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**Research Note**

# Rapid Depletion of Marbofloxacin Residues in Rabbit after Therapeutic Treatment

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## ABSTRACT

Although rabbit meat production represents a very small percentage of the world meat market, this percentage has been growing continuously during the last 30 years. Rabbit is considered a minor food species, and therefore no drugs are specifically registered for this animal. This situation encourages rabbit farmers to make off-label use of antibacterial drugs authorized for food-producing animal species other than rabbits. In the present study, the distribution and elimination of the fluoroquinolone antibacterial agent marbofloxacin in rabbit muscle, liver, and kidney was investigated. Marbofloxacin was chosen as a representative of a new generation of antibacterial drugs active against most gram-positive and gram-negative bacteria and mycoplasms; it is well tolerated and has short elimination times in bovine and swine species. Rabbits were treated with marbofloxacin at 2 mg kg of body weight$^{-1}$ for 5 days. Residual concentrations in liver, kidney, and muscle tissues were determined posttreatment with high-performance liquid chromatography and fluorescence detection. Marbofloxacin was rapidly distributed and eliminated from rabbit tissues. Concentrations were higher in the liver and kidney than in muscle. However, 48 h after the end of treatment, marbofloxacin concentrations dropped below the maximum residue level fixed for this antibacterial drug in cattle and pigs. Considering the efficacy of marbofloxacin for the treatment of the most common rabbit diseases, its tolerability, and its short elimination time as verified in the present study, use of this antibacterial drug could be extended to therapeutic treatment of rabbits.

During the last decade, rapid changes in food habits of people in the European Union (EU) have occurred for nutritional reasons and because of unpredictable factors (e.g., bovine spongiform encephalopathy in cattle and dioxins in poultry). As a result, there has been a notable increase in the production of alternative kinds of meat *(10)*. Rabbit production for human consumption has increased because rabbit meat is highly nutritious; it is low in fat and cholesterol and rich in proteins and certain vitamins and minerals. Currently, rabbit meat production represents about 3.6% of the Italian meat market.

Many experts believe that rabbit production will continue to increase substantially because it is a low-cost activity, the animals are highly productive (up to 40 offspring per year compared to 0.8 and 1.4 for cattle and sheep, respectively), the meat has high nutritional value, and there are few social, cultural, and religious restrictions associated with it. However, the success of any rabbit farming enterprise will depend on good management and veterinary practices. Because commercially farmed rabbits commonly have little or no coliforms in their digestive tract, they are very susceptible to infections in production units *(11)*. These infections lead to poor performance, dehydration, and ultimately death. Mortality is the major issue in rabbit production, and 50 to 70% mortality is not unusual *(2)*. To alleviate this problem, the administration of antimicrobial drugs and chemicals in rabbitry is sometimes unavoidable. Nonetheless, no drugs are specifically registered for use in these animals *(7)*. Farmers, in the absence of specific recommendations, often resort to off-label use of antibacterial drugs that are authorized for food-producing animal species other than rabbit. Specific recommendations are lacking for rabbit because this animal is considered a minor food-producing species in the EU *(6)*. Current knowledge of antibacterial drug pharmacokinetics in minor species is very limited. Therefore, cooperative efforts should be made in the EU to gain needed approval for the use in rabbit production of certain drugs that are registered currently only for use in other food-producing species *(7)*.

Quinolones are effective antibacterial drugs that are widely used in both human and veterinary medicine for treatment of various systemic bacterial infections *(1)*. They are generally well tolerated, although they can cause arthropathy in immature animals *(12)*. During the last few years, marbofloxacin, a molecule belonging to this antimicrobial family, has received much attention for its potential efficacy in animal therapy *(17, 19)*.

Marbofloxacin is a fluorinated quinolone derivative with broad-spectrum activity that has been developed ex-

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clusively for use in veterinary medicine. Some microbiological, metabolic, and pharmacokinetic aspects of this drug have been reported in previous studies in dogs, cats, cattle, swine, and horses *(16, 19)*; however, to the best of our knowledge, no data on marbofloxacin distribution and elimination in rabbits are available. The EU Commission establishes and periodically reviews drug maximum residue levels (MRLs) in food. MRLs for marbofloxacin have been established for muscle, fat, liver, and kidney of cattle and swine and for cow milk. The values range from 50 to 150 $\mu g\ kg^{-1}$ *(7)*.

