Pharmacokinetic and pharmacodynamic modelling of marbofloxacin administered alone and in combination with tolfenamic acid in calves

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In a four-period, cross-over study, the fluoroquinolone antibacterial drug marbofloxacin (MB) was administered to calves, alone and in combination with the nonsteroidal anti-inflammatory drug tolfenamic acid (TA). Both drugs were administered intramuscularly (IM) at doses of 2 mg/kg. A tissue cage model of inflammation, based on the actions of the mild irritant carrageenan, was used to evaluate the pharmacokinetics (PK) of MB and MB in combination with TA. MB mean values of area under concentration–time curve (AUC) were 15.1 μg·h/mL for serum, 12.1 μg·h/mL for inflamed tissue cage fluid (exudate) and 9.6 μg·h/mL for noninflamed tissue cage fluid (transudate). Values of C_{max} were 1.84, 0.35 and 0.31 μg/mL, respectively, for serum, exudate and transudate. Mean residence time (MRT) of 23.6 h (exudate) and 22.6 h (transudate) also differed significantly from serum MRT (8.6 h). Co-administration of TA did not affect the PK profile of MB. The pharmacodynamics of MB was investigated using a bovine strain of *Mannheimia haemolytica*. Time–kill curves were established *ex vivo* on serum, exudate and transudate samples. Modelling the *ex vivo* serum time–kill data to the sigmoid E_{max} equation provided $AUC_{24 h}/MIC$ values required for bacteriostatic (18.3 h) and bactericidal actions (92 h) of MB and for virtual eradication of the organism was 139 h. Corresponding values for MB + TA were 20.1, 69 and 106 h. These data were used to predict once daily dosage schedules for a bactericidal action, assuming a MIC_{90} value of 0.24 μg/mL, a dose of 2.6 mg/kg for MB and 2.19 mg/kg for MB + TA were determined, which are similar to the currently recommended dose of 2.0 mg/kg.

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INTRODUCTION

Marbofloxacin (MB) is a fluoroquinolone developed for use in veterinary medicine. Its action is bactericidal and it possesses a wide spectrum of antimicrobial activity, which includes mycoplasmas, most gram-negative and some gram-positive bacteria (Spreng *et al.*, 1995; Brown, 1996; Schneider *et al.*, 1996; Thomas *et al.*, 2003). Against most pathogens, its killing action is concentration dependent (Aliabadi & Lees, 2002). Plasma protein binding is relatively low (Ismail & El-Kattan, 2007). Clinical trials of MB for the treatment of bovine and porcine pneumonias have established its clinical efficacy under field conditions at a dose of 2 mg/kg once daily (Thomas *et al.*, 1998a,b, 2001). In addition, it is also licensed to use for bovine respiratory disease (BRD) at 8 mg/kg as a single dose.

Tolfenamic acid (TA) is a drug of the nonsteroidal anti-inflammatory (NSAID) class. It is used in human and animals for its antihyperalgesic actions (Robertson & Taylor, 2004). In addition, its anti-inflammatory, antipyretic and antihyperalgesic properties provide a pharmacological basis for its use in combination with antimicrobial drugs, in pneumonias of young calves and pigs. For example, TA has been used for BRD therapy in combination with oxytetracycline (Deleforge *et al.*, 1994). Moreover, direct antimicrobial activity of TA has been reported in humans against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Kruszewska *et al.*, 2002). Another drug

of the NSAID class, diclofenac, has been shown, in a murine model of tuberculosis, to possess direct antimycobacterial activity, and it also interacts synergistically with streptomycin (Dutta *et al.*, 2007).

As both MB and TA are licensed for the therapy of BRD and are marketed by the same manufacturer, it is probable that they are co-administered frequently in clinical use. Previous reports from our laboratory have described the effect of concurrent administration of MB on the PK of TA in calves (Sidhu *et al.*, 2006a) and goats (Sidhu *et al.*, 2006b). However, there are no published data on the influence of concurrent TA dosing on the PK of MB.

The aims of this investigation were to establish (i) the serum concentration–time profile and PK data; (ii) the rate and extent of penetration into and elimination from carrageenan-inflamed (exudate) and noninflamed (transudate) fluids in a tissue cage model; (iii) the *ex vivo* antibacterial activity in serum, exudate and transudate against a strain of *M. haemolytica* isolated from a clinical BRD case; and (iv) integration and modelling of PK and PD data as a basis for dosage determination of MB administered to calves both alone and in combination with TA.

MATERIALS AND METHODS

Animals and surgical procedures

The study was carried out in eight healthy male Holstein Friesian calves weighing 94–142 kg and aged 6–9 months. The animals had free access to hay and water and were given daily 1 kg of concentrate ration. Four cylindrical tissue cages prepared from silicone rubber tubing were inserted subcutaneously in each animal, two on each side of the flank as previously described (Sidhu *et al.*, 2003). Animals were allowed to recover from surgery for 7 weeks to permit wound healing and the growth of granulation tissue into and around the cages.

