

Tissue Cages in Calves for Studies on Pharmacokinetic/Pharmacodynamic Relationships of Antimicrobials

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Abstract

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Knowledge on pharmacokinetic/pharmacodynamic (PK/PD) relationships of antimicrobials may assist in defining a dose that maximises effect while minimising side effects such as selection of resistance. The aim of this thesis was to develop a model for studies of PK/PD relationships of antimicrobials in presence of the host defences. To this end, the usefulness of subcutaneously implanted tissue cages was explored.

Tissue cages implanted in calves were infected with *Escherichia coli* and the calves were treated iv with different doses of trimethoprim + sulfadoxine. Irrespective of dose, no effect was noted in cages infected before treatment. *In vitro* studies and analysis of thymidine indicate that the effect of trimethoprim was antagonised by thymidine in tissue cage fluid.

When the antimicrobial is administered systemically as above, the pharmacokinetics of the drug in tissue cage fluid is typically sluggish. It was shown that if tissue cages with a constant diffusion area but different volumes are used, and drug is injected directly into the cages, the volume of the cage will determine the elimination rate of the drug. Thereby, by varying the dose and cage-type, a range of concentration-time profiles can be simulated.

To explore the utility of this concept, the effects of penicillin and danofloxacin against infections with *Mannheimia haemolytica* in tissue cages in calves were studied. For penicillin, the area under the curve to minimum inhibitory concentration (MIC) and time above MIC were equally predictive for effect. For danofloxacin, the area under the curve to MIC predicted the effect, and the magnitude needed for near maximum effect was 240-244 h. These results are in line with information for other fluoroquinolone-bacterial combinations.

The advantage of the described model is that different concentration-time profiles can be simulated, and concentrations of drug at the site of infection and bacterial counts can be monitored over time in presence of the host defences. Its validity needs to be challenged by direct comparisons with other models, and by clinical studies. The results suggest it can be a useful intermediary step between *in vitro* studies and clinical trials aiming at drug dosage optimisation.

Keywords: pharmacodynamics, pharmacokinetics, trimethoprim, penicillin, danofloxacin, tissue cages, *Mannheimia*, *Escherichia*

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Appendix

Papers I-IV

This thesis is based on the following studies, which will be referred to by their Roman numerals:

- I Greko, C., Bengtsson, B., Franklin, A., Jacobsson, S.-O., Wiese, B. & Luthman, J. (2002). Efficacy of trimethoprim-sulfadoxine against *Escherichia coli* in a tissue cage model in calves. *Journal of veterinary pharmacology and therapeutics*, **25**, 413-423
- II Bengtsson, B. & Greko, C. (2002). Simulation of concentration-profiles of benzyl-penicillin, enrofloxacin and dihydrostreptomycin in tissue cages in calves. *Journal of veterinary pharmacology and therapeutics*, **25**, 389-392.
- III Greko, C., Finn, M., Öhagen, P., Franklin, A. & Bengtsson, B. A tissue cage model in calves for studies on pharmacokinetic/pharmacodynamic interactions of antimicrobials. *International journal of antimicrobial agents*. In press.
- IV Greko C, Finn M, Franklin A & Bengtsson B. Pharmacokinetic/pharmacodynamic interactions of danofloxacin against *Mannheimia haemolytica* in a tissue cage model in calves. *Manuscript*.

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Abbreviations

AUBC	area under the bacterial counts – time curve (log cfu/mL × h)
AUC	area under the antimicrobial concentration - time curve (mg/L × h)
cfu	colony forming units (of bacteria)
$\Delta\text{cfu}_{\text{max}}$	maximum difference in colony forming units per volume from time 0 (log cfu/mL)
C_{max}	maximum drug concentration (mg/L; $\mu\text{g/ml}$)
E_{max} model	non-linear mathematical model with a minimum and maximum effect level (a dose-response model)
I_E	intensity of effect; measure of antibacterial effect defined as the area between bacterial kill curve including regrowth and growth control curve (log cfu/mL × h)
K_{el}	elimination rate constant
MBC	minimum bactericidal concentration (of an antimicrobial) (mg/L; $\mu\text{g/ml}$)
MHB	Mueller–Hinton Broth
MIC	minimum inhibitory concentration (of an antimicrobial) (mg/L; $\mu\text{g/ml}$); note: free concentration of drug
MIC_{tot}	minimum total concentration needed for inhibition of growth; <i>i.e.</i> including both free and protein bound drug (mg/L; $\mu\text{g/ml}$)
PAE	post antibiotic effect
PD	pharmacodynamics
PK	pharmacokinetics
PME	post MIC effect
SA	surface-area
t	time (h)
$t_{1/2}$	half-life of drug (h)
$T_{>\text{MIC}}$	the time concentrations remain above MIC (% or h)
TCF	tissue cage fluid
V	volume

Background

Fifty years ago, Eagle, Fleichman & Levy (1953) concluded a report on the effect of different dosage intervals of penicillin on therapeutic efficacy with the following paragraph:

‘The data here reported, and the conclusions drawn from them, apply solely to penicillin. With this antibiotic there is a maximally effective concentration, varying from strain to strain, that kills the organisms at the fastest possible rate, and even a ten-thousandfold increase in penicillin concentration beyond this maximally effective level does not further accelerate its bactericidal action. The time-dosage relations discussed above follow directly from this property. With the other antibiotics, the bactericidal effect either increases continuously with the concentration used (as with streptomycin and bacitracin) or is so slow (as with aureomycin, chloramphenicol and terramycin) as to suggest that host factors contribute materially to their therapeutic effect. With streptomycin and bacitracin, the most rapidly effective schedule would, *a priori*, be one providing the maximum concentration, and for the longest continuous period, consistent with the toxicity of the drug. With the latter group of antibiotics the time-dosage relations remain to be determined. Studies on the degree to which the host contributes to their therapeutic action, and the nature of the host effect, are clearly pertinent in this connection.’

Since, the knowledge in this area has increased considerably. The essence of the statement regarding the degree of concentration dependency of different groups of antimicrobials and implications thereof, however, still holds true. The questions discussed in the citation are crucial for antimicrobial therapy: what is the optimal dose and what is the optimal dosage interval?

Pharmacokinetics and pharmacodynamics

The effect of an antimicrobial treatment is the result of a dynamic three-way interaction between the host, the drug, and the infecting organism. Pharmacokinetics (PK) is what the host does to a drug, *i.e.* absorption, distribution, metabolism and elimination, as reflected in the time course of drug concentrations in plasma or tissue. Pharmacodynamics (PD) is the effect of the drug as a function of its concentration. For antimicrobials, the effect is often measured as killing or inhibition of bacteria, but it should be stressed that this is the result of the drug’s interaction with receptors in or on the bacterial cell. The study of PK/PD relationships links the concentration-time profile resulting from pharmacokinetics to the effect of these concentrations on bacteria. Such studies may, or may not, include the third component of interest, the interaction between the host and the pathogen.

Defining the optimal dose

Many of the currently used dosage regimens of antimicrobials are designed to maintain serum levels above the minimum concentration needed to inhibit growth of the target pathogen for the greatest possible proportion of the dosing interval (CPMP, 2000; Lees & Aliabadi, 2002). For penicillin, this is in line with the findings of Eagle, Fleichman & Levy (1953), but for other antimicrobials it

ignores the condition stressed by the authors; different strategies should apply for different classes of antimicrobials.

This early insight has been reinforced by knowledge gained in the last decades on how relevant PK/PD relationships are for dose setting. Even if clinical trials and experience show that a certain dose leads to a high degree of clinical success, this does not automatically mean that the efficacy of the antimicrobial is fully exploited. It may be that another dose or dosage interval would lead to an even higher probability of success (clinical or microbiological), or reduce the risk of adverse effects. Certain dosages of antimicrobials may also increase the risk for selection of resistant bacteria. The importance of optimised antimicrobial therapy has therefore been further emphasised by the need to contain the global increase in resistance among bacteria (WHO, 2001).

Ideally, dose-ranging studies should define the minimum and the maximum effective dose and dose interval. For this, the responses to a variety of doses, ranging from sub-optimal to potentially toxic regimens should be compared (Toutain, del Castillo & Bousquet-Mélou, 2002). It is, however, economically unrealistic and unethical to fully explore this in clinical trials.

Studies on PK/PD relationships of antimicrobials by use of *in vitro* or animal models, or both, may assist in defining the pharmacokinetic parameter that best predicts the effect on specific target organisms, and its critical levels. For example, identification of the ratio of peak concentration and MIC as outcome predictor for aminoglycosides (*e.g.* Moore, Smith & Lietman, 1984) led to a shift from twice daily to once daily dosing for humans (Craig, 1998) and in veterinary medicine (Prescott, Baggot & Walker, 2000). There is also evidence that the likelihood of selection of bacteria with mutations conferring resistance can be predicted on basis of PK/PD relationships (*e.g.* Blaser *et al.*, 1987; Madaras-Kelly *et al.*, 1996; Hyatt & Schentag, 2000).

The optimal dose and dosage interval should maximise effect while minimising the risk for adverse effects such as resistance, toxicity and, for food-producing animals, residues in edible products. Knowledge of different aspects of PK/PD interactions, and of variability within the populations concerned (hosts and target bacteria), can assist the identification of a dose and schedule that has a high likelihood of meeting the criteria for optimum (Drusano *et al.*, 2001). With such an approach, currently used dosages may be challenged. For new drugs, a rational framework for selection of doses to be evaluated in clinical trials is provided.

Basic pharmacodynamic concepts

MIC and MBC

The simplest and most widely used measure of the intrinsic activity of antimicrobials is the minimum inhibitory concentration (MIC). Its determination is usually straightforward; bacteria are exposed to two-fold dilutions of the antimicrobial in broth or agar and, after incubation, the lowest concentration that inhibits visible growth is recorded as the MIC (NCCLS, 1997; EUCAST, 2000). The minimum bactericidal concentration (MBC) is defined as the concentration

that kills 99.9% of the initial inoculum (NCCLS, 1999; EUCAST, 2000). The MIC is influenced by factors such as density of the inoculum, medium, incubation temperature and time, *etc.* Therefore, the use of standardised protocols is imperative (*e.g.* White *et al.*, 2001).

The relevance of MICs for the *in vivo* situation has always been questioned. The standardised conditions of the test (*e.g.* defined inoculum size, artificial media and static concentrations) are truly different from those expected at the site of infection. Nonetheless, clinical and experimental evidence indicate a good correlation between MICs and effect (*e.g.* Schentag, 1991; Craig, 1993; Knudsen, Frimodt-Moller & Espersen, 1995; Preston *et al.*, 1998).

The MIC assumes a pivotal role when it is used to correlate pharmacokinetic parameters to potency, *i.e.* to calculate the PK/PD indices time above MIC ($T_{>MIC}$), area under the drug concentration-time curve to MIC (AUC/MIC) and peak concentration to MIC (C_{max}/MIC) (see below). Two important caveats relating to this must be stressed. First, MICs are generally determined by use of two-fold dilutions of the drug. The true MIC is mostly not the figure determined, but in between the observed MIC and the next lower dilution. The inherent error is one dilution step in either direction. Evidently, this imprecision may have a profound influence for the calculated PK/PD indices (Frimodt-Moller, 2002).

The second caveat relates to the use of MIC₉₀ (*i.e.* the 90th percentile of the MICs in a given material) as denominator to account for variation in MICs in a particular bacterial species. The MICs of a given drug for strains in a population of bacteria where resistance has emerged often assumes a bimodal distribution. In such cases, the MIC₉₀ will increase in a stepwise manner. For example, for *Staphylococcus intermedius*, an increase in the proportion of erythromycin resistant isolates from 9 to 10% would mean an increase of the MIC₉₀ from 1 (or less) to more than 8 mg/L [theoretical example based on MIC distributions given in SVARM 2001 (2002)]. Still, in most cases there would be no true difference in the sampled bacterial population. From this follows that if the MIC₉₀, or the MIC₅₀ (the median value), is used for PK/PD integration, only the wild (inherently susceptible), part of the population should be considered whenever there is a bimodal distribution (CVMP, 2002).

Notwithstanding, the MIC is still a reasonable approximate of the concentration of free drug needed at the site of infection. Prediction of effect based on pharmacokinetic parameters is improved if they are correlated to the MIC of the infecting strain (*e.g.* Drusano *et al.*, 1984; Leggett *et al.*, 1989; Schentag, Nix & Adelman, 1991; Preston *et al.*, 1998).

Time-kill curves

Bacterial time-kill curves are a simple and informative way to obtain basic information on the kinetics of the effect of an antimicrobial. The methodology is similar to the MBC determination procedures; bacteria are exposed to various static concentrations of the antimicrobial, and viable counts are performed at the start and at various times after inoculation (NCCLS, 1999; EUCAST, 2000). From such curves, it can be deduced whether the rate of killing increases with increasing

concentrations, or if the influence of increasing concentrations is marginal above low multiples of the MIC. For a third category, the bacteriostatic drugs, almost no killing is observed.

The concentrations of the antimicrobial in traditional time-kill curve experiments are static, and represent a situation at steady state. Models with variable drug concentrations are an extension of the time-kill curve methodology (see 'Experimental models for PK/PD studies').

Sub-inhibitory concentrations and post antibiotic effects

The MIC is, as defined above, the lowest concentration that inhibits growth but exposure of a bacterium to lower (sub-MIC) concentrations may result in a variety of effects, such as reduced growth rate, and altered morphology, ultrastructure, antigenicity, adherence, and toxin production (Lorian, 1993).

After exposure to concentrations above MIC a delay in regrowth can often be observed, even if the drug is completely removed. Various types of effects have been studied in this respect (for a review see Odenholt, 2001). Of these, the *in vitro* measure post-MIC effect (PME) and the corresponding *in vivo* post antibiotic effect (*in vivo* PAE), are probably the most clinically relevant (den Hollander *et al.*, 1998a; Odenholt, 2001). Both PME and *in vivo* PAE are defined as the time it takes for a culture to increase 1 log cfu/ml from the time when the concentrations of an antimicrobial have declined below MIC, *i.e.* the inhibition of regrowth (Mouton *et al.*, 2002). These effects will most likely be influenced by the time it takes for bacteria to repair sublethal damage caused at supra-MIC concentrations, but above all by sub-MIC effects (Löwdin *et al.*, 1996; den Hollander *et al.*, 1998a).

