UNIVERSITAT AUTÒNOMA DE BARCELONA

FACULTAT DE VETERINÀRIA

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CERTIFIQUEN:

Que En Carles Vilalta Sans ha realitzat aquest Treball de Recerca que duu per títol **Marbofloxacin achieves high concentration in pig tonsils according to a dose dependent fashion** sota la seva direcció als laboratoris del Centre de Recerca en Sanitat Animal (CReSA) i en col·laboració amb Laboratorios Vetoquinol internacional.

I perquè així consti, a efectes de ser presentat com a Treball de Recerca del Programa de Doctorat en Farmacologia signen aquest certificat.

Bellaterra, 20 d´agost de 2010.

Dr. Carles Cristòfol. Dr. Lorenzo José Fraile.

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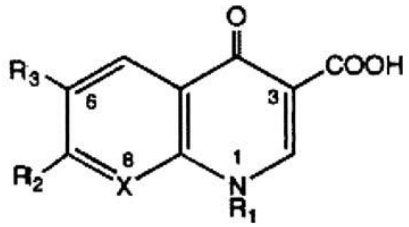
ABSTRACT

Marbofloxacin is a fluoroquinolone with a wide spectrum of activity. This spectrum includes *Actinobacillus pleuroneumoniae* (APP) that is the causative agent of porcine pleuroneumonia. APP can remain in the tonsils of asymptomatic pigs for a long period maintaining the disease in the herd. The penetration of marbofloxacin into tonsils was assessed in fattening pigs. Two different dosages were used to treat the animals: 2 mg/kg b.w. every 24 hours during 3 days (P1 group) and 4 mg/kg b.w. every 48 hours two times (P2 group). Marbofloxacin achieved a mean concentration in tonsils of 0.5 and 0.7 µg/mL 24 hours after the last administration in groups P1 and P2, respectively. Marbofloxacin achieved a tonsillar concentration three times greater than the plasma concentration whatever the dose administered 24 hours after the last administration. A ratio between the mean tonsillar concentration of marbofloxacin for both doses (0.5 and 0.7 µgr/mL) and its MIC_{90} for APP (0.03 µg/mL) was calculated. These Ratio values were 16.6 and 23.3 for P1 and P2 group, respectively and were also above the threshold established for PK/PD efficacy parameters in the case of fluoroquinolones. These findings suggest that marbofloxacin could be a good candidate to carry out additional studies focused on deciphering the potential use of this antibiotic for control or eradication programs in APP affected farms.

1. INTRODUCTION

QUINOLONES

Quinolones belong to a large and expanding group of synthetic antimicrobial agents. This group of antibiotics began with the discovery of the nalidixic acid in 1962 as a by-product of anti-malarial research in 1962 (Lesher et al., 1962). The nalixidic acid (figure 1) had a good activity against aerobic Gram negative, but reached solely the therapeutic concentration in urine. Owing to this particularity, the nalixidic acid was used for the treatment of urinary tract infections. The addition of a fluorine atom at the 6 position on the quinolone carboxylic acid nucleus enhanced the efficacy of the quinolones against gram negative pathogens and broadened the spectrum of activity against Gram positive pathogens. Moreover, the addition of piperazine derivatives in the 7-position enhanced tissue penetration and it also reduced its central nervous system toxicity. As a result of these improvements a second generation of drugs were born, the fluoroquinolones (FQ) (M. Martinez et al., 2006).



X = N; naphthyridone

X = CH ; quinolone ;

Nalidixic acid ; $R_1 = CH_2CH_3$;
R_2 = pyridyl
R_8 = H
X = N

Figure 1. Nalidixic acid. Martinez et al. (2006)

FLUOROQUINOLONES

The FQ have a good oral absorption (exceptions: ruminants and horses), complete parenteral absorption, and good tissue distribution owing to their physicochemical properties. Their metabolism and excretion occurs in the liver and in the kidney, respectively. These antimicrobials have a wide spectrum of action against gram-negative bacteria, mycoplasma and some gram positive bacteria. FQ affect the DNA supercoiling by inhibit an enzyme found in all bacteria, the DNA gyrase. This gyrase plays a vital role in DNA packing, replication and transcription. Furthermore, the FQ have a secondary target, the topoisomerase IV that acts in the ATP dependent relaxation of the DNA (M. Martinez et al., 2006). There are some FQ only used in animals (enrofloxacin, difloxacin, danofloxacin, marbofloxacin, orbifloxacin and sarafloxacin) (figure 2).

