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SULFADIMETHOXINE RESIDUES IN RABBIT MUSCLE AFTER EXTENDED ORAL TREATMENT AT THERAPEUTIC DOSAGE

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Abstract: Sulfadimethoxine is extensively used in rabbit breeding for preventive and curative purpose and residues are sometimes observed in carcasses at slaughter. It has been suggested this is due to dosage and/or duration of treatment not being in compliance with the manufacturer's recommendations, which probably induces residue levels in the meat above the maximum residue limit (MRL) value of 100 µg/kg. In order to test this hypothesis, a study was carried out on gravid rabbits and their progeny. The animals were subjected to an extended treatment with sulfadimethoxine at therapeutic level in the feed. The feed was supplemented before pelleting with a commercial veterinary product containing 20 g of trimethoprim and 93 g of sulfadimethoxine per kg. On the basis of the dosage indicated for this commercial veterinary product, the incorporation level in the feed was 5 kg/ton (i.e. 465 g of sulfadimethoxine/ton), providing oral daily therapeutic treatment of the animals of ca. 12.5 to 50 mg of sulfadimethoxine per kg bodyweight. The mothers were treated during the last 21 d of pregnancy and during the whole period of lactation (35 d). The animals were sacrificed after a wash-out period of 12 d with blank feed. The young rabbits received the supplemented feed after weaning during the first 40 d of the fattening period. These animals were also sacrificed after a wash-out period of 8, 12, 15 or 20 d, respectively, with a blank feed. A sample of the leg muscle was taken for analysis. An HPLC analytical method was used to determine the sulfadimethoxine concentrations in tissue, with a LLOQ (Lower Limit Of Quantification) of 50 µg/kg of muscle (trimethoprim was not considered in this study). Sulfadimethoxine concentrations above the MRL value of 100 µg/kg were registered only in muscle from 1 out of 8 mothers and in 2 out of 8 young rabbits sacrificed 12 d after cessation of the treatment. For other young rabbits sacrificed on the 8th, 15th or 20th d after cessation of treatment, Sulphonamide concentrations in muscle always remained below the MRL value (8 animals per slaughtering time). These results show that oral treatment of rabbits with veterinary products containing sulfadimethoxine administered for a long period at the daily therapeutic level of 12.5 to 50 mg/kg does not seem to induce the accumulation of this molecule in muscle.

Key words: rabbit, Sulfadimethoxine, residues, muscle, HPLC.

INTRODUCTION

Sulfadimethoxine is a long-acting sulphonamide of interest for the prevention and treatment of various rabbit diseases, including coccidiosis and colibacillosis, which cause serious problems in commercial rabbit breeding. The anti-coccidial activity of sulfadimethoxine has a positive effect on animal mortality, carcass weight and feed utilisation.

Sulfadimethoxine is an amphoteric molecule with high liposolubility. Its intestinal absorption is very rapid and its high rate of binding to plasma proteins (more than 90%) is responsible for its long-acting properties. The unbound fraction is widely distributed in tissues, secretions and fluids like milk, bile,

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amniotic liquid (Wohler *et al*., 1961). Sulfadimethoxine is metabolised in the liver and eliminated in the urine.

Preventive and curative activity of sulfadimethoxine has been demonstrated in rabbit coccidiosis with pathogen agents like *Eimeria stiedai*, *E. exigua, E. intestinalis, E. irresidua, E. magna, E. media, E. perforans* or *E. piriformis* (Dürr and Lämmler, 1970; Gòmez-Bautista and Rojo-Vázquez, 1986). The preventive and curative activity of sulfadimethoxine (in presence of trimethoprim) is generally claimed for a daily oral dose of 25 to 50 mg/kg body weight during 5-10 d. This oral dosage corresponds to a sulfadimethoxine supplementation of about 450 g/ton of feed.

Sulphonamides are widely used in farm animal nutrition as a veterinary drug for prophylactic and therapeutic purposes and their residues in edible tissues are an important concern due to the possibility of risk to human health, such as the development of resistance and toxicity (Kim and Lee, 2003; Niessen, 1998). The effects of Sulphonamides for human health concern the thyroid gland (inhibition of the iodine metabolism and subsequent thyroid hyperplasia), haematology (rare phenomena of agranulocytosis and aplasic anaemia) and hypersensitivity (OMS, 1989). From the chemical point of view, hypersensitivity reactions are induced by the sulfamyl group of the Sulphonamide molecules (Davis, 1984).