Drug concentrations of concern

Antimicrobials will bind to different degrees to proteins and other molecules in plasma or other body fluids to different degrees. Only the free (non-bound) fraction of the drug can interact with bacterial receptors (*e.g.* Rolinson, 1980; Odenholt, Holm & Cars, 1987; Garrison *et al.*, 1990). Therefore, it is only the concentration of free drug that is of concern for its PK/PD relationship.

When a drug is administered, it is first absorbed to the blood (unless given *i.v.*), then distributed into tissue. Ideally, the concentration of free drug in the extracellular space at the site of infection should be determined and related to effect (Liu, Muller & Derendorf, 2002). For tissues where there is no barrier to penetration, the level of free drug in serum is an adequate surrogate marker for these concentrations (Cars, 1991).

In veterinary medicine, total tissue concentrations are still frequently reported with the argument that high 'tissue levels' means superior antibacterial activity. This may be grossly misleading (Toutain, del Castillo & Bousquet-Mélou, 2002). Such total concentrations are mostly determined from homogenates of tissue, and represent a mean of concentrations of drug within and outside cells and in capillaries. About 80% of the volume of tissue is the intracellular space, and

therefore total tissue concentrations will largely reflect the degree of drug distribution into cells (Nix *et al.*, 1991). Most infections of interest are, however, located extracellularly and direct comparisons of total tissue concentrations with pharmacodynamic parameters are therefore meaningless (Cars, 1991).

PK/PD relationships – methodological aspects

Most studies on PK/PD relationships of antimicrobials are essentially designed to explain how the concentration-time profile of a drug relates to the magnitude and time course of effect on bacteria. The crux of the matter is how to assess the relation between one dynamic event and another. In the *in vivo* situation, this is further complicated by the fact that a third factor, the host, also influences outcome in a dynamic way. To simplify, the concentration-time curve of the drug is mostly 'reduced' to pharmacokinetic parameters, correlated to a relevant MIC and used as PK/PD indices. Likewise, the bacterial kill-curve can be expressed as different measures of effect.

The PK/PD field has evolved rapidly over the last two decades and a number of different definitions and expressions have been coined. Recently, a proposal of a standardised PK/PD terminology has been presented (Mouton *et al.*, 2002) by which some of the confusion in the area will hopefully be resolved.

PK/PD indices

The three PK/PD indices commonly used reflect different aspects of the concentration-time curve; the time ($T_{>MIC}$), the peak of the curve (C_{max}/MIC), and the integrate of time and concentrations calculated as area under the curve (AUC/MIC) (Figure 1). These indices will co-vary to a large extent. For example, with a given rate of elimination of a drug, and a fixed dosing interval, any increase in C_{max} will also increase the AUC and the $T_{>MIC}$. With a dose escalation and fractionation design, the co-linearity of the indices can be reduced.

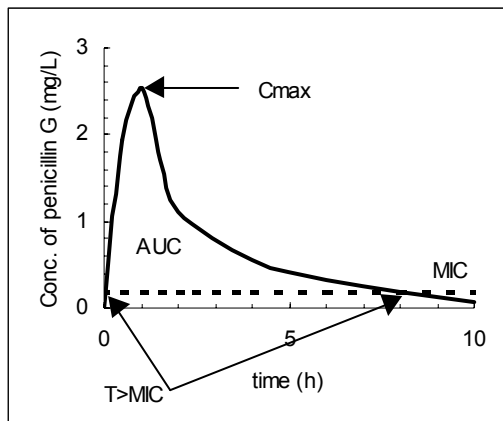


Figure 1. Illustration of the pharmacokinetic parameters that form the basis of common PK/PD indices, and of time above MIC (based on data from Bengtsson *et al.*, 1989; concentration-time profile of penicillin G after intramuscular injection; data from one calf).

Time of exposure

For antimicrobials showing minimal concentration-dependency (*e.g.* penicillins, cephalosporins), the time of exposure ($T_{>MIC}$) will determine the effect. The $T_{>MIC}$ is defined as the percentage of a 24 h period that the free concentration of drug exceeds MIC (Mouton *et al.*, 2002), but is also frequently expressed as the chronological time (h) (Frimodt-Moller, 2002). For drugs with these characteristics, frequent dosing or a slow release formulation may be considered, but little will be gained by increasing the doses.

The applicability of $T_{>MIC}$ for sustained release (long acting) formulations with dosing intervals of 48 h or more must be questioned. In those cases, then $T_{>MIC}$ is 50% the concentrations will be below MIC for 24 h or more. It is probable that this time is sufficient for regrowth to occur. For prediction of dosages of this type of formulations, identification of a maximum time in h below MIC might be of interest.

Degree of exposure

The effect of concentration dependent antimicrobials (*e.g.* aminoglycosides, fluoroquinolones) will depend on the degree of exposure, *i.e.* the concentrations in relation to MIC. The PK/PD indices of interest are C_{max}/MIC and AUC/MIC . The C_{max}/MIC is the ratio between the highest concentration of free drug reached in the compartment of interest and the MIC (Mouton *et al.*, 2002). The AUC/MIC is normally determined from the AUC over 24 h. When other time periods are used, this should be indicated by a subscript (Mouton *et al.*, 2002). The dimension of AUC/MIC is time (h), and it reflects the total exposure. For drugs that depend on C_{max}/MIC for effect, infrequent large doses are probably best, and for those who depend on AUC/MIC various approaches may be equally effective. A high C_{max}/MIC or a large AUC/MIC is probably needed to prevent selection of resistant mutants (Woodnutt, 2000).

The expression AUIC (area under the inhibitory curve) was originally used for the area under curve of the reciprocal serum inhibitory titres versus time (Flaherty *et al.*, 1988). The serum inhibitory titre is determined as the highest titre of the sample that inhibits growth of a chosen bacterial strain (NCCLS, 1999; EUCAST, 2000). The acronym AUIC is sometimes used to designate the AUC above MIC ($AUC > MIC$), or as a synonym to AUC/MIC . While these three indices are conceptually similar, they are not identical, and it has been recommended that AUIC should be reserved for its original definition (Mouton *et al.*, 2002).

There is some controversy as to which of C_{max}/MIC and AUC/MIC that best predicts the effect of, *e.g.*, the fluoroquinolones. Discrimination between these two indices is difficult because of their inherent co-linearity. Further, it is possible that they interact. For example, in a study by Drusano *et al.* (Drusano *et al.*, 1993) the C_{max}/MIC of fluoroquinolones was best linked to survival when this index was above 10, while AUC/MIC was a better predictor when C_{max}/MIC was below 10. Further, in a study by MacGowan *et al.* (MacGowan *et al.*, 2001), the initial slope of the bacterial kill-curve (rate of bacterial kill) was predicted by C_{max}/MIC , while the overall effect was predicted by AUC/MIC .

Outcome parameters, endpoints, or measures of antibacterial effect

The outcome of an antibacterial treatment can be measured clinically (success, survival, specific criteria) or microbiologically (eradication of bacteria, decrease in bacterial density). Lately, emergence, or non-emergence, of resistance has been added to the list. The conclusions in a particular study reached will, to some extent, be tied to the chosen measure of effect.

Most experimental models, and some types of clinical studies, allow for sampling at least before and after treatment. In these cases, the antibacterial effect can be measured directly. Depending on the study design and possibilities of repeated sampling, three different categories of measures may be considered; changes in bacterial counts, time-to-kill and integrated measures reflecting both bacterial counts and time (MacGowan & Bowker, 2002). For an illustration, see Figure 2.

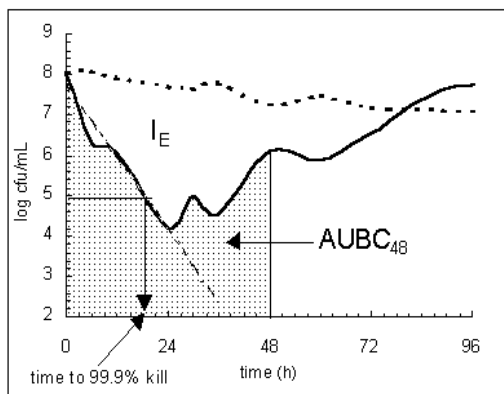


Figure 2. Illustration of measures of antibacterial effect. AUBC is the area under bacterial kill curve (dotted area), I_E is the area between kill-curve and control until regrowth, slope of kill from 0 to 24 h is shown as straight broken line [Data are means of viable counts of *M. haemolytica* in non-treated tissue cages (broken line) and small cages treated with high dose penicillin G (solid line); data from study III].

Change in bacterial counts

The simplest measures of effect reflect the change in bacterial counts from the start of the experiment to a predefined time-point, generally the end of the last dosing interval. When repeated sampling is possible, the maximum reduction in colony forming units ($\Delta\text{cfu}_{\text{max}}$) can also be defined.

Point-measures have the merit of being easy to understand, and are mostly easy to determine. However, they depend on one single measurement and are therefore sensitive to analytical errors (MacGowan *et al.*, 2000). A further disadvantage is that they do not fully reflect the effect before or after the predefined point, which negatively affects their discriminatory power (Firsov *et al.*, 1997; Firsov *et al.*, 1998; MacGowan *et al.*, 2000).

Time to event

Another group of frequently used measures is time-to kill measures. Generally 90, 99 or 99.9% reduction of the initial bacterial counts, *i.e.* a decrease by 1, 2 or 3 log cfu, is used (MacGowan, Rogers & Bowker, 2000). These measures reflect the speed of initial kill, and will probably for concentration-dependent antimicrobials, such as fluoroquinolones, depend on the peak concentrations of the antimicrobial (MacGowan *et al.*, 2001). The time to event measures will to varying degrees

depend on the chosen sampling scheme, as the lowest possible value will be the first sampling time. If killing is more rapid kill at higher concentrations, that will go unnoticed. Further, these measures do not reflect changes after the predefined event.

Bacterial counts and time

The use of measures integrating bacterial counts and time requires a study design that allows for repeated measurement of effect. Measures such as the slope of, or area below, or above, the kill curve are less dependent on individual time-points and are thereby more robust (MacGowan *et al.*, 2000). The area under the kill-curve to different arbitrarily chosen time-points, $AUBC_t$, is by far the most widely used variant. Another area measure is the intensity of effect, I_E , defined by Firsov *et al.* (e.g. Firsov *et al.*, 1997) as the area between the kill-curve and the control curve until complete regrowth.

The I_E will depend on the time it takes for bacteria to start regrowth. This in turn depends on the time during which inhibitory concentrations are maintained in the system ($T_{>MIC}$) plus any post-antibiotic effect, and I_E will thus be influenced by the $t_{1/2}$ of the antimicrobial (MacGowan, Rogers & Bowker, 2000). By contrast, $AUBC_t$ where time is the end of the last dosing interval will for, e.g. fluoroquinolones, depend mainly on AUC/MIC (MacGowan & Bowker, 2002).

In addition to better reproducibility compared with point-measures, integrated measures have the advantage that they always provide a numerical value to the endpoint, while changes in bacterial counts and time-to-kill are unchanged once a maximum is reached (*i.e.* eradication). Disadvantages are the need for repeated sampling and culture, and that area measures are less easy to understand and relate to the clinical situation (MacGowan & Bowker, 2002).

Analysis of data

Methods for relating the drug concentration-time curve to the bacterial kill curve range from entirely descriptive to the use of mathematical PK/PD models. Common objectives for the analysis are to define the PK/PD index that best predicts the effect, and the magnitude of that index that results in a certain degree of effect.

It is likely that, if studied over a sufficient range, the relation of the predictive index to effect is non-linear: At very low exposures, there is no effect. At higher exposures, the effect increases with dose but there is a biological maximum (*i.e.* total eradication of the target bacteria) and when this is reached there is no further benefit in increasing the exposure. Non-linear models used to describe such relations are based on the Hill equation and are often referred to as sigmoid E_{max} models (Holford & Sheiner, 1981). However, there are no easy ways to objectively compare the fit of a non-linear model to different data combinations and such models are not suited to discriminate between the predictive power of different PK/PD indices. Therefore, techniques based on linear regression are commonly employed as a first step. An E_{max} model may then be used to describe the relation between the most predictive index and outcome, and to estimate the

magnitude of the index producing a specified percentage of the maximum effect can be estimated.

As mentioned above, the indices and effect parameters are simplifications of the two curves. More elaborate PK/PD models that truly link the dynamic events would probably be more informative. Such models will, be limited by the assumptions underlying the model (CPMP, 2000). Hitherto, this approach has received limited attention. The utility of models of varying complexity has been explored (Zhi, Nightingale & Quintiliani, 1986; Zhi, Nightingale & Quintiliani, 1988; Mattie *et al.*, 1990; Hishikawa *et al.*, 1991; Li, Nix & Schentag, 1994; Renard *et al.*, 1996; Mattie *et al.*, 1997; Mouton, Vinks & Punt, 1997; Corvaisier *et al.*, 1998; Yano *et al.*, 1998). A further expansion of this area could increase the understanding of the dynamics of the interactions, leading to more refined predictions based on experimental data.

Experimental models for PK/PD studies

Most current knowledge of the PK/PD relationship of antimicrobials derives from a variety of *in vitro* and animal models.

In vitro models

In *in vitro* kinetic models, dilution or diffusion (through dialysis) is used to simulate the clearance of drug from the system (the body) (*e.g.* Grasso *et al.*, 1978; Zinner, Husson & Klastersky, 1980; Toothaker, Welling & Craig, 1982). Disease specific models using fibrin clots or glass beads to simulate endocarditis or device related infections, respectively, have also been described (*e.g.* Vergères & Blaser, 1992; McGrath, Kang & Kaatz, 1994). These *in vitro* kinetic models allow for experiments on the effect of different defined concentration-time profiles of a drug on selected bacterial strains in a controlled and reproducible environment. (MacGowan, Rogers & Bowker, 2000). Different aspects of PK/PD relationships, including selection of resistance, can be studied and critical factors identified.

