Pharmacokinetic/pharmacodynamic relationship of danofloxacin against *Mannheimia haemolytica* in a tissue-cage model in calves

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**Objective**: To evaluate an experimental model of the pharmacokinetic/pharmacodynamic (PK/PD) relationship of danofloxacin against *Mannheimia haemolytica* infection, using subcutaneously implanted tissue cages in calves.

**Methods**: Tissue cages implanted subcutaneously in calves were infected with *M. haemolytica* and different concentration–time profiles of danofloxacin were simulated. Drug concentrations and bacterial counts were monitored over time and various PK/PD parameters calculated.

**Results**: By using different types of cage and various doses, a range of PK/PD indices were simulated. The PK/PD index that best predicted the antimicrobial effect was the AUC/MIC ratio. The magnitude of this index needed for near-maximum effect (80%), assessed using the area under the bacterial kill curve to 48 h, was 244 h.

**Conclusions**: The model described enabled different concentration–time profiles to be simulated, and PK/PD interactions to be studied in the presence of the host’s defences. The validity of this model needs to be confirmed by clinical studies, but the results suggest that it may be a useful intermediary step between *in vitro* and clinical studies.

**Keywords**: pharmacodynamics, *in vivo* models, tissue cages, fluoroquinolones, *Mannheimia*

**Introduction**

Drug-dosage regimens ideally should be based on an understanding of the interaction between the drug, the infecting organism and the host. Although a variety of models have been developed to study the relationship between the pharmacokinetics (PK) and pharmacodynamics (PD) of antimicrobials,1,2 each model has its inherent merits and limitations. However, despite this there is good evidence that their outcomes have a bearing on the clinical situation.3

Tissue cages implanted subcutaneously have been used extensively to study the PK of antimicrobials.4 Initially, this type of model was believed to reflect the PK in interstitial fluids. Subsequently, however, the kinetics of free drug in tissue cages was found to be governed by the surface-area-to-volume of the specific cages used, rather than the serum PK.5 As a result, the clinical counterpart of tissue-cage models is not clearly defined and their value in PK studies is questionable.

In the study of the PD of antimicrobials, tissue cages have also been used to contain an infection, either focusing on device-related infections or more general objectives.4 This type of model allows for repeated sampling, and is an interesting approach in PK/PD studies.6,7 However, penetration and elimination of antimicrobials into and from the cages is slow following systemic administration, which limits the ability to vary the concentration–time profiles of the drug. In a previous study, we were able to show that by injecting drug directly into tissue cages, different concentration–time profiles of drug could be simulated.8 The utility of this concept for PK/PD interactions was confirmed in a study on the effect of a time-dependent antimicrobial (penicillin) against *Mannheimia haemolytica* infection in tissue cages in calves.9

In this paper, we evaluated the tissue-cage model in the PK/PD relationship of danofloxacin against *M. haemolytica*.

**Materials and methods**

**Bacterial strain and preparation of the inoculum**

The bacterial strain *M. haemolytica* serovar A1, Ab 35/85, also used in the previous study,9 was stored at ~70°C. Immediately before the start of the experiments, a culture grown for 16 h on horse blood agar was suspended in sterile isotonic saline to produce an inoculum of ~5 × 10⁶ cfu/mL.

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Determination of MIC and MBC

For the test strain, MIC and MBC of danofloxacin (Pfizer Animal Health, Pfizer Inc, New York, NY, USA) were determined by a microdilution method using cation-adjusted Mueller–Hinton broth (Difco, Boule Diagnostics AB, Huddinge, Sweden).10 The concentrations tested were 0.006, 0.012, 0.038, 0.050, 0.062 mg/L, and all determinations were made in duplicate on 3 different days.

The MIC determined in vitro (representing free drug) was adjusted for protein binding in tissue-cage fluid (TCF) using the following equation:

\[ \text{MIC}_{\text{TCF}} = \text{MIC}/f_u \]

where \( f_u \) is the fraction of unbound danofloxacin in TCF at the MIC, as determined by protein binding studies.

Animals and tissue cages

Eight male calves of the Swedish Red and White breed were used in the study. The experiments were approved by the Ethics Committee for Animal Experiments, Uppsala, Sweden.

Tissue cages were implanted subcutaneously in calves aged 13–16 weeks (body weight 91.4 ± 12.0 kg), after sedation and local anaesthesia. The procedures were essentially as described earlier.11 The cages were made of silastic rubber tubing with an inner diameter of 15 mm, perforated at each end and sealed with silastic rubber plugs. Two cage sizes were used: large cages with a theoretical volume (non-perforated part of the tube) of 10.6 mL, and small cages with a theoretical volume of 2.7 mL. Both cages had a surface area of 1.8 cm². Three cages of each size were implanted in each calf.