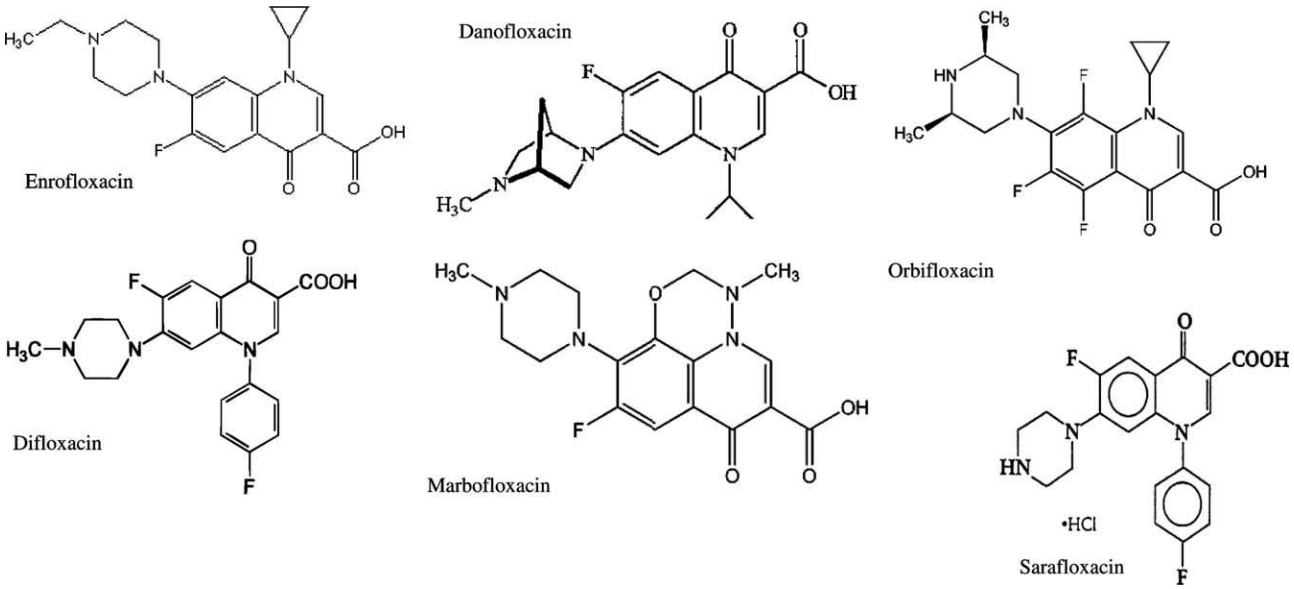


Figure 2. Chemical structures of different fluoroquinolones. Martínez et al. (2006)

MARBOFLOXACIN

Marbofloxacin (MB) (figure 3) is a fluoroquinolone widely used in veterinary medicine. This molecule is an organic acid with good tissue penetration. Its volume of distribution exceeds the body water volume and the degree of binding to plasma proteins is low (Sidhu et al., 2009). It possesses a broad spectrum of activity against mycoplasma, most Gram negative, some Gram positive bacteria and some intracellular pathogens such as *Brucella* and *Chlamydia* species, but with limited or no activity against anaerobes (Hannan et al., 1989; Spreng et al., 1995; Appelbaum and Hunter, 2000). This spectrum of activity includes most of the swine respiratory pathogens, including *Actinobacillus pleuropneumoniae* (APP).

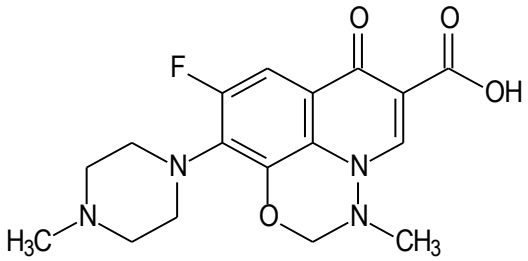


Figure 3. Chemical structure of Marbofloxacin. (Kindly provided by Vetoquinol International)

PORCINE RESPIRATORY DISEASE COMPLEX

Porcine respiratory disease complex (PRDC) is clinically characterized by dyspnoea, coughing, acute depression, anorexia, fever, and nasal discharge, specially affecting growing to finishing pigs (Dee, 1996). This complex disease is most often due to the interaction of multiple factors. Both viral and bacterial organisms play a role, as well as the environment and various management practices employed by producers. When in the right combination, these factors can sufficiently compromise the pig respiratory defense mechanisms, resulting in severe respiratory disease (Thacker et al., 2006). The most common viral pathogens associated with PRDC are porcine respiratory and reproductive syndrome virus (PRRSV), swine influenza virus (SIV), pseudorabies

virus (PRV) and porcine respiratory coronavirus (PRCV). The most common bacterial pathogens associated with this complex include: *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Pasteurella multocida, Haemophilus parasuis, Streptococcus suis, Arcanobacterium pyogenes, Salmonella choleraesuis* and *Actinobacillus suis* (Christensen et al., 1999).

PLEUROPNEUMONIA

APP is the causative agent of porcine pleuropneumonia, a worldwide disease with occasional clinical outbreaks that can have a severe economic impact (Gottschalk and Taylor, 2006). Attempts to control the disease have been made by vaccination, treatment with antibiotics and the establishment of herds free of the infection. Pigs can become asymptomatic carriers of the organism in their tonsils for long periods (Macinnes and Rosendahl, 1988; Vigre et al., 2002), thereby exposing susceptible animals and maintaining the disease in the herd. Moreover, pigs can carry APP in their tonsils for several months without seroconverting (Lavritsen et al., 2002).

Attempts to eradicate APP from pig herds have been made with different antibiotics. For example, Fittipaldi et al. (2005) used feed medicated with tilmicosin phosphate for 30 days but found that the tonsils of the majority of animals were still PCR-positive 30 days later. Most of the results have been published in case reports describing procedures applied to one or a few farms (Angen et al., 2008). On the other hand, an eradication program that includes sow medication with a fluorquinolone was successful (Bækbo, 2006). However, these field trials are not supported with the determination of antibiotic concentration in the tonsils (target tissue).

PK/PD RELATIONSHIP

The pharmacokinetics describes, through mathematic concepts, the kinetics of a drug inside the organism and defines a pattern of the ADME process (Absorption, Distribution, Metabolism and Elimination) of each drug. The most relevant pharmacokinetic parameters (C_{max}, t_{max}, AUC, bioavailability, volume of distribution at steady-state, and elimination half-lives) have been described for many antimicrobial drugs in several species of veterinary interest. These parameters are necessary to establish the posology regimen for each particular species.

One of the most important PK considerations is where to measure drug concentrations from which PK data are derived. It is evident that the antimicrobial should reach the locus of the offending bacteria to be effective and, therefore, it could be assumed that tissue or cellular concentrations would be the most appropriate for determining the required kinetic parameters (McKellar et al., 2004). Nevertheless, plasma concentrations have been shown to be the best predictors of clinic success even for most tissue infections (Schentag, 1989; Toutain et al., 2004). Otherwise, there are special cases, where a specific anatomical or pathological barrier exists, where it may be more appropriate to use concentrations derived from that site (Toutain et al., 2004).

