



EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

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Committee for Medicinal Products for Veterinary Use

European public MRL assessment report (EPMAR) Virginiamycin (Poultry)

On 3 September 2015 the European Commission adopted a Regulation¹ establishing maximum residue limits for virginiamycin in poultry species, valid throughout the European Union. These maximum residue limits were based on the favourable opinion and the assessment report adopted by the Committee for Medicinal Products for Veterinary Use.

Virginiamycin is intended for use in broiler chickens for the treatment of necrotic enteritis.

PHIBRO Animal Health Corporation submitted the application for the establishment of maximum residue limits to the European Medicines Agency, on 3 October 2013.

Based on the original and complementary data in the dossier, the Committee for Medicinal Products for Veterinary Use recommended on 6 November 2014 the establishment of maximum residue limits for virginiamycin in poultry species.

Subsequently the Commission recommended on 16 July 2015 that maximum residue limits in poultry species are established. This recommendation was confirmed on 6 August 2015 by the Standing Committee on Veterinary Medicinal Products and adopted by the European Commission on 3 September 2015.

¹ Commission Implementing Regulation (EU) No 2015/1491, O.J. L 231, of 03 September 2015



Summary of the scientific discussion for the establishment of MRLs

Substance name:	Virginiamycin
Therapeutic class:	Anti-infectious agents / Antibiotics
Procedure number:	EMA/V/MRL/003878/FULL/0001
Applicant:	PHIBRO Animal Health Corporation
Target species:	Poultry
Intended therapeutic indication:	Treatment of necrotic enteritis
Route(s) of administration:	Oral

1. Introduction

Virginiamycin belongs to a group of cyclic peptides (streptogramins) produced as secondary metabolites by *Streptomyces spp* (*Streptomyces virginiae*). Streptogramins are unique among antibiotics in that each member of the class consists of at least 2 structurally unrelated molecules: compound A streptogramins (macrolactones), including virginiamycin factor M1, and compound B streptogramins (cyclic hexadepsipeptides), including virginiamycin factor S1. Virginiamycin is composed of approximately 75% virginiamycin M1 (also known as ostreogrycin A), 20% virginiamycin S1 and 5% of less abundant S-analogues.

Virginiamycin has been used in animal production since 1975.

In the European Union virginiamycin was previously used as a feed additive but such use intended for growth promotion was banned in 1998 by Council Regulation (EC) No 2821/98.

The substance is approved in the USA as a feed supplement, alone or in combination with other agents, in cattle, swine and poultry for disease prevention, increased rate of weight gain and increased feed efficiency at concentrations ranging from 5 to 25 g/ton of feed.

In veterinary medicine virginiamycin is intended for use in broiler chickens for the treatment of necrotic enteritis, a common frequently fatal gastrointestinal disease in poultry caused by toxigenic strains of *Clostridium perfringens*. The product is intended to be orally administered at a rate of 80 mg/kg feed (corresponding to approximately 8 mg/kg bw) for 14 days.

2. Scientific risk assessment

2.1. Safety assessment

2.1.1. Overview of pharmacological properties

Pharmacodynamic properties including mode of action

The target of both components of virginiamycin is the 50S subunit of the bacterial ribosome.

The A-component (virginiamycin M1) binds to a tight pocket within the peptidyl transferase catalytic centre (PTC) of the ribosome whereby the attachment of transfer RNA (tRNA) to both the acceptor (A) site and the donor (P) site of the PTC is prevented. This leads to a shutoff of peptide bond formation and stops

the elongation of the growing polypeptide chain. This binding site and inhibition mechanism is similar to that of certain other antibiotics, such as chloramphenicol for example. In contrast, the B component (virginiamycin S1) does not affect the peptidyl transferase reaction but inhibits the peptide elongation process after a few cycles of peptide bond formation by binding to the 23S ribosomal RNA within the ribosomal exit tunnel. This is similar to the mode of action of macrolide antibiotics, such as erythromycin. Hereby, extension of the nascent protein chain is prevented and additionally the peptidyl-tRNA is released from the ribosome, which results in incomplete peptides.

The structural composition of the ribosomal complex is important as virginiamycin S1 acts synergistically to the conformational change of the peptidyl transferase centre of the 50S-ribosome induced by virginiamycin M1.

Individually, each component of virginiamycin exhibits only moderate bacteriostatic activity by binding to the 50S subunit of bacterial ribosomes, thereby blocking translational processes, whereas the combination of the two components leads to a synergistic effect with a hundred fold higher activity that results in a bactericidal activity.

As the mechanism of action of both factors is different, there are several possible mechanisms of resistance.

Secondary pharmacodynamics properties related to growth promotion have not been addressed. However no consistent effect on body weight and feed efficiency was observed in the toxicological studies reported below.

