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Pharmacokinetics and milk penetration of difloxacin after intravenous, subcutaneous and intramuscular administration to lactating goats

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The single-dose disposition kinetics of difloxacin were determined in clinically normal lactating goats ($n = 6$) after intravenous (i.v.), subcutaneous (s.c.) and intramuscular (i.m.) administration of 5 mg/kg. Difloxacin concentrations were determined by high performance liquid chromatography with fluorescence detection. The concentration–time data were analysed by compartmental and noncompartmental kinetic methods. Steady-state volume of distribution (V_{ss}) and total body clearance (Cl) of difloxacin after i.v. administration were estimated to be 1.16 ± 0.26 L/kg and 0.32 ± 0.05 L/h·kg respectively. Following s.c. and i.m. administration difloxacin achieved maximum plasma concentrations of 1.33 ± 0.25 and 1.97 ± 0.40 mg/L at 3.37 ± 0.36 and 1.79 ± 1.14 h respectively. The absolute bioavailabilities after s.c. and i.m. routes were 90.16 ± 11.99% and 106.79 ± 13.95% respectively. Difloxacin penetration from the blood into the milk was extensive and rapid, and the drug was detected for 36 h after i.v. and s.c. dosing, and for 72 h after i.m. administration.

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INTRODUCTION

Difloxacin is a fluoroquinolone antibacterial drug developed specifically for use in veterinary medicine. It has a high *in vitro* activity against a wide range of gram-positive and gram-negative aerobes and anaerobes (Granneman *et al.*, 1986), including most species and strains of *Klebsiella* spp., *Staphylococcus* spp., *Escherichia coli*, *Enterobacter*, *Campylobacter*, *Shigella*, *Proteus*, *Pasteurella* spp., *Mycoplasma* spp., *Rickettsia* spp. and *Chlamydia* (Stamm *et al.*, 1986; Abd El-Aty *et al.*, 2005). Fluoroquinolones exhibit bactericidal action by targeting the bacterial DNA topoisomerases II (gyrase) and IV (Wolfson & Hooper, 1989; Drlica & Zhao, 1997). Principal advantages of fluoroquinolones include good bioavailability, bactericidal activity at low tissue concentrations and good penetration into phagocytic cells (Giguere *et al.*, 1996). They have a large volume of distribution combined with low plasma protein binding, which allows them to reach tissue concentrations often higher than concurrent serum concentrations (Prescott & Baggot, 1993).

It is generally accepted that xenobiotics cross the blood–milk barrier in the udder by nonionic passive diffusion. The extent to which a drug has access into milk when given systemically depends on its main pharmacokinetic properties: lipid solubility, degree of ionization and extent of binding to serum and udder proteins (Ziv, 1980). Moreover difloxacin could be a substrate of efflux proteins, as protein ABCG2. Identification of this protein as a major factor in xenotoxin transfer to milk has immediate relevance for the use of drugs that are applied in dairy animals, so this transport could play a role in the pharmacokinetics of difloxacin (Jonker *et al.*, 2005).

The milk to serum concentration ratio for drugs is often unknown. Furthermore, knowledge of the pharmacokinetics and residues of antimicrobial agents in goats is still very limited. The pharmacokinetic properties of difloxacin have been evaluated in several species as nonlactating goats (Atef *et al.*, 2002), rabbits (Abd El-Aty *et al.*, 2005) and horses (Fernández-Varón *et al.*, 2005), but not yet in lactating goats.

Thus, to assess the efficacy of difloxacin in mammary gland infection in goats, we have measured the pharmacokinetic parameters relevant to its antibacterial activity in the milk and plasma of goats.

MATERIALS AND METHODS

Animals

Six clinically healthy Murciano–Granadina female goats weighing 50.2–63.4 kg and aged 5–6 years were obtained from the caprine farm of the University of Murcia. The animals were housed and fed an antibiotic-free diet for at least 30 days preceding the study. For each treatment period of the crossover, they were observed daily for general health, and clinical observations were made prior to injection and at 2, 10 and 24 h postinjection. Alfalfa hay and water was provided *ad libitum* together with a drug-free concentrate.

The study was approved by the Bioethics Committee of the University of Murcia.

Experimental design

A crossover design ($2 \times 2 \times 2$) was used in three phases. Each animal received single intravenous (i.v.), subcutaneous (s.c.) and intramuscular (i.m.) injections of 5% difloxacin (Dicural®, Fort Dodge Veterinaria, Madrid, Spain, SA) at a dose of 5 mg/kg with at least a 15-day washout period.

