Pharmacokinetic–pharmacodynamic integration and modelling of florfenicol in calves

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Florfenicol was administered subcutaneously to 10 calves at a dose of 40 mg/kg. Pharmacokinetic–pharmacodynamic (PK-PD) integration and modelling of the data were undertaken using a tissue cage model, which allowed comparison of microbial growth inhibition profiles in three fluids, serum, exudate and transudate. Terminal half-lives were relatively long, so that florfenicol concentrations were well maintained in all three fluids. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration were determined in vitro for six strains each of the calf pneumonia pathogens, Mannhemia haemolytica and Pasteurella multocida. An PK-PD integration for three serum indices provided mean values for P. multocida and M. haemolytica, respectively, of 12.6 and 10.4 for \( \frac{C_{\text{max}}}{\text{MIC}} \), 183 and 152 h for \( \frac{\text{AUC}_{0-24}}{\text{MIC}} \) and 78 and 76 h for \( T > \text{MIC} \). Average florfenicol concentrations in serum exceeded \( 4 \times \text{MIC} \) and \( 1.5 \times \text{MIC} \) for the periods 0–24 and 48–72 h, respectively. Ex vivo growth inhibition curves for M. haemolytica and P. multocida demonstrated a rapid (with 8 h of exposure) and marked (6 \( \log_{10} \) reduction in bacterial count or greater) killing response, suggesting a concentration-dependent killing action. During 24-h incubation periods, inhibition of growth to a bacteriostatic level or greater was maintained in serum samples collected up to 96 h and in transudate and exudate samples harvested up to 120 h. Based on the sigmoidal \( E_{\text{max}} \) relationship, PK-PD modelling of the ex vivo time–kill data provided \( \frac{\text{AUC}_{0-24}}{\text{MIC}} \) serum values for three levels of growth inhibition, bacteriostatic, bactericidal and \( 4 \log_{10} \) decrease in bacterial count; mean values were, respectively, 8.2, 26.6 and 39.0 h for M. haemolytica and 7.6, 18.1 and 25.0 h for P. multocida. Similar values were obtained for transudate and exudate. Based on pharmacokinetic and PK-PD modelled data obtained in this study and scientific literature values for MIC distributions, Monte Carlo simulations over 100 000 trials were undertaken to predict once daily dosages of florfenicol required to provide 50% and 90% target attainment rates for three levels of growth inhibition, namely, bacteriostasis, bactericidal action and \( 4 \log_{10} \) reduction in bacterial count.

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INTRODUCTION

In most countries, florfenicol has replaced for veterinary use the first drug of this class, chloramphenicol, which was introduced in 1947 as a broad spectrum antibiotic and classified as a bacteriostat. Florfenicol is now the major and, in many countries, the only remaining drug of the fenicol class in veterinary use. It is a fluorinated analogue of thiamphenicol with a similar mechanism of action to chloramphenicol and thiamphenicol, involving inhibition of the 30S ribosomal subunit of bacteria, leading to inhibition of protein synthesis (Cannon et al., 1990).
Clinically, the main use for florfenicol is treatment for respiratory tract infections in calves and young pigs by parenteral administration (Hoar et al., 1998; Varma et al., 1998; Jim et al., 1999; Aslan et al., 2002; De Haas et al., 2002; Catry et al., 2008). In calves, the spectrum of activity includes the pneumonia pathogens, Mannheimia haemolytica, Pasteurella multocida and Histophilus somni (Varma et al., 1998; De Haas et al., 2002; Catry et al., 2008). The latter authors reported no resistance to these species using Kirby–Bauer discs; of the isolates tested, all were sensitive to florfenicol, with the exception of two of 39 P. multocida isolates, which were intermediate in sensitivity. Similarly, Priebe and Schwarz (2003) reported high activity of florfenicol against these bovine respiratory tract pathogens. Reports of minimum inhibitory concentration (MIC) for P. multocida and M. haemolytica (MIC ≤ 2.0 µg/mL) indicate the relatively high potency of florfenicol for these organisms (Priebe & Schwarz, 2003; Kehrenberg et al., 2004; Shin et al., 2005; Dowling, 2006; Thiry et al., 2011). However, Ayling et al. (2000) reported high MIC₅₀ and MIC₉₀ values of 4 and 16 µg/mL, respectively, against Mycoplasma bovis.

It is of interest that, whilst florfenicol has been in widespread use in farm animals for 20 years and whilst resistance occurrence and mechanisms have been described (Kehrenberg et al., 2004; Schwarz et al., 2004; Berge et al., 2005; Kehrenberg & Schwarz, 2005), it has retained a high level of activity against respiratory tract pathogens of calves (Catry et al., 2005; Shin et al., 2005). Moreover, De Haas et al. (2002) demonstrated a clear bactericidal action against bovine and porcine respiratory tract pathogens, and they classified florfenicol as concentration dependent in its killing action.

The pharmacokinetic profile of florfenicol has been described in chickens (Shen et al., 2003; Anadon et al., 2008), turkeys (Switala et al., 2007), pigs (Li et al., 2002; Liu et al., 2003), dogs (Park et al., 2008), rabbits (Koc et al., 2009) and sheep (Lane et al., 2007). Florfenicol pharmacokinetics has also been reported in adult cattle (Bretzlfaff et al., 1987; Varma et al., 1994; Soback et al., 1995) and calves (Varma et al., 1986; Adams et al., 1987; Lobell et al., 1994; De Craene et al., 1997). The latter group reported an elimination half-life of 3.2 h in calves after intravenous dosing, similar to values of 2.7–3.2 h in adult cows (Bretzlfaff et al., 1987; Lobell et al., 1994; Soback et al., 1995). However, the rate of decline in plasma concentration was much slower in calves administered florfenicol intramuscularly or subcutaneously (Lobell et al., 1994; Varma et al., 1998), as a consequence of flip-flop pharmacokinetics. Similarly, Lacroix et al. (2011) reported a terminal half-life of florfenicol of 39.6 h after subcutaneous dosing of a combination product containing florfenicol and the nonsteroidal anti-inflammatory drug (NSAID), flunixin, at a dosage of the former of 40 mg/kg.