Pharmacokinetic properties

Studies were performed to extract and quantify total radioactive residues in liver and urine from rats administered ¹⁴C-virginiamycin by gavage (0 and 25 mg/kg bw/day for 14 days; 7 animals/sex/group) and to fractionate the extracts using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) in order to determine the extent of virginiamycin fragmentation. The average residue level in the liver was 6.1 µg equivalents /g (males: 6.82 µg equivalents /g, females: 5.35 µg equivalents /g), approximately 26% of total residues in the liver (22.8%, males and 29.1%, females) was extracted with methanol (14.7 to 20.7%) and phosphate buffer (8.1 to 8.4%). The majority of liver residues were considered non-extractable. Fractionation of tissue residues by HPLC indicated that no single component represented more than 3.3% of total liver residues. In urine, no single fraction (HPLC) represented more than 9.9%, and no region (TLC) represented more than 5.7% of the total urine residues.

The metabolites in liver from rat, turkey and cattle treated orally with ¹⁴C-virginiamycin were compared. Results demonstrate a similar metabolic profile in turkeys, cattle and rats, indicating that the rat is a suitable model for comparative purposes. The majority (43-65%) of the total liver residue from virginiamycin treated turkeys, cattle, and rats was found to be non-extractable (present as a bound residue). The extractable residues were shown to consist of multiple components indicating that virginiamycin is extensively metabolised in the tissue.

2.1.2. Calculation of pharmacological ADI, if relevant

In line with the CVMP Guideline on the approach to establish a pharmacological ADI (EMA/CVMP/SWP/355689/2006), a pharmacological ADI is not needed for substances for which the only expected pharmacological activity is an antimicrobial activity. For such substances the microbiological ADI covers relevant effects.

As well as exerting an antimicrobial effect virginiamycin is known to impact on body weight and feed efficiency. However, as no consistent effect on decrease or increase in body weight and feed efficiency was observed in the toxicological studies at the doses used (25 to 1000 mg/kg bw – i.e. doses well in excess of the intended therapeutic dose), the establishment of a pharmacological ADI for virginiamycin is not considered necessary.

2.1.3. Overview of toxicology

Single-dose toxicity

Oral administration of virginiamycin (suspended in 10% gum Arabic) in mice confirms the low acute oral toxicity of the substance, with LD₅₀ values greater than 7000 mg/kg bw in males and greater than 9000 mg/kg bw in females. Clinical signs seen following oral dosing included deep respiration, puritis, disappearance of sensory nerve reflexes and breathing difficulties. Following intraperitoneal administration with the same formulation, the LD₅₀ was greater than 570 mg/kg bw in males and greater than 700 mg/kg bw in females.

In chickens given a premix containing 2000 mg virginiamycin per kg feed (i.e. 25 times the intended therapeutic dose) for 24 hours no signs of toxicity considered to be compound related were seen.

Repeated dose toxicity

Repeated dose toxicity studies were carried out in rats and dogs as well as in chickens. A number of toxicology studies were conducted including two 3-month oral administration studies in rats (one pre-GLP study and one GLP study), a 3-month (pre-GLP) oral administration study in dogs and a 6-month sub-chronic (GLP) oral administration study in dogs. In the pre-GLP 3-month rat study and in the 3 month dog study no toxicity was evident at doses of up to 100 mg/kg/day, the highest dose tested.

In the 3-month GLP study in rats the toxic potential of virginiamycin was examined following dietary administration (10 rats /sex/group) at doses of 0, 100, 300 and 1000 mg/kg bw/day. Virginiamycin was tolerated at levels of up to 1000 mg/kg bw/day. A dose related decrease in feed efficiency linked to a decrease in body weights in males and females was observed. In females the decrease in body weight was statistically significant at 300 and 1000 mg/kg bw/day, and the decrease in feed efficiency was statistically significant at all doses. Group differences in haematology parameters were noticed at week 13 (end of trial), some of which achieved statistical significance ($p < 0.05$ to $p < 0.001$), but these were minor or occurred in only one sex group or lacked a dose relationship and as such were not considered to be attributable to virginiamycin administration. Differences in clinical chemistry or organ weights were not associated with concurrent histological abnormalities. Also, the small magnitudes or the direction of the change lacked a dose relationship and therefore did not support a toxicological effect. The LOEL from this study was 100 mg/kg bw/day based on decreased feed efficiency in females.

