. The induction of these tumours appears to be a function of adrenergic stimulation. Human epidemiology data indicated that the incidence of mesovarian leiomyomas was not increased in women following use of adrenergic agents. Therefore, it was concluded that clenbuterol was not carcinogenic.

20. Clenbuterol was not a sensitiser when in the Maximisation test and when tested by the Bühler technique.

21. Several clinical trials were carried out with clenbuterol in humans. These included studies in healthy human volunteers and in pregnant women with premature labour pains. In these studies the tolerance was rated good in most patients, the reported adverse effects at therapeutic dose levels were restlessness, palpitations and muscle tremors. In a study in patients with coronary heart disease, there were no unwanted effects on the cardiovascular system; respiratory functions

improved and a regression of the pathological electrocardiogramm-changes was observed. Patients with chronic obstructive airway disease were shown to be more susceptible than "normal" patients to the bronchodilatory effects of the substance. Two trials were carried out to examine the acute bronchospasmolytic effect in these patients; 1 trial involved a total of 20 patients and the other was a cross-over study in 6 patients. Bronchospasmolysis was determined by measuring airway resistance using whole body plethysmography. From the results of these 2 studies it was concluded that 2.5 µg/day was a pharmacological NOEL in humans.

22. A pharmacological ADI of 0.25 µg for a 60 kg adult (i.e. 0.0042 µg/kg bw) was calculated by applying a safety factor of 10 to the pharmacological NOEL of 2.5 µg/day in humans. The safety factor of 10 was justified because asthmatics are especially sensitive to the bronchodilatory effects of β-agonists.

23. A toxicological ADI of 0.15 µg/kg bw was calculated by applying a safety factor of 100 to the NOEL of 15 µg/kg bw per day which was established in the reproductive toxicity study, based on reduced pup weights at higher dose levels. This was very similar to the ADI of 0.2 µg/kg bw which was calculated by applying a safety factor of 500 to the dose level of 0.4 mg/kg bw per day which was the lowest dose level used in a 3-month repeated-dose study in the dog; the safety factor of 500 was justified because the dose level was not a NOEL and caused tachycardia but no other effects. It was considered that the pharmacological ADI derived from data in humans was the most relevant ADI for risk assessment.

24. Two studies were carried out in the horse in which the concentrations of total ^{14}C-residues in tissues were compared with residues of unmetabolised clenbuterol. In both studies, highest residues were found in liver and declined from 5.7 to 27.0 µg equivalents/kg, 12 hours after treatment, to 4.6 to 7.2 µg equivalents/kg, 9 days after treatment and 0.51 to 0.79 µg equivalents/kg 28 days after treatment. Significant residues were also found in kidney and declined from 1.7 to 8.0 µg equivalents/kg 12 hours after treatment to 0.17 to 0.59 µg equivalents/kg 9 days after treatment and 0.14 to 0.23 µg equivalents/kg 28 days after treatment. Residues in muscle were very low; in 1 study, 1 sample taken 12 hours after the end of treatment contained 1.05 µg equivalents/kg; residues in the 2 other samples taken at the same time point were undetectable. In the second study, a muscle sample taken 24 hours after the end of treatment contained 0.21 µg equivalents/kg but residues were below the limit of detection in subsequent samples (less than 0.05 µg equivalents/kg). Residues in fat were also low; in 1 study, 1 sample of renal fat taken 12 hours after the end of treatment contained 1.05 µg equivalents/kg; residues in the other 2 samples of renal fat and all 3 samples of ormental fat taken at the same time points were undetectable. For the first 48 hours after treatment, most of the residues in liver were unmetabolised clenbuterol (76% and 37% in 2 different animals at 12 hours in the first study; 90% and 78% in single animals at 24 and 48 hours in the second study). The percentage of residues present as clenbuterol declined to 8.5%, 9 days after treatment and to 6.4%, 12 days after treatment. The nature of the residues in horse kidney was investigated at only 1 time point: 24 hours after treatment, 88% of the total residues consisted of clenbuterol. Residues in muscle were too low for characterisation.

