

RESULTS OF AVILAMYCIN RESIDUES MONITORING PLANS FOR THE EXPERIMENTAL USE IN ITALY

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ABSTRACT

The digestive disease is the main cause of mortality in industrial fattening rabbit farms. Recently, avilamycin has been experimentally used by rabbit producers in Italy as a new option to control digestive syndrome. This experimental use was exceptionally authorized by the Italian Health Ministry in order to reduce the losses due to Epizootic Rabbit Enteropathy (ERE) in the rabbit breeding. Although the ERE pathogenesis is not yet completely known in all its aspects, the presence of *Clostridium perfringens* has been reported as associated agent in most of cases. The objective avilamycin experimental use was to evaluate the efficacy of the drug in feed for the control of digestive signs associated with *Clostridium* spp. in rabbits at a dose of 5 mg avilamycin/kg body weight/day, equivalent to 60÷120 g/1000 kg of feed on the basis of age, body weight and feed consumption for all animals in the weaning phase. During the experimental use some different residues monitoring plans were performed by Italian health authorities and Elanco Animal Health with the aim to ensure public health on treating food-producing animals with an experimental therapy. According to the guidelines of the Italian Health Ministry an official avilamycin residues monitoring plan was conducted by Elanco during the last two years and other experimental plans were performed by regional authorities and foodstuff producers. An HPLC-MS/MS method was developed by Elanco, performed and validated by the “Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna” according to European legislation (EEC/657/2002) and applied to monitor avilamycin residues in the different plans actuated in Italy. The Avilamycin was analyzed by hydrolysis to dichloroisovernic acid (DIA), the residual marker analyzed. The avilamycin linear range was from 50 to 250 µg/kg (approximately concentration of DIA from 10 to 50 µg/kg). Within laboratory data (reproducibility intra-laboratory) were from 11% to 17% for muscle and liver. Repeatability was included between 10% and 19% for both tissues. The mean recovery was 85% for muscle and 81% for liver. According to different monitoring plans, more than 250 samples of rabbit treated with avilamycin were collected and their tissues (muscles, livers or both tissues) were analyzed. The results obtained from these analyses demonstrate the very low risk due to residues and the high level of safety for avilamycin used in rabbit as food-producing animal.

Key words: Digestive syndrome, Rabbit, Avilamycin, Residues, LC-MS/MS.

INTRODUCTION

The digestive diseases are the main causes of mortality in industrial fattening rabbit farms. In a recent publication (Morel-Saives *et al.*, 2007), the economical impact of an episode of digestive disease was evaluated to be 0.78 € by produced rabbit. The losses due to Epizootic Rabbit Enteropathy (ERE) in the rabbit breeding are very common; the ERE pathogenesis is not yet completely known in all its aspects but it seems definitely to exclude the viral etiology (Licois *et al.*, 2007); in fact, *Clostridium perfringens* has been reported as associated agent in most of cases. Avilamycin (Sunderland *et al.*, 2004) was licensed for use as a growth promoter within the European Union in the swine and poultry

industries but not for therapeutic use and in any case, not for rabbit. In the last years the use as growth promoter was banned and it seemed necessary to improve experimental use under the authorization of the Italian Health Ministry for the registration as therapeutic drug for rabbit diseases. Recently, avilamycin has been experimentally used by rabbit producers in Italy as a new option to control digestive syndrome.

Avilamycin is an oligosaccharide mixture (Scott *et al.*, 1999) composed predominantly of the two compounds A and B that constitute 99% of the microbiological activity of avilamycin powder, belonging to the orthosomycin class, obtained by fermentation of a strain of *Streptomyces viridichromogenes*. This compound is active against Gram positive bacteria, including *Clostridium perfringens* (MIC₉₀=0.45 µg/ml). According to European dispositions it was necessary to develop an analytical method to monitor the avilamycin residues in rabbit to evaluate eventually risks due to concentration of drug in edible tissues. Although some methods have been published for the determination of avilamycin in animal feed (Scott *et al.*, 1999), only few disposition residues study using gas chromatography (Formica *et al.*, 1986) or radio labelled avilamycin (Magnussen *et al.*, 1991) or HPLC method (Sunderland *et al.*, 2004) have been reported; to date no LC-MS/MS method was for the determination of avilamycin in tissues.