Pharmacodynamics studies the action of drugs on microorganisms or on specific receptors in the body to modify a physiological action. One of the difference between PD studies on mammalian cells/ tissues and on microbes is that the response in the former system is normally quantified as an enhancement or reduction of some component of cell or body function (smooth muscle contraction, decrease in body temperature, etc.), whereas pharmacodynamics parameters for microorganism establish threshold values (MIC or MBC) to link the concentration of antibiotic, normally measured in serum or plasma, with the growth of the microorganism population. The most widely used in vitro PD parameter is the minimum inhibitory concentration (MIC). This parameter is determined under standard culture conditions either on a solid agar medium or, more usually, in liquid broth culture. It is defined as the lowest concentration of antimicrobial drug which prevents (as assessed

by visual examination) visible microorganism growth. MIC is a simple, versatile, readily performed measure, which enables large numbers of microorganisms to be screened. Because MIC varies considerably between strains of a single organism, it is usual practice to measure MIC on many (up to several hundred) isolates and then compute the MIC_{50} and MIC_{90} values. To register an active ingredient for antimicrobial use, it is usual to use MIC_{90} rather than MIC_{100} values as the main pharmacodynamic parameter to establish its posology regimen. This is because, for any given population of microorganisms, there will commonly be a small percentage of isolates which are not susceptible, even to very high drug concentrations. MIC_{90} provides the best available indicator to link with PK data and establish a posology regimen to be applied under field conditions (Lees et al., 2004). It is important to take into account that the distribution of MICs in a wide range of isolates is not always normally distributed (Alibadi et al., 2000).

The utility of PK/PD information is demonstrable in the development of new antimicrobials, the more specific selection of appropriate antimicrobials from formularies, the design of optimal dosage strategies and the reduction of the selection of antimicrobial resistance (Gunderson et al., 2001).
The PK/PD parameters which have been mostly investigated, and for which the most robust information is currently available, are $AUC_{0\text{-}24}$:MIC, C_{max}:MIC and T>MIC (Hyatt et al., 1995). Antimicrobial drugs have been classified as concentration-dependent where increasing concentrations at the locus of infection improve bacterial kill, or time-dependent where exceeding the MIC for a prolonged percentage of the inter-dosing interval correlates with improved efficacy. For the latter group, increasing the absolute concentration obtained above a threshold does not improve efficacy. The PK/PD relationship for each group of antimicrobial drugs is “bug and drug” specific, although ratios of 125 for $AUC_{0\text{-}24}$:MIC and 10 for C_{max}:MIC have been recommended to achieve high efficacy for concentration-dependent antimicrobial drugs, and exceeding MIC by 1-5 multiples for between 40 and 100% of the inter-dosing interval is appropriate for most time-dependent agents (McKellar et al., 2004).

Concentration-dependent	Time-dependent	Co-dependent
Aminoglycosides	Beta-lactams	Beta-lactams*
Fluoroquinolones	Macrolides (except azithromycin)	Fluoroquinolones**
Metronidazole	Clindamycin	Glycopeptides
(vs. Anaerobes)	Vancomycin	

Table 1. General classification of antimicrobial drugs for which information is available on concentration or time-dependent killing activity (*In relation to reduction in resistance selection pressure;**Some with anaerobic activity). McKellar et al., 2004.

PALATINE TONSIL

The Palatine Tonsil (PT) is the major immunological component of the oropharinx ubicated in the soft palate. This consists of organized lymphoid tissue covered by stratified squamous epithelium but penetrated by branching crypts covered with nonkeratinized epithelium. The organized tissue contains B cell follicles and T cells (Pastoret et al., 1998). The crypt epithelium is a lymphoepithelium containing goblet cells, microfold cells (M cells) and intraepithelial lymphoid cells (Belz and Heath, 1996). Some bacteria native to the oropharynx may inhabit the tonsils, resulting in subclinical carriers of, for example, *Actinobacillus Pleuroneumoniae* (Macinnes and Rosendahl, 1988; Vigre et al., 2002), *Erysipelothrix rhusiopathiae* (Takahashi et al., 1999), salmonellae, or some groups of streptococci (Pastoret et al., 1998).

Taking into account the previous information provided in the introduction, the goal of this study was to quantify the marbofloxacin (MB) penetration in tonsils after applying two different MB dose regimens to decipher the potential use of this antibiotic for control or eradication programs in APP affected farms.

2. MATERIALS AND METHODS

ANIMAL SAMPLING.

Thirty 2-month-old pigs weighting 17.4 to 27 kg were selected for this study coming from a farm with clinical cases of porcine pleuropneumonia. Animals were clinically healthy when the study began. All pigs included in the study received non-medicated commercial feed ad libitum and had free access to drinking water. Animals were randomly divided into three groups (control, P1 and P2) of ten animals. All the animals were weighed with a calibrated scale to guarantee an exact dose of marbofloxacin or saline and tagged on the ear in order to identify them (table 2). Each treatment group received Marbocyl® 2% applied at a dose of 2 mg MB/kg b.w. for three consecutive days (group P1) and at a dose of 4 mg MB/kg b.w. every 48 hours two times (group P2) intramuscularly. Animals of the control group were sham injected with the same volume of physiological saline (2cc). The animals were sacrificed by intravenous administration of pentobarbital sodium twenty four hours after the last administration. This sampling time was chosen to allow achieving distribution equilibrium in the tonsils even if this tissue would behave as a deep tissue in comparison with well-irrigated organs such as the lung. Blood sample were taken to obtain serum before the administration of pentobarbital. Tonsils were collected during the necropsy and frozen immediately with carbonic ice at -80ºC until analysis.

LABORATORY PROCEDURES

The concentration of marbofloxacin in serum and tonsils was quantified by HPLC according to standard procedures in Vetoquinol laboratories in Lure (France). This extraction method is based on a liquid-liquid process with chloroform. Ofloxacin was used as an internal standard. Ofloxacin is a molecule that posses a molecular structure similar to marbofloxacin but it has different molecular weight. These characteristics are shown in figure 4.