In the 6-month GLP study in dogs (6/sex/group) animals were administered virginiamycin at doses of 0, 25, 200 and 750 mg/kg bw/day. Toxicity was seen at 750 mg/kg bw/day including one premature death, low body weight gain and feed intake as well as histopathological findings in the liver. Low erythrocyte counts, haemoglobin concentrations and haematocrit were observed during the treatment period at 750 mg/kg bw/day and were more marked in males than in females. At 200 and 750 mg/kg bw/day no dogs (out of 6 females) underwent oestrus, as opposed to 3 of 6 and 4 of 6 females in the control and low dose groups, respectively. However the effect on oestrus incidence was not statistically significant and is not considered to have been due to virginiamycin. The NOAEL from this study was 200 mg/kg bw/day.

A 21 week follow-up haematology study was performed in dogs to investigate the erythrocyte changes seen in the 6 month study. This study used a maximum dose of 750 mg/kg bw/day. No effects were observed.

In a subacute toxicity study in broilers, no signs of toxicity were observed when virginiamycin was administered in the feed at 22, 66 or 110 mg/kg feed for 7 weeks.

Reproductive toxicity, including developmental toxicity

A one generation GLP rat study with limited toxicological investigations was performed at dose levels of up to 594 mg/kg bw/day to support dose level selection for the 2-generation rat study. Due to excessive mortality in the 2 highest dose groups (413 mg/kg bw/day and 594 mg/kg bw/day), both dose groups were sacrificed after approximately 4 weeks of treatment. No test substance related effects were evident in the pregnancy/fertility rates of the 96, 168 or 347 mg/kg bw/day dose level groups.

In a GLP 2-generation reproduction toxicity study in rats given 0, 25, 65, 300/100 mg/kg bw/day (due to low body weight gain the top dose was reduced in both sexes during mating and in females throughout gestation and lactation) (25/sex/group) no effect of virginiamycin on reproductive parameters was observed at up to 100 mg/kg bw/day. Low body weight gain was observed at 300 mg/kg bw/day and at 100 mg/kg bw/day in males and females during mating and in females throughout the gestation and lactation phases. In all dose groups, including the low dose group of 25 mg/kg bw/day, an effect of treatment on feed consumption (increase) in male rats could not be excluded and was also noticeable in females during growth at the low dose. This was not a consistent finding as, for example, the food consumption data for treated F1 male pups during the 11-week growth period were considered unremarkable. It can be concluded that no substance related effect on the reproductive performance of rats was observed at the 100 mg/kg/day which is therefore retained as the NOEL for reproductive effects in this study.

From a GLP developmental toxicity study in rats (24 females/group) given doses of 0, 25, 75 and 200 mg/kg bw/day via gavage between days 6 to 15 of gestation virginiamycin was maternotoxic and embryotoxic at 200 mg/kg bw/day but not foetotoxic or teratogenic. At 75 mg/kg bw/day and 200 mg/kg bw/day reductions in the mean number of implantations and viable foetuses were observed. However, at 75 mg/kg bw/day the findings were not considered significantly different to those seen in controls and were within the range of historical control data. Therefore 75 mg/kg bw/day was retained as the NOEL for this study.

In a GLP embryotoxicity/foetotoxicity study in mice (25 females/group) given 0, 25, 160 and 1000 mg/kg bw/day via gavage between days 6 to 15 of gestation, a NOEL of 160 mg/kg bw/day was retained based on findings in the high dose group of 1000 mg/kg bw/day (female weight, renal pelvis dilatation) considered as having biological significance. Otherwise, no teratogenic effects were observed in mice at doses up to 1000 mg/kg/ bw/day.

Overall the NOEL for reproductive toxicity is 75 mg/kg/day.

Genotoxicity

Virginiamycin was evaluated for mutagenic activity in an Ames Salmonella/microsome plate incorporation test using tester strains TA98, TA100, TA1535, TA1537 and TA1538 of *Salmonella typhimurium* over a dose range of 0.08 to 8 µg/plate. Virginiamycin was tested in the presence and absence of mammalian liver microsomal enzyme preparations from arochlor-induced (S9) rats. There were no significant increases in revertant frequencies either in the presence or absence of S9. The results of the Ames test can be considered as negative.

Virginiamycin was examined for its mutagenic potential in a L5178Y TK+/- mouse lymphoma mutagenesis assay in the presence and absence of aroclor induced rat liver S9. Virginiamycin was tested at concentrations from 200 µg/ml down to 2.7 µg/ml in non metabolically-activated cultures and from 1000 down to 13 µg/ml in S9 activated cultures. Two non metabolically-activated cloned cultures (63.3 and 47.5 µg/ml) exhibited mutant frequencies 5.8 and 2.8 times, respectively, the mean mutant frequency of solvent controls. Total growth of these cultures was 4% and 28% of control growth, respectively. Two S9 activated cloned cultures (133 and 100 µg/ml) exhibited mutant frequencies 5.5 and 2.9 times, respectively, the mean mutant frequency of solvent controls. Total growth of these cultures was 15% and 54% of control growth, respectively. It is concluded that virginiamycin produced a positive response in the presence and absence of exogenous metabolic activation.