Because the pharmacokinetic properties of antibacterial agents differ significantly among species, the aim of this study was to determine the elimination trend of marbofloxacin in rabbits over time after intravenous administration of the drug. In this investigation, we evaluated rabbit muscle, liver, and kidney.

## MATERIALS AND METHODS

**Materials.** Marbofloxacin standard was purchased from Vetoquinol Laboratoire Pharmaceutique Veterinaire (Lure, France). The 2% Marbocyl (a commercial veterinary formulation whose active ingredient is marbofloxacin) was purchased from Azienda Terapeutica Italiana Srl (A.T.I., Bologna, Italy). Standard stock solutions were prepared by dissolving 10 mg of marbofloxacin in methanol and 1 M NaOH (999:1, vol/vol) and adjusting the final volume to 10 ml. This solution was stable at 4°C for 3 months. Working standard solutions were prepared by diluting the stock solution 1:10 (vol/vol) with Tris buffer solution (pH 9.1). Working solutions were stable at 4°C for 2 weeks. Acetonitrile, methanol, and tetrahydrofuran were high-performance liquid chromatography (HPLC) grade, and the other reagents used were analytical grade.

**Animals and trials.** Fifty-seven male New Zealand White rabbits (90 days old; Charles River S.p.A., Como, Italy) were used in the investigation. On their arrival at the laboratory, the animals were examined, quarantined, and housed in accordance with standard good laboratory practice.

Before starting drug administration, the animals were acclimatized for 15 days and then randomly divided into four groups of 10 animals each, H12, H24, H48, and H96, plus one group of 7 animals as a control (blanks).

Marbofloxacin was administered as Marbocyl 2% to the four groups for five consecutive days, and the control group was treated with the administration medium (physiologic solution) for the same period. Because no information on marbofloxacin therapeutic regimens for rabbits was available, dose and route of administration were chosen following the drug insert descriptions and suggestions for therapeutic treatments of other animal species. For all the species considered (dogs, cats, cattle, and swine) the recommended dose was 2 mg kg of body $weight^{-1}\ day^{-1}$ by injection. Thus, the same dosage was chosen for the rabbits, and the drug was administered by subcutaneous injection in the neck region with a 1-ml syringe and sterile hypodermic needle. Each rabbit was weighed to calculate the exact dose for each individual.

**Sample collection and preparation.** At the end of the treatment period, all the animals were sacrificed by vertebral dislocation and immediate jugulation. Rabbits in the H12, H24, H48, and H96 groups were sacrificed 12, 24, 48, and 96 h, respectively, after the end of treatment. Control rabbits (blanks) were sacrificed 96 h after the end of treatment. Muscle (thigh), liver, and kidney samples were collected from each animal and stored at −80°C until analysis.

**Analytical procedures.** The sample pretreatment procedure and chromatographic conditions followed those described by Yorke and Froc *(21)*, with some modifications. Methanol–acetic acid (10 ml, 98:2, vol/vol) was added to the homogenized tissue sample (about 1 g). After magnetic agitation for 15 min, the sample was transferred to a 15-ml tube, and 3 ml of methanol (used to wash beaker and magnetic stirrer) was added. After sonication for 15 min, the sample was centrifuged at 4,640 × *g* for 10 min at 5°C. The supernatant was transferred into a 50-ml flask. The residue in the tube was extracted two more times by the addition of two fresh 5-ml portions of methanol–acetic acid (98:2, vol/vol), agitation on a vortex mixer for 2 min, and then sonication and centrifugation as described above. The resulting supernatants were added to the first supernatant, 3 ml of methanol (used to wash the tube) was added, and the combined extracts were evaporated on a rotary evaporator (50°C, 200 mbar, 100 rpm) to about 2 ml. Water–acetic acid (10 ml, 98:2, vol/vol) was added to the sample solution. Solid-phase extraction (SPE) cartridges (500 mg, 6 ml; Speed SPE $C_{18}$, Applied Separations, Allentown, Pa.), previously conditioned with methanol (6 ml) and then with water (6 ml), were used for the purification of the quinolone drug (cartridges were kept under vacuum at 10 in. Hg during the whole procedure). The whole sample solution was deposited on top of the cartridge, which was then washed with water–1 M phosphoric acid (6 ml, 3:2, vol/vol). The drug residues were eluted from the SPE column with methanol–1 M phosphoric acid (10 ml, 9:1, vol/vol). The flask walls were washed with methanol (3 ml), and the collected fractions were evaporated to dryness on a rotary evaporator (50°C, 200 to 72 mbar, 100 rpm). The dried pellet was reconstituted with Tris buffer solution (1 ml, pH 9.1). After filtration with 0.45-μm-pore-size nylon filters, 20 μl of the final solution was injected into an HPLC system that was assembled as follows. A pump with an autosampler device (model Alliance 2690, Waters S.p.A., Vimodrone, Milan, Italy) was fitted with a reverse-phase PLRP-S column (100 Å, 150 by 4.6 mm, 5 μm; Polymer Laboratories, Church Stretton, UK) and a reverse-phase RP-18-E guard cartridge (40 by 4 mm; Polymer). A Croco-cil oven (CIL, Cluzeau, Sainte-Foy-la-Grande, France) was heated to 40°C, and a fluorescence detector (model 474, Waters) was set at 294 and 514 nm for the excitation and the emission wavelengths, respectively. The eluents for isocratic elution were 25 mM *orto*-phosphoric acid (92.5%), acetonitrile (6.0%), and tetrahydrofuran (1.5%). The flow rate was 1 $ml\ min^{-1}$.