For the i.v. administration, the solution was injected into the left jugular vein and blood samples (4 mL) were collected from the contralateral jugular vein into heparinized tubes. Subcutaneous injections were administered under the skin of the back at a single location in the thoraco-lumbar region lateral of the midline and i.m. injections into the semimembranous muscle. Blood samples were collected at 0 (pretreatment), 0.083, 0.167, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h postdosing. Samples were centrifuged at 1500 ***g*** for 15 min and the plasma taken and stored at −45 °C until assay.

Milk samples were collected before and at 1, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h after complete evacuation of the udder.

Analytical method

Plasma and milk concentrations of difloxacin were measured using a modified high-performance liquid chromatography (HPLC) method previously reported (Siefert *et al.*, 1999). The HPLC system was equipped with a model LC-10ASvp pump, a RF-10AXL Fluorescence Detector and a model SIL-10ADvp autoinjector (Shimadzu, Kyoto, Japan). The above-mentioned system was connected to a computer with a Shimadzu Class-VP™ Chromatography Data System programme (Shimadzu, DC, USA).

Difloxacin pure substance (Fort-Dodge, Madrid, Spain) was used for quality controls. Ciprofloxacin (Vita Pharma, Madrid, Spain) was used as internal standard. After addition of 10 μL of the internal standard to 200 μL of plasma or milk, 200 μL acetonitrile was added. Plasma and milk proteins were precipitated by shaking in an ultrasonic bath followed by centrifugation for 10 min at 1600 ***g***. The supernatant was diluted fourfold with 0.067 M disodium hydrogen phosphate buffer pH, 7.5 and transferred to HPLC autosampler vials. The HPLC separation was performed using a reverse-phase Discovery C_{18} column (250×4 mm; 5 μm particle size; Supelco, Bellefonte, Philadelphia, PA, USA) with an injection volume of 60 μL. Autosampler vials and column temperature was set at 5 °C. The mobile phase consisted of acetonitrile (20%) and tetrabutylammonium hydrogensulphate solution (5 g/L) (80%) using an isocratic method with a flow rate of 1.0 mL/min. Difloxacin eluted at approximately 6.4 min. The fluorescence detection was performed at an excitation wavelength of 280 nm and an emission wavelength of 445 nm.

Method validation

Quality controls were prepared from a pool of blank goat plasma or milk spiked with nine concentrations of difloxacin between 5 and 2000 μg/L. Plasma and milk aliquots were stored at −45 °C until assay. Aliquots of quality controls were extracted as above and 60 μL was injected into the chromatographic system. Standard curves were obtained by unweighed linear regression of difloxacin peak areas vs. known concentrations. Each point was established from an average of five determinations. Correlations coefficients (r) were >0.99% for calibration curves. The percentage recovery was determined by comparing the peak areas of plasma and milk blank samples spiked with different amounts of drug and treated as any samples, with the peak areas of the same standards prepared in phosphate buffer. Each point was established from an average of five determinations. The mean percentage recoveries of difloxacin from plasma and milk were 97.35 ± 2.51% and 92.95 ± 4.68% respectively. The assay precision (RSD) was assessed by expressing the standard deviation of repeated measurements as a percentage of the mean value. Intra-day precision was estimated from six replicates of three standard samples (plasma or milk) used for calibration curves (RSD < 4.9%). Inter-day precision was estimated from the analysis of standard samples (plasma or milk) on three separate days (RSD < 5.2%). The limit of quantification was 5 μg/L for plasma and milk.

Pharmacokinetic analysis

Plasma

The concentration–time data obtained after each treatment in each individual animal were initially fitted to one, two, three and four exponential equations by the retroprojection method (Gibaldi & Perrier, 1982). The PKCALC computer program (Shumaker, 1986) was used to obtain the best estimates of the parameters of these equations. The final curve fitting was carried out using nonlinear regression with the MULTI computer program and the Gauss-Newton damping algorithm (Yamaoka *et al.*, 1981). Akaike's Information Criterion (Yamaoka *et al.*, 1978) was used to determine the number of compartments used in the pharmacokinetic analysis and the most appropriate weighting for the data. The data points were weighted with the inverse of the squared fitted value. Pharmacokinetic parameters were obtained from the individual fitted equations (Gibaldi & Perrier, 1982). The absorption and disposition half-

lives were calculated as $t_{(1/2)ka} = \ln 2/k_a$, $t_{(1/2)\lambda 1} = \ln 2/\lambda_1$ and $t_{(1/2)\lambda z} = \ln 2/\lambda_z$ respectively.