Results obtained in these models are affected by the same factors as MIC determinations, *e.g.* medium, growth phase of inoculum and its density. Further, model specific factors such as dilution effects and presence of sessile (adherent) bacteria may influence outcomes. Such factors, coupled with varying study designs and analytical procedures probably explain the conflicting results that are sometimes reported (MacGowan, Rogers & Bowker, 2000). Nonetheless, a significant amount of knowledge has been generated through use of *in vitro* models (for a review see MacGowan & Bowker, 2002). A limited number of studies using such models deal specifically with antimicrobial-pathogen combinations of veterinary interest (Koritz, Kilroy & Beville, 1994; Lindencrona, Friis & Jensen, 1999; Lindencrona, Friis & Aarestrup, 2000).

These models are flexible, reproducible and relatively low cost. Thereby, they are excellent tools for studies on most aspects of PK/PD relationships of antimicrobials. They do not, however, account for effects of the interaction between the host and the infecting organism.

Experimental infections in small rodents

Experimental infections in animals have been used extensively to study the pathogenesis of infections. A variety of infection models in small rodents (mice, rats, rabbits or guinea pigs) have also been developed for studies on the pharmacodynamics of antimicrobials (Zak & O'Reilly, 1991; Andes & Craig, 2002a). These models are either general (thigh infections, peritonitis-septicemia) or reflect more specific disease conditions (pneumonia, meningitis, endocarditis, pyelonephritis, abscesses, foreign-body infections etc). Each model has its own limitations, but there are also more general factors that affect the conclusions, such as choice of bacterial strains, inoculum size, time between infection and treatment and dosing regimen (Cleeland & Squires, 1991).

The main shortcoming of these models is the difference in pharmacokinetics between small rodents and man, or larger animals. This may, to some extent, be overcome through dose fractionation or prolongation of the elimination rate through, *e.g.*, renal impairment (Zak & O'Reilly, 1991; Andes & Craig, 2002a). Some other factors are also of concern regarding extrapolation of results to the clinical situation. Treatment is often initiated soon after infection and it has been questioned whether results are representative of therapy of established infections (Barza, 1978; Zak & O'Reilly, 1991). Further, the animals are often rendered neutropenic before infection, which nullifies the effect of the host defences. Finally, the measures of effect used range from bacterial counts over time to survival, depending on the possibilities of repeated sampling. This may in turn may influence the conclusions (Hyatt *et al.*, 1995).

As for the *in vitro* models, conflicting results are probably to a large extent explained by factors discussed above. Still, studies designed specifically to compare results from different animal models (Erlendsdottir *et al.*, 2001), or from an *in vitro* and an animal model (Blaser *et al.*, 1995; den Hollander *et al.*, 1998b; Bonapace *et al.*, 2002) indicate that the results from the different models agree on a general level. The relatively few clinical studies (in man) on PK/PD relationships and dose optimisation also confirm the general findings from both *in vitro* and animal models (for a review see Frimodt-Moller, 2002).

Most research relating to PK/PD relationships of antimicrobials in small rodent models has been focused on antimicrobial therapy of man. One example of a veterinary application of a model in small rodents is a series of studies relating results from a thigh infection model in guinea pigs to pharmacokinetic profiles of different drugs in dogs (Meinen, McClure & Rosin, 1995; Campbell, Bartholow & Rosin, 1996; McClure & Rosin, 1998). Still, conclusions valid for human medicine should to a large extent be valid also for veterinary medicine as the target for the drug is bacteria and not the host.

Models in larger animals, with emphasis on tissue cages

In veterinary medicine, dose-titration studies can often be performed in models of infection in the animal species of direct interest (*e.g.* Yancey *et al.*, 1990; Hibbard *et al.*, 2002). Mostly, the results are only presented in terms of effect *versus* dose. Analysis of data in studies relating pharmacokinetic parameters to effect directly

in the animal host of concern range from descriptive (*e.g.* White, Piercy & Gibbs, 1981; Franklin *et al.*, 1984; Sarasola *et al.*, 2002) to mathematical modeling of results (Renard *et al.*, 1996). One experimental model that has attracted some attention for pharmacodynamic studies is the tissue cage model (Clarke, 1989).

Tissue cages are perforated hollow devices, mostly implanted subcutaneously. After implantation, the cages fill up with a fluid with about half the protein content of serum (Bengtsson, Luthman & Jacobsson, 1984). Such models have been used extensively for studies on pharmacokinetics of antimicrobials in animal species of direct veterinary interest (for a review see Clarke, 1989). Their clinical counterpart has, however, not been clearly defined (Bengtsson, 1990).

Tissue cages can also be used to contain an infection, and can thereby be used to study the pharmacodynamics of antimicrobials. Most studies have been conducted in small rodents (Rylander *et al.*, 1981). The cage in itself represents a foreign-body, and this type of model has been used to study various aspects of device related infections (for a review see Zimmerli, 1993).

The pharmacodynamics of different antimicrobial-pathogen combinations has also been studied in tissue cage models in calves, dogs, and horses (*e.g.* Powers, Varma & Powers, 1984; Wagner *et al.*, 1986; Beadle *et al.*, 1989; Clarke *et al.*, 1989c; McDevitt *et al.*, 1992; Ensink *et al.*, 1996a; Gruet, Richard & Autefage, 1997). The measured effects have generally been related to drug concentrations at the site of infection in a descriptive way. Lately, this type of model has also been used for more explicit studies of PK/PD relationships (Aliabadi & Lees, 2001; Aliabadi & Lees, 2002). In those studies, however, the effect of the drug was measured *ex vivo* through conventional kill curve experiments in samples of tissue cage fluid (TCF) or serum taken at various intervals during treatment.

A model for studies on PK/PD relationships should, ideally, enable a detailed description of the pharmacokinetics of the drug, the pharmacodynamic effect on bacteria, and preferably also of the host contribution to the measured effects. Tissue cage models have the advantage of allowing for repeated sampling, thereby permitting close measurement of the drug concentration time profile at the site of infection and of antibacterial effect, in presence of the host defence (for a review see Clarke, 1989).

Objective

The aim of the studies underlying this thesis was to develop and evaluate a model for studies of PK/PD relationships in presence of the host defences. The model should allow for close determination of the concentration-time curve of the antimicrobial at the site of infection, and of the antibacterial effect. Further, it should be possible to study the effect of a range of PK/PD indices.

Comments on materials and methods

Animals

In study I, crossbred Swedish calves of both sexes were used. The animals were purchased through a livestock-marketing organisation and originated from different herds. In study II, III and IV, male Swedish Red and White breed calves were used. They were acquired from the experimental herd of the Swedish University of Agricultural Sciences, where they were born and reared until delivery. The animals were fed according to Swedish standards, and water was freely available.

Assay of antimicrobial concentrations

In study I, high pressure liquid chromatography was used to determine concentrations of trimethoprim and sulfadoxine in plasma and tissue cage fluid (TCF) while in studies II-IV, bioassays based on agar diffusion were used (Chapin-Robertsson & Edberg, 1991). To minimise the volume of sample needed, a paper disk diffusion assay was used for penicillin in study III. Preliminary studies on danofloxacin determination indicated that this approach would result in an unsatisfactory detection limit, and a standard cut-well technique was used in study II and IV.

In all microbiological drug assays, standards were prepared in pooled uninfected TCF sampled prior to the experiments. Large assay trays (225 × 225 mm) were used. A series of standards was applied to each tray, and concentrations in samples were calculated from the regression equation specific for that tray.

Assay of thymidine concentrations

In study I, thymidine concentrations in calf serum and TCF were analysed using a bioassay described by Nottebrock & Then (1977). This is a variant of the agar well method used for drug assay. In brief, an agar medium free from thymidine but supplemented with trimethoprim is used and *Lactobacillus plantarum* ATCC 8014, susceptible to trimethoprim, is used as test strain. Samples are added to wells cut in the agar, and if a sample contains thymidine, trimethoprim will be antagonised and the test strain can grow over an area proportional to the thymidine concentration. The concentration of thymidine in samples was calculated from a log-linear plot of concentration *versus* zone-diameter of a range of standards.

Bacterial strains, preparation of inoculum and bacterial counts

The bacterial strains used, *Escherichia (E.) coli* M17094/83 (study I) and *Mannheimia (M.) haemolytica* serovar A1, Ab 35/85 (study III and IV), were isolated from cattle with mastitis and pneumonia, respectively. In both cases, the strains were isolated in pure culture and the symptoms described were consistent with disease caused by the respective bacterial species. Confirmatory identification and serotyping of the *M. haemolytica* strain was kindly performed

by the French Agency for Food Safety (Agence Française de Sécurité Sanitaire des Aliments) in Lyon.

E. coli isolated from bovine mastitis are generally serum-resistant, and this was also the case for the strain used in the present experiments. No specific virulence factors have (yet) been associated with *E. coli* from bovine mastitis, and the strain used was not further investigated in this respect.

M. haemolytica (previously *Pasteurella haemolytica*) is a small gram-negative rod belonging to the family Pasteurellaceae, *i.e.* related to the genera *Actinobacillus*, *Pasteurella* and *Haemophilus*. It is an important causative agent of pneumonia in calves, and the natural infection is characterised by a rapid fibrinosuppurative and necrotising response (Ackermann & Brogden, 2000).

In the study with *E. coli* (I), the inoculum for the tissue cage experiments was prepared from a culture in logarithmic growth phase but for the studies with *M. haemolytica* (III and IV) a stationary growth phase culture was used. For *in vitro* studies, use of cultures in logarithmic growth phase is standard as the mode of action of some antimicrobial groups, such as the beta-lactams, depend on dividing cells for effect. However, the transfer of an *in vitro* culture to an *in vivo* setting will inevitably induce a lag-phase during which the bacteria adapt to the new medium and environment. It was therefore assumed that the difference between inoculation with bacteria in logarithmic and stationary growth phase would be at most marginal. A procedure similar to that in studies III and IV was also used by Clarke (1989b) to infect tissue cages with *M. haemolytica*.

The effect of the various treatments was monitored over time by use of viable counts of bacteria using 10-fold dilutions (I, III, IV). Carry-over of antimicrobial from the samples to the culture plates may influence the bacterial counts if the concentrations in samples are high enough to inhibit growth of bacteria on the plates (NCCLS, 1999). In the present studies, the highest measured concentrations of antimicrobial in relation to the MIC of the strain used were in study IV, where the maximum concentrations of danofloxacin were around 3 mg/L. The samples were first diluted 10-fold, and from this, 0.1 ml was cultured. This means that a maximum amount spread over the surface of any agar plate was 0.03 µg. The volume of agar in a plate is about 20 ml, and the MIC of danofloxacin for the strain of *M. haemolytica* used was 0.04 mg/L. The maximum concentration in the plates would then have been less than 1/10 of the MIC of the strain. Thus, carry-over was a negligible problem.

***In vitro* pharmacodynamic studies**

Reproducibility and comparability of MIC and MBC determinations is assured through use of standardised media, methods and quality control strains. In study I, III and IV, internationally recognised standards were followed where appropriate (NCCLS, 1997; NCCLS, 1999).

In study I, results from MIC and MBC determinations as well as killing kinetics of trimethoprim and sulfamethoxazole in standard medium (Mueller-Hinton broth) were compared with results obtained in broth added with different concentrations

of thymidine, bovine serum, or thymidine depleted serum. The purpose of this was to screen for the presence of an antagonist that could influence the results in the *in vivo* experiments.

As mentioned in the section on Background, the inherent error of standard two-fold dilutions for determination of MICs can have a profound influence on the calculated PK/PD indices. To overcome this, arithmetic dilution steps were used to determine MIC and MBC of penicillin and danofloxacin on *M. haemolytica* in study III and IV.

Tissue cages

A previously described model with subcutaneously implanted tissue cages (Bengtsson, Luthman & Jacobsson, 1984) was employed. The cages were of silastic rubber tubing with an internal diameter of 15 mm, perforated at both ends but with a non-perforated mid-section. The inner diameter determines the surface-area where diffusion of antimicrobials can take place and the non-perforated mid-section will define the volume of the cage lumen.

When this type of cage is implanted subcutaneously, highly vascularised tissue grows in and fills the perforated end sections (Bengtsson, 1990). The tissue is initially infiltrated with inflammatory cells but with time becomes more collagen rich. This is paralleled by a gradual change in composition of tissue cage fluid over the first 6 weeks (Bengtsson, Luthman & Jacobsson, 1984). Notably, the concentrations of total protein and albumin decrease to approximately 50% of the serum levels (Bengtsson, 1990). In the present studies, the experiments were performed at 8 to 12 (study I) or 5 to 6 (study II, III and IV) weeks after surgery, *i.e.* at times when it could be assumed that the tissue in each cage was mature and the fluid composition was stable.

The tissue cages were injected or sampled through percutaneous puncture with a 0.6-mm needle and a syringe. To minimise the influence of sampling on elimination of drug from the tissue cages, sample volumes were kept to a minimum in the small cages used in study III and IV. The large cages in the same studies had a volume four times that of the small ones, and sampling was at all times kept proportional to the size of the cages.

Tissue cages with membranes

In study III, tissue cages fitted with semi-permeable membranes were implanted, in addition to the standard cages. The intention was to allow for assessment of the effects of cellular defences on infection and treatment. By the end of the experiments it was obvious that the membranes were often ruptured and unfortunately, this part of the experiment had to be dropped. Still, it was decided to let the data from membrane cages remain in the data set for further analysis as the study aim primarily was to explore its usefulness for studies on PK/PD relationships, and not to conclude on some specific issue.