Before sample analysis, the HPLC method was validated in house according to recommendations from European Community (EC) Commission decision no. 657/2002 *(9)*.

**Validation of the in-house method.** Because no certified reference materials were available for marbofloxacin, the accuracy of the method was evaluated on the basis of the recovery from in-house standard materials (fortified blank matrix). To determine accuracy and precision, 18 blank tissue samples for each kind of matrix (muscle, liver, and kidney) were selected, and six aliquots were fortified with marbofloxacin at each of 0.5, 1, and 2 times (25, 50, and 100 $\mu g\ kg^{-1}$) the lowest MRL (50 $\mu g\ kg^{-1}$) fixed for cattle and swine tissues. Accuracy and precision, expressed as recovery and repeatability, respectively, were calculated as described in EC Commission decision no. 657/2002 *(9)*. In particular, recovery was estimated by analysis of the 18 fortified blank tissue samples (6 for each of the three fortification concentrations) for each kind of matrix. Repeatability was calculated by marbo-

TABLE 1. *Performance of the analytical method for determination of marbofloxacin concentrations in rabbit tissues*

| Marbofloxacin fortification ($\mu g\ kg^{-1}$) | Accuracy[a] | | Precision (CV [%]) | |
|---|---|---|---|---|
| | Concentration of recovered marbofloxacin ($\mu g\ kg^{-1}$) | % recovered | Intraday[b] | Interday[c] |
| Muscle | | | | |
| 0 | 0.8 ± 0.2 | | 25 | 37 |
| 5 | 5 ± 0.8 | 100 | 16 | 20 |
| 25 | 26 ± 1.1 | 104 | 4.2 | 6.9 |
| 50 | 50 ± 3.4 | 100 | 6.8 | 7.2 |
| 100 | 102 ± 7.7 | 102 | 7.6 | 7.8 |
| Kidney | | | | |
| 0 | 1 ± 0.5 | | 50 | 70 |
| 15 | 16 ± 2.4 | 107 | 15 | 18 |
| 25 | 28 ± 2.3 | 110 | 8.2 | 8.9 |
| 50 | 48 ± 3.5 | 96 | 7.3 | 8.7 |
| 100 | 100 ± 4.8 | 100 | 4.8 | 9.2 |
| Liver | | | | |
| 0 | 1.2 ± 0.8 | | 67 | 78 |
| 25 | 21 ± 1.9 | 84 | 9.0 | 10 |
| 50 | 45 ± 2.9 | 90 | 6.4 | 7.8 |
| 75 | 71 ± 3.8 | 95 | 5.3 | 6.1 |
| 100 | 96 ± 4.5 | 96 | 4.7 | 5.8 |

[a] Values are means ± SDs for six independent samples.
[b] Values are average CVs (%) for six independent samples analyzed within 1 day.
[c] Values are average CVs (%) for 18 samples (six independent samples repeated on three different days).

floxacin determinations on the same samples except that the analyses were repeated on two other occasions (different days).