There are no major reports linking the pharmacokinetics and pharmacodynamics of florfenicol in calves within a single investigation. Therefore, the aims of this study were to: (i) establish the serum concentration–time profile and to derive pharmacokinetic data for florfenicol in calves after subcutaneous administration at the dose of 40 mg/kg, (ii) determine the rate and extent of florfenicol penetration into and elimination from carrageenan-inflamed (exudate) and noninflamed (transudate) fluids in a tissue cage model, (iii) investigate ex vivo time–kill curves for florfenicol in samples of serum, exudate and transudate collected from florfenicol-treated calves against clinical isolates of each of two calf pathogens. M. haemolytica and P. multocida, (iv) integrate and model the data as a basis for determination of the magnitude and duration of action of florfenicol at the 40 mg/kg dose and (v) use the data generated as a basis for calculating daily dosage rates for three levels of growth inhibition.

MATERIALS AND METHODS

Animals, surgical procedures and tissue cage model

The study was carried out in 10 healthy female Aberdeen Angus calves weighing 145–204 kg (mean = 179 kg, SD = 16.7) and aged 79–131 days (mean = 108 days, SD = 15 days). Silicon tissue cages were implanted subcutaneously in the flank under general anaesthesia, as previously described (Sidhu et al., 2003). A period of 35 days after surgery was allowed for wound healing and to permit the growth of granulation tissue into the cages, prior to dosing.

At zero time (immediately preceding the injection of florfenicol), 0.5 mL of 1% w/v sterile lambda carrageenan solution in saline (Viscarin; FMC biopolymers, Philadelphia, PA, USA) was injected into a single tissue cage. This was subsequently used to harvest exudate. An unstimulated cage was used to collect noninflammatory fluid (transudate).

Drug administration

Florfenicol was administered as Nuflor® (Schering Plough Animal Health, Milton Keynes, Bucks, UK) subcutaneously at a dose of 40 mg/kg over the rib cage with a maximum volume of 10 mL per site. Florfenicol was administered immediately before carrageenan injection into the tissue cage.

Sampling procedures

Blood samples (10 mL) were collected from a jugular vein into vacuumers (Becton, Dickinson and Company, Oxford, Oxon, UK) without anticoagulant at times of 15, 30 and 45 min and 1, 2, 3, 4, 6, 8, 10, 12, 24, 29, 33, 48, 54, 72, 80, 96 and 120 h after administration of florfenicol (Nuflor Injectable Solution: Intervet Schering Plough Animal Health, Boxmeer, The Netherlands). Samples were kept at room temperature for 30 min and then placed on ice for 30–60 min to facilitate clot contraction. The supernatant serum was harvested after centrifugation (2000 g for 10 min at 4 °C) and aliquoted into four polypropylene tubes for subsequent analyses or retention as reserve samples. All samples were stored at −70 °C until either analysed for florfenicol concentration or used for measurement of ex vivo antibacterial activity.
Exudate and transudate samples (1.5 mL) were collected before and at predetermined times (2, 4, 6, 8, 10, 12, 24, 29, 33, 48, 54, 72, 80, 96 and 120 h) after drug administration. Samples were placed on ice for 30–60 min and then centrifuged at 2000 × g for 10 min at 4 °C to remove leucocytes. Supernatants were divided into three aliquots and stored at −70 °C until analysed for florfenicol concentration, used for measurement of ex vivo antibacterial activity or retained as a reserve sample.

Analysis of florfenicol in serum, exudate and transudate

Serum, exudate and transudate samples were assayed for florfenicol by a high-performance liquid chromatography (HPLC) method with UV detection. The method was adapted from one previously published (De Craene et al., 1997). Briefly, 250 μL of 0.1 M sodium phosphate buffer (pH 7) and 10 μL of a 250 μg/mL stock solution of chloramphenicol (internal standard) were added to 250 μL of serum, exudate or transudate, and the solutions vortexed for 30 sec. After mixing, 2.5 mL ethyl acetate was added, and the solution was vortexed for 1 min. It was then sonicated for 5 min. After centrifugation at 3500 × g for 10 min at 24 °C, 2 mL supernatant was evaporated to dryness at 45 °C under nitrogen. The residue was reconstituted in a volume of 250 μL of water:acetonitrile (80:20 v/v), vortexed for 30 sec and then sonicated for 3 min. A 20 μL aliquot of the reconstituted sample was injected onto the HPLC column. The system comprised a 125 solvent module pump, a Rheodyne manual injector (Rheodyne, Cotati, CA, USA) and a System Gold 166 Detector at 224 nm. The column was an Ultrasphere C18, 5 μm, 250 × 4.6 μm (Beckman Coulter Ltd., High Wycombe, Bucks, UK). The flow rate was 1.0 mL/min, and the run-time was 8 min. The mobile phase was a methanol: water mixture (50:50 v/v). All reagents, of HPLC grade, were obtained from Sigma-Aldrich Chemicals (Poole, Dorset, UK).