Pharmacokinetics in tissue cages –theoretical considerations

The distribution of systemically administered antimicrobials into the tissue cage model used has been studied previously (Luthman, Bengtsson & Jacobsson, 1984; Bengtsson, Luthman & Jacobsson, 1986; Luthman & Jacobsson, 1986; Bengtsson *et al.*, 1989; Bengtsson *et al.*, 1991). Irrespective of drug, peak concentrations are lower and delayed compared with those in serum, and the rate of elimination is slower. This is also a typical feature of other tissue cage models (Bergan & Versland, 1978; Carbon *et al.*, 1978; Piercy, 1978; Cars, Henning & Holm, 1981; Stanton, Bywater & Palmer, 1982; Ziv, Wanner & Nicolet, 1982; Short *et al.*, 1987; Clarke *et al.*, 1989a; Walker *et al.*, 1990; Ensink *et al.*, 1996b; McKellar *et al.*, 1999). The distribution kinetics differ markedly from that in synovial fluid, subcutaneous tissue fluid or granuloma pouch fluid (Bengtsson *et al.*, 1989; Bengtsson *et al.*, 1991). Thus, from a pharmacokinetic point of view there is no obvious clinical counterpart to this model.

For studies on PK/PD relationships, it is often desirable to include a wide range of PK/PD indices. The possibility to obtain such ranges in tissue cages after systemic administration of an antimicrobial is limited by the general pharmacokinetics of the drug in the animal, by the dose range that can be safely administered and by the above described sluggishness of the kinetics in the tissue cages.

From a previously elaborated mathematical model linking drug kinetics in serum to kinetics in TCF (Bengtsson, Bredberg & Luthman, 1992), it can be inferred that transfer of a drug to and from tissue cages follows first-order kinetics and is directly proportional to the ratio of the diffusion area (SA) to the volume (V) of the cage. It was hypothesised that if a sufficiently small dose of drug is administered directly into the tissue cage, diffusion will be from TCF to serum only. The elimination of drug from the tissue cages will be a first-order process, and the rate of elimination will then depend on the fraction of unbound drug in TCF, on the permeability coefficient for drug transfer from TCF to serum and on the SA/V ratio. For a given drug, protein binding and permeability will be a constant characteristic, and the elimination will be directly proportional to the SA/V ratio.

Based on the reasoning and findings above, it was postulated that different concentration-time profiles of an antimicrobial can be simulated through injection of drug directly into tissue cages with different SA/V ratios (study II). Such a procedure would increase the flexibility of the model, making it more suitable for PK/PD studies.

General design of tissue cage experiments

Study I

A total of 23 calves were used to study the efficacy of trimethoprim and sulfadoxine on *E. coli* (study I). Two tissue cages in each calf were inoculated with *E. coli*. Twenty-four hours after inoculation, the calves were injected iv. with different doses of trimethoprim + sulfadoxine (Borgal®, 40 mg trimethoprim +

200 mg sulfadoxine per ml, Hoechst). First, the label dose (2.5 + 12.5 mg/kg) was given as a single injection. The preliminary results indicated that there was no observable antibacterial effect, and that concentrations of drug in TCF were below MIC. Therefore, in another group of calves, the dose was increased to 5 + 25mg/kg. Again, no obvious antibacterial effect could be noted. Finally, 7.5 + 37.5 mg/kg (three times the label dose) was given five times with 12-h interval. In three of the calves from this highest dose group, an additional tissue cage on each of the three calves was inoculated at 3 h after administration of the first injection of drug (*i.e.* the treatment was prophylactic). Throughout, untreated but infected calves were kept as controls. Plasma and TCF was sampled at different times for drug assay, and TCF also for viable counts of bacteria.

Study II

Study II was designed to find out whether different concentration-time profiles could be simulated in tissue cages according to the theoretical considerations discussed above. Six calves were implanted with cages of the same diameter, *i.e.* the same surface-area, but with differing volumes. The cages were injected with penicillin G, enrofloxacin and dihydrostreptomycin in sequence, with a washout period in between. Samples were taken for determination of drug concentrations.

Study III

In study III, the concept from study II was used to develop a PK/PD model. Eighteen calves were used for the experiments. In each calf, tissue cages of two different volumes but with the same surface area were infected with *M. haemolytica*. Twenty-four h after infection, each tissue cage was injected with penicillin G to yield one of three different initial concentrations; 1.1, 2.8 and 5.6 times the MIC_{tot} of the strain used for infection. In addition, one cage in each calf was injected with saline and kept as growth control. Only one dose category was used in each calf. Injection of drug or saline was repeated either after 24 h, or for the lowest and highest dose in the small cages, after 12, 24 and 36 h. Samples were taken for determination of drug concentration and bacterial counts.

Study IV

To further evaluate the model from study III, an antimicrobial with a different mode of action, danofloxacin, was chosen. Eight calves were implanted with cages of two different sizes and infected with *M. haemolytica*, as in study III. Two different initial concentrations of danofloxacin were targeted: 13.5 and 27 times the MIC of the strain used for infection. One cage of each size in each of the calves was injected with each of the doses and one cage of each size was kept untreated as control. Injection of drug or saline was repeated after 24 h. Samples were taken for drug concentration assay and bacterial counts.

Measures of effect

In the present studies, various variants of the categories of measures of antibacterial effect that were mentioned in the Background were considered and

explored. For the final analysis, only maximum reduction of bacterial counts ($\Delta\text{cfu}_{\text{max}}$) and the area under the bacterial kill curve (AUBC) to different times were used (I, III, IV).

PK/PD indices

Protein binding

Penicillin (study III)

Previous data indicate that the unbound fraction of penicillin in bovine serum is 0.5 and the concentration of albumin in bovine serum and TCF is 40 and 20 g/L, respectively (Bengtsson, Bredberg & Luthman, 1992). No indications of concentration-dependency were noted in that study. Based on these figures, in study III the MIC of penicillin against *M. haemolytica* determined in Mueller-Hinton broth (reflecting free drug only) was calculated to the total concentration of drug needed for inhibition, MIC_{tot} , as $\text{MIC}_{\text{tot}} = \text{MIC}/f_u$, where f_u is the fraction of unbound penicillin in TCF. The calculated figure was confirmed though MIC determinations in Mueller-Hinton broth added with bovine albumin to a concentration of 20 g/L. The MIC_{tot} was used for calculation of PK/PD indices (see below), and should provide a good estimate of these indices based on free concentrations.

Danofloxacin (study IV)

For danofloxacin (study IV), available information suggested that the free fraction of drug in bovine plasma depends on the concentration of the drug in a clinically relevant range (Friis, 1993). The method used for penicillin (III) could therefore not be applied.

The free fraction of danofloxacin at different concentrations in bovine serum was determined through ultracentrifugation. The results were compared to those reported by Friis (1993) using ultrafiltration, and as similar figures were obtained, it was assumed that adsorption of drug to the filter membrane is negligible. This was also reported for other fluoroquinolones (Okezaki *et al.*, 1989; Zlotos *et al.*, 1998a). The volume of TCF available was insufficient for ultracentrifugation, and ultrafiltration was therefore used to determine the free fraction of danofloxacin in TCF. The results were used to calculate the free concentration of drug from the measured total concentrations.

An MIC_{tot} for danofloxacin against *M. haemolytica* was also calculated as described for penicillin.

Calculation of PK/PD indices

Pharmacokinetic parameters were calculated for each calf or tissue cage using standard non-compartmental techniques on basis of measured total drug concentrations (I-IV).

As the drug is injected directly into the cages the true maximum concentration of drug in TCF is C_0 . However, in study III C_{max} was taken as the highest measured concentration. In study IV, C_{max} was taken as C_0 estimated through backward

extrapolation of the slope of the concentration-time curve. For further analysis, the mean C_{\max} from the different dosage intervals was used.

The recommended standard measure for PK/PD integration is AUC_{24} (Mouton *et al.*, 2002). Thus, for reasons of comparability the average AUC_{24} , *i.e.* $AUC_{48/2}$, was used as one of the explored indices.

For penicillin (III) MIC_{tot} was used as denominator to estimate AUC/MIC and C_{\max}/MIC based on free drug. For danofloxacin (IV), AUC and C_{\max} were calculated from estimated free concentrations, and indexed with the MIC .

In study I, $T_{>MIC}$ was calculated directly from data. In study III and IV this index was estimated assuming first-order kinetics. The model $C_t = C_0 \times e^{-K_{el}t}$, where C_0 = dose/volume, K_{el} is the elimination rate constant and t is time, was fitted to the measured concentrations of penicillin or danofloxacin. From the resulting fit, the total time that the concentration exceeded MIC_{tot} , or multiples of MIC_{tot} , in each cage was estimated. The results were compared to corresponding estimates made visually from the plots of concentration-time curves. When an estimate based on the regression deviated notably from figures calculated directly from these curves, it was assumed that the fit of the model was poor and the visual estimate was chosen for analysis.

Analysis of data

To analyse possible differences between groups, analysis of variance with a general linear model procedure was used. The main issue assessed was the influence of dose and cage-size on $t_{1/2}$ in the tissue cages (II, III, IV).

In study III and IV, data from different cages on an individual calf are not statistically independent. Data were nonetheless pooled for analysis, as the primary objective was to assess the usefulness of the model for studies PK/PD relationships. To assess whether this influenced outcomes, different procedures were used in study III and IV (for details see study III and IV).

The relation between measures of effect and different variants of PK/PD indices were first explored through inspection of plots. Data from non-treated cages were included with PK/PD indices set to 0. In both studies, the relation between the AUC_t to AUC_t/MIC group of indices appeared curvilinear. A logarithmic transform of AUC/MIC is commonly used in analyses of this type, but for this data from the control cages would have to be excluded. As second order polynomial models seemed to fit reasonably well, both simple and polynomial regressions were fitted to the data sets. The strength of the relations between measures of effect and different indices were evaluated by their adjusted coefficient of determination (adjusted R^2) and inspection of plots of residuals. Best subset regression for effect parameters *versus* the indices and their quadratic terms was used to explore the influence of multiple predictors.

The PK/PD indices that were best correlated with the effect parameter in the analyses described above were selected for further analysis through non-linear regression with an inhibitory four-parameter E_{\max} model (Holford & Sheiner, 1981). The fit of the model was assessed by the biological relevance of the

estimated constants (*i.e.* whether they made sense), the confidence intervals, inspection of plots observed *versus* predicted and of residuals.

Results and discussion

Infections in tissue cages (I, III, IV)

Growth of bacteria in non-treated tissue cages

Immediately after inoculation, the geometric means for bacterial counts in the tissue cages were close to the intended concentrations (Table 1). For comparison, unpublished data on growth of *Streptococcus (S.) dysgalactiae* and *Staphylococcus (S.) aureus* in tissue cages are also shown. The counts of *M. haemolytica* (III, IV) increased by more than 3 log cfu/mL over the first 24 h (Table 1). Thereafter, the numbers decreased slowly to about 7 log cfu/mL over time. Clarke *et al.* (1989c) observed similar growth patterns for *M. haemolytica* in another subcutaneous tissue cage type in calves. By contrast, for *E. coli* (I), the mean bacterial counts in non-treated cages were almost unchanged to the end of the experiment. In individual cages, the counts never exceeded 7 log cfu/mL. This is lower than what has been reported for *E. coli* in tissue cage models in other animals (Wagner *et al.*, 1986; Widmer *et al.*, 1991). An apparently limited growth rate was also observed for *S. dysgalactiae* and *S. aureus* (Table 1).

Table 1. Bacterial counts (log cfu/mL) in non-treated tissue cages infected with different bacterial strains (geometric means \pm standard deviation)

Bacterial species (study)	No. ¹	Sampling time (h) ²		
		-24 ³	0	96
<i>M. haemolytica</i> (III)	28	4.6 \pm 0.7	8.1 \pm 0.3	7.0 \pm 0.6
<i>M. haemolytica</i> (IV)	15	4.6 \pm 0.7	7.7 \pm 0.6	7.2 \pm 0.7
<i>E. coli</i> (I)	11	5.2 \pm 0.5	5.8 \pm 0.8	5.6 \pm 0.6
<i>S. aureus</i> (unpubl.) ⁴	5	4.6 \pm 0.4	5.1 \pm 0.8	6.3 \pm 1.6
<i>S. dysgalactiae</i> (unpubl.) ⁴	11	4.7 \pm 0.5	6.3 \pm 0.9	5.4 \pm 1.0

¹Number of samples included; ² -24 h is immediately after inoculation; ³ Target concentration for inoculation was 4.8 for *M. haemolytica* and *S. aureus* and 5.7 for *E. coli* and *S. dysgalactiae*; ⁴ Unpublished data, same type of tissue cages implanted in calves of similar age and breed inoculated with *S. aureus* or *S. dysgalactiae*. Both strains were isolated at the National Veterinary Institute, Uppsala, from bovine mastitis.

In Figure 3, the bacterial counts over the first 24 h for the strains of *E. coli* and *M. haemolytica* in tissue cages are compared with data from *in vitro* studies in Mueller-Hinton broth and broth supplemented with 40 or 50% heat inactivated bovine serum. The generation times of the two strains were estimated from the slope of the line between bacterial counts immediately after inoculation and 8 h later. For tissue cages, no data were available between these times and it should be noted that the estimates therefore include a potential lag-phase. The mean generation time of *E. coli* in tissue cages was negative over the first 8 h (-143 min), while in broth and broth with serum it was 26 min and 46 min, respectively. If the generation time is measured from 4 to 8 h, *i.e.* without lag-phase, it is similar in serum-supplemented broth and in standard broth, as shown in Figure 3.

Growth of *E. coli* is apparently well supported in 40% serum. As this medium is similar to uninfected TCF, the limited growth of *E. coli* in tissue cages was

probably caused by factors other than medium. The *in vitro* figures were obtained from cultures grown aerobically, but in the tissue cages the oxygen tension will be reduced. However, as *E. coli* is facultative in its oxygen requirements, it is more likely that other factors, e.g. the host defence, played a role.