For these reasons, both EU as well as US regulations have set a maximum residue limit (MRL) of 100 µg/kg for Sulphonamides in edible tissues and organs from treated animals (AFSSA/ANMV-French Agency for Veterinary Medicinal Products). With the exception of certain veterinary medicinal products, a withdrawal time of 12 d after the end of oral treatment is usually proposed for sulfadimethoxine and more generally for any product containing a Sulphonamide administered by the oral route.

However, field experience shows that Sulphonamide residues are sometimes detected in rabbit carcasses following controls at slaughter. This observation may be correlated to the fact that, according to their low toxicity and the extremely rare adverse reactions observed in rabbits, the oral utilisation of these molecules as preventive agents is sometimes in the form of addition to drinking water or feed during prolonged periods.

The aim of this work was to study the possible influence of prolonged oral treatment with sulfadimethoxine at a therapeutic dosage level on the elimination of residues by the treated rabbits. Sulphonamide was thus given orally to mother rabbits during pregnancy and the suckling period and also to the young rabbits during the post-weaning period. The veterinary medicinal product was used in the feed under field conditions, at the dosage recommended by the manufacturer and different withdrawal periods after cessation of treatment were considered.

MATERIALS AND METHODS

Experimental phase

A total of 20 primiparous female Hycole rabbits were used for the experiment, with body weight from 3.5 to 4.3 kg. Animals were housed in individual cages during the whole period of the study, with free access to feed and water.

After a period of acclimatization, the animals were artificially inseminated and pregnant status was verified by palpation 11 d later. After dropping (32 d after insemination), 4 mothers and their progeny were not treated and were used as controls for the study. Of the 16 remaining animals, only 6 young rabbits were maintained with each mother to ensure litter viability. The young remained with their mother during the whole lactation period of 5 weeks (date of weaning). Eight mothers were randomly sacrificed 12 d after weaning. The 6 young rabbits from the same litter were kept in the same cage during a fattening period of 48, 52, 55 or 60 d, at each of which times 8 animals were randomly sacrificed.

During the study all the animals were fed with two complete pelleted feeds: one was a maternity feed given to the mothers during pregnancy and lactation, the other was a fattening feed given to the young during the fattening period and also to the mothers during the period between weaning and sacrifice 12 d later.

Maternity and fattening feeds were given to animals either as blank feed or as feed supplemented with an authorized commercial veterinary medicinal product containing sulfadimethoxine (93 g/kg) and trimethoprim (20 g/kg).

The veterinary medicinal product was incorporated in the feed before pelleting at the supplementation level of 5 kg/ton, which meant that sulfadimethoxine was present in the feed at a level of 465 g/ton. At this supplementation level and according to the field conditions of use, the daily dosage of sulfadimethoxine was about 12.5 to 50 mg/kg bodyweight, which is the normal dosage proposed for preventive and curative rabbit treatments. The withdrawal time proposed for edible rabbit tissues under these conditions for a treatment duration of 5-10 d is 12 d from the end of treatment. Table 1 presents the different feed treatments carried out during the study on the mothers and young.

Sacrifice of rabbits was performed by CO_2 narcosis followed by exsanguination by cardiac puncture. Muscle was taken from the rear leg and minced with a homogenizer. This location was chosen for questions of ease of sampling, repeatability between animals and quantities large enough for the analysis. Aliquots of minced tissues were frozen and kept below −75°C until analysis.

Analytical phase

The analytical method used for the quantification of sulfadimethoxine in the rabbit muscle was developed in our laboratory using information from numerous publications concerning the analysis of Sulphonamides in biological matrices.

Standard and reagents

Methanol and chloroform (Sigma-Aldrich, Saint Quentin Fallavier, France) and acetonitrile (VWR, Fontenay sous Bois, France) were HPLC grade. Sodium hydroxide, phosphoric acid and n-hexane (Riedel-de Haën, Saint Quentin Fallavier, France) were reagent grade. The sulfadimethoxine basis active ingredient used for the assays had a purity of 99.4%. The sulfadimethoxine stock standard solution at 1000 µg/mL was prepared by dissolving in a mixture of methanol, water and sodium hydroxide 1 M (125/250/1 v/v/v). The working solutions of sulfadimethoxine (2.5 to 37.5 µg/mL) were obtained by dilution in ultrapure water. These solutions remained stable during the whole analysis period.

Sample preparation

Sulfadimethoxine (but not trimethoprim) was determined in the muscle samples. Five grams of minced rabbit muscle were homogenised with a mixture of acetonitrile/water and centrifuged (10 min, 3000 rpm).