Retention times for florfenicol and the internal standard were approximately 4.5 and 6.0 min, respectively. The lower limit of quantification (LLOQ) of florfenicol in serum, exudate and transudate was 0.25 μg/mL. Samples of serum, exudate and transudate samples collected from drug naïve animals were spiked with florfenicol (a gift from Merck Animal Health) to 0.1 M sodium phosphate buffer (pH 70) were added to 250 μL of serum, exudate or transudate, and the solutions vortexed for 30 sec. After mixing, 2.5 mL ethyl acetate was added, and the solution was vortexed for 1 min. It was then sonicated for 5 min. After centrifugation at 3500 × g for 10 min at 24 °C, 2 mL supernatant was evaporated to dryness at 45 °C under nitrogen. The residue was reconstituted in a volume of 250 μL of water:acetonitrile (80:20 v/v), vortexed for 30 sec and then sonicated for 3 min. A 20 μL aliquot of the reconstituted sample was injected onto the HPLC column. The system comprised a 125 solvent module pump, a Rheodyne manual injector (Rheodyne, Cotati, CA, USA) and a System Gold 166 Detector at 224 nm. The column was an Ultrasphere C18, 5 μm, 250 × 4.6 μm (Beckman Coulter Ltd., High Wycombe, Bucks, UK). The flow rate was 1.0 mL/min, and the run-time was 8 min. The mobile phase was a methanol: water mixture (50:50 v/v). All reagents, of HPLC grade, were obtained from Sigma-Aldrich Chemicals (Poole, Dorset, UK).

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Florfenicol binding to serum protein

Serum protein binding of florfenicol was determined in triplicate on each of nine pooled blood samples, harvested at predetermined times from the 10 calves used in the pharmacokinetic study. For each sample, total concentration of florfenicol was determined as described above. Samples were then centrifuged at 4000 × g for 20 min at 25 °C using an Amicon Ultra Centrifugal filter (Ultracel 10K; Millipore (UK) Limited, Watford, Hertfordshire, UK) and florfenicol concentration redetermined on the ultrafiltrate.

Pharmacokinetic analyses

Florfenicol concentration–time data in serum, exudate and transudate for individual calves were analysed by noncompartmental analysis (WIN-NONLIN; Pharsight Corporation, Mountain View, CA, USA) using the statistical moment approach (Yamaoka et al., 1978). The linear trapezoidal rule was used to calculate areas under concentration–time curves (AUC) and areas under the first moment curves (AUMC). The mean residence time (MRT) was determined as AUMC/AUC.

In vitro microbiology

Twenty isolates each of the calf pneumonia pathogens, M. haemolytica and P. multocida, were supplied on swabs by the United Kingdom Veterinary Laboratories Agency (VLA). All isolates had been obtained at post-mortem from clinical cases of calf pneumonia in various geographical regions of the United Kingdom. Following receipt, they were stored at −70 °C in a fluid of composition glycerol:milk:water in the ratio 20:10:70. This was prepared by mixing 2 g Marvel milk powder (Premier Foods, Dublin, Ireland) with 10 mL distilled water, and 4 g of glycerol was added. Distilled water was added to provide a final volume of 20 mL. To sterilize, the fluid was boiled for 5 sec, left to cool for 12 h and then boiled again for a further 5 sec. The solution was stored at −20 °C until used.

Two criteria were used to select six isolates of each organism for detailed study. First, each isolate was tested for ability to grow logarithmically in four fluids: Mueller–Hinton Broth (MHB) and calf serum, exudate and transudate. Second, each isolate was investigated for sensitivity to florfenicol by an initial screen, involving disc diffusion test and measurement of diameter of zone of growth inhibition conducted using CLSI guidelines (data not shown). Sensitivity was then confirmed by determination of MIC in MHB, using doubling dilutions. This preselection procedure ensured that all isolates could be used to determine MIC and minimum bactericidal concentration (MBC) in both MHB and the three calf fluids. MICs and MBCs of each of six selected isolates of both species were then determined in MHB and calf fluids by a microdilution method based on CLSI methodology, but using five overlapping sets of doubling dilutions for MIC, to improve accuracy, as described by the study of Aliabadi and Lees (2001, 2002). MBCs were determined on agar plates using doubling dilutions.

Ex vivo microbiology

To establish ex vivo time-kill curves, aliquots of serum, transudate and exudate samples were pooled from the 10 florfenicol-treated calves for specific time points, as indicated below:
An aliquot from each pooled sample was analysed by HPLC to determine the florfenicol concentration. *Ex vivo* bacterial time-kill curves were established on a second aliquot of the pooled serum, exudate and transudate samples, as described by the study of Allabadi and Lees (2001). Briefly, for each experiment, four isolates each of *M. haemolytica* and *P. multocida* were freshly grown from stocks previously stored at −70 °C. A starter culture was prepared by inoculating 4 mL of MHB with a few colonies of the strain to be tested and then incubating overnight at 37 °C in an orbital shaking incubator to provide an estimated growth of 1 × 10⁸ colony forming units/mL (cfu/mL). Fifty microlitre of this culture was diluted 1:50 in freshly prepared prewarmed MHB and incubated statically for 1 h at 37 °C. The cells were centrifuged at 3100 g for 2 min. The supernatant was discarded, and the cells resuspended in 50 μL phosphate-buffered saline. The counts of viable cells were determined by serial dilution and spot-plate counts. Volumes of 396 μL serum, exudate or transudate were prewarmed in an incubator at 37 °C, and each was inoculated with 4 μL of the starter culture to give a final volume of 400 μL. The cultures were then placed in an orbital shaking incubator at 37 °C. Forty microlitre of each culture was sampled, and the viable count was determined by serial dilution and spot-plate counts after 0, 1, 2, 4, 8 and 24 h incubation. The lowest detectable count was 33 cfu/mL.