25. In cattle, the pattern of residue depletion was similar to that of horses with the highest residues encountered in liver and kidney and very low residues in muscle and fat. In a study in ruminating calves given intramuscular injections of 0.8 µg/kg bw per day of clenbuterol hydrochloride every 12 hours for a total of 21 doses, total ^{14}C-residues in the liver declined from 28.7 to 47.2 µg equivalents/kg, 6 hours after treatment to 5.7 to 9.9 µg equivalents/kg 6 days after treatment and 3.8 to 4.6 µg equivalents/kg 10 days after treatment. The corresponding residues in kidney were 30.2 to 46.9 µg equivalents/kg, 2.7 to 3.7 µg/kg and 1.7 to 2.9 µg equivalents/kg. Residues in muscle were in the range of 1.7 to 2.5 µg equivalents/kg 6 hours after treatment, to 0 to 0.2 µg equivalents/kg 6 days after treatment and 0 to 0.1 µg equivalents/kg 10 days after treatment. Residues at the last injection site range from 2.6 to 3.1 µg equivalents/kg 6 hours after treatment to 0.2 to 0.6 µg equivalents/kg 6 days after treatment and 0.15 to 0.3 µg equivalents/kg 10 days after treatment. Residues in fat were in the range 0.3 to 1.6 µg equivalents/kg 6 hours after treatment but were only in 1 out of 9 fat samples taken 6 days after treatment (0.02 µg equivalents/kg). The relationship between residues of clenbuterol and total residues was determined 6 hours, 3 and 6 days after treatment. Residues in muscle (including injection site)

consisted mostly of clenbuterol. At the 6-hour time point, residues in liver consisted almost entirely of clenbuterol; after 6 days the percentage of clenbuterol had declined to around 16%.

26. Residues in cows' milk were determined following oral and intramuscular administration of the recommended tocolytic dose regime. Total ^{14}C-residues declined from a peak mean value of 0.7 µg equivalents/ml to less than 0.05 µg/ml, 72 hours after treatment. For the first 2 to 3 days after treatment, residues in milk consisted almost entirely of unmetabolised clenbuterol. The very low concentrations of radioactivity at subsequent time points did not appear to be clenbuterol.

27. The 4 metabolites of clenbuterol, which had been shown to be present as residues in livers and kidneys of treated animals, were tested for pharmacological activity. Evidence of pharmacological activity was found for only one: 1-(4-amino-3,5-dichlorophenyl)-2-hydroxy-tert.butylamino)-ethanol-HCl. Its broncholytic effect in the guinea pig was less than 20% that of clenbuterol. It comprised only a small percentage of the residues. In calves it accounted for only around 1 to 2% of the extractable residues in liver and less than 1% of the residues in kidney, 6 hours after treatment; residues at later time points were generally undetectable. The metabolite accounted for 9.7% of the extractable residues in one sample of horse liver, 48 hours after treatment but was not identified in samples of horse liver and kidney taken at other time points. Because of the low pharmacological activity of this metabolite and its low concentration in tissues, it was concluded that only residues of clenbuterol were important in the assessment of consumer risk. There was no evidence for the presence of bound residues; extraction efficiencies were very good and approached 100% for tissues samples during the first week after treatment.

28. The proposed routine analytical method for the determination of clenbuterol in bovine and equine tissues and in cow's milk was based on HPLC with MS detection. The method used deuterium labelled clenbuterol as an internal standard. The presence of clenbuterol and deuterium labelled clenbuterol in chromatograms were characterised by daughter ions at m/z values of 203.1 and 204.1 respectively. The method was of acceptable accuracy and precision and the limits of quantification were 0.05 µg/kg for bovine and equine muscle; 0.25 µg/kg for bovine and equine liver and kidney and 0.025 µg/kg for bovine milk.

Conclusions and recommendation

Having considered that:

- an ADI of 0.0042 µg/kg bw (i.e. 0.25 µg/person) was established,
- clenbuterol was identified as marker residue and considering that the marker residue represents 100% pharmacologically active residue at 48 hours after treatment,
- a validated analytical method for monitoring residues based on HPLC-MS is available;

the Committee for Veterinary Medicinal Products recommends the inclusion of clenbuterol 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
Clenbuterol	Clenbuterol	Bovine	0.1 µg/kg 0.5 µg/kg 0.5 µg/kg 0.05 µg/kg	Muscle Liver Kidney Milk	
		Equidae	0.1 µg/kg 0.5 µg/kg 0.5 µg/kg	Muscle Liver Kidney	

Based on these MRLs values, the daily intake will represent about 72% of the ADI.