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Population pharmacokinetics and pharmacokinetic/pharmacodynamic evaluation of marbofloxacin against Coagulase-negative staphylococci, *Staphylococcus aureus* and *Mycoplasma agalactiae* pathogens in goats.

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Abstract

Marbofloxacin is a broad-spectrum fluoroquinolone, and an extra-label use has been reported in horse, sheep and goat. However, extrapolation of dosage regimens from cattle to horse and small ruminants could lead to incorrect dosing due to pharmacokinetic differences among species, increasing the risk of antimicrobial resistance or toxicity. Pharmacokinetic properties of marbofloxacin, including PK/PD analysis, have been studied by intravenous, intramuscular and subcutaneous administration in lactating and non-lactating goats. A population pharmacokinetic model of marbofloxacin in goats was built using 10 pharmacokinetic studies after intravenous, intramuscular, and subcutaneous administration at a dose of 2, 5 and 10 mg/kg. Serum or plasma and milk concentration-time profiles were simultaneously fitted with a non-linear mixed effect model with Monolix software. Level of milk production (lactating and non-lactating) and health status (healthy and un-healthy) were retained as covariates on volume of distribution and clearance. Marbofloxacin concentrations were well described in plasma serum and milk by the population model. Simulated dose regimens of marbofloxacin administered at 2, 5 and 10 mg/kg by intramuscular route for five days were evaluated (n = 5000 per group). Steady-state *f*AUCs for each dose regimen were obtained. Probability of target attainment of *f*AUC/MIC ratios were determined and PK/PDco values (highest MIC for which 90% of individuals can achieve a prior numerical value of the *f*AUC/MIC index) were established using Monte Carlo simulations (n=50000). MIC values for wild type isolates of *Staphylococcus aureus*, coagulase negative staphylococci, and *Mycoplasma agalactiae* were determined and tentative epidemiological cutoff (TECOFF) were obtained at 1.0, 0.5 and 0.5 mg/L, respectively. The PK/PDco for the dose regimen of 2 mg/kg/24h and 5 mg/kg/24h (0.125 and 0.25 mg/L) were lower than TECOFF (0.5 and 1

mg/L). The dosage regimen of 10 mg/kg/24h was adequate for intermediate MIC values of 0.125-0.50 mg/L and could be effective for a population with a target *f*AUC/MIC ratio < 48 for Coagulase negative staphylococci and *Mycoplasma agalactiae*, but not for *Staphylococcus aureus*. Results obtained in this study could be taken as a starting point by committees that set the clinical breakpoints and justifies expert rules to optimize marbofloxacin dose regimens.

keywords

Population pharmacokinetic, marbofloxacin, goats, milk, Monte Carlo simulations.

1. Introduction

Marbofloxacin is a broad-spectrum fluoroquinolone licensed to use in cattle, pigs in Europe and, dogs and cats in Europe and United States. The drug is considered a critically important antimicrobial by the World Health Organization (WHO) and World Organization for Animal Health (OIE) (OIE, 2019; WHO, 2018). In the European Union (EU), marbofloxacin has been incorporated into the B category antibiotics. Therefore, it can be used when no antibiotics of category C & D are clinically effective, and its use should be based on the results of antibiotic susceptibility testing (AST) (EMA, 2020). Moreover, extra-label use of marbofloxacin has been reported in other species like horse, sheep and goat to treat respiratory infections, mastitis or endotoxemia between 2 and 10 mg/kg dose (Bousquet-Mélou et al., 2021; Lorenzutti, et al., 2021a; Coskun et al., 2020). However, extrapolation of dosage regimens from cattle to horse and small ruminants could lead to incorrect dosing due

to pharmacokinetic (PK) and pharmacodynamic (PD) differences among species, increasing the risk of antimicrobial resistance or toxicity (EMA, 2018).

Pharmacokinetic properties of marbofloxacin, including PK/PD analysis, have been studied by intravenous (IV), intramuscular (IM) and subcutaneous (SC) administration in lactating and non-lactating goats (Bhardwaj et al., 2018; Bhardwaj et al., 2019; Dova et al., 2007; Fernández-Varón et al., 2021; Lorenzutti et al., 2017; Lorenzutti et al., 2021a; Sánchez et al., 2019; Sidhu et al., 2010; Waxman et al., 2001; Waxman et al., 2003; Waxman et al., 2004). These studies were conducted with 6-7 animals, dense sampling schedule and PK data were analyzed by conventional compartmental and non-compartmental methods. Consequently, individual parameter estimates were used to calculate population mean and standard deviation (SD) using the two-stage approach. Nevertheless, this analysis presents known disadvantages; inter-individual variability of the PK parameters (IIV) and their correlation are not explained, the effect of covariates that can influence the estimation of the parameters or the identification of subpopulations with different drug disposition is unknown, and finally, the simulations obtained are less robust. In this context, the use of population PK modeling (POP-PK) is a powerful strategy to address these issues (Mould and Upton, 2012).

A POP-PK model comprises a structural model describing the time course of drug concentrations, a stochastic model describing sources of variability such as inter-individual variabilty (IIV) and residual variability, and a covariate model (Mould and Upton, 2013). Finally, a POP-PK model can be used for further simulations with higher sample size, different scenarios, and populations (Bon et al., 2018; Lavielle, 2015; Owen and Fiedler-Kelly, 2014). This is especially pertinent for antimicrobials because the identification of subpopulations with different drug disposition is crucial to conduct PK/PD analysis, which

allows to individualize dose regimens representative of the patient population in which the antimicrobial will be used (de Velde et al., 2018). According to European Medicines Agency recommendations (EMA), POP-PK models should be used for antimicrobial PK/PD analysis, in order to include subpopulations of interest (unhealthy subpopulations whenever possible is recommended) and Monte Carlo simulations (Cómitre et al., 2021; EMA, 2015).

POP-PK models are also used for PK meta-analysis, a retrospective study consists of collecting raw PK data from individual small studies (increasing the number of individuals) to obtain new estimations of population parameters and addressing IIV, including the effects of covariates. This approach is gaining popularity in veterinary pharmacology because it can be used for unbalanced designs, like different sample size, limits of quantification or subpopulations of animals and breeds (Bousquet-Mélou et al., 2021; Paulin et al., 2018; Pelligand et al., 2016; Wang et al., 2019).

POP-PK models are not frequent in veterinary pharmacology, however its use has been increased in recent years due to its ability to analyze complex data sets, with multiple doses and routes, combined with the effect of modeling different covariates of veterinary interest. In the same way, more robust simulations than with the classic two-stage methods can be obtained and more efficient analysis as have been recommended (Mould & Upton. 2013; Wang et al., 2019).