Control Group			Group P1 (2mg/Kg)			Group P2 (4mg/Kg)		
Animal	**Weight (Kg)**	**Dosage (cc)**	**Animal**	**Weight (Kg)**	**Dosage (cc)**	**Animal**	**Weight (Kg)**	**Dosage (cc)**
1	24.5	2	**11**	23.7	2.37	**21**	19.2	3.84
2	25.4	2	**12**	23.1	2.31	**22**	26	5.2
3	23.1	2	**13**	27	2.7	**23**	24.6	4.92
4	24.9	2	**14**	25.7	2.57	**24**	24.1	4.82
5	25.2	2	**15**	25.5	2.55	**25**	25.7	5.14
6	25.3	2	**16**	23.9	2.4	**26**	24.6	4.9
7	23.1	2	**17**	22.6	2.3	**27**	20.6	4.1
8	18.8	2	**18**	17.4	1.75	**28**	26.2	5.2
9	22.9	2	**19**	23.3	2.3	**29**	18.5	3.7
10	25.1	2	**20**	17.8	1.8	**30**	18	3.6

Table 2. Weight and administered volume of saline (control group) and marbofloxacin (P1 and P2 group).

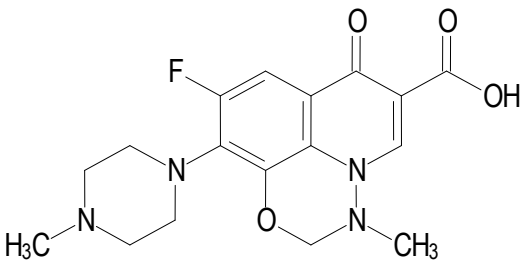
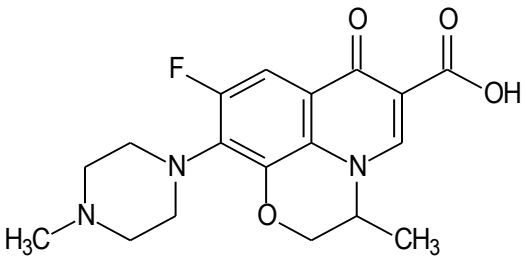
MARBOFLOXACIN	**OFLOXACIN**
Empirical formula: $C_{17}H_{19}FN_4O_4$	Empirical formula: $C_{18}H_{20}FN_3O_4$
Molecular weight: 362.4	Molecular weight: 361.4
	

Figure 4. Main similarities and differences between marbofloxacin and ofloxacin. Vetoquinol

The next solvents and reagents were used to prepare the different solutions needed in the extraction procedure: Acetonitrile, acetic acid, ortophosphoric acid, chloroform, potassium dihydrogen phosphate, di-ammonium hydrogen phosphate, di-sodium hydrogen phosphate, methanol, tetrabutylammonium hydrogen sulphate, triethylamine and ultra-pure water (Milli-Q Millipore). Thus, reference substances marbofloxacin and ofloxacin (Sigma).
Further details of solutions and equipment used are shown in Annex 1.

After thawing the tonsils, 500 mg of porcine tonsil sample were weighted and treated with 1 mL of enzymatic solution (protease). The mixture was agitated moderately during 90 min at about 60ºC. After cooling, 50 µL of internal standard solution IS2 (Ofloxacin at 1µg/mL) was added, except for the blank porcine tonsil and mixed. The samples were adjusted to pH 7 with buffer solution pH 2 HCl – Citrate (about 300 µL) and 4 mL of chloroform. After the agitation (10 min on a reciprocating agitator) and centrifugation (at 5000 rpm for 10 min at about 5ºC), the organic phase was transferred in a conical-bottomed inactinic glass tube and evaporated under a nitrogen stream at about 38ºC. The dry extract was solved with 200 µL of mobile phase A, mixtured on the vortex, sonicated in an ultrasonic bath and mixture on vortex again. The mixture was transferred in an Eppendorf tube, it was added 1mL of hexane, mixtured on the vortex and sonicated in an ultrasonic bath. Finally, the mixture was centrifugated (1300 rpm for 10 min at about 5ºC) and the bottom phase was transferred in vial for injection.
Calibration samples from 0 to 5 µg/g or 5 µg/mL were prepared with 500 mg of porcine tonsil or 1 mL of serum (see table 3 below), respectively in order to obtain:

- 8 samples of blank porcine tonsil or serum with different amounts of marbofloxacin.
- a blank porcine tonsil or serum with internal standard.
- a blank porcine tonsil and serum.

Marbofloxacin concentration of the calibration samples (µg/g)	**Weight of tonsil (mg)**	**Used marbofloxacin standard solution**	**Spiking volume (µL)**
0	500	NA	NA
0.005	500	A4	25
0.01	500	A4	50
0.025	500	A4	125
0.05	500	A3	25
0.1	500	A3	50
1	500	A2	50
2.5	500	A2	125
5	500	A1	25

Table 3.

NA : Not applicable

Briefly, serum samples were extracted with chloroform after adding ofloxacin as internal standard, following the same method as described previously from the tonsils.

The limit of quantification was 0.005 µg/mL for serum and 0.005 µg/gr for tonsil. To prepare standards, control serum and tonsils from animals which had received no treatment were spiked with marbofloxacin, the spiked standard concentrations ranging from 0.005 to 5 µg mL^{-1} or µg/g. Both methods were highly linear with coefficients of correlation of the standard curves (r) better than 0.99. Accuracy and reproducibility were determined from inter-day and intra-day variances of assays with spiked concentrations. For the serum samples, accuracy was within the range of 100 to 102 % and precision was better than 5 %. For tonsil samples, accuracy was within the range of 95 to 103 % and precision was better than 11 %.

HPLC-reversed phase with a C18 stationary phase (analytical column: Merck Lichrospher 100RP18 (250 x 4) mm. 5µm) with fluorescence detection set to 295 nm for excitation and 500 nm for emission was used. The mobile phase was a mixture of phosphate buffer (pH 2.7), methanol, acetonitrile, acetic acid and triethylamine (86.5/10/2.5/1/0.3 v/v/v/v/v). The method is linear from 0.005 to 5 µg/g with a weighting factor of $1/C^2$.