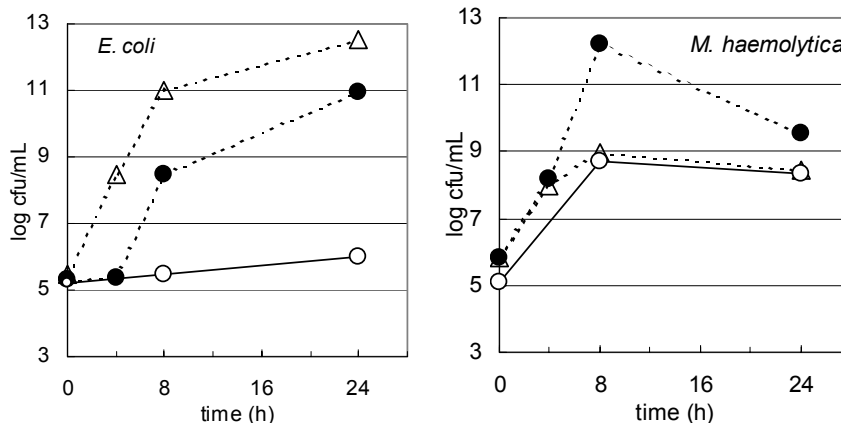


Figure 3. Viable counts of bacteria *in vitro* in Mueller-Hinton (triangles), Mueller-Hinton with serum (filled circles) and *in vivo* in tissue cages (open circles) after different times. Data from study I (*E. coli*), study III (*M. haemolytica* in tissue cages) and unpublished results (*M. haemolytica* *in vitro*).

For *M. haemolytica* the generation times in tissue cages, broth and broth with serum were 41, 46 and 23 min, respectively. It appears that this species, or strain, is well adapted to growth in this environment, and that host factors were initially inefficient in limiting the growth.

Host responses

In study I, the calves reacted to the *E. coli* infection with a mild to moderate fever (39.6 - 40.5 °C). By 24 h after inoculation, swelling around the cages was apparent. From the third day post-infection, the body temperatures were normalised. Thus, the reaction to the infection was mild.

In a separate unpublished study, the local inflammatory response in tissue cages in calves infected with *E. coli* was monitored through cell counts and determination of prostaglandin E₂ (PGE₂) concentrations (Table 2). Leukocyte counts in tissue cage fluid before infection was about 1/10 of that in blood. After infection, a numerical increase in mean cell counts could be noted, but the response was very variable between calves. The concentration of PGE₂ in TCF increased markedly after infection. Prostaglandins are released in tissue as a part of the inflammatory response, and the observed increases are consistent with those reported for experimental infections with *M. haemolytica* and *Streptococcus uberis* in intraperitoneally implanted dialysis sacs in calves (Thomas, Haddock & Lees, 1997). Similar increases of PGE₂ in tissue cages in calves have also been reported for inflammatory agents such as endotoxin (Luthman *et al.*, 1988) and carrageenan (e.g. Landoni, Cunningham & Lees, 1995).

Table 2. *Bacterial counts, leukocytes and concentration of PGE₂ in tissue cage fluid after infection with E. coli (means from 6 calves ± standard deviation)¹*

Time ² (h)	Bacterial counts (log cfu/mL)	Leukocytes (×10 ⁹ /L)	PGE ₂ (nmol/L)
0	4.4 ±0.7	0.6 ±0.3	<0.5
6	4.0 ±0.8	1.0 ±0.9	8.7 ±11.2
12	5.2 ±1.3	4.2 ±3.9	85.6 ±122.3
24	5.3 ±0.8	4.4 ±4.1	112.0 ±115.4
30	5.8 ±1.2	5.0 ±4.5	156.5 ±104.3
36	6.1 ±1.0	4.1 ±2.9	189.1 ±128.0
48	5.9 ±1.1	1.9 ±1.3	144.6 ±116.3
72	5.9 ±1.2	5.2 ±3.6	110.1 ±116.5

¹ Unpublished data. Infection, sampling and viable counts of bacteria were performed as in study I. Leukocytes were counted in a Bürker chamber and prostaglandin E₂ (PGE₂) was measured as described in Luthman *et al.* (1988); ²Inoculated at time = 0 h.

In calves with tissue cages infected with *M. haemolytica* (III and IV), the general signs of infection in terms of fever and haematological changes were more pronounced compared with that of calves with *E. coli*. The swelling around the cages was more marked, and the body temperature mostly remained elevated throughout the experiments. Clarke *et al.* (1989c) reported transient mild fever when *M. haemolytica* was used to infect tissue cages in calves. Also, the local signs of inflammation were less intense than in the present study, indicating that the strain used in the present studies was more virulent.

In study III, efforts were made to follow the concentration of cells in TCF (unpublished). An example of cell counts from untreated cages in individual calves is shown in Table 3. Cell counts were variable and within 24 h after infection, aggregates of cells were often observed. Following infection, many cells had a swollen, foamy appearance. These cells did not stain with Trypan blue, which indicates that they were still viable. A similar morphology of leukocytes exposed to *M. haemolytica* in tissue cages has been described previously (Clarke, Confer & Mosier, 1998), and was ascribed to the membrane damage caused by the leukotoxin of *M. haemolytica*.

Table 3. *Examples of total leukocyte counts in tissue cage fluid from individual calves after infection with M. haemolytica¹*

Time (h) ²	Leukocytes × 10 ⁹ /L (% non viable)			
	Calf 76	Calf 77	Calf 78	Calf 81
0	0.53 (19)	0.48 (16)	1.14 (8)	1.90 (7)
4	0.11 (56)	0.56 (0)	2.24 (14)	1.34 (6)
10	0.23 (27)	0.67 (7)	0.39 (24)	0.56 (20)
16	0.04 (29)	0.86 (4)	0.62 (15)	1.09 (21)
24	0.29 (35)	0.58 (16)	0.75 (36)	0.61 (40)
28	3.18 (4)	0.71 (1)	1.51 (28)	-

¹ Leukocytes were counted in Bürker chambers after staining with Trypan blue. Stained cells were counted as non-viable; ² time = 0 h is time of infection.

Implications for the model

The results discussed above show that infections with different bacterial species are easily established in the tissue cages. Growth of *M. haemolytica* in non-treated tissue cages was very similar in study III and IV, indicating that the time-course of bacterial counts is reproducible. The maximum bacterial counts reached will, however, differ between bacterial species. The infections in the tissue cages elicit an inflammatory response from the host, as indicated by both systemic and local signs. Thus, bacterial growth in the tissue cages is subject to a dynamic response from the host, which opens for possibilities to monitor pharmacodynamics of antimicrobials in a realistic environment.

The main limitation of the model with respect to infection and host defences is that the tissue cage will act as a foreign body (Zimmerli, 1993). In the presence of foreign material, host defence mechanisms will be impaired (Zimmerli *et al.*, 1982; Zimmerli, Lew & Waldvogel, 1984). Further, bacteria can adhere to the surface to form biofilms (Cheng, Irwin & Costerton, 1981; Gristina *et al.*, 1987; Widmer *et al.*, 1991). This should be borne in mind if comparisons between efficacy of different antimicrobial classes are attempted, as the model is likely to favour antimicrobials that are able to kill slowly growing bacteria.

Measures of effect (I, III, IV)

For many infections, bacterial eradication will correlate to clinical success (Dagan *et al.*, 2001; Toutain, del Castillo & Bousquet-Mélou, 2002). Eradication will reduce the risk for selection and spread of resistance (Dagan *et al.*, 2001), at least when only the target pathogen is considered. Thus, direct measures of antibacterial effect (*i.e.* bacterial killing) are likely to predict clinical success. For a discussion on advantages and disadvantages of different measures of antibacterial effect, see the section Background.

Table 4. Coefficient of variation (%) of measures of bacterial growth for non-treated tissue cages in study I, III and IV

Effect measure ¹	Study		
	I (No. = 11)	III (No. = 28)	IV (No. = 15)
$\Delta\text{cfu}_{\text{max}}$	67	58	92
Δcfu_{48}	224	73	111
Δcfu_{96}	590	70	112
AUBC ₀	15	5	9
AUBC ₂₄	10	3	4
AUBC ₉₆	9	6	4

¹ $\Delta\text{cfu}_{\text{max}}$, maximum difference from $t=0$ h in bacterial counts; Δcfu_{48} , Δcfu_{96} , difference in bacterial counts from $t=0$ to $t=48$ and 96 h, respectively; AUBC₀, area under the bacterial curve from inoculation, $t=-24$ h until treatment, $t=0$ h; AUBC₂₄, AUBC₉₆ = area under the bacterial curve from $t=0$ to $t=24$ and 96 h, respectively.

In the present studies, bacterial counts were followed over time, resulting in individual growth or kill curves for each tissue cage. Initially, different variants of the above mentioned categories of measures of effect were explored. In Table 4, the coefficients of variation, CV, of some of these measures for non-treated tissue cages are shown (data from I, III and IV). The variability of the area measures (AUBC_t) was much less than that for the point-measures (variants of Δcfu). This is well in line with the findings of MacGowan *et al.* (MacGowan *et al.*, 2000), who studied the within strain reproducibility of different measures of antibacterial effect in an in vitro pharmacodynamic model.

In study III and IV the correlation of AUBC_t to PK/PD indices was stronger than for the point-measures. As an example, plots of AUBC₉₆ or $\Delta\text{cfu}_{\text{max}}$ versus AUC_{24m}/MIC are shown in Figure 4 (data from IV). A better correlation between area measures of effect and PK/PD indices than point-measures was also described by Firsov *et al.* (1997).

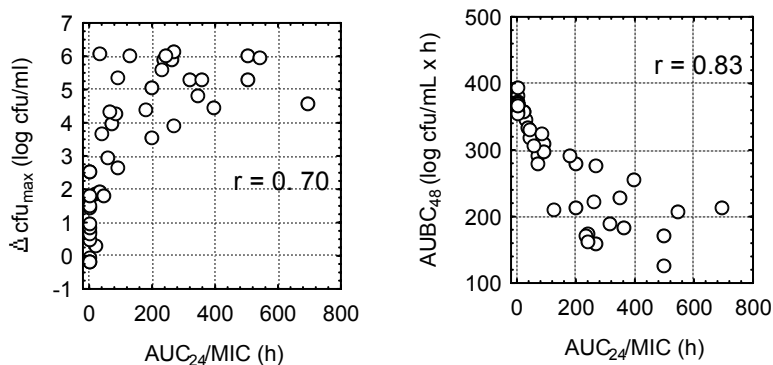


Figure 4. Examples of correlation between effect parameters and PK/PD indices for danofloxacin against *M. haemolytica*; plots of $\Delta\text{cfu}_{\text{max}}$ and AUBC_{96} versus $\text{AUC}_{24}/\text{MIC}$ (data from study IV).

Based on the above, AUBC_t was used as main effect measure for analysis of PK/PD relationships. In study III and IV, AUBC₄₈ reflects the total effect during treatment. To account for possible effects beyond that time, AUBC₉₆ was also used. With that measure, any regrowth before the end of the experiment will be included. Similar to I_E , the later part of that area will probably be influenced by the $T_{>\text{MIC}}$.

Systemic treatment with trimethoprim and sulfadoxine (I)

Dosage recommendations for parenteral trimethoprim + sulfonamide combinations to cattle vary from 2.5 + 12.5 mg/kg bodyweight once daily (label dose in Sweden and many other countries) to two, or even three times that dose twice daily (Prescott, 2000). The selection of dose for the combination is complicated as for cattle, the half-life in plasma is markedly shorter for trimethoprim than for currently used sulfonamides (Prescott, 2000).

The lowest of the doses above agrees with the findings in a classical dose titration study (White, Piercy & Gibbs, 1981), where a dose of 2 + 10 mg/kg of trimethoprim + sulfadiazine given iv once daily for five days clearly prevented mortality from experimentally induced salmonellosis in week-old calves. However, treatment of experimentally induced *E. coli* mastitis in lactating cows with the same drugs dosed 7.5 + 37.5 mg/kg every 12 h four times offered no advantage over no treatment (Pyörälä *et al.*, 1994). Data on distribution of trimethoprim to milk in healthy udders indicate that, with the doses used, concentrations slightly above the MIC of the strain used were maintained for 50-95 % of the dosage intervals (Kaartinen *et al.*, 1999). Still, mastitis in cattle is among the authorised indications for the label dose of these combinations.

Study I was conceived with the above in mind. Tissue cages were infected with *E. coli* and, 24 h later, the calves were treated with different doses of trimethoprim + sulfadoxine iv. No effect of treatment could be observed, even when a dose corresponding to three times the label dose was given five times with a 12-h interval. In a subset of the calves in this dose-group, an additional tissue cage was infected 3 h after initiation of treatment. The AUBC to 48 and 96 h in each of these cages from the treated calves was lower than in the corresponding cages from non-treated animals, but none of the cages was cleared from the infection.

PK/PD relationships

The PK/PD index that determines the effect of trimethoprim-sulfonamide combinations has not been fully elucidated, but some deductions can be made from knowledge on the mode of action. Trimethoprim and sulfonamides inhibit the tetrahydrofolate-synthesis pathway of many prokaryotes and some protozoa (for a review, see Burman, 1986a). Sulfonamides compete with *p*-aminobenzoate (PABA) for the enzyme dihydropteroase, and trimethoprim competitively inhibits dihydrofolate reductase, an enzyme later in the pathway. The bacterial cell requires tetrahydrofolates for synthesis of purines and certain amino acids, and a block in the pathway leads, amongst other things, to a shortage of thymine. If protein synthesis continues, this leads to death of the bacterial cell. This bactericidal effect, however, is at best only noted for trimethoprim and for the weakly synergistic combination of trimethoprim and sulfonamides (Burman, 1986b). A paradox effect is often observed, which means that when static concentrations approach 10 times the MIC, the cidal effect wanes (Angehrn & Then, 1973). Taken together, it is probable that concentrations of trimethoprim and sulfonamides must be maintained above MIC for a certain time to produce a bactericidal effect. High concentrations do not seem to offer an advantage, rather the opposite. In other words, the clinical effect most probably depends on the time above MIC.

In the highest dose group in study I, the mean $T_{>MIC}$ (MIC of trimethoprim when tested in combination with sulfadoxine) in the tissue cages was 94 to 101 h. This figure was calculated from the total concentrations of drug in the tissue cages, *i.e.* including the non-active protein bound fraction. The protein binding of trimethoprim in bovine serum is around 57% (FASS VET., 2002). From this, the MIC_{tot} (total concentration in TCF of drug needed for effect) of trimethoprim for

the strain used can be calculated to 0.20 mg/L (calculated as for penicillin, see 'Comments on materials and methods'). This is close to twice the MIC of free drug, and $T_{>2\times MIC}$ (86 to 94 h) therefore serves as a good estimate of the time during which active concentrations were maintained. It is unlikely that this time was too short for any observable effect to occur.