The aim of this work was to evaluate the efficacy of avilamycin added in rabbit feed at a dose of 5 mg avilamycin/kg bw/day for the control of digestive signs associated with *Clostridium spp.*, and to monitor the residual concentration of drug metabolites after a withdrawal period. An HPLC-MS/MS method was developed by Elanco (sponsor of monitoring plan authorized by the Italian Health Ministry), performed and validated according to European legislation (EEC/657/2002) by IZSLER (Official laboratory authorized to analyze avilamycin residues) and applied to monitor avilamycin residues in the different plans actuated in Italy.

MATERIALS AND METHODS

Experimental phase

Preliminary residual depletion study

A first avilamycin residues depletion study was conducted by IZSLER during 2006 with the objective to evaluate the avilamycin residues depletion kinetic in rabbit tissues at three different dosages in medicated feed (60-120-160 mg of avilamycin per kg of feed) and at 2 different length of treatments (13 days and 5 days). The avilamycin residues depletion study conducted by IZSLER has been splitted in two phases: Phase I and Phase II. In the phase I 20 rabbits were treated with a medicated feed at dosage of 60 mg of avilamycin per kg of feed; after 5 days of treatment 2 animals were sacrificed and their livers and muscles were analyzed. At the 13th day of treatment other two animals were sacrificed and their livers and muscles analyzed and the remaining 16 rabbits were splitted into two groups of 8 animals each one. A group was treated with a medicated feed at dosage of 120 mg of avilamycin per kg of feed for 5 days and the other group was treated with a medicated feed at dosage of 160 mg of avilamycin per kg of feed for the same time period. At the end of the treatment all 16 animals were sacrificed and their tissues (liver and muscle) were analyzed.

Official monitoring plan

During the experimental use different residues monitoring plans were performed by Italian health authorities and Elanco Animal Health with the aim to ensure public health on treating food-producing animals with an experimental therapy. The objective of this experimental use was to evaluate the efficacy of avilamycin added in feed for the control of digestive signs associated with *Clostridium spp.* in rabbit. The feed was medicated at the dose of 60÷120 g/1000 kg on the basis of age, body weight and feed consumption for all animals in the weaning phase: the aim was to obtain a dosage of 5 mg avilamycin/kg bw/day. The treatment was continuous for 18 days.

The Elanco avilamycin residues monitoring plan was developed in two different years according to the guidelines of the Italian Health Ministry and samples were collected from 3 most important Italian regions where the rabbit breeding represent a widespread zootechnical practice: Veneto region, Emilia Romagna region and Lombardia region.

During 2006, 116 samples (58 muscle samples and 58 liver samples) were collected from 5 different rabbit farms, 2 from Veneto region, 2 from Emilia Romagna region and one from Lombardia region respecting a withdrawal period of 3 days after the last treatment. In the same way, in 2007, a total of 216 samples (108 muscle samples and 108 liver samples) from 6 different rabbit farms, 3 from Veneto region, 2 from Emilia Romagna region and one from Lombardia region were collected respecting a withdrawal period of 3 days after the last treatment.

Regional and other plans

Two avilamycin residues monitoring plans were performed in authorized farms and slaughterhouses by two different regional health authorities in 2006 and 2007 respectively. In 2006 a total of 77 samples (54 muscle samples and 23 liver samples) were collected from Emilia Romagna region, while in 2007 a total of 27 samples (all muscles) were collected from Toscana region.

Finally, two avilamycin residues monitoring plans were performed by two different foodstuff producers to implement their self control plans during the last two years. 15 rabbit muscle samples were collected in 2006 and 44 samples were checked during 2007.