**PK-PD integration**

For PK-PD integration, four surrogates of bacteriological outcome, T > MIC (time for which florfenicol concentration exceeded MIC), maximum concentration (C_{max})/MIC ratio and area under concentration–time curve over 24 h (AUC_{0–24 h})/MIC ratio and AUC_{0–24 h}/MIC ratio were calculated for each fluid (serum, exudate and transudate) and each organism, *M. haemolytica* and *P. multocida*. Results were expressed as mean ± SEM. In addition, a fifth PK-PD variable (partial areas under in vivo concentration–time curves) was used to express the ratio of average serum, exudate or transudate concentration (C_{av}) relative to MIC for three consecutive 24 h periods (serum) or four consecutive 24 h periods (exudate and transudate) after dose administration, which is 0–24, 24–48, 48–72 and 72–96 h.

**PK-PD modelling**

For PK-PD modelling, AUC_{0–24 h}/MIC data obtained after 24 h incubation from *ex vivo* bacterial growth inhibition curves for four isolates of each species, *M. haemolytica* and *P. multocida*, were modelled to the sigmoidal \( E_{\text{max}} \), equation (1):

\[
E = \frac{E_0 + E_{\text{max}} \cdot X^N}{E_{C_0}^{0.5} \cdot X^N}
\]

where \( E_0 \) is the change in log_{10} cfu/mL of sample (in serum, exudate or transudate) after 24 h incubation in the control sample (no florfenicol) subtracted from the initial inoculum log_{10} count, \( E_{\text{max}} \) is the maximum growth inhibition determined as the change in log_{10} cfu/mL over 24 h in samples incubated with florfenicol between time 0 and 24 h, \( E_{C_0} \) is the AUC_{0–24 h}/MIC value providing 50% of the maximum antibacterial effect, \( X \) is the AUC_{0–24 h}/MIC in the effect compartment (*ex vivo* site), and N is the Hill coefficient which describes the slope of the AUC_{0–24 h}/MIC effect curve. These pharmacodynamic parameters were calculated using a nonlinear regression programme (WIN-NONLIN 5.2; Pharsight Corporation).

PK-PD modelling provided a predicted plot of log_{10} change in cfu/mL vs. AUC_{0–24 h}/MIC. From this plot, the antibacterial effect of florfenicol was quantified for three levels of growth inhibition: bacteriostatic action (no change from initial inoculum count, \( E = 0 \)), bactericidal action (3 log_{10} reduction in count, \( E = −3 \)) and 4 log_{10} reduction in count (\( E = −4 \)).

**Daily dosage prediction**

Assuming pharmacokinetics linearity, predicted daily doses were calculated from equation (2):

\[
\text{Dose} = \frac{\text{Cl} \times (\frac{\text{AUC}_{0–24 h}}{\text{MIC}}) \times \text{MIC}}{F \times f_u}
\]

where, \( \text{Cl} = \) clearance, \( F = \) bioavailability, \( f_u = \) unbound fraction, \( \text{AUC}_{0–24 h}/\text{MIC} = \) experimentally determined ratio of area under the serum concentration–time curve over 24 h to MIC for experimental strains of *M. haemolytica* and *P. multocida*, which is the breakpoint PK/PD index to be achieved, MIC\text{t} distribution of MICs from the scientific literature. The daily dose was computed using Monte Carlo simulations in Oracle Crystal Ball (Oracle Corporation, Redwood Shores, CA, USA) with the following data input: (i) the distribution of Cl/F obtained for 10 individual calves in the study of Allabadi and Lees (2001), 2011). For MIC\text{t} values, values were corrected to allow for florfenicol protein binding in serum, as the reported literature values were determined in broth. The probabilities of distribution for daily doses were run for 100 000 trials. The daily dose to achieve target attainment rates of 50% and 90% for bacteriostatic, bactericidal and 4 log_{10} reduction in bacterial count was determined.
**Statistical analyses**

Data are presented as mean ± SEM or SD. Arithmetic, geometric and harmonic means were determined, as appropriate, for each pharmacokinetic variable. Differences between fluids were determined by ANOVA using the Prism software package (Graph pad Software Inc., London, UK).

**RESULTS**

**Analytical method validation**

The analytical method for florfenicol was validated in bovine serum, exudate and transudate samples. Selectivity was assessed for each matrix on six blank samples from a matrix pool from untreated calves: no interfering peaks were detected at the retention times of florfenicol and the internal standard, chloramphenicol. The method was linear over the calibration range 0.25–25 μg/mL; the linearity of the standard curve was \( r^2 > 0.999 \) for all three fluids. The LLOQ of florfenicol in serum, exudate and transudate was 0.25 μg/mL. The intra-assay and inter-assay repeatability and reproducibility of the method were evaluated using spiked concentrations. Intra-assay and inter-assay coefficients of variation (CV%) were <8% and <5.6% for serum, <8.2% and <7.0% for exudate and <5.9% and <7.6% for transudate. Percentage recoveries were 95.2 ± 2.49% for serum, 98.0 ± 4.01% for exudate and 94.6 ± 3.69% for transudate.

**Florfenicol concentrations in serum, exudate and transudate**

The mean (± SEM) concentrations of florfenicol in serum up to 24 h after administration at a dose of 40 mg/kg are presented in Fig. 1. Florfenicol concentrations (±SEM) in serum, exudate and transudate up to 120 h after dosing are presented in Fig. 2. Compartmental pharmacokinetic analysis indicated that serum concentration–time data best fitted a two compartment open model, for exudate and transudate the data best fitted a one compartment model. Pharmacokinetic variables derived by noncompartmental analysis are presented in Table 1 (serum) and Table 2 (exudate and transudate).

Florfenicol was present in serum in detectable concentrations at all sampling times in all calves between 15 min and 80 h.