The limit of detection (LOD) and the limit of quantification (LOQ) for muscle, liver, and kidney samples were calculated according to the requirements of the International Conference of Harmonization *(5, 13)*. Twenty-one blank tissue samples (triple independent samples from seven different rabbits in the control group) for each tissue were fortified with an amount of standard marbofloxacin able to produce signal-to-noise ratios ranging from 2.5 to 5. LODs were determined by multiplying the standard deviations (SDs), calculated for the 21 blank tissue samples fortified with low drug concentrations, by the Student $t$ test value ($n - 1$; $1 - \alpha = 0.99$). The exact equation of the calculation is LOD = SD × Student $t$ test value ($n - 1$; $1 - \alpha = 0.99$), where SD is the standard deviation for each tissue, Student $t$ test value ($n - 1$, $1 - \alpha = 0.99$), where SD is for the 21 blank tissue samples fortified with low concentrations. In our case, $n = 21$; therefore, the Student $t$ test value was 2.845. The LOQs were estimated to be 10 times the same SD (i.e., LOQ = SD × 10, where SD is the standard deviation for the 21 blank tissue samples fortified with low drug concentrations).

Calibration curves for each kind of matrix were constructed using five concentrations of spiked marbofloxacin: 0, 5, 25, 50, and 100 $\mu g\ kg^{-1}$ for muscle; 0, 15, 25, 50, and 100 $\mu g\ kg^{-1}$ for kidney; and 0, 25, 50, 75, and 100 $\mu g\ kg^{-1}$ for liver.

## RESULTS

**Animal health.** Rabbits treated with the chosen doses of Marbocyl 2% showed no manifest reactions to the drug. No evidence of macroscopic alterations in organs and tissues were reported after necropsy. During and after the treatment period, animals maintained the same eating and drinking habits as those observed during the acclimatizing period, and no other behavioral alterations were noted.

TABLE 2. *Marbofloxacin depletion in rabbit tissues at different times*

| Time (h) | Marbofloxacin concentration ($\mu g\ kg^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Muscle | | Kidney | | Liver | |
| | Mean ± SD[a] | CV (%) | Mean ± SD | CV (%) | Mean ± SD | CV (%) |
| 12 | 36 ± 15 | 42 | 101 ± 36 | 36 | 132 ± 66 | 50 |
| 24 | 3 ± 1 | 33 | 16 ± 6 | 37 | 32 ± 10 | 31 |
| 48 | 1 ± 0.7 | 70 | <LOD[b] | | <LOD | |
| 96 | <LOD | | <LOD | | <LOD | |

[a] Values are means ± SDs for 10 independent samples (10 different animals).
[b] LOD for marbofloxacin was 1 $\mu g\ kg^{-1}$ in muscle, 4 $\mu g\ kg^{-1}$ in kidney, and 7 $\mu g\ kg^{-1}$ in liver.

**Performance of the analytical method.** Results of validation of the in-house method are shown in Table 1. The coefficients of variation (CVs) for marbofloxacin concentrations, equal to 0.5, 1, and 2 times (25, 50, and 100 $\mu g\ kg^{-1}$) the lowest MRL (50 $\mu g\ kg^{-1}$) fixed for cattle and swine tissues, ranged from 4.2 to 7.8%, from 4.8 to 9.2%, and from 4.7 to 10% for muscle, kidney, and liver, respectively. These data indicate that the intraday and interday repeatability of the method was high; the CVs are all below the recommended limits reported in EC Commission decision no. 657/2002 *(9)*.

Recovery percentages also were high, with values of 100 to 104%, 96 to 110%, and 84 to 96% for muscle, kidney, and liver, respectively. These values fall within the guideline range (−20 to +10%) for mass fractions ≥10 $\mu g\ kg^{-1}$ reported in EC Commission decision no. 657/2002.

LODs and LOQs for marbofloxacin in rabbit were 1 and 4 $\mu g\ kg^{-1}$ in muscle, 4 and 15 $\mu g\ kg^{-1}$ in kidney, and 7 and 23 $\mu g\ kg^{-1}$ in liver, respectively.

The calibration curves corrected for recovery in rabbit tissue samples were used for the quantification of marbofloxacin: for muscle, $y = 2 \times 10^{-4}x - 10.356$ ($R^2 = 0.9998$); for kidney, $y = 2 \times 10^{-4}x - 11.387$ ($R^2 = 0.9965$); and for liver, $y = 2 \times 10^{-4}x - 6.1958$ ($R^2 = 0.9999$). For samples with marbofloxacin concentrations above the highest value of the calibration curve, samples were diluted so that the concentrations were within the linear range.