The ratio between MB concentration in tonsils and MIC_{90} value (0.03 µg/mL) was calculated to obtain a PK/PD parameter as previously described (Mckellar et al., 2004). Moreover, MIC_{90} for APP was determined following CLSI (CLSI M31-A2. 2002) recommendations (Valle et al., 2006). The MB tonsil concentration: MIC_{90} ratio calculated did not correspond to the Cmax/MIC ratio due to that the sample time chosen was 24 hours after the intramuscular administration and the Tmax described for pigs after intramuscular administration is 0.8 hours (Anonym, 1997). This ratio of tissue concentrations versus MIC_{90} values is one of the PK/PD efficacy parameters described for fluoroquinolones (Sarasola et al., 2002).

STATISTICAL ANALYSIS

A non-parametric test (Mann-Whitney) was used to compare the marbofloxacin concentration achieved in serum and tonsils between the P1 and P2 groups. The SPSS 15.0 software was used (SPSS Inc., Chicago, IL, USA) to carry out this statistical analysis and the level of significance (α) was set to $P < 0.05$.

3. RESULTS

The results of the quantification of marbofloxacin in serum and tonsils are showed in the following table:

Control Group			Group P1			Group P2		
Animal	**Serum (µg/mL)**	**Tonsils (µg/g)**	**Animal**	**Serum (µg/mL)**	**Tonsils (µg/g)**	**Animal**	**Serum (µg/mL)**	**Tonsils (µg/g)**
1	ND	ND	**11**	0.1988	0.5872	**21**	0.3394	1.020
2	ND	ND	**12**	0.1745	0.5399	**22**	0.1612	0.4693
3	ND	ND	**13**	0.1797	0.5599	**23**	0.1876	0.6751
4	ND	ND	**14**	0.1702	0.5436	**24**	0.3144	0.9843
5	ND	ND	**15**	0.1611	0.5691	**25**	0.1366	0.4270
6	ND	ND	**16**	0.1235	0.3374	**26**	0.2533	0.7178
7	ND	ND	**17**	0.2080	0.6353	**27**	0.2906	0.7965
8	ND	ND	**18**	0.1752	0.5212	**28**	0.2369	0.6745
9	ND	ND	**19**	0.1230	0.3576	**29**	0.2285	0.6179
10	ND	ND	**20**	0.1499	0.3973	**30**	0.2285	0.6609

Table 4. Results of quantification of marbofloxacin in serum and tonsils (ND: Under the limit of detection).

Average marbofloxacin serum concentration was 0.16 and 0.24 µg/mL. 24 hours after administering the last Marbocyl® 2% dose for the P1 and P2 group, respectively. Moreover, average marbofloxacin tonsil concentration was 0.50 and 0.70 µg/gr for the P1 and P2 group, respectively (figure 5). Marbofloxacin concentration were significantly higher for the P2 group than for the P1 group in plasma ($p=0.01$) and tonsils ($p=0.009$). Thus, serum and tonsil tissue concentrations

increased in a dose-dependent fashion but the tonsil MB versus serum MB concentration ratio was close to 3 independent of the dose administered to the animals. The MB tonsil concentration: APP MIC_{90} ratio was 16.6 and 23.3 for P1 and P2 group, respectively.