Analytical phase

Standard and reagents

Methanol and acetone (Carlo Erba), ethyl acetate (Fluka), acetonitrile (BDH), and formic acid were HPLC grade. Phosphoric acid and natrium idroxiide (Merck) were reagent grade. Avilamycin, dichloroisoeverninic acid (DIA), were from Ely Lilly Elanco (USA). Dicamba (used as internal standard) was from Chem-Service (USA). The Avilamycin stock standard solutions were prepared by dissolving in acetone; DIA and Dicamba stock standard solutions were prepared by dissolving in methanol; all the working solutions were diluite with methanol. IST AL-N SPE columns (6 ml, 500 mg) were from Isolute (UK): the SPE column was conditioned by passing through 10 ml of ethyl acetate.

Sample preparation

One gram of rabbit tissue was collect into a 50 ml polypropylene tube and spike with a working solution of Dicamba (IS). The sample was sonicated, homogenised and centrifuged, the supernatant was decanted in a glass bottle and the residue was extract again. The solution was evaporated under a nitrogen stream. Then 4 ml of NaOH 1N was added and the solution was heated at 70°C for two hours; the extract was acidified and extracted with ethyl acetate twice. The extract was purified by SPE, the solvent evaporated and reconstituted with methanol for LC-MS/MS analysis.

LC-MS/MS analysis

The analysis was performed on a Waters Alliance 2795 HPLC system (Milford MA USA) with a Synergy Polar column (2.0 mm x 150 mm; 4µm) (Phenomenex, USA), coupled with a Quattro Ultima Platinum mass spectrometer (Micromass Manchester UK). The flow rate was 0.2 ml/min and the injection volume was 10 µl. Acetonitrile and water were used as mobile phase containing 0.1% formic acid. The mass spectrometer operated in electrospray negative mode. The capillary voltage was held at 2.8 kV and the cone voltage was 35 eV. All quantitative analysis were performed using multiple reaction monitoring (MRM). Two precursor –product were chosen for DIA (marker residue 249—190.1; 249—205.1) and one for Dicamba (IS 219—175.1).

RESULTS AND DISCUSSIONS

The aim of the residues depletion study conducted by IZSLER in 2006 was to know residues depletion kinetic in rabbit tissues because only few data were present. During the phase I of the experiment the medium dosage of avilamycin was 4.46 mg avilamycin/kg bw/day: two rabbits were sacrificed at the medium time of the treatment (after 5 days) and two rabbits were sacrificed at the end of the first phase (after 13 days). Residues of DIA (avilamycin) were under the limit of quantification (9 µg/kg) in both the samples. In the second phase of the experiment the rabbits were treated for a shorter time (5 days) but with two different higher dosage of avilamycin in feed. The medium dosage of the treated group with feed at dosage of 120 mg per kg was 6.24 mg avilamycin/kg bw/day: at the end of experiment all the rabbits were sacrificed and residues of DIA (avilamycin) were over the limit of quantification in only one liver sample (10 µg/kg DIA). The medium dosage of the treated group with feed at dosage of 160 mg per kg was 8.87 mg avilamycin/kg bw/day: at the end of experiment all the rabbits were sacrificed and residues of DIA (avilamycin) were under limit of quantification in all the samples. The rabbits were analyzed sampling *longissimus dorsi* muscle and liver: all the experimental analysis were performed by IZSLER, official laboratory authorized to analyze avilamycin residues.

The results of Elanco avilamycin residues monitoring plan results are summarized in Table 1. In 2006 year no muscle was founded over the limit of quantification of the method (50 µg/kg for avilamycin - 9 µg/kg for DIA), and six liver samples were over the limit of quantification, but no one of them was founded over the MRL. The six samples came from four different rabbit farms. In the same case all of the muscle samples collected during 2007 were negative: only two liver samples coming from 2 different rabbit farms were positive, but under the MRL.