A noncompartmental model was used to determine the area under the concentration–time curve (*AUC*), area under the first moment curve (*AUMC*), using the linear trapezoidal rule with extrapolation to time infinity. Mean residence time was calculated as $MRT = AUMC/AUC$. Mean absorption times were calculated as $MAT = MRT_{\text{s.c., i.m.}} - MRT_{\text{i.v.}}$. The systemic clearance was estimated as $Cl = \text{Dose}/AUC$. The apparent volumes of distribution at steady-state were calculated as $V_{ss} = (\text{Dose} \times AUMC)/AUC^2$.

Bioavailability (F) was calculated by the method of corresponding areas:

$$F\ (\%) = \frac{AUC_{\text{s.c.,i.m.}}}{AUC_{\text{i.v.}}} \times 100$$

Milk

The milk concentration–time data were analysed by noncompartmental methods using WinNonlin Professional program (version 5.1; Pharsight Corporation, Mountain View, CA, USA). Drug concentration at each sampling time interval and the volume of milk at each time interval were used to calculate the cumulative amount of difloxacin eliminated in milk, recovery (%) and terminal half-life. AUC_{milk} was calculated using the linear trapezoidal rule with extrapolation to infinity.

Statistical analysis

Descriptive statistical parameters as mean, standard deviation and coefficient of variation were calculated. Harmonic means were calculated for the half-lives of elimination and absorption. The Wilcoxon Rank Sum test and Student's *t*-test were used to test parameters for significant differences between i.v., s.c. and i.m. administration (Powers, 1990). The statistical software used was SPSS (Version 11.0; SPSS Inc, Chicago, IL, USA).

RESULTS

All animals remained in good health throughout the acclimatization and study periods. The drug was distributed according to an open two-compartment model after i.v., s.c. and i.m. administration. The mean (±SD) plasma and milk concentrations of difloxacin following i.v., s.c. and i.m. administration are plotted in Figs 1 & 2 respectively. The mean (±SD) pharmacokinetic parameters based on compartmental and noncompartmental pharmacokinetic analysis are presented in Table 1.

Milk production per day was (mean ± SD) 1.68 ± 0.51 L.

DISCUSSION

After i.v. administration, the half-life ($t_{(1/2)\lambda z} = 4.92$ h) in the present study was shorter than that reported in nonlactating goats after i.v. dosing of difloxacin ($t_{(1/2)\lambda z} = 6.3$ h; Atef *et al.*, 2002), and plasma clearance ($Cl = 0.32 \pm 0.05$ L/h·kg) was higher than the data reported by the same author. These differences may be due possible because there are changes in drug disposition between lactating and nonlactating animals (Petracca *et al.*, 1993).

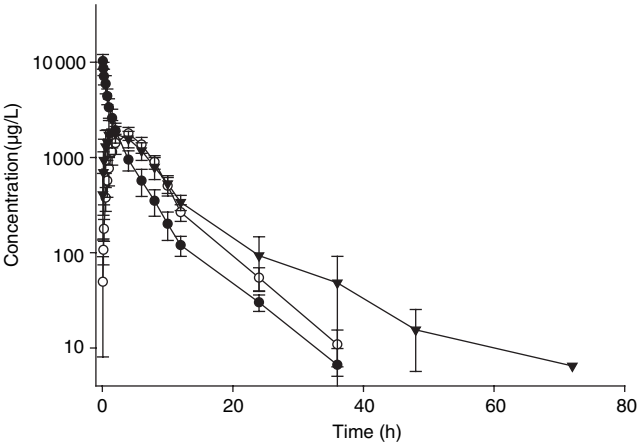

Fig. 1. Semilogarithmic plot of the plasma concentrations (mean ± SD) of difloxacin following a single intravenous (-●-), subcutaneous (-○-) and intramuscular (--) dose of 5 mg/kg bodyweight ($n = 6$).