In vitro, static concentrations of similar magnitude as those maintained during this time produced a marked bactericidal effect in standard media (Figure 5). It is therefore not probable that a paradox effect due to too high concentrations led to loss of the cidal effect of the drug combination *in vivo*.

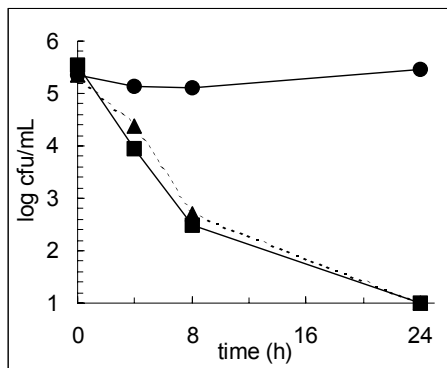


Figure 5. Effect of 1+20 mg/L of trimethoprim+sulfamethoxazole against *E. coli* in Mueller-Hinton broth (triangles), 40 % bovine serum (circles) and 40 % thymidine depleted bovine serum (squares).

The failure to clear infection from the cages inoculated after initiation of treatment can be explained by the poor ability of trimethoprim-sulfonamide combinations to kill adherent bacteria, as suggested by Widmer (1991). However, this does not fully account for the lack of measurable effect in tissue cages infected 24 h before treatment, as at least some reduction of planctonic, *i.e.* non-adherent, bacteria would be expected. Thus, explanations for the treatment failure must be sought elsewhere.

Antagonistic effect of thymidine

Exogenous thymidine can be used by many bacterial species to bypass the inhibitory effect of trimethoprim (Burman, 1986b). It has long been known that the activity of trimethoprim *in vitro* is inversely correlated to thymidine concentrations in the medium (Koch & Burchall, 1971; Dornbusch, 1976). Therefore, the effect of trimethoprim - sulfamethoxazole in bovine serum against the *E. coli* strain was compared with the effect in thymidine depleted serum (I). In the latter medium, the effect was bactericidal and equal to that in standard Mueller-Hinton broth while in normal serum, this bactericidal effect was inhibited (Figure 5). This shows that the concentration of thymidine in normal bovine serum is high enough to antagonise the effect of trimethoprim. Addition of a concentration of 0.08 mg/L of thymidine to Mueller-Hinton broth increased the MBC of trimethoprim-sulfamethoxazole for *E. coli* one dilution step, and 0.25 completely abolished the cidal effect (I). The concentration of thymidine in serum and uninfected TCF from 10 calves ranged from 0.2 to 0.5 and 0.7 to 4.0 mg/L, respectively, which is in agreement with previously published data on calf serum (Nottebrock & Then, 1977) and bovine exudates and tissues (Indiveri & Hirsh,

1992). These concentrations are clearly sufficient to inhibit the activity of trimethoprim-sulfonamide combinations, at least *in vitro*.

Clarke *et al.* (1989c) studied the effect of trimethoprim + sulfadiazine at a dose of 5 + 25 mg/kg body-weight once daily for five days on *M. haemolytica* in a tissue cage model in calves. By 48 h, the bacterial counts in treated cages had decreased by about 2 log cfu/mL and at the end of treatment (120 h), they were about 4 log cfu/mL lower than the initial values. This pronounced decrease seemingly contradicts the hypothesis that thymidine levels in bovine TCF are high enough to antagonise the effect of trimethoprim. However, the ability of *M. haemolytica* and *E. coli* to utilise thymidine as an exogenous source may differ. Of greater importance is probably that the growth of *M. haemolytica* in tissue cages differed markedly from that of *E. coli*, and the response of the calves to the infection was more intense (III, IV; see 'Infection in tissue cages' above). It is therefore likely that host responses contributed to a greater extent to the observed effect in the cited study by Clarke *et al.* (1989c).

Taken together, these findings suggest that trimethoprim was antagonised by high thymidine concentrations in TCF which explains why no bactericidal effect was observed even when the drug concentrations were above MIC for around 90 h.

Clinical implications

The possible implications of extracellular levels of thymidine in tissues and body fluids for the clinical efficacy of trimethoprim in man has been the subject of debate (for a review see Burman, 1986b). Levels of thymidine in serum and urine from healthy humans are low (Nottebrock & Then, 1977), but higher levels have occasionally been reported for infected urine (Stokes & Lacey, 1978). Reports on thymidine requiring mutants isolated from infected sites provide indirect evidence of occasional antagonistic effects *in vivo* are (eg. Koch & Burchall, 1971; Stokes & Lacey, 1978; Platt, Guthrie & Langan, 1983).

Regarding animals, Nottebrock & Then (1977) reported widely differing concentrations of thymidine in serum from different species, with cattle, mice and rats having by far the highest levels. These authors also showed that infections, both bacterial and viral, further increased the levels in experimentally infected mice. Then (1993) argued that studies on potency of trimethoprim in mice always resulted in high 50% effective doses (ED₅₀), which might be explained by the high thymidine levels of that animal species. Indiveri & Hirsch (1992) reported high levels of thymidine in fluids and exudates from infected sites of various animal species, and the authors cautioned against use of trimethoprim - sulfonamide combinations for treatment of infections where anaerobic bacteria play a role.

More recently, the issue of *in vivo* antagonistic effects of thymidine was addressed in an elegant series of experiments (Tokunaga *et al.*, 1997). A thymidine kinase-deficient mutant, incapable of utilising exogenous thymidine, was derived from an *E. coli* strain with known murine virulence. The mutant and the parent strain were used to produce systemic infections in mice, and the effects of treatments with trimethoprim and other antimicrobials were compared. The

ED₅₀ of trimethoprim was more than 20 times higher for infections with the parent strain (capable of using exogenous thymidine) than for the mutant, while no differences in doses required for effect were observed for the other antimicrobials tested, including sulfamethoxazole. This clearly demonstrates that the activity of trimethoprim is inhibited by thymidine *in vivo* in a manner similar to that observed *in vitro*.

The above highlights the need for caution when extrapolating results from studies in one animal species to another, and further support the conclusion that the lack of observed effect in study I was related by high levels of thymidine in TCF. This suggests that deep-seated purulent infections in cattle (*e.g.* abscesses) are refractory to treatment with trimethoprim - sulfonamides. The influence of the naturally high thymidine levels of cattle on the effect of trimethoprim-sulfonamides when used for other indications remains to be investigated.

Simulation of concentration-time profiles in tissue cages (II-IV)

Studies on PK/PD relationships often aim to determine the PK/PD index that best predicts the effect, and the magnitude of this index needed for a certain outcome. The analysis of data depends on regression techniques, and it is desirable that the range of PK/PD indices studied is as wide as possible.

In study I, the observed $t_{1/2}$ for trimethoprim in serum and uninfected TCF was 1.2 ± 0.3 h and 14.1 ± 4.7 h, respectively. This exemplifies the sluggish pharmacokinetics that is typical of tissue cages (see Comments on materials and methods). After systemic administration of a drug, the concentration-time curve will be flat, and the possibility to vary the PK/PD indices will be limited.

To increase the usefulness of the model for PK/PD studies, a way was sought to circumvent this problem. On basis of a theoretical reasoning (see “Comments on materials and methods”), it was hypothesised that if tissue cages of different sizes are used, and drug is injected directly into these, it would be possible to simulate different concentration-time profiles of the drug. This was confirmed in study II, where penicillin, enrofloxacin or dihydrostreptomycin were injected into tissue cages with different SA/V ratios. As predicted, the elimination rate of the drugs from TCF was proportional to the SA/V ratio of the cages. Distinctly different concentration-time profiles could be simulated, but the variation in pharmacokinetic parameters between individual cages of each cage-size and dose-group was wide in uninfected (II) as well as infected tissue cages (III, IV). This is at least partly explained by different amount of tissue in-growth in the cages, which implies that the true SA/V ratio will vary and that each cage is unique. Nonetheless, the means of $t_{1/2}$ differed significantly between the different cage-sizes, but not between drugs (II) or between different doses of the same drug (III, IV).

When fluid is removed through sampling, drug is also removed from the tissue cage. This will influence the elimination rate to varying degrees depending on the volume of the cage, the sample size and the sampling frequency. For study II, the elimination induced by sampling was estimated assuming that the tissue cages fill up with fluid after each sampling. The calculated impact on $t_{1/2}$ was marginal, but

for further studies it was decided to keep sample volumes to a minimum and proportional to the volume of the cages used.

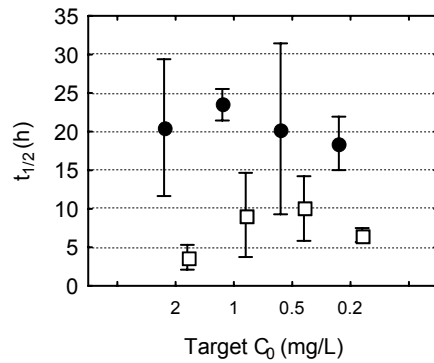


Figure 6. Mean $t_{1/2}$ of penicillin from small (squares) and large (circles) tissue cages following different target initial concentrations (C_0). Data are from study II (2 mg/L; uninfected cages, one dose only) and III (0.2, 0.5 and 1 mg/L; infected cages, multiple doses, $t_{1/2}$ calculated after last dose). Vertical bars indicate standard deviation.

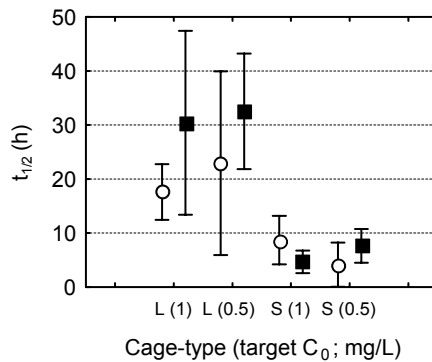


Figure 7. Mean $t_{1/2}$ of danofloxacin during first (circles) and second (squares) dosage interval in different cage-types (L= large; S= small) following different target concentrations (C_0). Data are from study IV. Vertical bars indicate standard deviation.

Infection may influence vascular supply and other factors that determine the elimination rate from the tissue cages (Ryan, 1993). The means of $t_{1/2}$ for different doses of penicillin injected into uninfected (II) or infected (III) tissue cages are compared in Figure 6. These means were calculated after a single dose in study II and after the last of multiple doses in study III and IV. The $t_{1/2}$ in infected small cages was conspicuously longer than in uninfected cages, but not so for the large cages. In study IV, the opposite was noted. The $t_{1/2}$ of danofloxacin was longer in the infected large cages, but not in the small, compared to what was expected for uninfected cages based on data for enrofloxacin in study II (Figure 7).

It is probable that in both studies (III, IV) the diffusion area of the tissue cages was reduced by tissue damage caused by the infection, resulting in slower elimination of drug. However, the influence of an infection on $t_{1/2}$ is expected to affect both cage-sizes similarly. The number of observations for each dose and cage-type combination is small, and the standard deviation of $t_{1/2}$ was wide. It is therefore likely that these seemingly conflicting results simply reflect a variation around the true means.

To assess whether the infection leads to a gradually decreased elimination rate, the $t_{1/2}$ of danofloxacin during the first and second (last) dosage intervals in study

IV were compared (Figure 7). The number of data-points for the first interval was insufficient to analyse data within calf, but a repeated measures analysis of variance was performed with cage-size and dose as between groups factors, and $t_{1/2}$ as repeated measure.

As previously, there was a significant effect of cage-size, but the difference between the $t_{1/2}$ from the two intervals also approached significance ($P = 0.07$). Post-hoc comparison through plots and Tukey's HSD for unequal numbers indicated that the $t_{1/2}$ was longer in the second dosage interval in the large cages. There was a considerable variance, however, and the results should be interpreted with caution.

To summarise, by use of the described approach, it is possible to simulate distinctly different concentration-time profiles, but it will not be possible to define, nor to repeat, exactly the same profile as in *in vitro* models. On the other hand, the variation leads to a wider range of PK/PD indices, which may be an advantage in the analysis. The main limitation of the model in this respect is that, with the type of tissue cages used, a $t_{1/2}$ shorter than around 4 to 6 h cannot be simulated, as smaller cages would be difficult to sample. A further limitation is the need to minimise sample volume.

PK/PD indices of penicillin and danofloxacin (III, IV)

Through the concept of simulated concentration-time profiles, an important prerequisite for the desired model was met. To explore the potential of this model for PK/PD studies, the cages were infected with *M. haemolytica* and the effect of different concentration-time profiles of two antimicrobials for which the PK/PD relationships are comparatively well known was studied.

The effect of penicillin (III), a beta-lactam, depends primarily on the time above MIC, while that of danofloxacin (IV), a fluoroquinolone, is concentration-dependent (Frimodt-Moller, 2002). Fluoroquinolones are by far the most studied antimicrobials with respect to PK/PD relationships. A more limited number of studies relate specifically to veterinary medicine, but among those are some recent studies on danofloxacin (Lindencrona, Friis & Aarestrup, 2000; Aliabadi & Lees, 2001; Sarasola *et al.*, 2002). For both drugs, treatment of pneumonia in cattle caused by *M. haemolytica* is among the authorised indications in Sweden (FASS VET., 2002) and other countries (Prescott, Baggot & Walker, 2000).

Free concentrations of drug

The clinical relevance of protein binding is an ever-debated topic. As mentioned in 'Background', only the free fraction is active against bacteria (Rolinson, 1980). Further, the free concentration in serum is a reasonable surrogate marker for the free concentrations at the site of infection (Cars, 1997). Thus, the recommendation that PK/PD indices should be calculated from the free concentration of the drug (Mouton *et al.*, 2002) is well founded. For this, different approaches were used in study III and IV, as explained in 'Comments on materials and methods'.