Sampling of TCF was performed by percutaneous puncture. Sterility of TCF was assessed by aerobic and anaerobic culture of samples taken from each cage immediately before the experiments.

Experimental design and sampling

The experiment started 6 weeks after implantation of the cages. At 24 h before the first treatment (t = −24 h), 0.8 or 0.2 mL of TCF was aspirated from the large and small cages, respectively, followed by injection of equivalent volumes of inoculum. Twenty-four hours after inoculation (t = 0 h), one large and one small cage in each calf was injected with a solution of danofloxacin to produce an initial concentration in TCF of 1 mg/L (high dose). Two other large and small cages were injected with a lower dose (0.5 mg/L) and the remaining cages with isotonic saline. The volume injected was 0.8 mL for the large cages and 0.2 mL for the small ones. Injection with danofloxacin or saline was repeated at 24 h.

TCF was sampled 30 min after inoculation (t − 24 h), at the time of the first injection of drug (0 h) and thereafter 0.5, 2, 5, 8, 10, 19, 24, 25, 26, 29, 32, 34, 43, 48, 54, 72 and 96 h after the first injection of drug.

The volume sampled was kept to a minimum and varied between 0.2 and 0.8 mL for the large cages and 0.05 and 0.2 mL for the small cages, depending on whether both assays and counts or just one of these analyses were being performed. Samples for assay of danofloxacin were kept at −70°C until analysis.

Bacteriological examination of TCF

Within 1 h of sampling, 50 µL of TCF was serially diluted 10-fold in PBS (pH 7.2). From each dilution, 0.1 mL was cultured on horse blood agar (5% v/v), and the plates were incubated for 24 h at 37°C. Colony-forming units were counted from plates; the theoretical limit of detection of this procedure is 200 cfu/mL.

Drug assay and protein binding

Total concentrations of danofloxacin in TCF were determined with an agar diffusion method12 using Escherichia coli ICB 4004 as the test organism and Mueller–Hinton agar (Difco) as the medium. Standards were prepared in pooled TCF collected before the experiments were started. The coefficient of determination, \( R^2 \), for the standard curves was >0.97 for all assays, and the limit of quantification 0.06 mg/L.

For determination of protein binding of danofloxacin in TCF, pooled uninfected TCF was adjusted to pH 7.2 and spiked with danofloxacin 0.08, 0.10, 0.21, 0.31 and 0.83 mg/L. Protein-bound drug was removed by ultracentrifugation using centrifugal filter devices (Amicon Ultra-4, Amicon, Lexington, MA, USA) and centrifugation at 4000g for 15 min at room temperature.13 Protein binding of danofloxacin was also determined in bovine serum adjusted to pH 7.2 and spiked with the same concentrations as TCF, but in this case protein-bound drug was removed by ultrafiltration at 295 000g for 3 h at +25°C.13

The concentration of danofloxacin before and after ultrafiltration or centrifugation was determined as described under drug assay (see above) using standards in protein-free TCF or serum, as appropriate.

Data analysis

Antibacterial effect. Bacterial counts were normalized by logarithmic transformation, with bacterial numbers below the detection limit arbitrarily set to 100 cfu/mL, for calculations where such a value was necessary. The area under the bacterial curve, AUBC, to \( t = 48 \) (AUBC₄₈) was calculated from the log cfu versus time plots starting from \( t = 0 \) using the trapezoidal method.

PK parameters. PK parameters of danofloxacin in TCF were calculated for each cage. The elimination rate constant \( (k_e) \) was determined by linear least squares regression from a semi-logarithmic plot of concentration versus time after each dose, and the elimination half-life \( (t_{1/2}) \) was calculated as \( \ln 2/k_e \). The initial concentration in TCF, \( C_{\text{inoc}} \), was obtained by backwards extrapolation of the regression lines for each dose and corrected to free drug, as based on results from the protein-binding studies. All measured concentrations of drug in TCF were corrected to free drug, and these figures were used to estimate the area under the concentration curve (AUC) of free drug using the linear trapezoidal rule until 48 h had elapsed. The mean AUC over 24 h, \( \text{AUC}_{24\text{hr}} \) was calculated as \( \text{AUC}_{24\text{hr}} = 2 \).