**Elimination from tissue.** The results for marbofloxacin elimination from rabbit tissues at different times are shown in Table 2. At 96 h after the end of treatment, marbofloxacin concentrations were less than the LOD of the analytical method in all the analyzed tissues. At 48 h after the end of treatment, marbofloxacin concentrations were not yet detectable in kidney and liver tissues and equal to the LOD in muscle tissues (1 $\mu g\ kg^{-1}$). At 24 h after the end

of treatment, the three tissues had very low marbofloxacin concentrations (3, 16, and 32 μg $kg^{-1}$ in muscle, kidney, and liver, respectively). At 12 h after the end of treatment, the residue concentrations were high in kidney and liver (101 and 132 μg $kg^{-1}$, respectively), but in muscle tissue the concentration (36 μg $kg^{-1}$) was already below the lowest MRL fixed by the EU *(7)* for marbofloxacin in other animal species (50 μg $kg^{-1}$ in bovine and swine fat).

## DISCUSSION

Drug depletion from food-producing animal species must be assessed to determine the time needed before the drug disappears from the animal tissue and to estimate when the treated animal can be safely consumed. Suitable and sensitive analytical methods are needed to monitor quinolone residues in food and to establish withdrawal times in rabbit after pharmacological treatments *(7).* The analytical method developed in the present study had LODs and LOQs for rabbit liver and kidney that were higher than those found in previous studies for the same tissues (5 μg $kg^{-1}$) but in different animal species (cattle and swine). In contrast, for rabbit muscle tissue the LODs and LOQs were comparable to those previously reported. The pharmacokinetics of a single drug generally changes in different animals, and methodologies developed for the analysis of a certain drug in a single species cannot simply be extended to a different species. Often, extraction and analysis procedures must be adapted to the new tissue and proper validation studies of the method must be conducted.

The results obtained in the present study confirm that the absorption and distribution of marbofloxacin administrated to a rabbit via subcutaneous injection reach high levels in a short time, as occurs in other animal species, at least in muscle, liver, and kidney. At 24 h after the end of treatment, marbofloxacin concentrations in the three tested tissues were below the lowest MRL fixed for bovine and swine fat tissues (50 μg $kg^{-1}$) *(8).* Between 12 and 24 h after the end of treatment, marbofloxacin concentrations markedly decreased (−92, −84, and −76% in muscle, kidney, and liver, respectively), which is indicative of high clearance and low elimination half-life values of the fluoroquinolone drug.

At all the times after the end of treatment, marbofloxacin residues probably were higher in the liver than in the other two tissues. Although marbofloxacin concentrations in liver were undetectable 48 h after the end of treatment, the LOD in liver is much higher than the LODs in kidney and muscle. However, previous depletion studies carried out with pigs and preruminant calves indicate the same trend, with marbofloxacin concentrations always higher in kidney and liver than in muscle tissues.

In the kidney, marbofloxacin concentrations fell under detectable levels 48 h after the end of treatment. Therefore, it is likely that the elimination half-life of this fluoroquinolone drug in the rabbit is very similar to or perhaps shorter than that described previously for other animal species (i.e., 8 to 10 h) *(16).*

In rabbit muscle, where fat levels are very low and where no significant metabolism or elimination takes place, marbofloxacin concentrations were always lower than those measured in the liver and kidney. However, marbofloxacin was efficiently distributed in muscle tissues, in accordance with previous data for dogs *(16).*

The CVs of the marbofloxacin concentrations measured in the three tissues of treated animals were much higher than those calculated for corresponding fortified blank matrix. This finding is not surprising considering the influence of intraspecies variability on drug metabolism and, consequently, on marbofloxacin concentrations in tissues of different rabbits.

Overall, the results of the present study confirm marbofloxacin as a viable candidate for registration as an antibacterial drug in the treatment of rabbit diseases. This fluoroquinolone drug is characterized by excellent antibacterial properties *(19),* and it is eliminated very rapidly in the rabbit, with clearance times comparable to those typical of other species (cattle and pigs) for which it is already registered and employed.

Rabbit meat, although representing a small market percentage in most European countries, has been a valuable meat resource especially during periods of sudden changes in food trends (e.g., during problems caused by bovine spongiform encephalopathy or dioxin in poultry). The availability of additional safe drugs for minor food species such as the rabbit is important for consumer health. When new molecules become registered, illegal and off-label uses of drugs are generally reduced *(3, 15),* and incidences of antibiotic resistance are less frequent *(4, 20).* When fluoroquinolones are not adequately administrated to food-producing animals following the legal indications for registered drugs, they can foster drug-resistance phenomena *(14)* and, if absorbed at certain concentrations, might cause serious pathologic changes in the joints of children and teenagers *(18).*

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