From a PD perspective, clinical breakpoints (CBP) for interpretation of antimicrobial susceptibility tests (AST) necessary to evaluate the marbofloxacin susceptibility in goats are unknown (Serrano-Rodríguez et al., 2017). Furthermore, minimum inhibitory concentration (MIC) threshold values of marbofloxacin used to classify bacterial populations as susceptible (S), intermediate (I) or resistant (R) are 1, 2 and 4 mg/L, respectively (Gajic et a.,2023;

Leclerg et al., 2013). These data were derived from dogs, cats, and cattle studies. Using these MIC criterion, epidemiological cut-off (ECOFF) and PK/PD-cutoff (PK/PDco) values of marbofloxacin calculated for goats could be tentative, like has been recently described in horses following VETCAST recommendations (Bousquet-Mélou et al., 2021; Toutain et al., 2017). PK/PDco is the highest possible MIC for which a pre-defined target PK/PD endpoint is achieved in a determined percentage (typically 90%) of animals in the target population for a given dose regimen. For fluoroquinolones, the PK/PD index is the ratio of area under the free-drug plasma concentration-time curve (AUC) divided by MIC, expressed as *f*AUC/MIC, with *f* representing the free plasma drug concentration (EMA, 2015; Toutain et al., 2021). Typical values for *f*AUC/MIC ratios close to 100–125 for gram-negative and 30–55 for gram-positive pathogens have been described as the most appropriate to predict clinical efficacy (Papich, 2014). However, some authors have indicated that different numerical values that those ratios might be applicable to ruminant species, with ratios lower than 30 or 100 for gram positive and negative pathogens (Fernández-Varón et al., 2021; Paulin et al., 2007). These issues combined with the fact that classic *f*AUC/MIC values have been obtained from clinical trials with humans or laboratory animals suggesting that would not necessarily always be applicable to other animal species such as ruminants, so other *f*AUC/MIC endpoints should be investigated (Aliabadi and Lees, 2002).

There are many pathogens that can cause clinical and subclinical mastitis in goats such as Coagulase-negative staphylococci, *Staphylococcus aureus* or *Mycoplasma agalactiae* (Gautier-Bouchardon, 2018; Serrano-Rodríguez et al., 2017). These diseases have a great impact in countries of the Mediterranean basin such as Spain or France, in others (Fernández-Varón et al., 2021). However, recent studies have shown a high resistance for

these bacteria to antibiotics such as macrolides or beta-lactams, with fluoroquinolones being an alternative to consider (Nelli et al., 2022). Consequently, susceptibility assessment as well as the development of PKPD population models can be a useful tool to determine tentative breakpoints for these pathogens in goats (EMA, 2015).

The current research aims: a) to conduct a PK meta-analysis by a POP-PK model of marbofloxacin in goats using data derived from different individual studies by IV, IM and SC administration in lactating and non-lactating animals, including healthy and non-healthy states; b) to determine the probability of target attainment (PTA) and PK/PDco values by Monte Carlo simulations from different simulated dose regimens of 2 mg/kg/day, 5 mg/kg/day and 10 mg/kg/day; and c) to evaluate the TECOFF values for *Mycoplasma agalactiae*, *Staphylococcus aureus* and coagulase negative staphylococci wild-type isolates from goats with mastitis.

2. Materials and methods

2.1. Raw marbofloxacin concentration-time data and animals

Individual goat PK data were obtained from ten different PK studies of marbofloxacin (a total of 64 individuals and 6-7 animals per study), with a rich sample design. Data used were obtained from healthy female lactating and non-lactating goats following marbofloxacin administration by IV (n=38) and IM (n=19) routes at 2 and 5 mg/kg, and by SC route (n=20) at 2 mg/kg, respectively. On the other hand, data from an endotoxin-induced fever model in non-lactating adult goats (n=7) by IV administration at 2 mg/kg, and data from coagulase-negative staphylococci induced mastitis (n=7) by IM administration of 10

mg/kg were also included. All studies administered a 10% aqueous solution of marbofloxacin (Marbocyl®, Vetoquinol, from laboratories of France, UK and Spain). Relevant data of each study are presented in Table 1.

2.2. *Population pharmacokinetic analysis*

Marbofloxacin plasma/serum and milk concentrations were simultaneously modelled by nonlinear mixed effect models with Monolix 2021R1® suite software (Simulations Plus/Lixoft, Ltd., Lancaster, CA, US).

Different structural models were evaluated (one, two and three compartments) with different absorption rates. Finally, the best model was selected according to the goodness-of-fit plots, the reduced variability, the likelihood ratio tests as −2·log-likelihood (−2 LL), Akaike information criterion (AIC), and the Bayesian information criterion (BIC) (Mould and Upton, 2013; Weisskopf et al., 2020). The final model included two extravascular administrations with first-order absorption rates for IM and SC routes, respectively, three compartments defined as central and peripheral for plasma/serum concentrations and a specific milk compartment for milk concentrations. The parameters estimated were: k_{aIM} (absorption rate constant for IM route), k_{aSC} (absorption rate constant for SC route), F_{IM} (bioavailability for IM route), F_{SC} (bioavailability for SC route), V_c (volume of central compartment), Cl (clearance of the central compartment), Q (inter-compartmental clearance between central and peripheral compartment), V_p (volume of peripheral compartment) Q_{milk} (inter-compartmental clearance between central and milk compartment) and $V_{milking}$ (hypothetical volume of milk compartment). A log-normal distribution was assumed for all parameters of the model, excepting F_{IM} and F_{SC}, which assumed a logit-normal distribution (Wang et al., 2019). However, to assess drug loss in milk in a semi-physiological context,

the volume of the milk compartment was a priori fixed to the mean amount of milk that can be taken between two milkings (Salman et al., 2011). A final volume of 0.16 L was used following the data described by Fernández-Varón et al., 2021. In the same way, an emptying effect was established in the milk compartment in order to include the milking effect of the udder (Woodward and Whittem, 2019). Additionally, final data frame was built taking into account the LLOQ of each study as censored data (Mould and Upton, 2013).

Each parameter obtained was described in the general form as: $\theta_i = \theta_{pop} \cdot e^{\eta\theta\omega}$. Where θ_i was the parameter estimated for the *ith* animal, θ_{pop} was the population value, $\eta^{\theta\omega}$ was the IIV associated with the *ith* animal of the corresponding population value (Mould and Upton, 2012). The model was written as ordinary differential equations system (ODE) and is described from equation 1 to equation 5, a schematic diagram is showed in Figure 1 (see supplementary material for the code of the model).

$$\frac{dA1}{dt} = ka_{IM} \cdot A_2 + ka_{SC} \cdot A_3 - \frac{Cl}{V_c} \cdot A_1 - \frac{Q}{V_c} \cdot A_1 + \frac{Q}{V_p} \cdot A_5 - \frac{Q_{milk}}{V_C} \cdot A_1 + \frac{Q_{milk}}{V_{milking}} \cdot A_4 \quad [1]$$

$$\frac{dA2}{dt} = -ka_{IM} \cdot A_2 \quad [2]$$

$$\frac{dA3}{dt} = -ka_{SC} \cdot A_3 \quad [3]$$

$$\frac{dA41}{dt} = \frac{Q_{milk}}{V_C} \cdot A_1 - \frac{Q_{milk}}{V_{milking}} \cdot A_4 \quad [4]$$

$$\frac{dA5}{dt} = \frac{Q}{V_c} \cdot A_1 - \frac{Q}{V_p} \cdot A_5 \quad [5]$$

The calculation of area under the curve (AUC) from plasma/serum and milk were obtained directly by integration of the model, AUC from zero to 24 hours (AUC_{24}) after administration were also obtained (see supplementary section for more information).