Table 1: Summary of treatments according to the different steps of the study.

Period of the study	Nature of feed
Acclimatization period (10 d) and first 11 d of the pregnancy period (mothers)	Maternity/blank
Late part of the pregnancy period (21 d) and whole period of lactation (35 d) (mothers and, to a lesser extent, young rabbits)	Maternity/supplemented
From weaning up to 12 d post-weaning (12 d) (mothers)	Fattening/blank
From weaning up to 40 d post-weaning (40 d) (young rabbits)	Fattening/supplemented
From 40 d after weaning to sacrifice (young rabbits)	Fattening/blank

Table 2: Main results obtained during the control step of the analytical method.

Parameter	Assay conditions	Results
Specificity	Comparison of chromatograms	Presence of a peak on chromatograms resulting from the analysis of muscle specimens spiked with sulfadimethoxine and absence of this peak for the blank specimens
Linearity	Range of the tested concentrations: 50 to 750 µg/kg rabbit muscle	Calibration curve: Concentration = Peak area/300901 $R^2 = 0.99803$[1]
Precision	Tested concentrations: 50 and 600 µg/kg rabbit muscle (6 preparations of each)	%RSD: 4.3 and 5.4%[2] (for 50 and 600 µg/kg, respectively)
Accuracy	Tested concentrations: 50 and 600 µg/kg rabbit muscle (6 preparations of each)	Mean error with the theoretical concentration: 14.0 and 8.0% (for 50 and 600 µg/kg, respectively)
Lower limit of detection (= LLOD)	Estimation from the baseline noise after analysis of blank rabbit muscle (6 different preparations)	LLOD = 30 µg/kg rabbit muscle
Lower limit of quantification (= LLOQ)	Lowest concentration tested for linearity	LLOQ = 50 µg/kg rabbit muscle
Upper limit of quantification (= ULOQ)	Highest concentration tested for linearity	ULOQ = 750 µg/kg rabbit muscle
Stability of the preparations	Tested concentrations: 50 and 600 µg/kg rabbit muscle (3 preparations of each)	1/ Before analysis, the reconstituted preparation remained stable at room temperature for 24 h. 2/ Spiked preparations stored at a temperature below −75°C remained stable for 66 d before extraction and analysis.

[1] R^2 = determination coefficient. [2] RSD: residual standard deviation.

N-hexane was added to the supernatant, vortex-mixed and centrifuged (5 min, 1000 rpm). Chloroform was added to the aqueous layer, vortex-mixed and centrifuged (5 min, 1000 rpm). The organic phase was evaporated to dryness at approximately 40°C and reconstituted with the mobile phase for HPLC analysis.

Each analytical run was accompanied with a daily calibration curve prepared with blank rabbit muscle fortified with the working solutions of sulfadimethoxine.

Quality controls (QC) were prepared with blank rabbit muscle spiked with sulfadimethoxine stock standards in order to obtain 100 or 600 µg/kg tissue concentration of Sulphonamide.

These preparations were extracted and analysed during the same analytical runs as for the experimental specimens, in order to validate the obtained results.

HPLC analysis

The analysis was performed according to our own method with Waters HPLC materials (Milford, MA, USA): 515 pump, 717+ autosampler, 2487 detector and Empower 1 integration system. Separation was carried out with a Kromasil C_{18} column (4.6 mm × 250 mm; 5 µm) (Macherey-Nagel, Düren, Germany) protected by a guard column µBondapak C_{18} (Waters, Milford, MA, USA). The mobile phase was a mixture of acetonitrile and 0.02 M phosphoric acid solution (50/50 v/v). The flow rate was 1 mL/min and the injection volume was 20 µL. Wavelength for detection was 272 nm. Analysis was performed at room temperature.

The analytical method used for the sulfadimethoxine determination in the rabbit muscle was carried out without the addition of an Internal Standard in the samples before extraction and was not completely validated according to the regulatory specifications in force for the residues of veterinary medicinal products in foodstuffs of animal origin. However, different criteria were studied in order to verify that this method was well adapted to the expected scope of the study. According to the results obtained (see Table 2), it should be emphasized that the analytical method used for the study allows the quantification of sulfadimethoxine in rabbit muscle at concentrations above the LLOQ of 50 µg/kg, which is half of the MRL related to this tissue. The analytical method presented good precision and accuracy in the tested interval of linearity (between 50 and 750 µg/kg muscle). The Sulphonamide remained stable for 66 d in spiked muscle homogenates kept below −75°C until analysis; similarly, the muscle extracts were stable at room temperature for 24 h before analysis.