PK/PD indices. \( C_{\text{inoc}}/\text{MIC} \) and \( \text{AUBC}_{48}/\text{MIC} \) ratios were calculated from the MIC and the PK parameters based on free drug defined above. To estimate time >MIC \( (T_{\text{MIC}}) \), a first order model was fitted to the measured concentrations of danofloxacin. Results were compared with estimates made visually from plots of concentration–time curves. When an estimate based on the first-order model deviated notably from the figure taken visually from the plot, the latter was chosen for analysis.

Statistical analysis. Co-variation between the PK/PD indices was assessed by Pearson’s correlation. To determine which of the PK/PD indices best predicted AUBC₄₈, linear and second-order polynomial regression, with calf as a between-group factor, were used. Adjusted \( R^2 \), inspection of residuals, and plots of observed versus predicted values were used to evaluate the fits of the regression lines.

To describe the relationship between AUBC₄₈ and the PK/PD indices, an inhibitory four-parameter \( E_{\text{max}} \), model (sigmoid dose-response with variable slope) was also used. The model was fitted both to pooled data from all calves, and separately to data from each calf. In the latter case, if the four-parameter model failed to converge, a three-parameter model was tried with either top (no effect) or bottom (maximum effect) set as arbitrary. The four-parameter model failed to converge, a three-parameter model was tried with either top (no effect) or bottom (maximum effect) set as arbitrary. The biological relevance of the estimated constants was considered for each model, and CIs, significance of the constants, inspection of residuals, and plots of observed versus predicted values were used to evaluate the fit.

STATISTICA (StatSoft Inc., Tulsa, USA) software was used for the statistical analysis and Microsoft Excel to estimate \( T_{\text{MIC}}\text{Min} \). Means are
Danofloxacin against *Mannheimia haemolytica* in a tissue-cage model

given ± s.d. and estimates from *E*<sub>max</sub> models with 95% CIs. A *P* value <0.05 was considered significant.

**Results**

**MIC and MBC**

The MIC and MBC of danofloxacin for the test strain were both equal to 0.04 mg/L, and the calculated MIC<sub>calc</sub>, allowing for protein binding, was 0.06 mg/L.

**Protein binding**

The protein binding of danofloxacin in TCF and serum was concentration-dependent in the range 0.08–0.31 mg/L (Figure 1). Linear regression of protein binding versus concentration in TCF (protein-bound fraction = 0.43 – 0.88 × concentration) was used to estimate the concentration of free drug from the measured total concentrations up to 0.4 mg/L. For danofloxacin concentrations above that, a protein-binding figure of 10% was used.

**Animal experiments**

All tissue cages were sterile before the start of the experiments, and both local and general signs of infection after inoculation were as previously described. Two small cages (one low-dose and one non-treated) in one calf, and one large cage (low dose) in another calf, were excluded because of haemorrhaging. The geometric mean bacterial counts in TCF from all cages at 0.5 h after inoculation were 4.6 ± 0.6 log cfu/mL, increasing to 7.7 ± 0.4 log cfu/mL by the time of treatment. At the end of the experiment (96 h), the geometric mean for all non-treated cages was 7.1 ± 0.6 log cfu/mL.

**PK parameters and PK/PD indices.** The mean *t*<sub>1/2</sub> for the large and small cages following the first dose were 19.9 ± 11.5 h and 6.8 ± 14 h, respectively.

The ranges of *C*<sub>max</sub>/MIC and AUC<sub>24h</sub>/MIC based on free drug concentrations, and of *T*<sub>96>MICtot</sub>, are shown on the x-axis in Figure 2. The variation between individual cages of the same type and dose group was wide. The co-variation between AUC<sub>24h</sub>/MIC and *C*<sub>max</sub>/MIC, and AUC<sub>24h</sub>/MIC and *T*<sub>96>MICtot</sub> was significant (*r* = 0.78 and 0.72, respectively). *C*<sub>max</sub>/MIC and *T*<sub>96>MICtot</sub> were not significantly correlated (*r* = 0.29).

**Predictive PK/PD index.** The relationship between AUBC<sub>48</sub> versus AUC<sub>24h</sub>/MIC was curvilinear, and best described by a second order polynomial equation (adjusted *R*<sup>2</sup> of 0.89). However, there appeared to be differences depending on subject, in that for data from two

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**Figure 1.** Protein-bound fraction of danofloxacin as a function of drug concentration in serum (open squares, broken line) and TCF (solid circles, solid line) The linear fit to data for serum reported by Friis<sup>16</sup> is shown as a dotted line.