Virginiamycin was tested in a second L5178Y TK+/- mouse lymphoma mutagenesis assay over a dose range of 97 down to 3.1 µg/ml in non metabolically-activated cultures and from 969 down to 13 µg/ml in the S9 activated cultures. In this test, two non metabolically-activated cloned cultures (41 and 31 µg/ml) exhibited mutant frequencies 7 and 2 times, respectively, the mean mutant frequency of solvent controls. The total growth of these cultures was 3% and 31% respectively. Two S9 activated cloned cultures (172 and 129 µg/ml) exhibited mutant frequencies 4 and 2.1 times, respectively, the mean mutant frequency of solvent controls. The total growth of these cultures was 14% and 38% respectively. Virginiamycin increased the mutation frequency at the TK locus in the L5178Y mouse lymphoma cells and, therefore, is considered to be mutagenic in this assay system.

Virginiamycin was tested in the sister chromatid exchange assay using Chinese hamster ovary (CHO) cells in the absence and presence of an aroclor-induced rat liver S9 activation system. Based on growth inhibition and cell cycle delay, dose levels of 1.25, 2.5, 5, 10 and 20 µg/ml were selected in the non metabolically-activated system and 25, 50, 100, 200 and 400 µg/ml were selected in the S9 system. Virginiamycin was negative in the non metabolically-activated system but marginally positive in the S9 activated system. A dose-related increase in sister chromatid exchange was apparent at 100 and 200 µg/ml and the concentration of 400 µg/ml was toxic. It was concluded that virginiamycin was positive in the S9 activated assay.

Virginiamycin was evaluated for its potential to induce chromosomal damage in CHO cells in the presence and absence of mammalian liver microsomal enzyme preparations from aroclor-induced rats (S9). Virginiamycin was tested at levels of 25, 50, 100 and 200 µg/ml with S9 activation and at levels of 13, 25, 50 and 100 µg/ml in the absence of the S9 activation system. A clastogenic activity of virginiamycin cannot be excluded based on a tendency towards a dose related effect - both on aberrations per cell as well as on percentage of cells with aberrations - for the 3 highest doses (in case of S9 activation) and on the statistically significant increase in mean aberrations per cell also seen at the second highest dose (100 µg/ml and 50 µg/ml in the metabolically activated and non metabolically-activated test system) respectively, at the 24 hour harvest.

Virginiamycin was evaluated for its ability to induce unscheduled DNA synthesis (UDS assay) in primary rat hepatocytes, measured by autoradiographic methods. The unscheduled DNA synthesis assay indicates that over a range of 5 concentrations (1 to 100 µg/ml) virginiamycin did not cause a significant increase or demonstrate a concentration dependent response in the average net nuclear count of silver grains over the solvent control. Virginiamycin did not induce detectable increases in unscheduled DNA synthesis over the applied concentration range of 1 up to 100 µg/ml suggesting that it is not capable of causing DNA damage. The results of the *in vitro* UDS test can be considered as negative.

Virginiamycin was tested for its mutagenic potential through its ability to induce sister chromatid exchanges (SCE) in mouse bone marrow cells *in vivo* in mice. Doses used in the assay were 500, 2500 and 5000 mg/kg bw. The results did not show a significant increase in bone marrow SCEs. Although no

direct assessment of the exposure of bone marrow cells to the test material was performed toxicological data in mice indicate systemic exposure following oral administration of virginiamycin and pharmacokinetic studies indicate systemic exposure and similar metabolism among species (turkey, cattle and rats). Therefore exposure to bone marrow can be assumed.

Virginiamycin was tested in an *in vivo* micronucleus cytogenetic assay in mice for its genotoxic potency in which bone marrow was examined. Doses used were 500, 2500, and 5000 mg/kg bw administered as a single oral gavage. No positive results associated with cytogenetic damage or aneugenic/clastogenic potential were observed. Although no direct assessment of the exposure of bone marrow cells to the test material was performed toxicological data in mice indicate systemic exposure following oral administration of virginiamycin and pharmacokinetic studies indicate systemic exposure and similar metabolism among species (turkey, cattle and rats). Therefore exposure to bone marrow can be assumed.

Virginiamycin was tested in an *in vivo* rat hepatocyte unscheduled DNA synthesis assay by single administration via oral gavage of 200, 600 and 2000 mg/kg bw. Hepatocytes were harvested 2 to 4 and 12 to 18 hours after administration. Results did not show a significant increase in mean net nuclear grain counts (i.e. an increase of at least 5 counts over the negative control) in isolated hepatocytes. Although no direct assessment of the exposure of bone marrow cells to the test material was performed as part of this study pharmacokinetic and metabolism studies have demonstrated the exposure of hepatocytes cells to the active substance following oral administration. It can be concluded that no genotoxic potential was shown in this test.