After the final model was selected, different error models (additive, proportional, or combined) were used to describe the residual unexplained variability (ε), defined as the difference between predicted and observed concentrations (Mould and Upton, 2012). Finally, the effect of covariates was also evaluated. Age and weight as continuous covariates. Breed (Murciano Granadina, Anglo Nubian and Beetal), level of milk production (lactating and non-lactating) and health status (healthy and unhealthy) as categorical covariates. For continuous covariates the relations between parameters were evaluated as $\theta_i = \theta_{pop} \cdot (Cov\theta_i / Cov_{mean})^{\beta} \cdot e^{\eta\theta\omega}$. Where $Cov\theta_i$ is the covariate for the *ith* animal and Cov_{mean} is the mean value of the covariate, and β is the regression coefficient to be determined. Categorical covariates were described as $\theta_i = \theta_{pop} \cdot e^{\beta Cov\theta i} \cdot e^{\eta\theta\omega}$ where $\beta Cov\theta_i$ is exponent for the covariate effect. Finally, covariates were included in the model if presented statistical significance ($p < 0.05$) and reduced the IIV and the likelihood ratio tests (LRT) as −2·log-likelihood (−2LL), Akaike information criterion (AIC), and Bayesian information criterion (BIC). A covariate was retained in the final model if produced a ≥10 reduction in BIC (Mould and Upton, 2013; Paulin et al., 2017).

The model was evaluated at each step of the analysis: goodness-of-fit diagnostics were supported by scatter plots of population/individual predicted versus observed concentrations in logarithmic and arithmetic scale, population/individual-weighted residuals (PWRES and IWRES) versus predictions/time and visual predictive check (VPC) plots (Bousquet-Mélou et al., 2021). In this way, standard errors of parameter estimates were generated based on the full variance–covariance matrix whereas that the correlation matrix was used to detect overparameterizations into the model and is reported in the supplementary material (Mould and Upton, 2013). Finally, the robustness of the model was verified using a

convergence assessment in Monolix taking into account 500 replicates, the shrinkage value and a non-parametric Bootstrap analysis (1000 replicates) with a 90% confidence interval. This analysis was performed in RStudio using the package Rsmlx (R Speaks Monolix) (Goutelle et al., 2020).

2.3. *Determination of PK/PDco and Monte Carlo simulations*

The results obtained after data analysis were imported to Simulx, a simulation package included in the Monolix 2021R1 suite software, and the individual predictions (IPRED) were used to carry out the simulations.

In a first step, multi-dose regimens of marbofloxacin administered IM at 2, 5 and 10 mg/kg for five days were simulated in plasma/serum and milk, therefore, a total of six subpopulation groups were obtained (n = 5000 per group) and were graphically represented. In a second step, simulated steady-state concentration-time profiles corresponding to the first marbofloxacin dose (AUC_{24}) in plasma/serum and milk were obtained and corrected by protein-binding data published in goats to obtain $fAUC_{24}$ (Fernández-Varón et al., 2021), and in a final step, these simulated areas for each dose regimen were used to determine PK/PDco values by PTA calculation by Monte Carlo simulation. The PTA values for a MIC range of 0.025-8 mg/L were determined (n=50000 simulations for each MIC tested), and PK/PDco were established using a wide range of pharmacodynamic target (PDT) values of 24, 48, 72, 96 and 120 (Paulin et al., 2017).

2.4. *MIC measurements and ECOFF determination*

MIC susceptibility testing was determined by microdilution method using CLSI guidelines for *Staphylococcus aureus* (n=44) and coagulase negative staphylococci (n=109)

isolates (CLSI, 2017). MIC values for *Mycoplasma agalactiae* isolates (n=36) were obtained with the methodology described by Fernández-Varón et al., 2021. Since no ECOFF of marbofloxacin have been reported for these microorganisms in goats, tentative ECOFFs (TECOFF) were calculated with ECOFFinder version 2.0 (Turnidge et al., 2006). The calculation of the TECOFFs and PK/PDco could eventually help to determine the clinical break-point of marbofloxacin for goats (Bousquet-Mélou et al., 2021; Toutain et al., 2017; Vegas et al., 2021).

3. Results

3.1. Population pharmacokinetic analysis

The final POP-PK model for IV, IM and SC administration with first-order absorption rate constants accurately described the disposition of marbofloxacin in goats. The VPC plots stratified by route and physiological status for plasma/serum and milk concentrations are showed in Figures 2 and 3, respectively. Most observed values fell into the prediction intervals and were centered around 50% (median). Model parameters are presented in Table 2. Precision of the estimates was good (RSE ≤20% in all cases) with shrinkage values from -12.0 to 9.83%, consequently, the individual parameters were well distributed throughout the entire population distribution, adequately reflecting their variability. In addition, plots observations versus predictions, residuals, distribution of the residuals and bootstrap analysis, suggested a good description of the observed data (Figures S1-S5 in supplementary material).

Marbofloxacin exhibited a fast absorption and complete bioavailability by both IM and SC routes. It showed a high volume of distribution, where the sum of volume compartments (Vc and Vp) exceeded 1 L/kg. Total body clearance of marbofloxacin was low with extraction ratio close to 0.043, suggested that can be classified as a drug with low blood clearance in goats, according to previously established veterinary breakpoints (Toutain and Bousquet-Mélou, 2004).

The residual variability (ε) was described by a combined error model for plasma and serum concentrations as $CONC_{obs} = CONC_{pred} + (a + b \cdot CONC_{pred}) \cdot \varepsilon$, and by a proportional error model for milk concentrations as $CONC_{obs} = CONC_{pred} + b \cdot CONC_{pred} \cdot \varepsilon$, where $CONC_{obs}$ is the observed concentration, $CONC_{pred}$ is the predicted concentration and a and b are the additive and multiplicative components for the residual error, respectively (Mould and Upton, 2013).

The effect of covariates was determined, and the final model was influenced by level of milk production (lactating and non-lactating) and health status (healthy and unhealthy) and were included (Table 2). In fact, unhealthy animals showed a mean 47.27% reduction in Cl and 29.53% in V_c and lactation reduced V_c by 18.13% (Figure 4). Moreover, the inclusion of covariates in the model reduced BIC in -164.7 respect to the base model that is largely significant compared with the recommended reduction ≥ 10 to consider the inclusion of a covariate in the final model (Kass and Raftery, 1995).

Determination of PK/PDco with Monte Carlo simulations

The concentration-time profiles in plasma/serum and milk corresponding to the simulated dose regimens of marbofloxacin administered at 2 mg/kg, 5 mg/kg and 10 mg/kg

by IM route for five days, including goat subpopulations with different health status and level of milk production were evaluated (Figures S6 in supplementary section). From these simulated curves, *f*AUC_{24} values in plasma/serum and milk were obtained and PTAs for PDTs ranging from 24-120 were determined using Monte Carlo simulations (Figure S7, supplementary material). Finally, PK/PDco values of marbofloxacin for each dose regimen tested were determined (Table 3).

3.2. *Evaluation of the TECOFF values for goat pathogens*

MIC distribution values, including MIC_{50} and MIC_{90} data, for each pathogen are shown in Table 4. The TECOFF values for *Mycoplasma agalactiae*, *Staphylococcus aureus* and coagulase negative staphylococci calculated from the distribution fitted curves were 0.5, 1 and 0.5 mg/L, respectively (Table 4). The observed and fitted distributions used to calculate the TECOFF values are shown in Figure S8 (supplementary section).

The PK/PDco values previously determined for each PDT were compared with obtained TECOFF of each pathogen. Based on TECOFFs, a dose regimen of 10 mg/kg/24h could achieve a PDT of 24 in plasma/serum and milk for coagulase negative staphylococci and *Mycoplasma agalactiae*, but this dose regimen failed to guarantee an appropriate MIC in 90% of goat for *Staphylococcus aureus*, even for the lower PDT of 24. Dose regimens of 2 and 5 mg/kg/day failed to achieve the lower PDT of 24 for the three investigated pathogens.