Penicillin (study III)

The MIC of penicillin for *M. haemolytica* determined in standard media was 0.12 mg/L. The total concentration of penicillin needed in TCF to yield a free concentration equal to that, MIC_{tot} , was calculated to 0.18 mg/L, and this was also confirmed by *in vitro* determinations in protein enriched media. This MIC_{tot} was then used to calculate the PK/PD indices.

Danofloxacin (study IV)

The protein binding of danofloxacin in serum was concentration dependent in the range from 0.08 to 0.31 mg/L with a maximum binding of 57% at very low concentrations and 30% at the higher concentration of drug. Above the latter concentration, the fraction bound seemed to be constant (Figure 8). This concurs with the findings of Friis (1993).

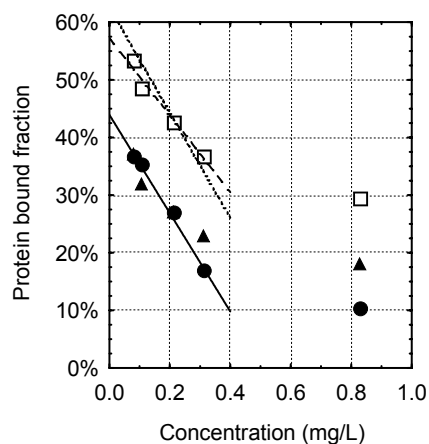


Figure 8. Protein bound fraction of danofloxacin as a function of drug concentration in serum (open squares, broken line) TCF (filled circles, solid line), and TCF calculated from data for serum (filled triangles). The linear fit to data reported for serum by Friis (1991) is shown as dotted line.

It has been suggested that quinolones, as most drugs, bind primarily to albumin (Okezaki *et al.*, 1989; Zlotos *et al.*, 1998a; Zlotos *et al.*, 1998b). In above cited study by Friis (1993), protein binding in bronchial secretions was around 30% irrespective of drug concentration. In these secretions, the drug concentrations were similar but the albumin concentration higher than in plasma. This indicates that in addition to albumin, danofloxacin binds to at least one other molecule in bovine serum (Friis, 1993). Interestingly, protein binding of danofloxacin in goat serum does not seem to be concentration dependent (Atef *et al.*, 2001).

A similar concentration dependency was found also for binding of danofloxacin in TCF. The difference in percentage bound at the respective drug concentrations is fully explained by the difference in protein concentration between serum and TCF (Figure 8). Thus, whatever the molecular background to the observed concentration dependency, the conditions are probably similar in serum and TCF. Visual inspection of a plot of data indicated that a linear approximation of the bound fraction *versus* concentrations from 0.08 and 0.31 mg/L would yield reasonable estimates within that range. Further, it was assumed that above 0.4 mg/L of drug, the fraction of protein bound drug in TCF would be constant (10%).

The procedure with a linear approximation will slightly overestimate the protein bound fraction at concentrations close to the saturation level, but as binding at those levels is below 20%, this was considered negligible. Of greater importance is probably that the approximation is valid for uninfected TCF. Following infection, the protein content will increase, and other changes in the chemical composition of TCF, such as a decrease in pH, will take place (Clarke *et al.*, 1989c). This means that the true free concentration of drug may differ from the estimates.

Simulated PK/PD indices

As mentioned in “Background”, PK/PD indices are mostly correlated with efficacy through use of various regression techniques. A commonly encountered problem is co-linearity, or interdependency, of the indices. In animal models, this interdependency can be reduced by varying dose and dosing interval, and by use of strains with different MICs (Vogelman *et al.*, 1988).

Table 5. *Correlation between selected PK/PD indices in treated cages in study III and IV (Pearson’s coefficient of correlation, r)¹*

		C_{\max}/MIC	AUC_{24m}/MIC
Study III	AUC_{24m}/MIC	0.88	-
	$T_{96>MIC}$	0.80	0.93
Study IV	AUC_{24m}/MIC	0.78	-
	$T_{96>MIC}$	0.29	0.72

¹ All coefficients of correlation except C_{\max}/MIC to $T_{96>MIC}$ in study IV were significant ($P < 0.05$).

In study III, the correlation between all PK/PD indices in treated cages was significant (Table 5). As an example, a plot of AUC_{24m}/MIC versus $T_{96>MIC}$ is shown in Figure 9. The chosen study design was not optimal to separate the indices. For two of the doses, the dosage interval was 12 h in the tissue cages with shorter $t_{1/2}$ and 24 h in those with longer $t_{1/2}$. The more frequent dosing in cages with more rapid elimination compensates the fact that a single dose of a certain magnitude leads to smaller AUC/MIC and shorter $T_{>MIC}$ in these cages, compared with those with longer $t_{1/2}$. This was most pronounced in the cages given the target C_{\max}/MIC of 1.1, where the AUC_{24m}/MIC was 22 ± 5 h in the cages with shorter $t_{1/2}$, compared with 21 ± 8 h in cages with longer $t_{1/2}$. For the medium dose (target C_{\max}/MIC of 2.8), where all cages were dosed every 24 h, the corresponding figures were 20 ± 6 h and 46 ± 20 h, respectively.

The correlation between indices was less pronounced in study IV, where danofloxacin was dosed twice with 24 h interval in both cage-types (Table 5 and Figure 9). Still, all coefficients of correlation except that for C_{\max}/MIC to $T_{96>MIC}$ were significant. The figures are comparable with those reported by Vogelmann *et al.* (1988) in the first study that successfully minimised the interdependency of the PK/PD indices in an animal model. They reported coefficients of determination, R^2 , for $T_{>MIC}$ to the other indices of 0-46% (8 and 52% in study IV), and 39-87% for AUC to C_{\max} (61% in study IV).

Another factor of importance in analysis of outcomes is the range of the PK/PD indices. In study I, the ratio between 10th and 90th percentile were 6, 7 and 11 for

C_{\max}/MIC , $\text{AUC}_{24\text{m}}/\text{MIC}$ and $T_{96>\text{MIC}}$, respectively. For study IV, the corresponding figures were 7, 16 and 6. In the subsequent analyses, data from untreated cages where the PK/PD indices = 0 were also included which means that the total range studied was wider.

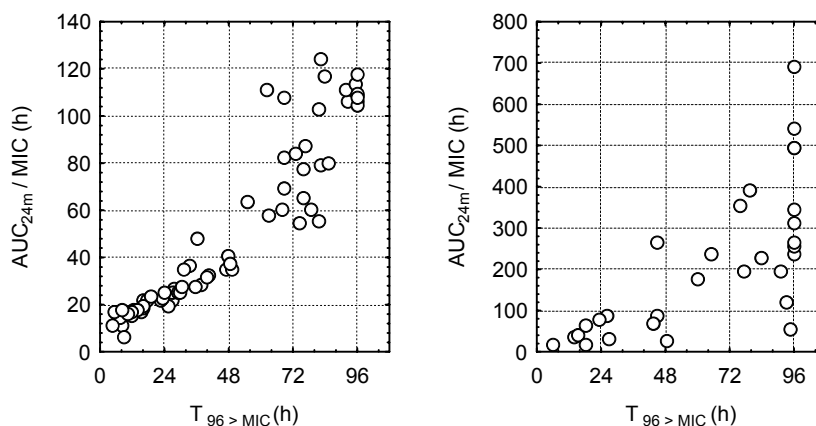


Figure 9. Correlation between $\text{AUC}_{24\text{m}}/\text{MIC}$ and $T_{96>\text{MIC}}$ in study III (penicillin; left panel) and study IV (danofloxacin, right panel).

This shows that the model allows for simulation of a wide range of PK/PD indices, and that the interdependency of the indices can be reduced provided that doses and dosage intervals are carefully chosen.

PK/PD relationships of penicillin and danofloxacin (III, IV)

Influence of dose and cage-type on effect

In study III and IV the variance of PK/PD indices within each category (group) of dose and cage-type was wide and data were not further analysed on this basis. Nonetheless, to illustrate the influence of dose, cage-type and the interaction between these factors on the effect parameter AUBC_{96} , analysis of variance was performed on the two data sets. For this analysis, the membrane cages were excluded from study III.

In Figure 10, plots of the least square means of AUBC_{96} for the different dose categories per cage-type are shown. For data from study III (penicillin), only the effect of dose was significant. The lack of influence of cage-type is explained by the fact that the PK/PD indices did not differ markedly between some of the categories, for reasons discussed above. In contrast, for study IV (danofloxacin) no difference was observed between the control cages, but treatment led to a more marked effect in the cages with longer $t_{1/2}$ than in those with shorter $t_{1/2}$ in both dose-groups.

The analysis above confirms that the simulated concentration-time profiles lead to different degrees of effect. It is, however, only an indirect way to compare the effect of different PK/PD indices defined by dose, dosage-interval and cage-type (*i.e.* rate of elimination).

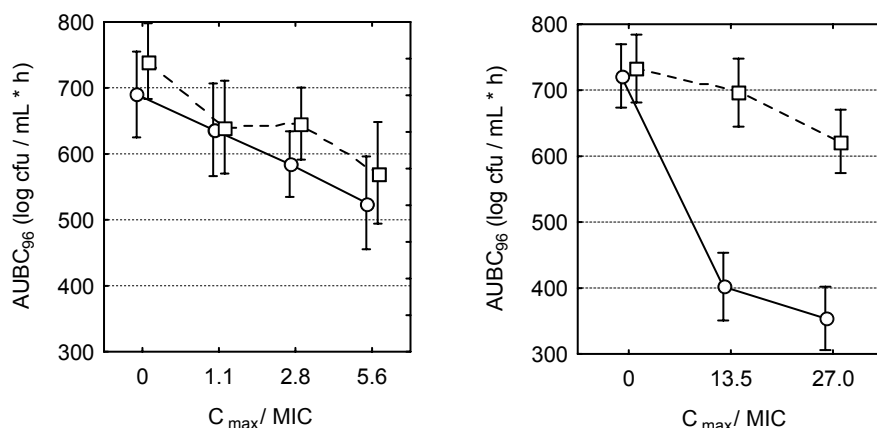


Figure 10. Least square means of AUBC₉₆ of *M. haemolytica* for different dose categories (target C_{max}/MIC) in study III (penicillin; left panel) and IV (danofloxacin; right panel) in small (squares) and large (circles) tissue cages. Bars indicate 95% confidence interval.

Predictive PK/PD index

Regression techniques were used to explore data, and to define the PK/PD index that best explained the effect (III, IV). A curvilinear pattern revealed in plots of AUC/MIC to AUBC_t was dealt with through use of second order polynomial regression. Polynomials are linear in their parameters, and can therefore be compared to simple linear regressions. To account for the difference in number of variables, adjusted R^2 was used for comparisons. As can be seen in Figure 11, the fitted polynomial line is quite close to that of the E_{max} model.

Penicillin (III)

In the study on penicillin (III), initial exploration of data using simple and polynomial regression indicated that C_{max}/MIC_{tot} was not a good predictor of the effect measured as AUBC₉₆. For the remaining indices, AUC_t/MIC, $T_{96>MIC_{tot}}$, and $T_{96>0.25 \times MIC_{tot}}$, adjusted R^2 of the best models ranged from 0.52 to 0.58 with the highest figures for AUC_t/MIC_{tot}. The fit of the E_{max} model was adequate for AUBC₉₆ versus AUC_{24h}/MIC_{tot} or AUC₉₆/MIC_{tot} (Figure 11). The fit to $T_{>0.25 \times MIC_{tot}}$ also yielded reasonable estimates but the confidence interval for the Hill slope constant was wide. For $T_{96>MIC_{tot}}$ the fit was considered inferior, as the estimate of maximum effect (the bottom of the curve in an inhibitory model) was poor. The scatter of data was pronounced for all variables over the whole range, and the collinearity of the predictors strong. Nonetheless, the analysis slightly favours AUC_t/MIC_{tot} as predictor of effect of penicillin on *M. haemolytica*.

For beta-lactam antibiotics, a clear correlation between the time above MIC and effect has been demonstrated in various models (Cars, 1997). This is also supported by clinical evidence (for a review see Turnidge, 1998). However, Lavoie and Bergeron (1985) showed a better correlation between bacterial killing

and the ratio of AUC in serum and clots than with $T_{>MIC}$ in a fibrin clot model in rabbits. The authors suggested that a large gradient is needed to penetrate into the core of the clot, where bacteria might otherwise be protected. It is possible that such penetration barriers also played a role in study III.

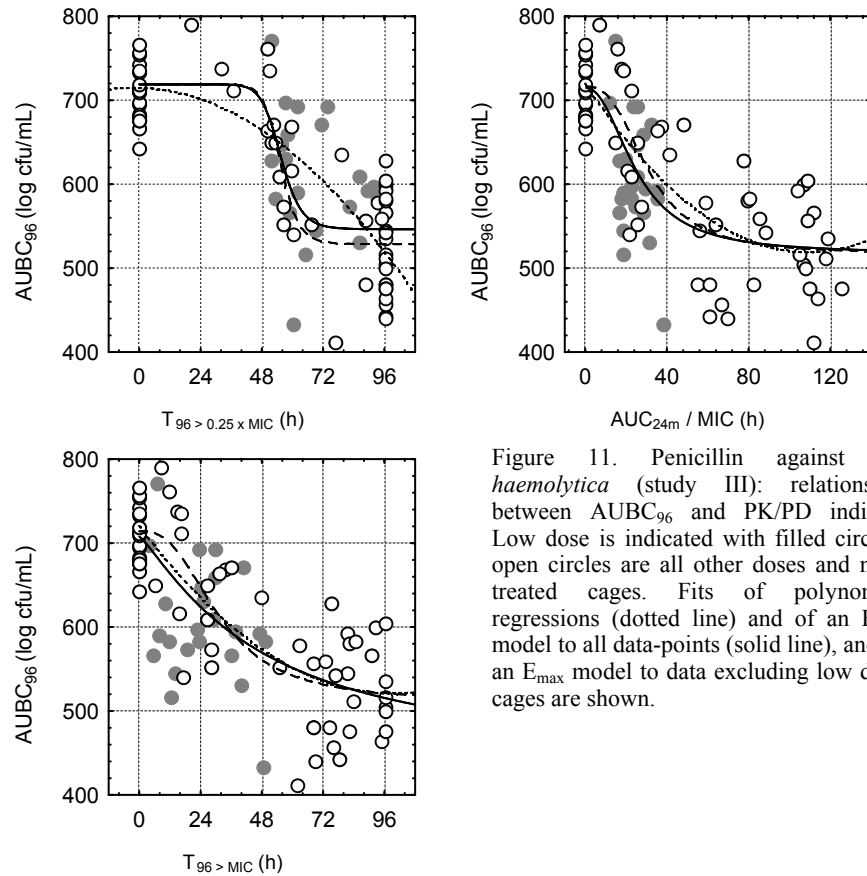


Figure 11. Penicillin against *M. haemolytica* (study III): relationship between AUC_{96} and PK/PD indices. Low dose is indicated with filled circles, open circles are all other doses and non-treated cages. Fits of polynomial regressions (dotted line) and of an E_{max} model to all data-points (solid line), and of an E_{max} model to data excluding low dose cages are shown.