Overall, virginiamycin produced positive results in a number of *in vitro* genotoxicity tests, particularly in the mouse lymphoma test and in the Chinese hamster ovary cell sister chromatid exchange assay, and the possibility of clastogenic activity in an *in vitro* chromosomal aberration test could not be ruled out. However in the *in vivo* tests all results were negative. Therefore it can be concluded that virginiamycin is not genotoxic *in vivo*.

Carcinogenicity / Chronic toxicity

Studies to evaluate chronic toxicity and/or the carcinogenic potential of virginiamycin were conducted in mice and rats.

Virginiamycin was administered orally to rats (70/sex/group) in the diet at doses of 25, 50 or 250 mg/kg bw/day (males) / 300 mg/kg bw/day (females) for 24 months. At dose levels of 250 or 300 mg/kg bw/day, low body weights, high food intake values and changes in some clinical chemistry parameters (total protein, globulin, (decreased) aspartate transaminase, (decreased) alkaline phosphatase) were observed. There were no significant neoplastic findings noted at any dose level. The NOAEL for this study was determined to be 50 mg/kg/day.

In a 2-year oral toxicity study in mice (males and females; 70/sex/group), virginiamycin was given in the diet at dose levels of 25, 75 or 1000 mg/kg bw/day. No treatment-related effects were observed in mortality, physical observations, gross or microscopic pathology, body weight or blood or clinical chemistry data. No evidence of carcinogenicity was seen in this study. A higher food intake was observed at 75 and 1000 mg/kg.

2.1.4. Calculation of the toxicological ADI or alternative limit

The lowest NOAEL observed in the toxicological studies was seen in the carcinogenicity study in rats and was 50 mg/kg bw/day. Reduced body weight, increased food consumption and statistically significant

differences in clinical chemistry parameters were observed at the next lowest dose. Applying a safety factor of 100 results in a toxicological ADI of 500 µg/kg bw (30000 µg/person).

2.1.5. Overview of microbiological properties of residues

The investigation of the microbiological ADI for virginiamycin was carried out in accordance with the VICH GL36 guideline on the general approach to establish a microbiological ADI (EMA/CVMP/VICH/467/03). A revised version of guideline VICH GL36(R) came into effect in May 2012, and consequently the submission has been evaluated in line with the recommendations provided in the updated version of the guideline.

Disruption of the colonisation barrier

Minimum inhibitory concentration (MIC)-data from an appropriate range and number of strains of relevant predominant genera present in the normal human intestinal microflora were provided. From a minimum of 10 isolates of at least 9 different groups of gut flora MIC data were generated for virginiamycin parent compound and for both virginiamycin components M1 and S1. A MICcalc was determined: 0.579 µg/ml for virginiamycin, 1.943 µg/ml for factor M1 and 6.788 µg/ml for factor S1.

Human pharmacokinetic data regarding virginiamycin are not available. Radiolabelled residue studies in chickens show that virginiamycin is extensively metabolised.

For the calculation of the microbiological ADI, the applicant assumed that 100% of ingested residue enters the colon.

Data from ¹⁴C-labelled studies in chickens were used to determine the fraction of residues available to microorganisms. These studies indicate that extractable residues of virginiamycin factor M1 were present in tissues at levels below 0.0051 mg equivalents/kg (i.e. below the limit of detection) and extractable residues of virginiamycin factor S1 were present at maximum levels in the range of 0.005 to 0.012 mg equivalents/kg. The level of radiolabelled virginiamycin residues seen in excreta was 5.63% of the administered dose (on day 20 after the first dose) and 5.04% of the administered dose (on day 12 after the first dose) for virginiamycin factor M1 and S1 respectively. Combined M1 and S1 factors can therefore be considered to be present in excreta at a level corresponding to approximately 10% of the administered dose.

Increase of the population of resistant bacteria

Virginiamycin factor S1 is considered to be the only residue that may have antimicrobial activity and this would only be present in ingested tissues at a concentration in the range of 0.005 to 0.012 mg equivalents/kg (low parts per billion). Data from the radiolabelled study in chickens show that residues of virginiamycin factor M1 were not detectable in tissues. Based on the MIC data, it can be concluded that the levels of virginiamycin factor S1 residues are orders of magnitude below the concentration of factor S that would have any selective impact against selected gastro-intestinal flora. MIC data for *Enterococcus spp.* indicate a pre-existing population of resistant *Enterococcus faecium* and consequently resistance genes in the gut flora of healthy individuals (based on an MIC₅₀ greater than 128 µg/ml for virginiamycin factor M1). Furthermore it can be considered that:

- Streptogramins have a Gram-positive spectrum of activity and so the genus of public health importance is *Enterococcus*.
- Prevalence of existing macrolide, lincosamide, streptogramin B (MLSB) resistance in clinical medicine is at such high levels that residue concentrations of all related antibiotics will not contribute further to the susceptibility profile of those organisms of public health significance.