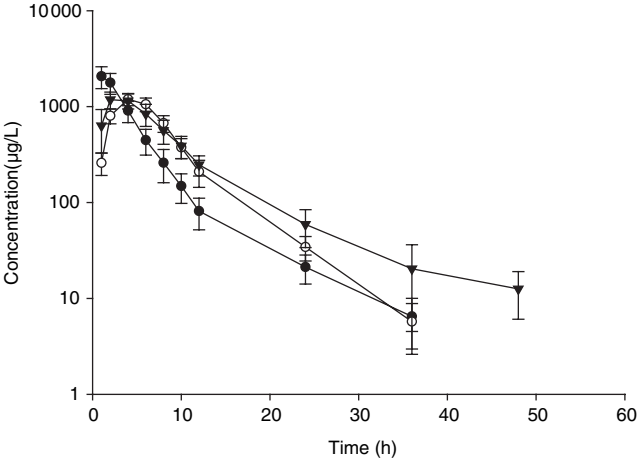

Fig. 2. Semilogarithmic plot of the milk concentrations (mean ± SD) of difloxacin following a single intravenous (-●-), subcutaneous (-○-) and intramuscular (--) dose of 5 mg/kg bodyweight ($n = 6$).

The volume of distribution at steady-state (V_{ss}) is a clearance independent volume of distribution that is used to calculate the drug amount in the body under equilibrium conditions (Toutain & Bousquet-Mélou, 2004a). Difloxacin was widely distributed in tissues as could be expected for fluoroquinolones antibiotics and as has been found in other species (Atef *et al.*, 2002; Abd El-Aty *et al.*, 2005; Fernández-Varón *et al.*, 2005).

Elimination half-lives ($t_{(1/2)\lambda z}$) after s.c. and i.m. dosing were 7.33 and 10.44 h respectively. These values are close to those reported in pigs and horses for difloxacin with an elimination half-life after i.m. dosing of 7.92 (Inui *et al.*, 1998) and 5.72 h

Table 1. Pharmacokinetic parameters (mean ± SD) of difloxacin in goats after intravenous (i.v.), subcutaneous (s.c.) and intramuscular (i.m.) administration at a dose of 5 mg/kg bodyweight ($n = 6$)

Parameters	Units	i.v.	s.c.	i.m.
Plasma				
K_a	Per h	–	0.32 ± 0.04	1.91 ± 1.42
λ_1	Per h	0.92 ± 0.18	0.32 ± 0.04*	0.29 ± 0.08*
λ_z	Per h	0.14 ± 0.02	0.09 ± 0.02*	0.07 ± 0.01*
$t_{(1/2)ka}$	h	–	2.19	0.36
$t_{(1/2)\lambda 1}$	h	0.76	2.19*	2.38*
$t_{(1/2)\lambda z}$	h	4.92	7.33*	10.44*
V_{ss}	L/kg	1.16 ± 0.26	–	–
AUC	mg·h/L	15.84 ± 2.61	14.17 ± 2.32**	16.70 ± 1.89
AUMC	mg·h^2/L	56.70 ± 12.22	102.46 ± 16.04*,**	149.31 ± 36.93*
MRT	h	3.59 ± 0.55	7.26 ± 0.61*	8.92 ± 1.90*
Cl	L/h·kg	0.32 ± 0.05	–	–
MAT	h	–	3.67 ± 0.95	5.33 ± 2.36
C_{max}	mg/L	–	1.33 ± 0.25**	1.97 ± 0.40
T_{max}	h	–	3.37 ± 0.36	1.79 ± 1.14
F (%)	–	–	90.16 ± 11.99**	106.79 ± 13.95
Milk				
C_{max}	mg/L	2.12 ± 0.50	1.20 ± 0.17*	1.28 ± 0.19*
T_{max}	h	1.17 ± 0.41	4.00 ± 0.00*	2.67 ± 1.03*
Recovery (%)	–	0.46 ± 0.16	0.35 ± 0.15*,**	0.39 ± 0.14*
$t_{(1/2)\lambda z}$	h	5.62	4.50	6.38
AUC_{milk}	mg·h/L	10.42 ± 2.19	9.58 ± 1.58	10.75 ± 1.65
$C_{max\text{-}milk}/C_{max\text{-}plasma}$	–	–	0.90 ± 0.07	0.67 ± 0.14
AUC_{milk}/AUC_{plasma}	–	–	0.68 ± 0.03	0.65 ± 0.09