4. Discussion

In this study, a POP-PK meta-analysis of marbofloxacin was conducted by non-linear mixed-effects models using raw data obtained from ten different pharmacokinetic studies

(n=64) in goats, including different population characteristics defined as covariates: breed, age, weight and, level of milk production and health status (22% of animals were unhealthy), three administration routes and doses of marbofloxacin with plasma/serum and milk concentrations, being this our first objective.

Bioavailability of marbofloxacin by IM and SC routes was complete, therefore, only IM route was used to conduct multi-dose simulations because of commonly used route in the clinical setting. Marbofloxacin showed a high volume of distribution with a good passage from blood to milk with $AUC_{plasma/milk}$ ratios close to 1 (Fernández-Varón et al., 2021; Lorenzutti et al., 2017). Two covariates, level of milk production and health status were retained, and had a significant effect on V_c and Cl (Table 2, Figure 4). In fact, drug distribution was influenced by level of milk production (non-lactating and lactating goats) with lower Vc values for lactating goats, indicating a reduced intravascular space for lactating goats in comparison to non-lactating group. Additionally, $Q_{milking}$ was higher than Q, suggesting a faster equilibration in the mammary gland compartment than peripheral compartment (Stec and Atkinson, 1981). A specific ABC transporter, the "breast cancer resistance protein" (BCRP) present in the mammary gland could contribute to transfer of fluoroquinolones into mother's milk (Merino et al., 2006). It has been reported that enrofloxacin and danofloxacin are substrates of BCRP in sheep and goats, so a possible role of BCRP for marbofloxacin excretion into milk of goats can't be denied. In this context, these transporters could in part drive the effect of level of milk production status on Vc (Mealey, 2012; Perez et al., 2013; Pulido et al., 2006; Real et al., 2011; Schrickx, and Fink-Gremmels, 2008; Wu et al., 2008).

The unhealthy status was included as covariate using data from animals having *E. coli*-endotoxin induced fever and mastitis produced by coagulase negative staphylococci. This covariate significantly reduced both V_c and Cl. These findings indicated that distribution and elimination of marbofloxacin were highly influenced by the health status of animal, highlighting the importance of including subpopulations in the population model, as recommended by EMA (EMA, 2016). As discussed by Lorenzutti et al. (2021a) and Waxman et al. (2003), it is reported that inflammation and infection could induce lower peripheral blood flow and downregulation of metabolizing enzymes and transporters (principally ABC superfamily), resulting in higher plasma drug disposition and affecting some distribution processes (Schmith and Foss, 2008). Inflammatory mediators (interleukins, cytokines, transforming growth factor, tumor necrosis factor or interferons) decreased cytochrome P450 complex expression, which caused reduced elimination in unhealthy as compared to healthy animals (Aitken et al., 2006; Morgan et al., 2008; Petrovic, and Piquette-Miller, 2007; Renton, 2005; Schmith and Foss, 2008; Waxman et al., 2003). It is interesting to note that marbofloxacin presented a context sensitive PK in goats with mastitis (Lorenzutti et al., 2021a), showing higher plasma and milk disposition on the first day of treatment. However, it could be a limitation of this model as it was constructed with single dose data. Lack of context sensitive information has direct implications on further PK simulations and highlights the importance of conducting multi-dose studies to determine CBP of antimicrobials.

Our second objective was to determine the PK/PDco values of marbofloxacin administered by IM route for multi-dose regimens of 2 mg/kg/24h, 5 mg/kg/24h and 10 mg/kg/24h using Monte Carlo simulations. Both the POP-PK model and PK/PDco values

follow the VetCAST and EMA recommendations and could help to further CBP determination for marbofloxacin in goats (EMA 2015, Toutain et al., 2017, Toutain et al., 2019).

Plasma/serum PK/PDco of marbofloxacin showed that the dose regimen of 2 mg/kg/24h and 5 mg/kg/24h were only predicting efficacy for relatively lower MIC values (0.025-0.125 mg/L and 0.05-0.25 mg/L, respectively). On the other hand, the dosage regimen of 10 mg/kg/24h was adequate for intermediate MIC values of 0.125-0.50 mg/L (Table 3 and Table 4). Based on these results, dosage regimens of 2 and 5 mg/kg/24h could be adequate for highly susceptible bacteria (mostly gram-negative microorganisms that are usually related with lower MIC values) but higher dosage 10 mg/kg/24h should be used for intermediate to low susceptible microorganisms (usually related to gram-positive bacteria or *Mycoplasmas*). It is significant to note that reported MIC and *f*AUC/MIC cut-off values for marbofloxacin in milk against coagulase negative staphylococci and *Staphylococcus aureus* were higher than reported for serum: a *f*AUC/MIC value of 41.48 (35.29–58.73) and 54.68 (27.69–160.3) was necessary for a −2 $\log_{10}$ reduction, respectively (Lorenzutti et al., 2021a-b). These results suggest that a dose of 10 mg/kg in a 5-day treatment could be the most effective option for the MIC range tested in this study. However, these data must be compared with MIC values obtained from wild-type strains to determine effectiveness and suggest tentative cut-off points (Bousquet-Mélou et al., 2021; Toutain et al., 2019; Vegas et al., 2021).

Our third objective was to assess the TECOFF values for pathogens obtained from mastitis goat´s milk. Values of 0.5, 1 and 0.5 mg/L for *Mycoplasma agalactiae*, *Staphylococcus aureus* and coagulase negative staphylococci from the distribution fitted curves were calculated, respectively. Susceptibility data were compared with the PDT values

for fluoroquinolones; a *f*AUC/MIC of 30-55 is usually recommended for gram-positive bacteria, similar to those tested in this study of 24-48 (Papich, 2014). It is pertinent to mention that these ratios are applicable against these specific pathogens, however, these ratios were used as a reference for reporting breakpoint values for *Mycoplasma agalactiae* (Fernández-Varón et al., 2021). In fact, *Mycoplasma* spp. are presumably evolved by degenerative evolution from gram-positive bacteria and are phylogenetically most closely related to some clostridia (Gautier-Bouchardon, 2018). For that reason, the use of *f*AUC/MIC ratios of 30-55 or 100-125 to determine the optimum dosage regimen for *Mycoplasma agalactiae* should be taken with caution (Mitchel et al., 2012; Zhang et al., 2016).

Results showed that the simulated dosage regimens of 2 and 5 mg/kg/24h will not achieve the corresponding TECOFF values. However, a 10 mg/kg/24h dose regimen could be effective for a population with a target *f*AUC/MIC ratio < 48 for Coagulase negative staphylococci and *Mycoplasma agalactiae*, but not for *Staphylococcus aureus*. It is important to note that PK/PDco were determined from simulations including both healthy and non-healthy individuals and more higher cut-off values could be achieved in unhealthy subpopulations with low Cl values. It is an important issue, since antimicrobials are used in unhealthy animals, and this model could help to determine PK/PDco values in this subpopulation. This information could be taken into account by committees that set the CBP and justifies expert rules such as "a germ declared "I" by the AST will in fact be category "S" for sick animals because a reduction in clearance is equivalent to an increase in antimicrobial exposition, which is the EUCAST definition of "I" (increased exposure). Consequently, TECOFF values should be taken into consideration for CBP determination for these pathogens in goats (Vegas et al., 2021).