- Available risk assessments show there to be minimal impact of streptogramin use in animals on treatment failure in human medicine.
- Streptogramin use in clinical medicine has declined significantly in recent years.

In light of the above it is considered appropriate to base the microbiological ADI on disruption of the colonisation barrier

Calculation of microbiological ADI

For the calculation of the ADI for colonisation barrier effects the revised formula according to VICH GL 36, was used:

$$\text{ADI} = \frac{\text{MIC}_{\text{calc}} (\mu\text{g/ml}) \times \text{daily faecal bolus (220 ml)}}{\text{fraction of an oral dose available for microorganisms} \times \text{weight of human (60 kg)}} (\mu\text{g/kg bw})$$

Where,

- MIC_{calc} is the lower 90% confidence limit for the mean MIC_{50} of the most relevant genera. MIC_{calc} for parent virginiamycin = 0.579 $\mu\text{g/ml}$
- the fraction of an oral dose available for microorganisms in the intestinal tract is considered to be approximately 10%, based on data indicating that a maximum of 5.63% and 5.04% of an administered dose of virginiamycin factors S1 and M1, respectively, were seen in excreta (on any individual day)
- the standard weight of the daily faecal bolus is considered to be 220 ml (g)
- the weight of a human is considered to be 60 kg.

The microbiological ADI for colonisation barrier effects was therefore calculated as follows:

$$\text{ADI} = \frac{0.579 \mu\text{g/ml} \times 220 \text{ g}}{0.1 \times 60 \text{ kg}} = 21.23 \mu\text{g/kg bw (i.e. 1273 } \mu\text{g/60 kg person)}$$

2.1.6. Observations in humans

No studies have been performed in humans but data on adverse effects are available. A major problem following human exposure to antibiotic drugs is sensitisation and subsequent hypersensitivity reactions. However very few allergic reactions to virginiamycin have been reported.

2.1.7. Findings of EU or international scientific bodies

In Europe the authorisation of virginiamycin for use in feedingstuffs was withdrawn in 1998 by Council Regulation (EC) No 2821/98 due to concerns over the potential to impair the efficacy of streptogramin antibiotics used for treating human infections through resistance emergence. The withdrawal followed a review of the use of virginiamycin in feedingstuffs by the Scientific Committee on Animal Nutrition (SCAN).

Council Regulation (EC) No 2821/98 does not apply to authorisation of for veterinary medicinal products containing virginiamycin for therapeutic use.

2.1.8. Overall conclusions on the ADI

As the microbiological ADI of 21.23 µg/kg bw (1273 µg/person) is lower than the toxicological ADI of 500 µg/kg bw (30000 µg/person), and as a pharmacological ADI is not considered necessary, the microbiological ADI is established as the overall ADI.

2.2. Residues assessment

2.2.1. Pharmacokinetics in target species

Studies were performed in the target species following oral administration of virginiamycin in feed. Due to the nature of virginiamycin, absorption, metabolism, distribution and elimination of factor M1 and S1 was investigated in two separate studies. This approach is considered appropriate as it captures the potential effect(s) of any interactions. In one study ¹⁴C-virginiamycin factor M1 was mixed with non-radiolabelled virginiamycin factor S1 to produce ¹⁴C-virginiamycin factor M1 with a final specific activity of 9.6 µCi/mg. In the other study ¹⁴C-virginiamycin factor S1 was mixed with non-radiolabelled factor virginiamycin M1 to produce ¹⁴C-virginiamycin factor S1 with a specific activity of 8.8 µCi/mg. In both studies the virginiamycin was administered in capsules 3 times per day. The dose was equivalent to 100 mg virginiamycin/kg feed, administered to 2 groups of 6 broilers (3/sex/group) for a period of 21 days.

The first study confirmed a rapid depletion of ¹⁴C-virginiamycin factor M1 residues in tissues. Factor M1 was observed in liver at greater levels than in kidney and in kidney at greater levels than in skin plus fat, and only in minor quantities in muscle. Between 2 hours (group 1) and 32 hours (group 2) after the end of dosing, the depletion of virginiamycin factor M1 was rapid. The extraction of residues / metabolites was limited compared to total radioactivity in tissues (a maximum of 20.2% of total radioactive residues was extracted). Different virginiamycin factor M1 components were not consistently recovered from all tissues by HPLC. The limit of quantification of the HPLC method for virginiamycin factor M1 was 2.2 µg/kg. The mean total radioactive residue in all edible tissues was low: 371, 278, 134, and 47 µg equivalents/kg in liver, kidney, skin+fat and muscle respectively 2 hours after the last (day 21) dose; 219, 152, 127 and 40 µg equivalents/kg in liver, kidney, skin plus fat and muscle, respectively, 32 hours after the last (day 21) dose.