RESULTS AND DISCUSSION

The pelleted feeds were analyzed with and without the veterinary medicinal product. Blank feed did not contain sulfadimethoxine and the results on supplemented feed confirmed their conformity with the theoretical rate of supplementation: 477 and 485 g of sulfadimethoxine per ton of feed for maternity and fattening feeds, respectively (theoretical rate of supplementation for a therapeutic effect: 465 g/ton).

The mothers continuously received the maternity feed spiked with sulfadimethoxine at this therapeutic level for 56 d, comprising 21 d of gestation and 35 d lactation. The young rabbits continuously received the fattening feed spiked with sulfadimethoxine at this therapeutic level for the first 40 d of the post-weaning period. Since Sulphonamides transfer through the placental barrier and are also present in the milk of the treated mother (Von Fust *et al*., 1960; Paget *et al*., 1964), foetuses were likely indirectly in contact with the Sulphonamide during the 21 d of the gestation period and young rabbits during the whole lactation period (35 d). Moreover, during the lactation period, the young rabbits certainly consumed a certain part of the supplemented maternity feed freely accessible in the cage. Finally, it can be considered that the young rabbits remained in contact with sulfadimethoxine for a very long period, which can be estimated at 96 d: the 21 last d of the gestation period (placental transfer), 35 d of lactation (milk transfer and supplemented feed of the mother) and the first 40 d of post-weaning (for this period, at the therapeutic daily dosage of 12.5 to 50 mg/kg bodyweight).

As indicated in Table 1, after the end of treatment with the supplemented feed, the mothers were fed blank feed for 12 d before slaughter. The young rabbits were also fed blank feed for 8, 12, 15 or 20 d before sacrifice. At each slaughtering time, 8 young rabbits were randomly chosen from among the 16 litters.

The determination of sulfadimethoxine in rabbit muscle after extraction and HPLC analysis were carried out with reference to a daily calibration curve (range 50 to 750 µg/kg). The Sulphonamide concentrations in QC presented variations between −30 and +10% with the theoretical concentration values.

Table 3 presents the results of sulfadimethoxine concentration in muscle according to the treatment applied and slaughtering time.

Among the mothers, only 1 out of the 8 animals presented a Sulphonamide concentration in muscle above the MRL value of 100 µg/kg.

In the young rabbits, the results of Table 3 lead to the 2 following observations:

1) At the time of first slaughtering (8 d), all the 8 analyzed muscle samples showed sulfadimethoxine concentrations below the MRL value, despite the length of treatment at therapeutic level. This observation, confirmed by the results obtained on the 15th and 20th d, underlines the considerable ability of rabbits to eliminate Sulphonamide residues.

2) The results observed for slaughter at 12 d show variations: of the 8 young rabbits, 2 animals presented Sulphonamide concentrations above the MRL value.

The rabbit is an animal species which seems to present a high capacity to eliminate sulfadimethoxine from its organism and numerous considerations on the metabolism of Sulphonamides have been reported (Porter, 1964; Bridges *et al*., 1968; Adamson *et al*., 1970). Biotransformation occurs mainly in the liver, where the Sulphonamides are subjected to acetylation, glucuronidation and oxidation. The biotransformation of such molecules is affected by enzymatic systems (N-acetyltransferases, cytochromes P450, glutathione-transferases, epoxide hydrolases), whose activity is governed by genetic factors.

Of the metabolic pathways of hepatic transformation, acetylation is the most important degradation mode in the rabbit (Adamson *et al*., 1970; Bridges *et al*., 1968; Porter, 1964). In this species, the speed of the Sulphonamide acetylation varies individually, with genetic control. There are thus fast and slow phenotype animals for the acetylation pathways of Sulphonamides (Bridges *et al*., 1968; Inamura *et al*., 1990; Porter, 1964; Song *et al*., 1999; WHO, 2004).

Sulphonamides are also subjected to glucuronidation in the liver cells. This form of metabolism is present to a minor extent in the rabbit species: only sulfadimethoxine-N4 glucuronide is detected in the urine of treated animals (not the sulfadimethoxine-N1 glucuronide), and the quantified levels observed in urine are very low (0.4-0.8% of the oral dose during a period of 24 h) (Abou-El-Makarem *et al*., 1967; Adamson *et al*., 1970; Bridges *et al*., 1968).