The bactericidal effect of penicillin is slow, and increased killing with increasing concentrations is only reported for concentrations up to about four times the MIC (Vogelman & Craig, 1986; Li, Nix & Schentag, 1994). This concentration-dependency at levels close to the MIC offers another explanation to the findings in study III. In treated cages, the C_{max}/MIC ranged from 1.1 to 12.9, with a median of 1.9. Thus, in most cases the concentrations were in the range where a certain concentration-dependency would be expected. The $T_{96>2 \times MIC_{tot}}$ did not seem to correlate well with AUC_{96} , but $T_{96>0.25 \times MIC_{tot}}$ did, so it is probable that the overall effect was influenced by concentrations below MIC.

Löwdin *et al.* (Löwdin *et al.*, 1996) studied PME in an *in vitro* dynamic model. They showed that the PME of penicillin on *Streptococcus pyogenes* was longest in experiments where the $t_{1/2}$ was shortest, while for longer $t_{1/2}$ the effect was less pronounced. The authors hypothesised that when concentrations decline slowly, the penicillin binding proteins are not fully saturated when the drug concentration

approaches MIC, and growth can resume earlier. Similar findings regarding correlation between PME and $t_{1/2}$ have also been reported for aminoglycosides (den Hollander *et al.*, 1998a).

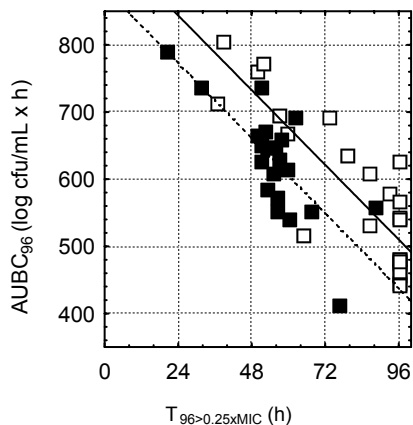


Figure 12. Penicillin against *M. haemolytica* (study III): relationship between $AUBC_{96}$ versus $T_{96>0.25 \times MIC}$ in small cages with rapid elimination rate (filled squares) and large cages with longer elimination rate (open squares).

In study III, the effect of a given $T_{96>0.25 \times MIC_{tot}}$ was slightly more pronounced in the small cages with shorter $t_{1/2}$ than in the large cages (Figure 12), which indicates a similar phenomenon.

According to the above, it is possible that in study III the PK/PD indices and pharmacokinetic parameters interacted in their influence on effect. If so, this could in part explain the scattered appearance of data in plots. This would be most pronounced for the dose group with a target C_{max}/MIC close to MIC. Re-examination of data revealed that the treated tissue cages in the lowest target C_{max}/MIC , all had a $T_{96>MIC}$ below 50 h and $T_{96>0.25 \times MIC}$ above that value. After exclusion of this dose group, the fit of the E_{max} model to $T_{96>MIC}$ was improved, but the confidence interval of the Hill slope was still wide. For the remaining indices, the fits were similar to those to the complete data set (Figure 11).

Danofloxacin (IV)

Like in study III, initial exploration of data in study IV (danofloxacin) indicated that C_{max}/MIC was not a good predictor of either $AUBC_{48}$ or $AUBC_{96}$. The AUC_{96}/MIC and AUC_{24m}/MIC were equally good predictors of $AUBC_{48}$ (adjusted R^2 of 0.89 for both regressions) while for prediction of $AUBC_{96}$, AUC_{96}/MIC was somewhat better than AUC_{24m}/MIC (adjusted R^2 of 0.87 and 0.81, respectively). As AUC_{96}/MIC reflects the drug exposure both during and beyond the treatment intervals, it is logical that this index is a better predictor of the effect measure that includes the re-growth phase. The $T_{96>MIC_{tot}}$ was inferior to both AUC_{96}/MIC and AUC_{24m}/MIC for prediction of $AUBC_{48}$ ($R^2 = 0.81$) but equal to AUC_{24m}/MIC for $AUBC_{96}$ ($R^2=0.78$). Adequate fits of the E_{max} model were obtained for both AUC_t/MIC indices related to $AUBC_{48}$ (Figure 13), and to $AUBC_{96}$. For the other indices, the fit of the E_{max} model was clearly inferior. Taken together, the analyses indicate that AUC_t/MIC was the group of indices that correlated best with the effect of danofloxacin against *M. haemolytica* in the tissue cages.

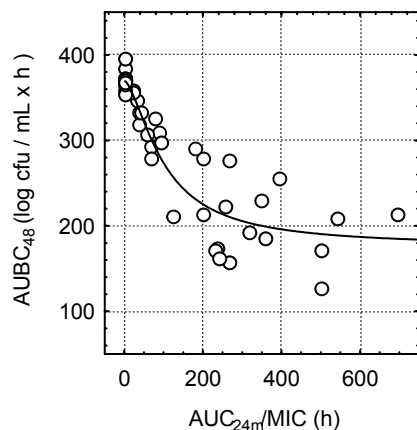


Figure 13. Danofloxacin against *M. haemolytica*. Fit of a four-parameter inhibitory E_{max} model to $AUBC_{48}$ versus AUC_{24h}/MIC (data from study IV).

The PK/PD relationships of fluoroquinolones have been studied extensively. The effect is concentration-dependent. In *in vitro* and *in vivo* models, AUC/MIC (e.g. Madaras-Kelly *et al.*, 1996; Bowker *et al.*, 1999; Andes & Craig, 2002b) or C_{max}/MIC (e.g. Blaser *et al.*, 1987), or both (e.g. Drusano *et al.*, 1993; MacGowan *et al.*, 2001), have been identified as the most important indicators of efficacy.

Hence, in this respect the findings in study IV are consistent with those of others. Results from studies where models with tissue cages or abscesses were used to study the relative importance of the different PK/PD indices of fluoroquinolones are, however, somewhat more contradictory. In a tissue cage model in guinea pigs designed to mimic device related infections, none of the PK/PD indices were correlated with reduction of bacterial counts over 48 h (Blaser *et al.*, 1995). Xuan *et al.* (2001), reported a good correlation of both AUC/MIC and C_{max}/MIC to bacterial killing in a rabbit tissue cage model. In a model with some resemblance to tissue cages, *i.e.* established subcutaneous abscesses in mice, C_{max} was more predictive for effect than AUC (Sterne *et al.*, 2001).

It is possible that these seemingly conflicting results are explained by differences in study design. It was suggested by Drusano *et al.* (1993) that C_{max}/MIC is the best predictor of effect when this index is above 10, while below that value AUC/MIC is more important. According to this, the proportion of data with values of C_{max}/MIC below 10 could influence the conclusions of a specific study. Further, in a study on the effect of gemifloxacin on *Streptococcus pneumoniae*, MacGowan *et al.* (2001) showed that the rate of kill, measured as time to a 99.9% decrease in bacterial counts, was best predicted by C_{max}/MIC , while AUC/MIC was predictive of the overall effect measured as $AUBC$. This illustrates how the choice of effect parameters may affect the results, as they measure different events that may relate differently to the PK/PD indices.

Magnitude of the predictive index

For drug dosage prediction, not only the PK/PD index that determines the effect but also its magnitude must be identified. In study III, the results concerning the

best predictive index were ambiguous which precludes further estimations. Therefore, in the following only results from study IV and other findings for fluoroquinolones will be discussed.

In experimental as well as in clinical studies, the estimate of the magnitude of the PK/PD index needed for effect will depend on, amongst other things, the chosen endpoint. This is illustrated by data reported by Forrest *et al.* (1993). In a retrospective study on nosocomial pneumonia treated with ciprofloxacin, the probability of treatment success was high for patients where AUC/MIC was above 125 h. However, bacterial eradication was more rapid when the AUC/MIC was above 250 h than when this figure was 125 to 250 h. Figures of the same order of magnitude for rapid bacterial eradication have also been reported for other fluoroquinolones from other clinical studies (Forrest *et al.*, 1997; Preston *et al.*, 1998; Highet *et al.*, 1999; Meinel *et al.*, 2000; Tran *et al.*, 2000). It has been suggested that to reduce the emergence and spread of resistant bacteria, antimicrobial therapy should optimise the potential for bacterial eradication in human respiratory tract infections (Dagan *et al.*, 2001). That concept is probably valid for other types of infections in man as well as in animals. Hence, toxicology permitting, the higher level (250 h) in the study by Forrest *et al.* (1993) may be closer to the optimal target of AUC/MIC than 125 h, which is a commonly cited figure that rather represents the minimum.

In study IV, the level of AUC_{24m}/MIC that produced 80% of the response measured as $AUBC_{48}$ was 244 h which is in line with these higher figures. Similar levels for 80% or near maximal response have been reported in studies on different fluoroquinolones on various bacterial species in *in vitro* dynamic models (Madaras-Kelly *et al.*, 1996; MacGowan *et al.*, 1999b; MacGowan, Wootton & Holt, 1999) and in animal models (Andes & Craig, 1998; Bédos *et al.*, 1998; Andes & Craig, 2002b). For studies using tissue cages to contain an infection (Fernandez *et al.*, 1999; Xuan *et al.*, 2001), an AUC/MIC in TCF producing near maximum bacterial killing can be estimated to above 150 h.

Lower figures (35-65 h) are reported in some studies for *S. pneumoniae* (Lacy *et al.*, 1999; Lister & Sanders, 1999; Hershberger & Rybak, 2000; Zhanel *et al.*, 2001) but not in others (Bédos *et al.*, 1998; MacGowan *et al.*, 1999a; MacGowan *et al.*, 2001). It is probable that the conflicting data reflect differences in study design.

This may also explain the lower figures found in studies relating specifically to danofloxacin. Lindencrona, Friis & Aarestrup (2000) reported on the effect of danofloxacin on *Salmonella* Typhimurium in an *in vitro* dynamic model and from presented data, an AUC/MIC of 80 h producing 80% response can be estimated. Aliabadi & Lees (2001) studied the killing kinetics of danofloxacin on *M. haemolytica* *in vitro* in TCF from goats sampled at various times during treatment. The AUC/MIC at the concentrations obtained in the samples (*i.e.* static concentrations) were calculated, and for elimination of bacteria 52-59 h was needed. The pharmacodynamics of danofloxacin against *M. haemolytica* has also been studied in a model of calf pneumonia (Sarasola *et al.*, 2002). Although no dose-titration was made, it can be noted that a bolus dose giving an $AUC_{0-\infty}/MIC$ of 43 h was sufficient to prevent development of pneumonia.

In conclusion, the observations on PK/PD relationships of penicillin (III) were unclear, which could partly be explained by an interaction of PK/PD indices and pharmacokinetic parameters. For danofloxacin (IV), the results concur with reports for other fluoroquinolone – bacterial species combinations from experimental models and clinical studies in man.

Concluding remarks

The understanding of PK/PD relationships of antimicrobials, and implications thereof for dose setting, has increased considerably over the last decades. Most of the research has been focused on human medicine. It is probable that the general results are applicable also to the major animal species in veterinary medicine because the target for antimicrobial drugs is the bacterium, not the host. The antimicrobial-pathogen combinations of interest, however, differ between animal species. Clearly, veterinary medicine would benefit from an expansion of this area.

In the studies underlying this thesis, a tissue cage model in calves was adapted for studies of PK/PD relationships of antimicrobials. The advantage of tissue cages is that repeated sampling can be performed. Thereby, both concentrations of drug at the site of infection and the antibacterial effect can be monitored in presence of the host defences. Through use of tissue cages with different SA/V ratios, and injection of different doses of drug directly into the cages, a range of concentration-time profiles can be simulated. By use of this concept, the effects of a range of PK/PD indices of penicillin and danofloxacin could be studied. The validity of the developed model needs to be challenged by direct comparison with other models, and by clinical trials in relevant animal species.

So far, the results suggest the here described model can be a useful intermediary step between *in vitro* studies and more elaborate infection models in relevant animal species. Through use of different experimental models in sequence, factors of relevance for antimicrobial effect can be identified, and the magnitude of the relevant PK/PD index needed for effect can be estimated. Together with data on population pharmacokinetics and distribution of MICs of relevant target pathogens, this information can be used to select doses and dosage regimens for clinical trials.

A reduction of unnecessary use of antimicrobials is a natural component of strategies aiming to contain resistance. However, it is probable that the epidemiology of resistance is influenced not only by how often, but also by how we use these drugs. Evidence that the dose and dosage regimen of an antimicrobial influence the risk for emergence of resistant mutants of the target bacterium is accumulating. Hitherto, corresponding information regarding the risk for selection and transfer of resistance genes is lacking. Nevertheless, it is plausible that factors such as the route of administration, dose and treatment time play a role. The described model could be an interesting option for studies on the influence of host defences on the pharmacodynamics of resistance. The increase in resistance to antimicrobials among bacteria from man and animals is a threat to successful therapy of bacterial infections. Therefore, an understanding of the relation between PK/PD and the risk for emergence of resistance is probably the most urgent research need in this area.

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