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**Table 1.** Pharmacokinetic variables for florfenicol in serum (mean and SEM, \( n = 10 \))

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Geometric mean (unless stated)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}} ) (μg/mL)</td>
<td>6.04</td>
<td>0.49</td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>2.96*</td>
<td>0.15</td>
</tr>
<tr>
<td>( T_{1/2} ) (h)</td>
<td>27.54†</td>
<td>2.26</td>
</tr>
<tr>
<td>( \text{AUC}_{0-\text{last}} ) (μg·h/mL)</td>
<td>154.1</td>
<td>5.44</td>
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<tr>
<td>( \text{AUC}_{0-\infty} ) (μg·h/mL)</td>
<td>175.1</td>
<td>16.97</td>
</tr>
<tr>
<td>( \text{AUMC}_{0-\text{last}} ) (μg·h²/mL)</td>
<td>3856</td>
<td>120.1</td>
</tr>
<tr>
<td>( \text{AUMC}_{0-\infty} ) (μg·h²/mL)</td>
<td>6308</td>
<td>571.9</td>
</tr>
<tr>
<td>( \text{MRT}_{0-\text{last}} ) (h)</td>
<td>25.02</td>
<td>0.49</td>
</tr>
<tr>
<td>CI/F (mL/kg/h)</td>
<td>228.5</td>
<td>8.67</td>
</tr>
</tbody>
</table>

*Arithmetic mean.
†Harmonic mean.

**Table 2.** Pharmacokinetic variables for florfenicol in exudate and transudate (mean and SEM, \( n = 10 \))

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Exudate</th>
<th>Transudate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geometric mean (unless stated)</td>
<td>SEM</td>
<td>Geometric mean (unless stated)</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (μg/mL)</td>
<td>3.41</td>
<td>0.22</td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>15.2*</td>
<td>1.96</td>
</tr>
<tr>
<td>( T_{1/2} ) (h)</td>
<td>27.80†</td>
<td>2.29</td>
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<tr>
<td>( \text{AUC}_{0-\text{last}} ) (μg·h/mL)</td>
<td>190.2</td>
<td>10.12</td>
</tr>
<tr>
<td>( \text{AUC}_{0-\infty} ) (μg·h/mL)</td>
<td>7752</td>
<td>482.6</td>
</tr>
<tr>
<td>( \text{AUMC}_{0-\text{last}} ) (μg·h²/mL)</td>
<td>9972</td>
<td>752.3</td>
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<tr>
<td>( \text{MRT}_{0-\text{last}} ) (h)</td>
<td>40.76</td>
<td>1.25</td>
</tr>
<tr>
<td>CI/F (mL/kg/h)</td>
<td>195.7</td>
<td>10.37</td>
</tr>
</tbody>
</table>

*Arithmetic mean.
†Harmonic mean.

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Mean values of $C_{\text{max}}$ and $T_{\text{max}}$ were 6.04 µg/mL and 3.0 h, respectively, and mean terminal half-life was 27.5 h. In all calves, $C_{\text{max}}$ was represented as a single peak. Mean residence time (MRT) was 25.0 h.

In exudate and transudate, florfenicol was present in quantifiable concentrations at all sampling times up to and including 120 h. Rate of penetration of florfenicol was similar for exudate and transudate, with $C_{\text{max}}$ being achieved at 15.2 h and 15.4 h, respectively. In all calves, $C_{\text{max}}$ for both fluids was represented by a single peak. Mean ratio of $C_{\text{max}}$ serum: $C_{\text{max}}$ exudate was 1.77:1, and the corresponding serum:transudate ratio was 2.03:1. At 24 h and all times up to 120 h, florfenicol concentrations in tissue cage fluids were higher than serum concentrations (Fig. 2). Slightly higher florfenicol concentrations were attained in exudate compared with transudate, with a ratio of 2.03:1. At 24 h and all times up to 120 h, florfenicol concentrations in tissue cage fluids were higher than serum concentrations.

PK-PD integration

Pharmacokinetic variables established in vivo were integrated with in vitro MICs. Mean values for the integrated pharmacokinetic and pharmacodynamic variables, $C_{\text{max}}$/MIC, $AUC_{0-\infty}$/MIC, $AUC_{0-\infty}$/$\text{MIC}$ and $T > \text{MIC}$, are presented in Table 4. For each, means were slightly higher for MHB than for serum, corresponding to the lower MIC values in MHB for both organisms. The peak serum concentrations were some 10–13 times greater than MICs for the six tested strains of M. haemolytica and P. multocida. Peak exudate and transudate concentrations were 5–6 times MICs (data not shown).

Mean values of average concentrations ($C_{\text{av}}$) of florfenicol over successive 24 h periods after dosing, relative to MICs, are presented in Tables 5 (serum), 6 (exudate) and 7 (transudate). $C_{\text{av}}$/MIC ratios exceeded 1:1 for both bacterial species and both MHB and serum up to 48–72 h and up to 72–96 h for exudate and transudate, with one exception, a slightly lower ratio of 0.91:1 for the period 72–96 h for M. haemolytica in calf serum.

### In vitro MIC and MBC values

For the six strains each of M. haemolytica and P. multocida, MICs and MBCs are presented in Table 3. All ranges were narrow. Values in serum were slightly higher than in MHB for both MIC and MBC. MBC/MIC ratios were equal to or less than 2:1, suggesting a relatively steep concentration–effect relationship.

### Ex vivo growth inhibition curves

Ex vivo growth inhibition curves in serum against four strains each of P. multocida and M. haemolytica are illustrated in Fig. 3. With high florfenicol concentrations, relative to MIC, a greater than 6 log$_{10}$ reduction in bacterial count was obtained after 4 or 8 h incubation for M. haemolytica and P. multocida, respectively. The rapid reduction in bacterial count to <33 cfu/mL indicates a bactericidal action by a concentration-dependent killing mechanism. For M. haemolytica only, slight regrowth occurred between 8 and 24 h incubation at all concentrations investigated. For both organisms, serum samples collected up to 96 h produced a 1 log$_{10}$ or greater reduction from the initial inoculum count after 24 h incubation.