Table 3: Sulfadimethoxine concentration in muscle of mothers and young rabbits following therapeutic treatment in feed and wash-out periods of different lengths.

Animals and number	Duration of the withdrawal period before sacrifice	Sulfadimethoxine concentration in muscle[1]
Therapeutic concentration in feed for 56 d (21 d of gestation + 35 d of lactation)		
8 mothers	Blank feed for 12 d	< MRL for 7 animals 410 µg/kg for 1 animal
Therapeutic concentration in feed for 40 d (beginning at the weaning date)		
8 young rabbits	Blank feed for 8 d	< MRL for 8 animals
8 young rabbits	Blank feed for 12 d	< MRL for 6 animals 130 and 460 µg/kg for 2 animals
8 young rabbits	Blank feed for 15 d	< MRL for 8 animals
8 young rabbits	Blank feed for 20 d	< MRL for 8 animals

[1] MRL: maximum residue limit.

As in many other animal species, oxidation of Sulphonamides also occurs in the liver cells of the rabbit. The transformations are mediated by the cytochrome P450 family (Roujeau, 2003; Trepanier, 2004; Vyas *et al*., 2006; WHO, 2004). As for the acetyltransferases, it has been reported that cytochromes P450 are enzyme systems with genetic polymorphism; for this reason, the concept of slow and fast metabolizers is also claimed for this biotransformation pathway (WHO, 2004). Thus, individual variations in the speed of the hepatic rate of oxidative reactions are foreseeable for Sulphonamides.

The considerations on the hepatic biotransformation of sulfadimethoxine, with slow and fast metabolizers, partly explain the individual variations of the degradation rate which can be expected in the rabbit. In the case of an animal with a genetically induced slow acetylation and/or oxidation (slow metabolizer), sulfadimethoxine residues will be present during longer periods than in a fast metabolizer.

A total of 96 young rabbits were used for the study, of which 8 were randomly chosen for the first slaughtering time 8 d after cessation of treatment. According to the absence of Sulphonamide residues in muscle, these 8 animals could have been fast sulfadimethoxine metabolizers. On another hand, among the 8 animals randomly chosen for slaughter from the 88 remaining at 12 d after cessation of treatment, 2 presented residues in muscle above the MRL value and could therefore be considered as slow metabolizers.

The same observation may be made for the 8 mothers sacrificed on the 12th d after cessation of treatment and chosen from the 16 animals remaining: one of these animals presented sulfadimethoxine residues in muscle at a concentration above the MRL value (410 µg/kg) and could thus be considered a slow metabolizer.

Complementary information on sulfadimethoxine residue depletion should be obtained on tissue concentrations measured in animals sacrificed sooner after cessation of treatment and/or in liver and kidney, the organs involved in the elimination of Sulphonamides. The results obtained in these organs could possibly confirm the presence or absence of residues in muscle and thus follow the same pattern of residue depletion.

The Sulphonamide concentrations observed in muscle demonstrate that a period of treatment much longer than the proposed therapeutic duration (about 10 times the usual duration of a therapeutic treatment) with a feed containing a therapeutic dosage of sulfadimethoxine does not induce accumulation of Sulphonamide in the organism of young and adult rabbits. The presence of sulfadimethoxine residues in muscle at a concentration level above the MRL value of 100 µg/kg sometimes observed at slaughter at 12 d, both for mothers and their young, could perhaps be explained by the concept of fast and slow genetic metabolizers. This could also explain the presence of Sulphonamide residues sometimes observed in carcasses at slaughter for rabbits treated under field conditions. It should also be remembered that field conditions do not always follow the manufacturers' recommendations, particularly concerning the duration of treatment. A minimum wash-out period of 15 d before slaughter would be more appropriate for such animals.

CONCLUSIONS

These results show that the very long period of oral treatment at a therapeutic level under field conditions did not induce an accumulation of sulfadimethoxine residues in the muscle of the treated animals. The results observed on the 12th d of slaughter could be explained by possible individual genetic differences in the hepatic biotransformation of Sulphonamides. Additional studies are necessary to confirm this hypothesis. A wash-out period of 15 d or more could be proposed for rabbits receiving orally veterinary medicinal products containing sulfadimethoxine during a treatment at therapeutic level under field conditions, i.e longer than 5-10 d. At the present time, Sulphonamide is no longer detected in muscle at

concentrations above the MRL value and the individual considerations on metabolism are simply matters of topical interest.

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