The second study similarly confirmed rapid depletion of ¹⁴C-virginiamycin factor S1 residues in tissues. Virginiamycin factor S1 levels at each time point were generally highest in liver followed by skin plus fat, kidney and then muscle. Between 2 hours and 32 hours after the end of treatment (i.e. after day 21), the depletion of virginiamycin factor S1 was rapid. The extraction of residues was limited (a maximum of 48.6% of total radioactive residues was extracted). Different virginiamycin factor S1 components could not be consistently recovered from all tissues. Factor S1 was detected in skin plus fat and kidney samples. The limit of quantification of the HPLC method for virginiamycin factor S1 was 2.4 µg/kg. Concentrations of total radioactivity were highest 2 hours after the end of the dosing period and then rapidly declined up to 32 hours after the end of dosing period. Mean residue levels in all edible tissues were generally low: 123, 60, 70 and 12 µg equivalents/kg in liver, kidney, skin plus fat and muscle, respectively, 2 hours after the last (day 21) dose; 92, 21, 31 and 7 µg equivalents/kg in liver, kidney, skin plus fat and muscle, respectively, 32 hours after the last (day 21) dose.

2.2.2. Residue depletion studies

One study evaluated the depletion of virginiamycin factor S1 in tissues of broiler chickens after administration of virginiamycin for 14 days at 100 mg/kg of feed *ad libitum* to 5 groups of animals (the estimated dose received by each animal ranged from 4.9 to 11 mg virginiamycin/kg bw). Groups 1 to 4 included 6 animals (3/sex) and were sacrificed 0, 6, 12 and 24 hours after feed removal. Group 5 included 3 males and was sacrificed 48 hours after feed removal. The study demonstrates the low levels of virginiamycin factor S1 in tissues. Even in group 1 (sacrificed at 0 hours) no residues above the lower limit of quantification (2.4 µg/kg) could be detected except in one bird (6.11 µg/kg in kidney and 2.77 in skin plus fat).

Selection of marker residue and ratio of marker to total residues

Although total radioactive residue levels were higher following administration of ¹⁴C-virginiamycin factor M1 than after administration of ¹⁴C-virginiamycin factor S1, as determined by mass spectroscopy (multiple reaction monitorign), virginiamycin factor S1 was considered to be an appropriate marker residue since it was detectable in all tissues and quantifiable in kidney and skin plus fat samples by HPLC.

The ratio of marker to total residues was calculated by comparing measured virginiamycin factor S1 with the combined total radioactive residues seen for virginiamycin factor S1 and virginiamycin factor M1 2 hours after the last dose of virginiamycin. Considering the lowest virginiamycin factor S1 levels measured, the ratios of marker to total residues are 0.016 for kidney and 0.014 for skin plus fat.

As there was no measurable factor S1 residue in muscle and liver, a conservative ratio of 0.01 was arbitrarily assigned for these tissues as a worst case scenario.

2.2.3. Monitoring or exposure data

No monitoring or exposure data other than those described elsewhere in this report were available.

2.2.4. Analytical method for monitoring of residues

An analytical method for the analysis of virginiamycin factor S1 in poultry liver, kidney, muscle, and skin plus fat was developed. The method is presented in an internationally recognised format. Samples were prepared for LC-MS/MS by extraction with acetonitrile. The upper limit of quantification was 200 µg/kg (with dilution) and the lower limit of quantification was 5 µg/kg. The method was shown to be precise, linear, accurate, stable and robust. The assay specificity was evaluated for each matrix and the absence of interference was confirmed against a range of coccidiostats and veterinary medicines.

The method is considered to conform to the requirements of Volume 8 of The rules governing medicinal products in the European Union. The relevant European Union reference laboratory was consulted on the analytical method and is in agreement with the above conclusions.

2.2.5. Findings of EU or international scientific bodies

No information on maximum residue limits established by other scientific bodies was available.

3. Risk management considerations

3.1. Potential effects on the microorganisms used for industrial food processing

The substance is not intended for use in dairy cattle and therefore potential effects in dairy products were not investigated.

3.2. Other relevant risk management considerations for the establishment of maximum residue limits

In the absence of MRLs for eggs the use of virginiamycin is restricted to animals not producing eggs for human consumption.