Table 1: Summary of the Elanco avilamycin residues monitoring plan results respecting a withdrawal period of 3 days

Elanco Animal Health	2006	2007
Muscles and livers (each one)	58	108
Concentration min Avilamycin (µg/kg)	<50	<50
Concentration max Avilamycin (µg/kg)	187	92
Concentration minimus DIA(µg/kg)	<9	<9
Concentration max DIA(µg/kg)	33	17

The results of the two avilamycin residues monitoring plans performed by regional health authorities of two different Italian regions in 2006 and 2007 respectively are provided in Table 2. No samples were over the limit of quantification of the method.

Table 2: Results of the two avilamycin residues monitoring plans performed by regional health authorities

Regional Health Authorities	2006	2007
Muscles and Livers	77 (54 M and 23 L)	27 (M)
Concentration min Avilamycin (µg/kg)	<50	<50
Concentration max Avilamycin (µg/kg)	<50	<50
Concentration minimus DIA(µg/kg)	<50	<50
Concentration max DIA(µg/kg)	<50	<50

The results of the two avilamycin residues monitoring plans performed by two different foodstuff producers to implement their self control plans in 2006 and 2007 are provided in Table 3. No samples were over the limit of quantification of the method.

The analytical phase was very difficult to improve: there were some different problems, first of all the basic-hydrolysis of the compound avilamycin in its derivate dichloroisoeverninic acid (DIA). The conversion from DIA to avilamycin was performed by ELANCO animal health (concentration of DIAx5.6 = concentration of avilamycin). The method validation according to EEC legislation was carried out fortifying samples with an avilamycin solution and monitoring the DIA residues using Dicamba as internal standard. The marker residue (DIA) is common for A and B avilamycin so the results included all avilamycin compounds.

The avilamycin linear range was from 50 to 250 µg/kg (approximately concentration of DIA from 10 to 50 g/kg) for both matrices. The linear correlation coefficient was above 0.99. The recovery, reproducibility and repeatability data for each tissue were measured by the analysis of six blank samples fortified at three separate concentrations (50-100-150 µg/kg of avilamycin), on three separated occasions. Within laboratory data (reproducibility intra-laboratory) were from 11 to 15% for the muscle and from 12 to 17% for the liver. Repeatability was included from 10 to 14% for the muscle and from 10 to 19% for liver. The mean recovery was 85% for the muscle and 81% for the liver. The 2002/657/EC decision introduced the CC α (decision limit) and the CC β (detection capability) to replace the limit of detection and quantification respectively: the CC α values calculated from 5 curves obtained at five levels were 115 µg/kg of avilamycin for muscle and 123 µg/kg for liver. The CC β values calculated from 5 curves obtained at five levels were 130 µg/kg of avilamycin for muscle and 147 µg/kg for liver.

Table 3: Results of the two avilamycin residues monitoring plans performed by two different foodstuff producers as self control plans in 2006 and 2007

Foodstuff Producers: Self Control Plan	2006	2007
Muscles	15	44
Concentration min Avilamycin (µg/kg)	<50	<50
Concentration max Avilamycin (µg/kg)	<50	<50
Concentration minimus DIA(µg/kg)	<50	<50
Concentration max DIA(µg/kg)	<50	<50

CONCLUSIONS

A LC-MS/MS method was developed and validated to analyze the samples collected from the different experiment plans. The performance of the method was according to European legislation: the maximum residue limits recently approved from European community (1064/2007/EC Reg.) fixes DIA as marker residue and fixes as MRL 50 µg/kg for rabbit muscle and 300 µg/kg for rabbit liver. The different MRL from the preliminary limits fixed not allowed the perfect validation according to EEC (1/2 MRL, 1 MRL, 1.5 MRL for evaluation of repeatability and reproducibility) but permits to use the method at lower concentration than MRL. Some different plans were performed under the exceptionally authorization by the Italian Health Ministry to use avilamycin against digestive diseases associated with *Clostridium spp.* Totally about 250 samples of rabbit treated with avilamycin were collected and their tissues (muscles or livers or both) were analyzed. The results obtained from these analyses demonstrate the very low risk due to residues and the high level of safety for avilamycin used in rabbit as food-producing animal.

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