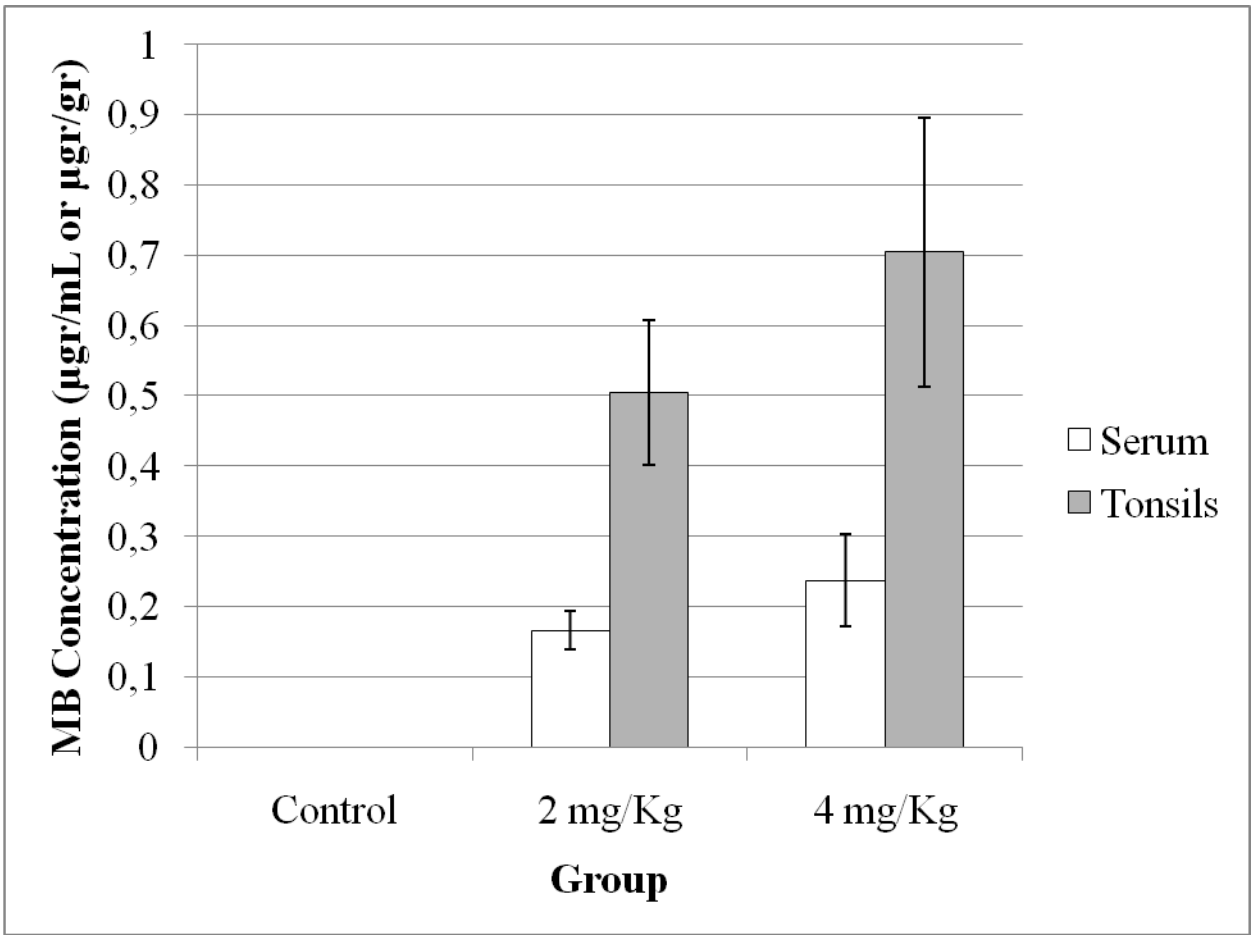


Figure 5. Mean (± standard deviation) concentration (µg/mL or µg/gr. respectively) of marbofloxacin (MB) in serum (white bars) and tonsil (grey bars) 24 hours after the last intramuscular application of marbofloxacin at 2 and 4 mg of MB/Kg administered three times (every 24 hours) and two times (every 48 hours) respectively in 10 pigs for experimental group.

4. DISCUSSION

The main goal of this study was to quantify the penetration of marbofloxacin in pig tonsils. It would have been ideal to define its pharmacokinetic tonsil profile using, at least, five sample times as it has been described for moxifloxacin in humans (Esposito et al., 2006). However, the quantification of this antibiotic in tonsil require to use the complete tonsil and, consequently, to sacrifice the animals. Thus, it was decided to use a representative number of animals (10) in one single sample time to minimize the number of animals used for welfare reasons. The sample time (24 hours after intramuscular administration) was chosen to allow the distribution equilibrium even whether the tonsil would behave as a deep tissue for antibiotic penetration in pigs.

The presented data indicates that MB achieves a good penetration into tonsillar tissue, which is comparable with tonsil/plasma ratios reported for other fluoroquinolones such as the ratio of 1.5–1.9 for ciprofloxacin, from 1 to 8 h after oral or intravenous doses of 200–500 mg; 2.02–2.08 for levofloxacin, from 1 to 9 h after single oral doses of 100 or 200 mg; and 1.4 for ofloxacin, 2 h after a single administration of an oral 200 mg dose (Fish and Chow, 1997; Falser et al.,1988). Tonsil/plasma ratio observed for MB was also very similar to that of moxifloxacin in humans as described by Esposito et al. (2006). MB tissue/plasma ratio, for other pig tissues at steady state (4 hours after a intramuscular dose of 2 mg of MB/body weight), such as the lung (1.8), liver (1.9), kidney (3.8), muscle (1.7) and skin (1), is equal or lower than the value observed for pig tonsils (Anonymus, 1997) clearly showing that tonsil did show a similar distribution pattern compared to most studied pig tissues.

In all the available bibliography, the tonsil concentration was measured taking into account the whole tissue as it was carried out in this study. Obviously, it would have been necessary to measure the concentration of unbound drug (marbofloxacin in this case) in the tonsil crypts because the determination of drug concentration derived from tissue homogenates could lead to an incorrect

conclusion, overestimating or underestimating, the amount of unbound drug in the site of action (Mouton et al., 2008). Unfortunately, it is almost impossible to describe the pharmacokinetics of this molecule in this target tissue. Thus, the only practical approach was analyzing marbofloxacin in the tonsil as a whole. On the other hand, this molecule is an organic acid with good tissue penetration and its volume of distribution exceeds the body water volume and the degree of binding to plasma proteins is low (Sidhu et al., 2009). All in all, it could be reasonably assumed that the concentration at tonsil crypts could be very close to the concentration present in the whole tonsil.

In PK/PD relationships for fluoroquinolones, the C_{max}:*MIC* ratio has been shown to have particular utility in determining optimal activity against Gram negative microorganisms (Drusano et al., 1993; Sarasola et al., 2002). A C_{max}:*MIC* ratio of >8 and an AUC_{0-24}:*MIC* ratio of >100 have been recommended to prevent resistance selection (Dudley, 1991; Thomas et al., 2001). The MB tonsil:MIC ratio described is above the threshold value (10) that is associated with clinical efficacy for all the doses studied (Drusano et al,. 1993; Sidhu et al,. 2009). According to the results it could be theoretically possible to eliminate *Actinobacillus pleuroneumoniae* from pig tonsils. Obviously, these threshold values have been established taking into account PK/PD parameters calculated from serum concentration values and pharmacodynamic parameters estimated using *in vitro* systems in different murine and veterinary models focus on different clinical end-points (for example pneumonia resolution). Moreover, it is assumed that the MIC90 value used to estimate PK/PD parameters is close to the MIC value that really has *Actinobacillus pleuropneumoniae* when it is located in the tonsil crypts of carrier animals. All in all, it can be discussed that the PK/PD parameters calculated are highly speculative and that there is no well-sustained information to be used as a reference. Authors might agree with this observation but it must be highlighted that there is neither specific PK/PD parameters to be used at tonsil level nor additional information in the public domain about pharmacodynamic parameters of this bacteria taking into account different biological forms. ThusIn conclusion, the PK/PD values described in this research paper must be

considered as the most reasonable approach at this stage. In any case, the information provided here should be confirmed by studies whose main goal should be focused on the bacteriological detection of APP in the tonsils of animals either by bacteriological isolation or by PCR confirmation.