**Table 3. Ranges of MIC and MBC values (µg/mL) of florfenicol for P. multocida and M. haemolytica in Mueller–Hinton broth and calf serum (n = 6)**

<table>
<thead>
<tr>
<th></th>
<th>P. multocida</th>
<th>M. haemolytica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHB</td>
<td>Serum</td>
</tr>
<tr>
<td>MIC</td>
<td>0.35–0.45</td>
<td>0.40–0.55</td>
</tr>
<tr>
<td>MBC</td>
<td>0.70–1.1</td>
<td>0.85–1.3</td>
</tr>
</tbody>
</table>

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Time period after dosing (h) | Predicted daily dosages
---|---
0–24 | 4.08 | 3.73 | 2.00 | 1.10
24–48 | 4.26 | 4.29 | 2.66 | 1.41
48–72 | 4.26 | 4.29 | 2.66 | 1.41
72–96 | 4.26 | 4.29 | 2.66 | 1.41

PK-PD modelling of ex vivo growth inhibition data

Data for PK-PD modelling of the ex vivo time–kill data are presented in Table 8. Three levels of growth inhibition, no change from initial inoculum count and 3 log$_{10}$ and 4 log$_{10}$ reductions in bacterial count were determined. Mean values for each level of growth inhibition were broadly similar for each of the three fluids, serum, exudate and transudate, and, overall, the means were similar for the two pathogens.

Predicted daily dosages

Based on the distributions of CI/F in the pharmacokinetic study (n = 10), AUC$_{0–24}$ h/MIC ratios for three levels of bacterial kill derived from PK/PD modelling (n = 4 for each organism) and distributions of MIC reported in the scientific literature (Thiry et al., 2011) predicted once daily dose requirements are presented in Table 9. Ninety% target attainment rate for P. multocida was achieved with predicted amounts (dosages) of 1.23 mg/kg (bacteriostasis), 2.87 mg/kg (bactericidal action) and 4.18 mg/kg (4 log$_{10}$ reduction in bacterial count). Comparative values for M. haemolytica were 3.09, 9.55 and 14.06 mg/kg. A worst-case scenario analysis (highest clearance, lowest protein binding, highest AUC$_{0–24}$ h/MIC and highest MIC values amongst reported distributions) yielded doses of 5.92 and 18.93 mg/kg per day for eradication of P. multocida and M. haemolytica, respectively.

Sensitivity analysis for M. haemolytica and P. multocida (mean, n = 4). SEM bars not shown for clarity.

Ex vivo growth inhibition curves in exudate (Fig. 4) and transudate (Fig. 5) were similar to those obtained in serum. For both organisms and both tissue cage fluids, a 5 log$_{10}$ reduction in count was obtained with high florfenicol concentrations, relative to MICs, after 8 h incubation with no apparent regrowth after 24 h incubation. With all three fluids, concentrations approximating to the in vitro determined MICs produced a 2–3 log$_{10}$ decrease in count after both 8 and 24 h incubation ex vivo.

**Table 5.** Average serum florfenicol concentration/MIC ratios for three 24 h periods after subcutaneous dosing at 40 mg/kg (n = 10)

**Table 6.** Average exudate florfenicol concentration/MIC ratios for four 24 h periods after subcutaneous dosing at 40 mg/kg (n = 10)

**Table 7.** Average transudate florfenicol concentration/MIC ratios for four 24 h periods after subcutaneous dosing at 40 mg/kg (n = 10)

**Table 8.** Average serum florfenicol concentration/MIC ratios for three 24 h periods after subcutaneous dosing at 40 mg/kg (n = 10)

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M. haemolytica in the calculated dose was attributable to the MIC and free fraction distributions, respectively. Sensitivity analysis for P. multocida indicated that 69% of the variability in the calculated dose was attributable to the MIC distribution.

Fig. 4. Ex vivo growth inhibition for florfenicol in exudate against (a) M. haemolytica and (b) P. multocida (n = 4). SEM bars not shown for clarity.

Fig. 5. Ex vivo growth inhibition for florfenicol in transudate against (a) M. haemolytica and (b) P. multocida (n = 4). SEM bars not shown for clarity.

26.8% was due to CI/F distribution and 4.1% was due to distribution of the fraction of florfenicol unbound.

DISCUSSION

The excellent responses in clinical use of florfenicol in bovine respiratory disease (Varma et al., 1998; Catry et al., 2008) can probably be ascribed to several factors. (i) Resistance of the principal bacterial pathogens, M. haemolytica and P. multocida, seems not to be widespread, despite clinical use for some 20 years. Shin et al. (2005) reported on the sensitivity of cattle and pig pathogens; all Actinobacillus pleuropneumoniae, P. multocida, M. haemolytica and 98.6% of isolates of Bordetella bronchiseptica were sensitive to florfenicol, with MIC values ≤1 µg/mL. Lizarazo et al. (2006) also reported MIC values and MIC values of 0.5 and 1.0 µg/mL and no resistance in 132 isolates of P. multocida harvested from cases of porcine pneumonia over the period 2003–2004. (ii) Recent studies have demonstrated that florfenicol exerts a protective effect in acute lung injury induced by lipopolysaccharide (Zhang et al., 2009), and it significantly increases survival time in murine endotoxaemia (Zhang et al., 2008). These beneficial effects were attributed to modulation of early cytokine responses in shock by blocking the NF-κB pathway. Florfenicol therefore possesses significant host animal immunomodulatory properties, which, in terms of bacterial kill, may be additive to or synergistic with its direct actions on bacterial protein synthesis. (iii) In clinical use, the drug is administered intramuscularly at a dosage of 20 mg/kg once daily or subcutaneously at a dose of 40 mg/kg as a single dose. For both parenteral routes (and the two dosages), the terminal half-life is much longer than the elimination half-life determined after intravenous dosing. Administration by the two nonvascular parenteral routes, therefore, in the commercially available ‘depot’ formulation results in slow release pharmacokinetics (Varma et al., 1998). The prolonged terminal half-life, especially after subcutaneous dosing at the 40 mg/kg dose, of 27.5 h in serum in this study thus ensures that concentrations in serum are maintained in excess of MICs of key pathogens for up to 3 days or longer. These high concentrations are likely to ensure a high level of efficacy and also minimize opportunities for the emergence of resistance.