Plasma: $t_{(1/2)\lambda 1}$, the disposition half-life associated with the initial slope (λ_1) of a semilogarithmic concentration–time curve (harmonic mean); $t_{(1/2)\lambda z}$, the elimination half-life associated with the terminal slope (λ_z) of a semilogarithmic concentration–time curve (harmonic mean); $t_{(1/2)ka}$, absorption half-life (harmonic mean); V_{ss}, the apparent volume of distribution at steady state; *AUC*, the area under the plasma concentration–time curve from zero to infinity; *AUMC*, area under the moment curve; *MRT*, mean residence time; *Cl*, the total body clearance of drug from the plasma; *F*, the fraction of the administered dose systemically available (bioavailability); T_{max}, the time to reach peak or maximum plasma concentration following s.c. and i.m. administration; k_a, absorption rate constant (first order); *MAT*, mean absorption time; C_{max}, the peak or maximum plasma concentration following s.c. and i.m. administration. *Milk*: T_{max}, the time to reach peak or maximum milk concentration following s.c. and i.m. administration; C_{max}, the peak or maximum milk concentration following s.c. and i.m. administration; Recovery (%); $t_{(1/2)\lambda z}$, the elimination half-life associated with the terminal slope (λ_z) of a semilogarithmic concentration–time curve; AUC_{milk}, the area under the milk concentration–time curve from zero to infinity.

*Significantly different from i.v. ($P < 0.05$). **Significantly different from i.m. ($P < 0.05$).

(Fernández-Varón *et al.*, 2005) respectively. The drug was eliminated from plasma after s.c. and i.m. treatment at a significant slower rate than after i.v. treatment, suggesting the presence of a 'flip-flop' effect at least after i.m. administration, because *MAT* was greater than $MRT_{i.v.}$ (Toutain & Bousquet-Mélou, 2004b). In that model, the last phase of the curve is determined by the absorption rate constant and not by the apparent elimination constant, because the rate of absorption is a limiting factor for the elimination process.

The pharmacokinetics of difloxacin after i.m. administration at 5 mg/kg bodyweight has been studied in horses (Fernández-Varón *et al.*, 2005) and nonlactating goats (Atef *et al.*, 2002). The C_{max} (1.97 ± 0.40 mg/L) and t_{max} (1.79 ± 1.14 h) values in the present study were intermediate between those reported in horses (1.48 mg/L and 2.79 h respectively) and nonlactating goats (4.1 mg/L and 1.00 h respectively). These data indicate similar maximum exposure and time to reach the maximum exposure to the antibiotic in these species (Toutain & Bousquet-Mélou, 2004b).

High values of systemic availability have been obtained after extravascular administration of difloxacin to goats. Similar high values have been obtained for this drug in other species (Atef *et al.*, 2002; Abd El-Aty *et al.*, 2005; Fernández-Varón *et al.*, 2005) and for other fluoroquinolones in lactating goats (Shem-Tov *et al.*, 1997; Fernández-Varón *et al.*, 2006).

The various solute transport and secretion processes involved in milk production offer pathways for the movement of the drugs molecules from plasma to milk (McManaman & Neville, 2003). For the movement of drugs from plasma to milk, however, passive diffusion appears to be the most likely route of transport.

Most drugs are either weak organic acids or bases and exist in solution (in plasma or milk) in both ionized and un-ionized forms. The proportion of the drug that is in the un-ionized state is

dependent of the pKa value of the drug and the pH of the environment, with the pKa of the drug being the pH at which 50% of the drug molecules are in the ionized state. Provided the drug molecule itself is sufficiently lipophilic, it is the un-ionized form that is able to diffuse across the lipid membranes. Other important factor that affect distribution of drugs into tissues is the extent of reversible binding to proteins in the solvent (plasma or milk) and tissues. This is a nonspecific interaction between drug and proteins that result in the formation of drug–proteins complexes, which are too large to cross tissue membranes.

Difloxacin, like other fluoroquinolones, is amphoteric due to the presence of a carboxylic acid and one or more basic amine functional groups. At a pH 6–8, this compound is sufficiently lipid-soluble to be able to penetrate tissues (Brown, 1996). So the extensive penetration of difloxacin from the blood to goat's milk was predictable on the basis of the ion trap mechanism (Atkinson & Begg, 1990). Other factor, as well, are the low binding of difloxacin to goat plasma proteins (Atef *et al.*, 2002) and the lipophilic nature of the molecule. It is well known that specialized drug transporters are also involved in drug passage from plasma into the milk (Jonker *et al.*, 2005).