The PK/PDco values obtained from POP-PK analysis and Monte Carlo simulations indicate that marbofloxacin at a dose of 10 mg/kg could be used successfully in goats for the treatment of mastitis caused by coagulase negative staphylococci. Similar results have been reported by Lorenzutti el al. (2017). The limitation of the study was the use of *f*AUC/MIC ratios derived from gram-positive pathogens due to non-availability of data associated with gram-negative pathogens in goats. Nevertheless, results obtained in the study provide critical information on rational usage of marbofloxacin against mastitis pathogens and can serve as starting point for further clinical trials in goats.

5. Conclusion

In conclusion, a) marbofloxacin concentrations were well described in plasma/serum and milk by the POP-PK model. b) The simulations we developed and Monte Carlo simulations predicted PK/PDco values ranging from 0.25-0.5 mg/L for Coagulase negative staphylococci and *Mycoplasma agalactiae* pathogens isolated from goats with mastitis, indicating that it could be useful in clinical settings. c) TECOFF obtained from our MIC distributions showed that Staphylococcus aureus presented a higher TECOFF than Coagulase negative staphylococci and Mycoplasma agalactiae, and this information could be useful for further ECOFF determinations of these pathogens in goats.

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Table 1: Description of each data set used to build a population model of marbofloxacin in goats (n = 64).

Study	Animals	Dose (mg/kg)	Route	Blood sampling times	Breed	Healthy	LLOQ (mg/L)
A	n = 7	2	IV	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48h	MG	Yes	0.017
		2	IM	1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48h	MG	Yes	
B	n = 7	2	IV	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48, 52h	MG	Yes	0.017
		2	IV	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48, 52h	MG	No	
C	n = 6	2	IV	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48h	MG	Yes	0.017
D	n = 6	2	SC	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48h	MG	Yes	0.017
E	n = 7	2	SC	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48h	MG	Yes	0.017
F	n = 6	5	IV	0, 2, 6, 12, 20, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48h	AN	Yes	0.025
		5	IV	Milk sampling times: 0, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48 h	AN	Yes	
		5	IM	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 29, 34, 48, 52h	AN	Yes	
		5	IM	Milk sampling times: 0, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48 h	AN	Yes	
G	n = 6	2	IV	5, 15, 30 min, 1, 2, 4, 6, 9, 12, 24h	B	Yes	0.01
H	n = 6	2	IM	5, 15, 30 min, 1, 2, 4, 6, 9, 12, 24 h	B	Yes	0.01
I	n = 6	2	IV	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 32, 48, 72, 96	MG	Yes	0.002
		2	IV	Milk sampling times: 0, 1, 2, 4, 6, 8, 10, 12, 24, 32, 48, 72, 96 h	MG	Yes	
		2	SC	0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 32, 48, 72, 96h	MG	Yes	
		2	SC	Milk sampling times: 0, 1, 2, 4, 6, 8, 10, 12, 24, 32, 48, 72, 96 h	MG	Yes	
J	n = 7	10	IM	0, 10, 20, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 10, 12, 24 h	AN	No	0.025
		10	IM	Milk sampling times: 0, 1, 4, 6, 8, 10, 12, 24 h	AN	No	

References: A: Waxman et al., 2001; B: Waxman et al., 2003; C: Waxman et al., 2004; D: Dova et al., 2007; E: San Andrés-Larrea. Personal Communication; F: Lorenzutti et al., 2017; G: Bhardwaj et al., 2018; H: Bhardwaj et al., 2019; I: Fernández-Varón et al., 2021; J: Lorenzutti et al., 2021. Breed: MG: Murciano-Granadina; AN: Anglo-Nubian; B: Beetal.

Table 2: Population pharmacokinetic parameters in plasma/serum and milk for marbofloxacin in goats after intravenous, intramuscular, and subcutaneous administrations at 2, 5 and 10 mg/kg. Data presented as estimates with relative standard error (%RSE), non-parametric Bootstrap is included.

Fixed Effects	Estimate (RSE; %)	Bootstrap Estimate (90% CI)	Shrinkage (%)
F_{IM}	0.92 (7.56)	0.92 (0.89 – 0.96)	8.92
k_{aIM} (1/h)	2.58 (9.14)	2.49 (2.29 – 2.70)	7.79
F_{SC}	0.91 (8.02)	0.95 (0.92 – 0.98)	-12.0
k_{aSC} (1/h)	1.58 (6.91)	1.60 (1.55 – 1.66)	4.78
Cl (L/h/Kg)	0.25 (3.24)	0.26 (0.25 – 0.27)	-0.591
V_c (L/Kg)	0.66 (3.68)	0.67 (0.65 – 0.69)	3.1
Q (L/h/Kg)	0.04 (11.2)	0.05 (0.05 – 0.06)	9.83
V_p (L/Kg)	0.40 (6.29)	0.40 (0.38 – 0.43)	2.72
Q_{milk} (L/h/Kg)	0.36 (11.4)	0.37 (0.32 – 0.43)	-8.50

Covariates	Estimate (RSE; %)	Bootstrap Estimate (90% CI)
$\beta_{(NONHEALTHY)}Cl$	-0.64 (13.5)	-0.66 (-0.75 – -0.57)
$\beta_{(NONHEALTHY)}V_c$	-0.35 (22.5)	-0.42 (-0.51 – -0.33)
$\beta_{(LACTATING)}V_c$	-0.20 (29.8)	-0.19 (-0.24 – -0.14)

Interindividual variabilities			Residual error model		
	Estimate (RSE; %)	Bootstrap Estimate (90% CI)	Estimate (RSE; %)		Bootstrap Estimate (90% CI)
IIV_F_{IM}	0.33 (16.2)	0.28 (0.24 – 0.32)	Plasma/Serum data		
IIV_k_{aIM}	0.43 (15.8)	0.36 (0.29 – 0.43)	a	0.004 (11.5)	0.004 (0.003 – 0.005)
IIV_F_{SC}	0.30 (18.8)	0.33 (0.28 – 0.38)	b	0.09 (2.68)	0.086 (0.083 – 0.089)
IIV_k_{aSC}	0.27 (20.9)	0.22 (0.16 – 0.28)			
IIV_Cl	0.30 (8.40)	0.31 (0.30 – 0.33)	Milk data		
IIV_V_c	0.21 (10.9)	0.22 (0.19 – 0.24)	b	0.13 (5.23)	0.13 (0.12 – 0.15)
IIV_Q	1.14 (8.27)	1.28 (1.09 – 1.48)			
IIV_V_p	0.40 (11.5)	0.38 (0.34 – 0.43)			
IIV_Q_M	0.77 (13.1)	0.84 (0.72 – 0.96)			

F_{IM}: bioavailability of IM route; k_{aIM}: absorption constant for IM route; F_{SC}: bioavailability of SC route; k_{aSC}: absorption constant for SC route; Cl: clearance of the central compartment; V_c: volume of central compartment; Q: inter-compartmental clearance between central and peripheral compartment; V_p: volume of peripheral compartment; Q_{milk}: inter-compartmental clearance between central and milk compartment; $\beta_{(NONHEALTHY)}Cl$: health status covariate effect on Cl; $\beta_{(NONHEALTHY)}V_c$: health status covariate effect on V_c; $\beta_{(LACTATING)}V_c$: productive status covariate effect on V_c; β values represent the effect of the categorical covariates studied for each pharmacokinetic parameter and are resolved with an exponential model. RSE %: relative standard error obtained in the estimation of each parameter (fixed or random) by the statistical model; CI: confidence interval; IIV: inter-individual variability represented as the standard deviation of the random effects; a and b are the components of the error model that describe the residual variability between observed and predicted concentrations.