Most standard texts describe drugs of the fenicol group, including florfenicol, as bacteriostatic agents. However, the studies of Varma et al. (1998), De Haas et al. (2002) and the data reported in the present investigation indicate that this is not so for the growth inhibiting action of florfenicol against isolates of M. haemolytica and P. multocida harvested from clinical cases of calf pneumonia. The ex vivo growth inhibition curves were characteristic of a concentration-dependent killing action; a high level of kill was attained within 4–8 h of exposure, and the concentration–response relationship was steep. Moreover, a bactericidal level of action (3 log10 reduction in bacterial count) was well maintained in all three fluids, serum, exudate and transudate. Furthermore, based on models of ex vivo serum AUC₀–₂₄ h/MIC ratios, the average florfenicol concentrations were well maintained in all three fluids, serum, exudate and transudate.
over the 24-h incubation period, expressed as multiples of MIC, were 0.34, 1.11 and 1.63 for *M. haemolytica* count reductions of 0.3 log_{10} and 4 log_{10}. Corresponding values of *P. multocida* were 0.32, 0.75 and 1.04, respectively. Thus, concentrations similar to or slightly greater than in *vitro* MICs reduced bacterial counts by 3 log_{10} cfu/mL. High levels of bacterial kill in time-kill studies were previously reported for florfenicol by De Haas et al. (2002) for single isolates of *M. haemolytica*, *P. multocida* and *H. somnus* at MIC concentrations.

In interpreting data from the tissue cage model, it should be noted that the rate and extent of penetration of any drug into intracavale fluids depend in part on the serum concentration-time profile, as this provides the driving concentration for passage into exudate and transudate. However, penetration depends also on whether the granulation tissue within the case has been stimulated with a mild irritant, such as carrageenan and, for several drugs of the NSAID class in several species, penetration rate, and extent into exudate exceeds penetration into transudate (Lees et al., 2004). A trend for greater penetration into exudate of florfenicol was noted in this study (AUC exudate:transudate ratio = 1.31:1), but this difference was not statistically significant. The lack of significance may be due to low percentage binding of florfenicol to serum proteins, because a factor favouring penetration of drugs such as NSAIDs is the very high degree of protein binding (Lees et al., 2004). In addition, a third factor influencing drug penetration into tissue cages is model dependency, varying with the shape of the tissue cage and, in particular, the surface area:volume ratio. Therefore, drug penetration into subcutaneous tissue cages cannot be equated to penetration into other tissues, whether inflamed or noninflamed, under clinical circumstances. In fact, tissue cages have been described, from a pharmacokinetic perspective, as a deep (as opposed to superficial) peripheral compartment (Clarke, 1989: Clarke et al., 1989). The fact that the terminal half-life of florfenicol was similar for both tissue cage fluids and plasma, whereas MRT was longer for the tissue cage fluids than for plasma, is explained by the fact that pseudo-steady state has been achieved; when the terminal half-life in serum is very long (as here), the drug exit rate from the tissue cage is no longer the limiting step for elimination from the tissue cage.

The principal value of tissue cages is, therefore, not to study extravascular drug distribution profiles per se but to provide a simple, ethically acceptable technique for undertaking PK-PD modelling. The latter technique enables determination of the whole sweep of concentration antimicrobial-effect relationship for three biological fluids (serum, exudate and transudate). As in previous investigations with fluoroquinolones in farm animal species (Aliabadi & Lees, 2001, 2002; Aliabadi et al., 2003), mean AUC_{0-24 h}/MIC ratios for three levels of growth inhibition were numerically broadly similar for each of the fluids. This similarity for the three fluids gives confidence in the

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**Table 8. PK-PD modelling of ex vivo florfenicol data (mean and SD, n = 4 for each organism) after subcutaneous administration at a dose of 40 mg/kg**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Parameter (units)</th>
<th>Serum</th>
<th>Exudate</th>
<th>Transudate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log E_{0} (cfu/mL)</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td><em>M. haemolytica</em></td>
<td>Log E_{max} (cfu/mL)</td>
<td>2.38</td>
<td>0.67</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>AUC_{24 h}/MIC for bacteriostatic action (h)</td>
<td>8.16</td>
<td>3.22</td>
<td>6.96</td>
</tr>
<tr>
<td>P. multocida</td>
<td>AUC_{24 h}/MIC for bactericidal action (h)</td>
<td>26.63</td>
<td>8.33</td>
<td>17.88</td>
</tr>
<tr>
<td></td>
<td>AUC_{24 h}/MIC for 4 log_{10} reduction in count (h)</td>
<td>39.04</td>
<td>12.10</td>
<td>26.78</td>
</tr>
</tbody>
</table>

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**Table 9. Predicted once daily dosages based on PK-PD modelling of ex vivo florfenicol data in serum**