The antibacterial activity of the fluoroquinolones is dependent on the drug concentration and the *MIC* of the micro-organisms (Walker, 2000). Fluoroquinolones exhibit concentration-dependent killing, so the peak milk concentration is a very important parameter. In this study, the milk C_{max} was higher following i.v., s.c. and i.m. dosing ($C_{max} = 2.12$, 1.20 and 1.28 mg/L respectively) than that reported for pefloxacin in lactating goats (Dose, 10 mg/kg; Abd El-Aty & Goudah, 2002) and for danofloxacin in ewes (Dose, 1.25 mg/kg; Shem-Tov *et al.*, 1997).The half-lives obtained in milk after i.v., s.c. and i.m. administrations were longer than that reported for moxifloxacin (Fernández-Varón *et al.*, 2006) and danofloxacin (Shem-Tov *et al.*, 1997, 1998) in lactating animals. Both parameters indicated, in principle, a favourable profile of difloxacin in the milk of the goats in this study.

Antimicrobial drugs which act predominantly by concentration-dependent mechanisms generally exert significant postantibiotic sub-*MIC* effects. Such drugs continue to inhibit bacterial growth for a period of hours after they have been completely removed from the system. Work in disease models has established that optimal outcome of therapy with this type of bactericide requires attainment of high concentrations and the success of therapy correlates with the *AUC*/*MIC* ratio, while prevention of the development of resistance correlates with the C_{max}/MIC ratio (Shojaee Aliabadi & Lees, 2000).

Consequently, the ratios C_{max}/MIC_{90} and AUC_{24}/MIC_{90} are the best parameters for predicting the antimicrobial effect of fluoroquinolones (Lode *et al.*, 1998). Previous investigations have shown that for fluoroquinolones $C_{max}/MIC_{90} > 3$ produced 99% reduction in bacterial count and C_{max}/MIC_{90} of 8 or greater prevented the emergence of resistant organisms (Craig, 1998). Furthermore, $AUC_{24}/MIC_{90} > 100$ h should be achieved to give maximum clinical and bacteriology efficacy (Turnidge, 1999). However, it is necessary to note that the numerical values of C_{max}/MIC_{90} and AUC_{24}/MIC_{90}, used as surrogate markers to predict optimal dosage, have been generated in experimental infections in laboratory animals or in human clinical trials (Toutain & Lees, 2004), and that these numerical values may or may not be applicable to goat infections or in general to animal infections. In fact, Lees and Shojaee Aliabadi (2002) have studied in *ex vivo* experiments the ratio of *AUC*/*MIC* producing bacteriostasis, bactericidal activity and elimination of bacteria with different fluoroquinolones. In all these cases, the surrogate markers for cattle, sheep, goat and camel were <100–125.

MIC data of difloxacin against caprine bacterial isolates have not been reported up to now. So if we take into account *MIC*s of other veterinary fluoroquinolones against sensitive strains of different micro-organisms isolated from field of veterinary importance (Hannan *et al.*, 1997; Watts *et al.*, 1997), and using the surrogate marker $C_{max}/MIC = 8$, difloxacin could be effective by the s.c. and i.m. routes at 5 mg/kg against bacterial isolates with $MIC \leq 0.17$ and $MIC \leq 0.25$ μg/mL, respectively, and against mastitis isolates with $MIC \leq 0.15$ μg/mL. Intravenous route can be used in acute mastitis for susceptible micro-organisms with a $MIC \leq 0.26$ μg/mL.

Using the surrogate marker $AUC_{24}/MIC_{90} = 100$, difloxacin would have success against micro-organisms capable to produce mastitis in goats with $MIC \leq 0.10$ μg/mL after i.v. and i.m. dosing, and $MIC \leq 0.09$ μg/mL after s.c. administration.

Although we have to take into account that mastitis produces physical and chemical changes both in the milk and the mammary gland itself that have the potential to alter the distribution of drugs. Inflammation of the mammary gland leads to vascular permeability changes and differences in milk composition. Milk pH generally increases, milk casein concentrations decrease, milk albumin concentrations increase, somatic cells increase and milk fat levels can decrease. All of these factors have the potential to impact on the pharmacokinetics of drugs; however, their exact importance is not well understood (Gehring & Smith, 2006). For example, the article of Fang and Pyörälä (1996) showed that normal milk reduced the activity of enrofloxacin against *E. coli* strains only by a factor of 2, but maintain similar activity in mastitic milk.

Consequently, difloxacin could be useful in the treatment of systemic severe infections and those affecting the udder in goats after specific assessment of susceptible micro-organisms as it exhibits, after s.c. and i.m. administration, high systemic availability and good distribution to the udder.

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