Table 3: PK/PDco values for different pharmacodynamic targets of marbofloxacin following intramuscular administration at 2, 5 and 10 mg/kg/24h in goats. Data expressed as MIC (mg/L) to achieve a *f*AUC/MIC ratio in the target population using a PTA = 90%.

	Dose regimen					
PDT	**2 mg/kg/24h**	**5 mg/kg/24h**	**10 mg/kg/24h**	**2 mg/kg/24h**	**5 mg/kg/24h**	**10 mg/kg/24h**
	Plasma/serum			*Milk*		
24	0.125	0.25	0.5	0.125	0.25	0.5
48	0.05	0.125	0.25	0.05	0.125	0.25
72	0.05	0.125	0.25	0.05	0.125	0.25
96	0.025	0.05	0.125	0.025	0.05	0.125
120	0.025	0.05	0.125	0.025	0.05	0.125

PDT: Pharmacodynamic target: *f*AUC/MIC (h). For a PDT of 24 (i.e. to achieve a mean marbofloxacin free-concentration equal to MIC over the 24h dosing interval) with a 2 mg/kg/24h dosing regimen, the PK/PDco (i.e. the maximum possible MIC value attained by marbofloxacin free-concentrations that can be reached in 90% of goat population, given a PDT of 24) is of 0.125 mg/L. Moreover, it is only 0.025 mg/L when the given PDT is 120 i.e. when the target marbofloxacin free-concentration is 5-folds the MIC of the pathogen in question

Table 4. MIC distributions and TECOFF values for marbofloxacin against goat pathogens.

Concentration (mg/L)	*Staphylococcus aureus*	Coagulase negative staphylococci	*Mycoplasma agalactiae*
0.0625	-	-	1
0.125	8	35	-
0.25	17	62	14
0.5	11	9	9
1	5	-	5
2	3	-	-
4	-	-	3
8	-	-	4
Total	44	109	36
MIC_{50}	0.25	0.25	0.5
MIC_{90}	1	0.5	4
95% Subset ECOFF (TECOFF)	1	0.5	0.5

95% Subset ECOFF is the tentative ECOFF (TECOFF).

Highlights

Plasma, serum and milk concentration of marbofloxacin in goats were analysed with a population pharmacokinetic model using 10 pharmacokinetic studies after intravenous, intramuscular, and subcutaneous administration at a dose of 2, 5 and 10 mg/kg.

Simulated dose regimens of marbofloxacin were obtained and probability of target attainment was determined using Monte Carlo simulation.

MIC of *Staphylococcus aureus*, coagulase negative staphylococci, and *Mycoplasma agalactiae* wild type isolates from goats were determined

Tentative epidemiological cutoff (TECOFF) and pharmacokinetic-pharmacodynamic *f*AUC/MIC ratios were obtained and investigated.

Dose regimen of 5 mg/kg/24h and 10 mg/kg/24h achieved *f*AUC/MIC ratios of 30 and 55, respectively and could be taken as a starting point for further clinical trials in lactating goats.

Population pharmacokinetics and pharmacokinetic/pharmacodynamic evaluation of marbofloxacin against Coagulase-negative staphylococci, *Staphylococcus aureus* and *Mycoplasma agalactiae* pathogens in goats.

1) Selection of raw data from manuscripts

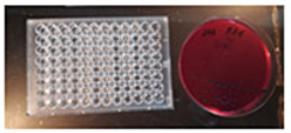
Published pharmacokinetic manuscripts: Rich sample design.

Breed: Anglo-Nubian, Beetal, Murciano-Granadina

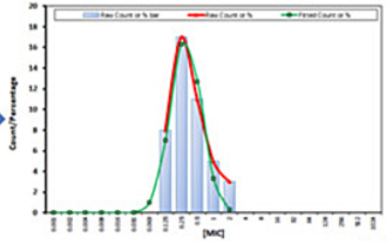
Healthy lactating goats	Unhealthy Non-lactating goats
non-lactating goats	IV (n=7) 2 mg/kg endotoxin-induced fever
IV (n=38) 2 mg/kg IV	
IM (n=19) 2 and 5 mg/kg	Unhealthy Non-lactating goats
SC (n=20) 2 mg/kg	IM (n = 7) 10 mg/kg mastitis

3) Pharmacodynamic: MIC and ECOFF determination

Staphylococcus aureus (n=44)
Coagulase negative staphylococci (n=109)
Mycoplasma agalactiae isolates (n=36)

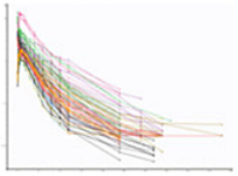


Tentative epidemiological cutoff (TECOFF)

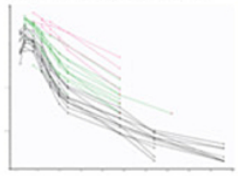


5) starting point for further clinical trials in goats.

Raw serum/plasma concentrations



Raw milk concentrations

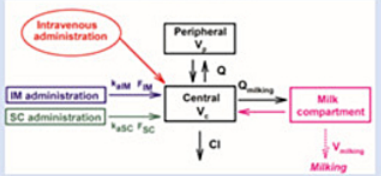


2) Modelling plasma/serum & milk concentrations: NLMEM Monolix ®

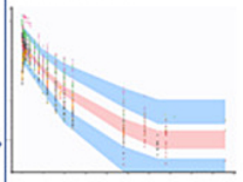
Population pharmacokinetic analysis

$\theta_i = \theta_{pop} \cdot e^{\beta Cov0i} \cdot e^{\eta 0\omega}$

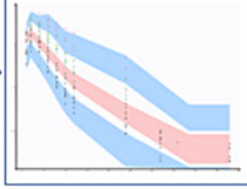
Monolix Suite ®

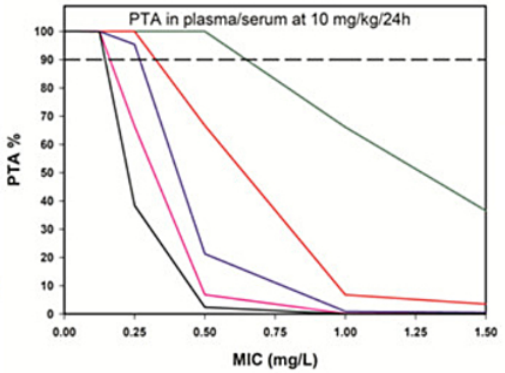


Modelled serum/plasma concentrations



Modelled milk concentrations





4) Monte Carlo Simulations (n=10000) Probability of target attainment (PTA): numbers of simulated $fAUC_{24}/MIC \geq 90\%$ for each dose regimen.

FINAL CONCLUSIONS:

1. Data were well described by the model for plasma/serum and milk concentrations.
2. Dose regimen simulated of 10 mg/kg/24h was adequate for intermediate MIC values of 0.125-0.50 mg/L
3. Results obtained in this study could be taken as a starting point by committees that set the clinical breakpoints and justifies expert rules to optimize marbofloxacin dose regimens in goats.