<table>
<thead>
<tr>
<th>Target attainment rate</th>
<th>90%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted daily doses for <em>P. multocida</em></td>
<td>1.23</td>
<td>0.82</td>
</tr>
<tr>
<td>Bacteriostatic</td>
<td>2.87</td>
<td>1.94</td>
</tr>
<tr>
<td>Bactericidal</td>
<td>4.18</td>
<td>2.63</td>
</tr>
<tr>
<td>4 log_{10} reduction in count</td>
<td>14.06</td>
<td>9.32</td>
</tr>
<tr>
<td>Predicted daily doses for <em>M. haemolytica</em></td>
<td>3.09</td>
<td>1.83</td>
</tr>
<tr>
<td>Bacteriostatic</td>
<td>9.55</td>
<td>6.36</td>
</tr>
<tr>
<td>Bactericidal</td>
<td>14.06</td>
<td>9.32</td>
</tr>
<tr>
<td>4 log_{10} reduction in count</td>
<td>14.06</td>
<td>9.32</td>
</tr>
</tbody>
</table>

Dosages calculated using equation 2: (i) with CI/F range of 163–272 mL/kg/h (n = 10); (ii) AUC_{0-24 h}/MIC distribution ranges (n = 4) of 5.8–9.4 h (*P. multocida*) and 4.8–11.0 h (*M. haemolytica*) for bacteriostasis, 15.2–21.3 h (*P. multocida*) and 17.2–33.8 h (*M. haemolytica*) for bactericidal action, and 19.4–12.0 h (*P. multocida*) and 25.6–51.2 h (*M. haemolytica*) for 4 log_{10} reduction in count; and (iii) MIC distributions as reported by Thiry et al. (2011) of 0.25–4.50 μg/mL (n = 59) for *P. multocida* and 0.50 to 1.00 μg/mL (n = 109) for *M. haemolytica*. For MICs, values were corrected by 18% to allow for florfenicol protein binding in serum, as the reported literature values were determined in broth.
methodology for predicting effective antimicrobial drug concentrations \textit{in vivo}. The present findings thus suggest that similar numerical values for the three levels of growth inhibition considered are likely to apply for florfenicol for most extracellular and transcellular fluids. The findings therefore lend support to the PK-PD modelling approach for estimating an effective \textit{in vivo} dosage regimen.

The present findings extend previous data from our laboratory in three respects. (i) The findings indicate that PK-PD modelling can be extended to florfenicol and previous studies in our laboratory have been restricted to drugs of the fluoroquinolone class in a range of farm animal species. (ii) Our previous studies investigated only one bacterial species, \textit{M. haemolytica}, whereas the present study provides information on a second clinically important calf pathogen, \textit{P. multocida}. (iii) Our previous investigations used a single isolate of \textit{M. haemolytica} and therefore provided no information on interstrain variability in levels of the surrogate marker AUC$_{0.24}$ h/MIC required for each level of bacterial growth inhibition. By using four to six strains of each bacterial species, the present investigation provided new data on the variability in response between those strains. Thus, CV\% AUC$_{0.24}$ h/MIC values for bacteriostatic, bactericidal and 4 log$_{10}$ count reduction responses were 39, 31 and 31, respectively, for \textit{M. haemolytica}. Corresponding values for \textit{P. multocida} were somewhat lower, 20, 17 and 23, respectively, indicating lesser variability between isolates for this species. This might ensure good consistency between animals in drug concentrations achieving therapeutic efficacy.

The florfenicol dosage, product formulation and administration route used in this investigation were designed by the manufacturer to achieve, for single-dose administration, a therapeutic effect over at least 72 h against most or all clinical isolates of \textit{M. haemolytica} and \textit{P. multocida}. PK-PD integration of data indicated that this objective was readily achieved against the 12 isolates (six strains of each bacterial species) investigated. Thus, for both MHB and calf serum, $C_{\text{avmax}}$/MIC exceeded 10:1, AUC$_{0.24}$ h/MIC exceeded 150 h, AUC$_{0.24}$/MIC exceeded 300 h and $T_{\text{MIC}}$ was greater than 75 h. As the MIC values for strains of both organisms used in this investigation were typical of those reported for field isolates (Piebre et al., 2005), these integrated variables are relevant to prediction of the efficacy of florfenicol in clinical use in calf pneumonia. Moreover, $C_{\text{av}}$ values over successive 24 h periods exceeded the MIC for serum for the three periods up to 72 h and for tissue cage fluids for all periods up to 96 h, except for a single value for transudate. The anticipated effectiveness of florfenicol over these extended periods, based on integrating \textit{in vivo} pharmacokinetic with \textit{in vitro} pharmacodynamic data, was confirmed by the \textit{ex vivo} growth inhibition curves on three counts: (i) with the high florfenicol concentrations achieved \textit{in vivo} reductions in bacterial count of 5 log$_{10}$ CFU/mL or greater were obtained; (ii) the speed of bacterial killing was rapid, indicating a concentration-dependent killing action; and (iii) high levels of growth inhibition were maintained for up to 72 h in serum and up to 96 h in exudate and transudate.

The product used in this study was a depot/slow release formulation of florfenicol, for which the manufacturer’s recommended dose is 40 mg/kg subcutaneously as a single dose (the dose and route used in the pharmacokinetic part of this study) and 20 mg/kg administered intramuscularly twice with an interval of 48 h. However, growth inhibition produced by \textit{M. haemolytica} and \textit{P. multocida} was established \textit{ex vivo} (in the pharmacodynamic part of this study) over a 24-h incubation period. Therefore, from these experimental data, it was possible for 24-h dosage regimens to be calculated for each bacterial species. The findings were thus used to design daily dosage schedules for the drug substance rather than the drug product. Based on Monte Carlo simulations providing a 4 log$_{10}$ reduction in bacterial count and literature reported MIC distributions, the estimated amounts (daily dose) for a 90% target attainment rate were 14.1 mg/kg for \textit{M. haemolytica} and 4.2 mg/kg for \textit{P. multocida}.

**ACKNOWLEDGMENTS**

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