FUTURE WORK

To our knowledge, this is the first study that describes the penetration of a fluoroquinolone in the pig tonsils. According to the results it could be theoretically possible to eliminate *Actinobacillus pleuroneumoniae* from pig tonsils. Some field studies (Miquel Colell, personal communication) have shown that the application of marbofloxacin (4 mg/Kg) every 48 hours two times in the whole farm (sows and piglets) reduces the mortality and the symptoms related to APP during the fattening period in farms suffering APP outbreaks during an unknown period of time (months or years). However, the disease could appear again in the fattening after this period of time after applying a marbofloxacin treatment as it has been observed by other authors (Marcelo Gottschalk, personal communication). More studies are required to confirm whether this decrease in the mortality would be the consequence of theoretical eradication APP or due to the reduction of the bacterial load in tonsil that could modify the transmission pattern of this bacterium at population level.

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ANNEX 1

I. SOLVENTS AND REAGENTS

1.1 Commercially available solvents and reagents

Unless specified for each particular product. all solvents and reagents were purchased from Sigma –Aldrich, France.

- Acetonitrile for HPLC
- Acetic acid 100% (CH_3COOH), for analysis
- Orthophosphoric acid 85% (H_3PO_4), for analysis
- Chloroform for analysis
- Potassium dihydrogen phosphate (KH_2PO_4) for analysis
- Di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) for analysis
- Di-sodium hydrogen phosphate (Na_2HPO_4. $2H_2O$) for analysis
- Methanol for HPLC
- Tetrabutylammonium hydrogen sulfate (TBA) for analysis
- Triethylamine (TEA) for analysis
- Water, ultra-pure.,Milli-Q Millipore, Billerica, MA, USA.

1.2 Reference substances

- Marbofloxacin, Vetoquinol R+D Department, Lure, France.
- Ofloxacin.

1.3 Prepared solution

1.3.1 Buffer solution pH 7.0

3.538 g of KH_2PO_4 and 7.3 g of Na_2HPO_4 ($2H_2O$) were dissolved in about 900 mL of ultra-pure water. The volume of the solution was adjusted to 1000 mL with ultra-pure water in a volumetric flask. The pH was checked.

1.3.2 Buffer solution pH 2.7

4 g of $(NH_4)_2HPO_4$ and 4 g of TBA were dissolved in about 900 mL of ultra-pure water. The pH is

adjusted to 2.7 with H_3PO_4. The volume of the solution was adjusted to 1000 mL with ultra-pure water in a volumetric flask. The pH was checked daily before use.

1.3.3 Mobile phases

- Mobile phase A

865 mL of buffer solution pH 2.7. 100 mL of methanol. 25 mL of acetonitrile. 10 mL of acetic acid and 2.8 mL of TEA were mixed and then degassed by vacuum filtration and ultrasonic bath.

-Mobile phase B

About 500 mL of methanol were degassed by vacuum filtration and ultrasonic bath.

1.3.4 Buffer solution pH 9.3

50 mL of NaOH at 0.1N were measured in a 100 mL volumetric flask. The volume of the solution was adjusted to 100 mL with ultra-pure water. The pH was adjusted to 9.3 with heated boric acid.

1.3.5 Enzymatic solution

The volume prepared depends on the number of samples.

10 mg of protease were dissolved with 10 mL of buffer solution pH 9.3

1.3.6 Marbofloxacin standard solutions

Solutions were prepared in volumetric inactinic glass flasks.

- ***Solution A1:*** accurately weighed 10 mg of marbofloxacin were dissolved in about 80 mL of buffer solution pH 7.0 contained in a 100 mL volumetric inactinic glass flask. The volume was completed to 100 mL with buffer solution pH 7.0.

Obtained concentration: 100 µg/mL

- ***Solution A2:*** accurately measured 5 mL of solution A1 in a 50 mL volumetric inactinic glass flask. The volume was completed to 50 mL with buffer solution pH 7.0.

Obtained concentration: 10 µg/mL

- ***Solution A3:*** accurately measured 5 mL of solution A2 in a 50 mL volumetric inactinic glass flask. The volume was completed to 50 mL with buffer solution pH 7.0.

Obtained concentration: 1 µg/mL

- ***Solution A4:*** accurately measured 5 mL of solution A3 in a 50 mL volumetric inactinic glass flask. The volume was completed to 50 mL with buffer solution pH 7.0.

Obtained concentration: 0.1 µg/mL

1.3.7 Internal standard solutions

Solutions were prepared in volumetric inactinic glass flasks.

- ***Solution ISm****:* accurately weighed 10 mg of ofloxacin were dissolved in about 80 mL of buffer solution pH 7.0 contained in a 100 mL volumetric inactinic glass flask. The volume was completed to 100 mL with buffer solution pH 7.0.

Obtained concentration: 100 µg/mL

- ***Solution IS1****:* accurately measured 5 mL of solution ISm in a 50 mL volumetric inactinic glass flask. The volume was completed to 50 mL with buffer solution pH 7.0.

Obtained concentration: 10 µg/mL

- ***Solution IS2:*** accurately measured 5 mL of solution IS1 in a 50 mL volumetric inactinic glass flask. The volume was completed to 50 mL with buffer solution pH 7.0

Obtained concentration: 1 µg/mL

1.3.8 Standard solution at 1 µg/mL of marbofloxacin and at 0.2 µg/mL of internal standard in mobile phase

Accurately measured 5 mL of solution A2 (10 µg/mL) and 10 mL of solution IS2 (1 µg/mL) were mixed in a 50 mL volumetric inactinic glass flask. The volume was completed to 50 mL with mobile phase A.

1.4 Stability of the standard solutions

The standard solutions of marbofloxacin at 100 µg/mL and at 0.1 µg/mL and of ofloxacin at 1µg/mL were stable for 6 hours when they were kept at ambient temperature.

The standard solutions of marbofloxacin at 100 µg/mL and the standard solution of ofloxacin at 1µg/mL were stable for 6 weeks when they were stored at about +4°C

Consequently, the standard solutions of ofloxacin at 100 and 10 µg/mL could be considered as stable for 6 weeks when they were stored at about +4°C.