Graphics Abstract

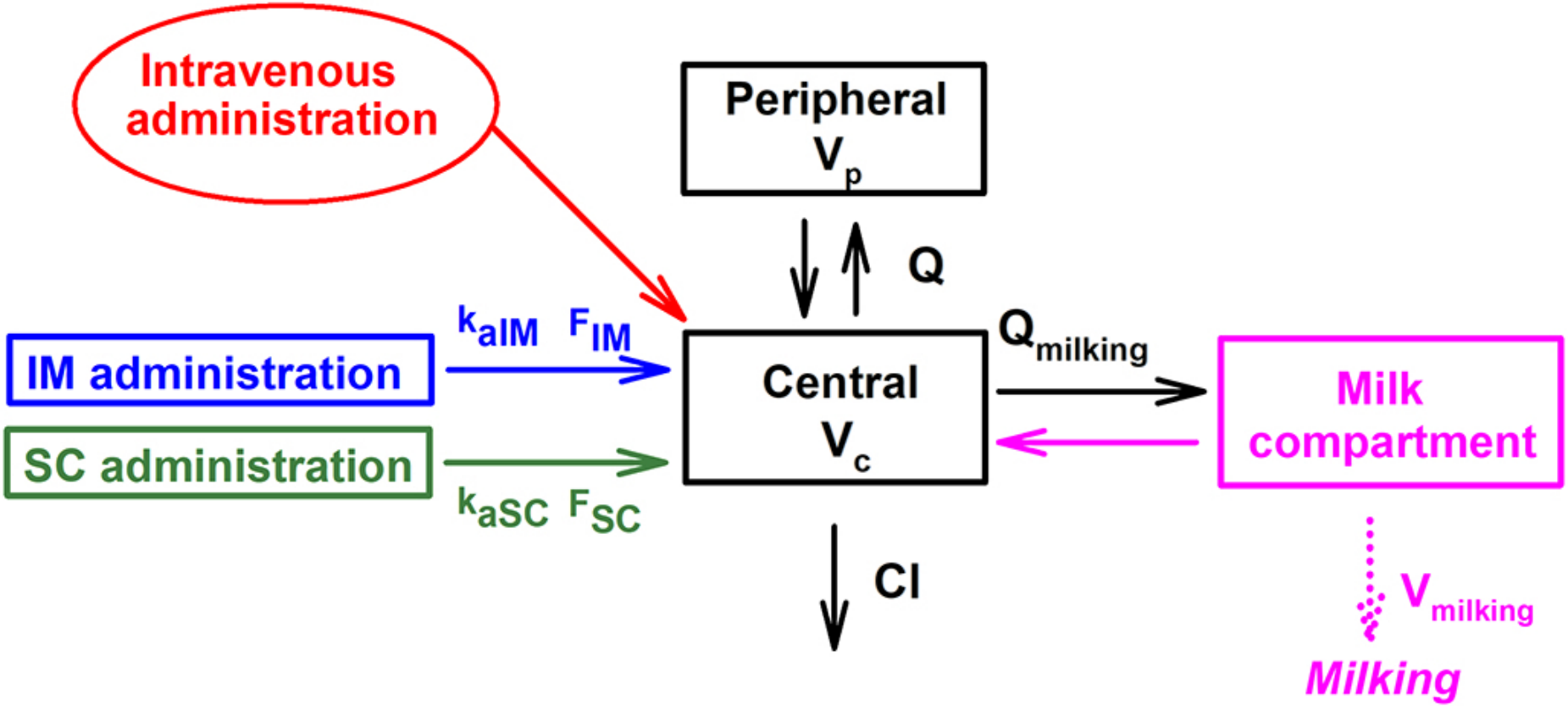
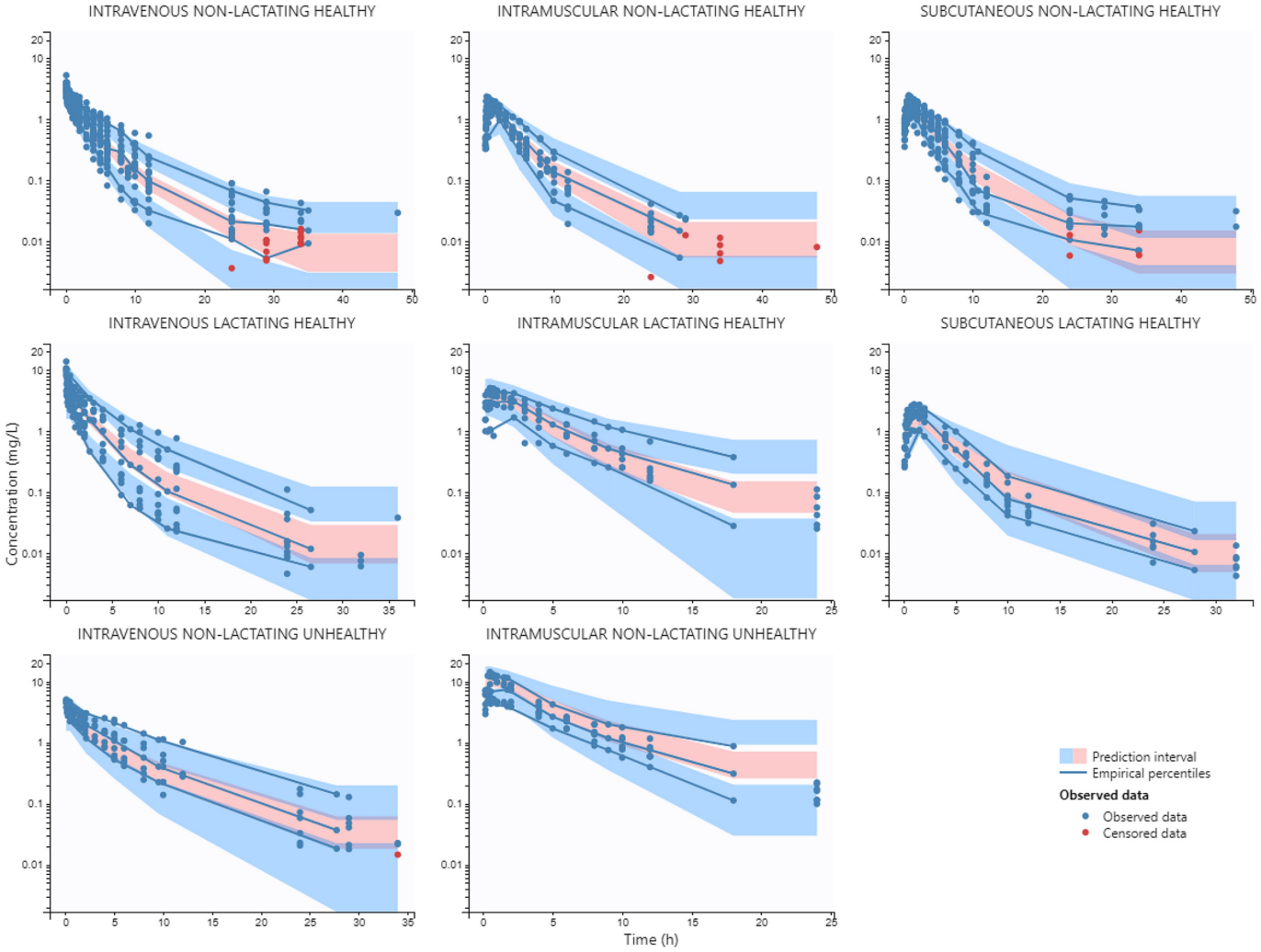