**Figure 2.** AUBC<sub>48</sub> versus PK/PD indices of danofloxacin against *M. haemolytica*. The solid line represents fit of an *E*<sub>max</sub> model to pooled data, the broken line is the mean of estimated parameters for data from individual calves (AUC<sub>24h</sub>/MIC only). Solid squares, large tissue cages/low dose; open squares, large cages/ high dose; solid circles, small cages/low dose; open circles, small cages/high dose; triangles, non-treated (control) cages.
calves, the slopes of the regressions differed from the others. Although comparisons between AUBC_{48} and T_{max,MIC} also gave good correlation coefficients (adjusted $R^2 = 0.81$), this value was lower than seen with AUBC_{48} versus AUC_{24h}/MIC. For T_{48h,AUC0-inf}, concentrations were >MIC throughout the dosing interval and this variable was therefore poorly discriminatory and was not considered further. Finally, regression of AUBC_{48} versus $C_{max}$/MIC gave poor correlation ($R^2 < 0.60$).

Adequate fits of the $E_{max}$ model were obtained for AUBC_{48} versus AUC_{24h}/MIC (Figure 2) with values for 50% and 80% of the response of 101 and 244 h, respectively.

When the model was fitted to data from each calf separately, one calf had to be excluded, as the number of datapoints was insufficient to estimate four parameters. For data from three calves, the four-parameter model failed to converge and either maximum or minimum effect was set to the value estimated from pooled data. The fit of the means of the estimated parameters for each of the seven calves is shown in Figure 2.

For AUBC_{48} versus $C_{max}$ or T_{96>MICtot}, the fit of the $E_{max}$ model was poorer than seen with AUBC_{48} versus AUC_{24h}/MIC (Figure 2). When attempts were made to fit the model to data from each calf separately for these indices, the model either did not converge, or yielded nonsensical estimates for one or more of the parameters.

Discussion

With the experimental model described, a wide range of values for the different PK/PD indices could be simulated (Figure 2). However, there was considerable variation between cages of the same size and dose group, which is consistent with earlier observations for other antimicrobials in both infected and uninfected cages. This variation is probably caused by differing amounts of tissue in-growth, leading to different true volumes of the cages and results in a wider range of values of PK/PD indices than would otherwise have been the case, which is an asset in studies aiming to identify and define the PK/PD index predictive of effect.

As it is generally accepted that it is only the free fraction of an antimicrobial that interacts with bacterial targets, PK/PD indices should be calculated from the non-protein-bound fraction of the drug. We therefore calculated the protein binding in TCF, after confirming the concentration-dependent protein binding in calf serum reported earlier by Friis, and demonstrating a similar concentration dependence in TCF.

The PK/PD relationships of different fluoroquinolones have been studied extensively both in vitro and in different animal models. Generally it has been found that the AUC/MIC best predicts the antimicrobial effect. This finding was also true in the present study, where AUC_{24h}/MIC was the PK/PD index that best explained AUBC_{48} as assessed both by linear regression and by an $E_{max}$ model.

In the tissue-cage model, the levels of AUC_{24h}/MIC that produced 50% and 80% of the response were 101 h and 244 h, respectively. Figures of similar magnitude were reported by Forrest et al. from a retrospective study on nosocomial pneumonia treated with ciprofloxacin. For patients where AUC/MIC was $>250$ h, bacterial eradication was more rapid than when this index was 125–250 h. When the AUC/MIC was $<125$ h, the probability of treatment failure was high. Figures of the same order of magnitude for rapid effects have also been reported for other fluoroquinolones from other clinical studies.

Similar or higher figures have also been reported for different combinations of fluoroquinolones and bacterial species, both in in vitro dynamic models and in animal models with either thigh infections or pneumonia. From studies using tissue cages, the AUC/MIC in TCF that produced the greatest rate of bacterial killing was estimated as $>150$ h, although lower figures are reported in some in vitro studies for Streptococcus pneumoniae, but not in others. Lower figures have also been reported from in vitro or ex vivo studies relating specifically to danofloxacin. Differences in study design, such as choice of measure of effect, differences in inoculum at the start of experiments and, in the case of tissue cages, the influence of a foreign body, may explain the different values for near-maximum effect reported in different studies.

The tissue-cage model used in this study is flexible, in that different concentration–time profiles can be simulated, and allows PK/PD interactions to be studied in the presence of the host’s defences. In the present study, the PK/PD index that best predicted the bacterial killing was AUC_{24h}/MIC, in line with findings from other studies. Although the validity of this model needs to be evaluated by clinical studies, the results suggest it may be a useful intermediary step between in vitro studies and clinical trials aiming at drug dosage optimization.

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References

Danofloxacin against Mannheimia haemolytica in a tissue-cage model