The existence of maximum residue limits for virginiamycin could, in principle, increase the risk that the substance would be used for its growth promoting effects. Such a use of the substance would not be expected to lead to noncompliant residue levels in poultry tissues and so would not represent a direct threat to consumer safety. However, this use would be forbidden by Council Regulation (EC) 2821/98.

The appropriate conditions of use for this substance will be fully addressed during the evaluation of any marketing authorisation application.

No additional relevant factors were identified for consideration of the risk management recommendations in respect to the establishment of MRLs.

3.3. Elaboration of MRLs

Based on the residue depletion data available and the ratios of marker to total residues 2 hours after the last administration of virginiamycin, MRL values can be derived as follows:

Tissue	MRL
Muscle	10 µg/kg
Skin and Fat	30 µg/kg
Liver	10 µg/kg
Kidney	60 µg/kg

Calculation of theoretical daily intake of residues

Edible tissue	Daily consumption (kg)	MRL (µg/kg)	Ratio of marker/total residue	Amount of virginiamycin residues per edible tissue (µg)
Muscle	0.300	10	0.01	300
Skin and	0.090	30	0.016	168
Liver	0.100	10	0.01	100
Kidney	0.010	60	0.014	42
Total				610 (48% ADI)

fat and skin in natural proportion

Based on the above figures the maximum theoretical consumer intake represents 48% of the ADI (of 1273 µg/person).

3.4. Considerations on possible extrapolation of MRLs

In line with Article 5 of Regulation (EC) No 470/2009 the CVMP considered the possibility of extrapolating its recommendation on maximum residue limits for virginiamycin to other food producing species on the basis of residue data in chickens. Taking into account the current scientific knowledge the recommendations on extrapolation are justified as follows:

Animal species/ food commodities	Extrapolation possible (Yes/No)	Justification
Poultry tissues	Yes	Existing data indicate that the pattern of metabolites seen in rats, chickens and turkeys is similar. Based on this existing inter-species metabolism data, it can be assumed that the virginiamycin factor S1 would be an appropriate marker residue in other poultry species as well as in chickens. No data are available to demonstrate that the analytical method used for monitoring of residues in chicken tissues is applicable for monitoring of residues in tissues of other poultry species but there is no reason for believing that it would not be.
Poultry eggs	No	No data are available that would allow conclusions to be drawn on the appropriate marker residue or marker to total residues ratio to use in eggs. No analytical method for monitoring of residues in eggs was available for evaluation.
Cattle (including milk), pigs, sheep, horses, rabbits, goats, fin fish, honey	No	Existing data indicate that the pattern of metabolites seen in rats, chickens and turkeys is similar and it seems likely that the virginiamycin factor S1 may be a suitable marker residue in some or all of these other species. However, no specific pharmacokinetic or residue data were available for these species and therefore the assumption related to the marker residue could not be verified. No data are available to demonstrate that the analytical method used for monitoring of residues in chicken tissues is applicable for monitoring of residues in commodities from these other species.

3.5. Conclusions and recommendation for the establishment of maximum residue limits

Having considered that:

- the microbiological ADI of 1273 µg/person (21.23 µg/kg bw) was established as the overall ADI,
- virginiamycin factor S1 was retained as the marker residue,

- the ratios of marker to total residues calculated 2 hours after the last dose were 0.016 for kidney, 0.014 for skin plus fat and 0.01 for muscle and liver,
- extrapolation of the maximum residue limits recommended for chickens to poultry is considered appropriate
- a validated analytical method for the monitoring of residues of virginiamycin in edible chicken tissues is available;
- although it was not specifically demonstrated, the analytical method for monitoring of residues in chicken tissues is expected to be basically applicable for monitoring of residues in poultry tissues,

the Committee recommends the establishment of maximum residue limits for virginiamycin in chicken tissues. Furthermore, and with reference to Article 5 of Regulation (EC) No 470/2009, the Committee agreed to extrapolate the conclusions to poultry tissues, in accordance with the following table:

Pharmacologically active substance	Marker residue	Animal species	MRLs	Target tissues	Other provisions	Therapeutic classification
Virginiamycin	Virginiamycin factor S1	Poultry	10 µg/kg 30 µg/kg 10 µg/kg 60 µg/kg	Muscle Skin and fat Liver Kidney	Not for use in animals from which eggs are produced for human consumption	Anti-infectious agents / Antibiotics

Based on the MRLs for edible poultry tissues the theoretical maximum daily intake (TMDI) is 1273 µg/person, which equates to 48% of the ADI.

4. Background information on the procedure

Submission of the dossier	3 October 2013
Steps taken for assessment of the substance	
Application validated:	16 October 2013
Clock started:	17 October 2013
List of questions adopted:	13 February 2013
Consolidated response to list of questions submitted:	8 August 2014
Clock re-started:	11 August 2014
CVMP opinion adopted:	6 November 2014