Intravenous administration
Peripheral
V_p
Q
k_{aIM} F_{IM}
IM administration
SC administration
k_{aSC} F_{SC}
Central
V_c
$Q_{milking}$
Milk compartment
Cl
$V_{milking}$
Milking

Figure 1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INTRAMUSCULAR NON-LACTATING HEALTHY
SUBCUTANEOUS NON-LACTATING HEALTHY
INTRAVENOUS LACTATING HEALTHY
INTRAMUSCULAR LACTATING HEALTHY
SUBCUTANEOUS LACTATING HEALTHY
INTRAVENOUS NON-LACTATING UNHEALTHY
INTRAMUSCULAR NON-LACTATING UNHEALTHY
Concentration (mg/L)
Time (h)
Prediction interval
Empirical percentiles
Observed data
Observed data
Censored data

Figure 2

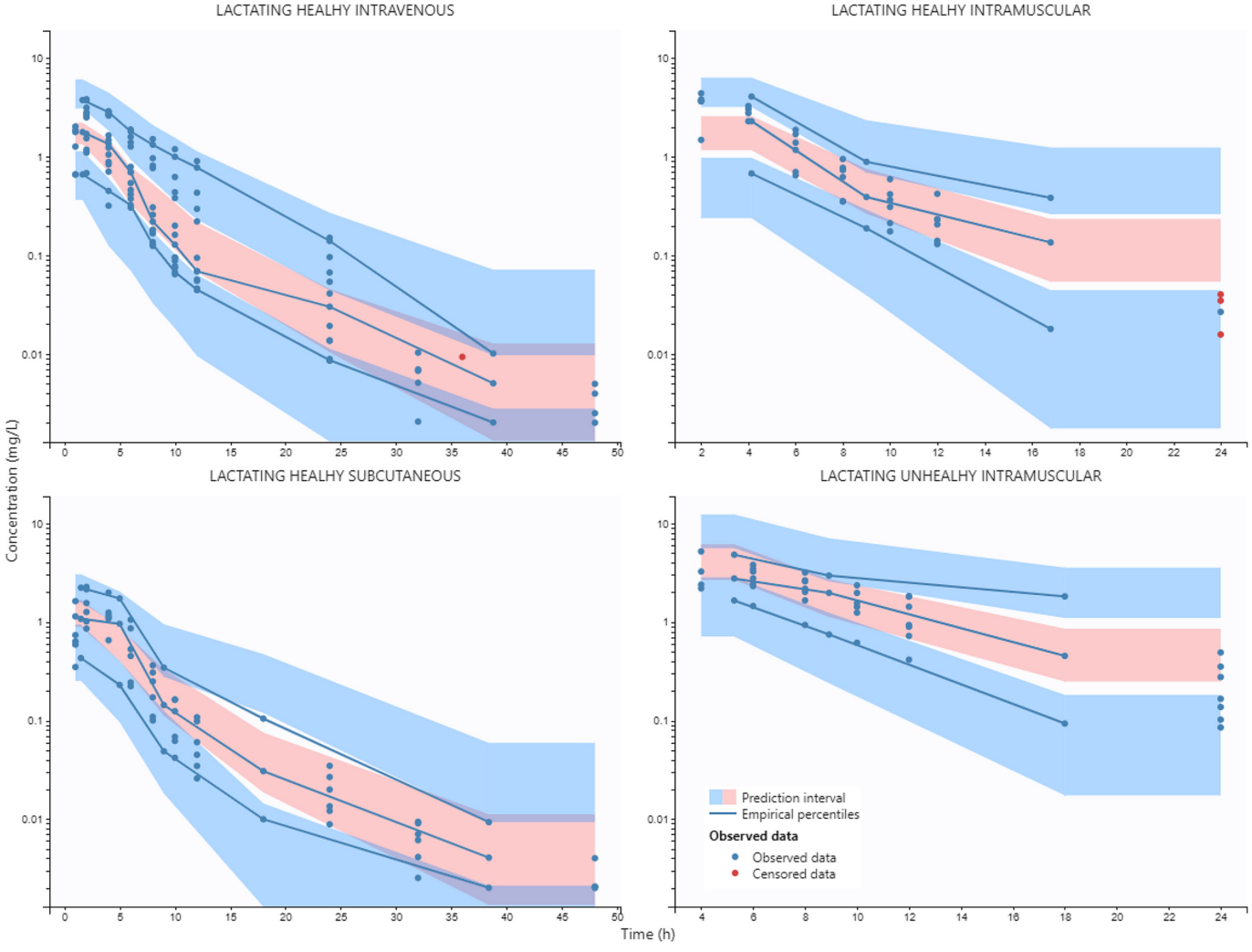
LACTATING HEALHY INTRAVENOUS
LACTATING HEALHY INTRAMUSCULAR
LACTATING HEALHY SUBCUTANEOUS
LACTATING UNHEALHY INTRAMUSCULAR
Concentration (mg/L)
Time (h)
Prediction interval
Empirical percentiles
Observed data
Observed data
Censored data

Figure 3

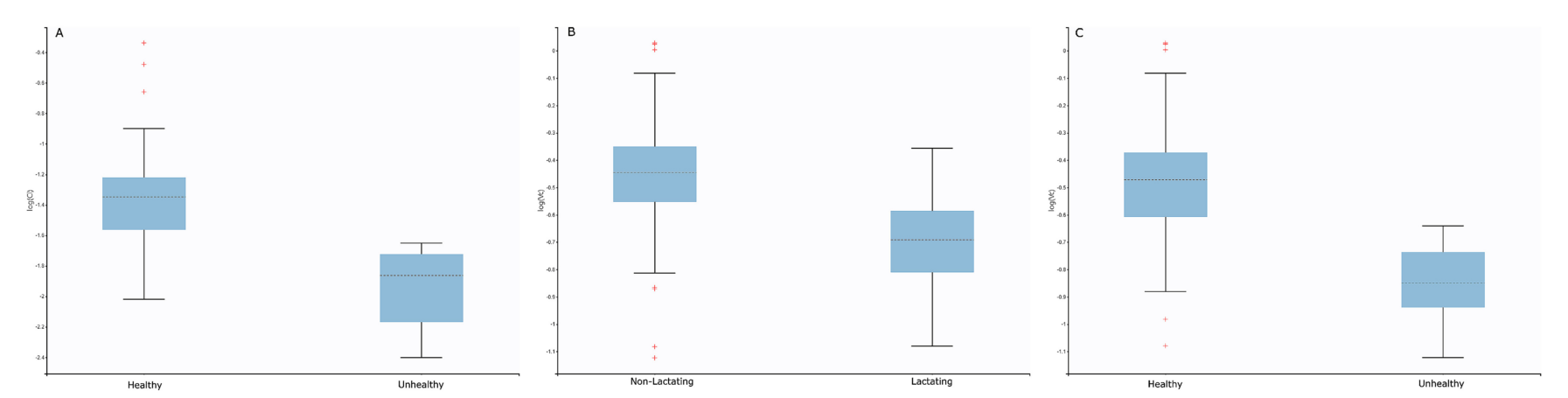
A
log(Ct)
Healthy
Unhealthy
B
log(Nc)
Non-Lactating
Lactating
C
log(Nc)
Healthy
Unhealthy

Figure 4