The standard solution of marbofloxacin at 0.1 µg/mL was stable for 4 weeks when it was stored at about +4°C.

Consequently, the standard solutions of marbofloxacin at 10 and 1 µg/mL could be considered as stable for 4 weeks when they were stored at about +4°C.

II. EQUIPMENT

The small equipment and instrumentation types and trademarks are detailed below:

2.1 Glassware and small equipment

2.1.1 Glassware

- Volumetric glass flasks, Class A, 1000 mL
- Volumetric inactinic glass flasks, Class A, 50 and 100 mL
- Volumetric pipettes. Class A, 5, 10 mL
- Graduated pipettes, 5, 10 mL
- Graduated cylinders, 25, 100 and 1000 mL
- Round-bottomed inactinic glass tubes, 25 mL
- Conical-bottomed inactinic glass tubes, 10 mL
- Autosampler vials in inactinic glass

2.1.2 Small equipment

- Pipettes, Gilson, P200 and P1000
- Pipette, Eppendorf 10-100 µL
- Pipette, Biohit, Proline 5-50 µL
- Pipettes, Biohit e-pet, 50-1200 µL
- Pipettes, Biohit e-pet, 200-5000 µL

- Ultrasonic bath
- Vortex mixer
- Hot-block evaporator
- Filters, Millipore HV, 0.45 µm
- pH paper 5.0 – 10.0
- Hot-bloc magnetic agitator

2.2 Instrumentation

- Reciprocating agitator
- Milli Q, Millipore for ultra pure water
- Scale, Mettler, Hospitalet de Llobregat, Spain
- Refrigerated centrifuge
- Freezer (-75°C)
- pH-meter
- Vacuum pump, Millipore, Billerica, MA, USA and Edwards, Tewksbury, MA, USA
- Fridge, Liebherr
- Solvent delivery pump, Waters, Milford, MA, USA
- Refrigerated autosampler, Waters, Milford, MA, USA
- Column oven, Waters, Milford, MA, USA
- Temperature Controle Module, Waters, Milford, MA, USA
- Fluorimetric detector, Waters, Milford, MA, USA
- Data acquisition software, Waters, Milford, MA, USA

III. CHROMATOGRAPHIC CONDITIONS

- *Chromatographic mode:* reversed-phase chromatography with C18 column
- *Guard and analytical columns:*

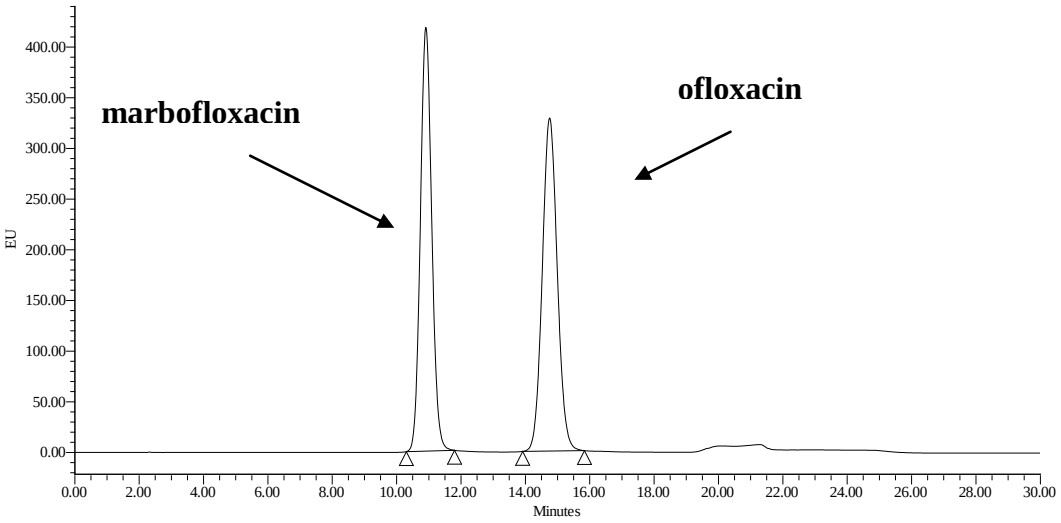
  Guard: Merck lichrospher100 RP18e, 4x4 mm, 5 µm.

  Analytical : Merck lichrospher100 RP18e (250x4) mm, 5 µm
- *Column temperature*: 35 °C

- *Elution : gradient*
- *Mobile phase :*

Time (min)	*Mobile phase A (%)*	*Mobile phase B(%)*
0	*100*	*0*
16	*100*	*0*
17	*75*	*25*
22	*75*	*25*
23	*100*	*0*
30	*100*	*0*

- *Flow rate* : 1 mL/min
- *Injection volume:* 50 µL except for calibration point at 5.0µg/g: 20µL.
- *Autosampler temperature*: About 10 °C
- *Detection*: fluorimetric Wavelengths: $\lambda_{excitation}$: 295 nm for excitation
  $\lambda_{emission}$: 500 nm for emission
- *Retention time :* about 9 to 12 min. for marbofloxacin
  about 13 à 15 min. for ofloxacin
- *Run time :* 30 minutes
- *Weighting factor:* $1/C^2$ (Calculation performed with the heights of the peaks)



Echantillon : Std à 1µg/mL marbo+0.2µg/mL oflo; / Tube : 1; / injection : 1; / Volume inj : 50.00; / Dilution 1.00; / Méthode acquisition : marbo_mset; / Date acquisition : 09/04/2009 14:05:59 CEST; / Date Retraitement : 10/04/2009 08:22:48 CEST

Chromatogramm of a standard solution at 1 µg/mL in mobile phase

IV. CALIBRATION SAMPLES

All the calibration samples from 0 to 5.0 µg/g were processed as described in the tonsil marbofloxacin extraction. 50 µL of solution IS2 were added to each calibration point sample, except for the blank porcine tonsil (ge0).