Experimental design and model of inflammation

A four-period, four-sequence, four-treatment, cross-over design was used, such that each of eight calves received MB, TA, MB + TA and placebo (PL) treatments in a randomized sequence, based on a Latin Square design. MB (Marbocyl 10%; Vetoquinol Ltd., Lure Cedex, France) and TA (Tolfedine 4%; Vetoquinol Ltd., UK) were supplied by the manufacturer. All injections were administered at zero time into the thigh muscles. Each drug was administered at the dose of 2 mg/kg, with each calf receiving two injections in each period, into opposite thigh muscles. The PL injection was sterile normal saline. Intervals of 10 days were allowed between each period.

A volume of 0.5 mL of 1% sterile lambda carrageenan solution (Viscarin GP 109; Marine Colloids, Springfield, NJ, USA) was administered into one tissue cage at zero time during each treatment period (Sidhu *et al.*, 2003). This cage was used to collect serial exudate samples (1.5 mL), and a noninjected tissue cage was used to collect serially samples of noninflamed tissue cage fluid (transudate; 1.5 mL).

Sampling procedures

Blood samples (10 mL) were collected from a jugular vein, into monovettes (Sarstedt, Leicester, UK) without anticoagulant at times of 5, 10, 15, 20, 30 and 45 min and 1, 1.5, 2, 3, 4, 6, 9, 12, 24, 30, 36, 48 and 72 h after administration of drugs. Serum was harvested after centrifugation (2000 *g* for 10 min at 4 °C) and aliquoted into two polypropylene tubes. Samples were stored at −20 °C until either assayed for MB concentration or for measurement of *ex vivo* antibacterial activity of MB.

Exudate and transudate samples were collected at the following times: 0, 1, 3, 6, 9, 12, 24, 30, 36, 48 and 72 h and centrifuged at 2000 *g* for 10 min at 4 °C to remove cells. Supernatants were divided into two aliquots and stored at −20 °C until either analysed for MB concentration or measurement of *ex vivo* antibacterial activity of MB.

Analysis of MB

Serum, exudate and transudate samples were assayed for MB by a high performance liquid chromatography (HPLC-Waters 600E; Milford, Boston, MA, USA) method with fluorescence detection as described by Petracca *et al.* (1993), with minor modifications as described by Aliabadi and Lees (2002). The precolumn and column (Toso Haas, Linton, Cambridge, UK) used were Lichrosorb RPC$_{18}$ 5 μm and Lichrosorb RPC$_{18}$ 7 μm (250 × 4 mm), respectively, with fluorescence detection. Reagents were obtained from Sigma-Aldrich Chemicals Ltd (Poole, Dorset, UK).

Retention times for MB and internal standard were approximately 8 and 10 min, respectively. The lower limit of quantification (LLOQ) of MB in serum, exudate and transudate was 0.005 μg/mL. Control serum, exudate and transudate collected from the animals which had received no drug treatment were spiked with pure MB to prepare standards, ranging from 0.0025 to 5 μg/mL. Linearity of the standard curve was $r^2 > 0.999$. In the MB assay, there was no interference from TA, and the percentage recovery of MB was 95.2 ± 3.87 (mean ± SEM, $n = 16$). The intra-assay and interassay repeatability and reproducibility of the method were evaluated using spiked concentrations. Intra-assay and interassay coefficients of variation (CV%) were 2.42% and 5.90%, respectively, at a concentration of 5 μg/mL and 7.65% and 12.5% at a concentration of 0.05 μg/mL. Percentage accuracies and precision were 93.4% and 5.2% in the absence of TA and 91.4% and 3.6% in the presence of TA.

Determination of MIC and MBC

For the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of MB, clinical calf United Kingdom isolate *M. haemolytica* 3575 was grown freshly from beads stored at −70 °C on tryptone soya blood agar (TSA; Oxoid, Basingstoke, UK). Eight to ten colonies were used to inoculate 30 mL of Mueller–Hinton broth (MHB), followed by incubation at 37 °C on a shaking incubator (220 rpm) for 3 h (final cell count approximately

1.0×10^6 cfu/mL). Further details of the method are described in Aliabadi and Lees (2002).

In vitro *and* ex vivo *antimicrobial activity of MB*

The isolate of *M. haemolytica* 3575 was grown freshly from beads stored at −70 °C on TSA. Eight to ten colonies were used to inoculate 9 mL of MHB, and the culture was allowed to grow overnight at 37 °C. *In vitro* time–kill curves were determined for MB in calf serum using multiples (0.25–8 times) of MIC. *Ex vivo* bacterial time–kill curves were determined in serum, exudate and transudate samples as described by Aliabadi and Lees (2002). The limit of detection was 10 cfu/mL.

PK analyses

Marbofloxacin concentration–time data in serum, exudate and transudate for individual calves were analysed using WinNonlin programmes (Pharsight Corporation, Mountain View, CA, USA). Serum data were submitted to compartmental analysis using nonlinear least squares regression. Schwarz criteria and Minimum Akaike Information Criteria Estimates were applied to discriminate the best fitting model and improved fit of data was achieved by re-weighting.

PK–PD integration and PK–PD modelling

For PK–PD integration, the surrogates C_{max}/MIC and $AUC_{24\,h}$/MIC were calculated for each fluid (serum, exudate and transudate) for each animal. Results were expressed as mean ± SEM. For PK–PD modelling, $AUC_{24\,h}$/MIC data from *ex vivo* bacterial growth inhibition curves were modelled to the Sigmoidal E-max equation:

$$E = E_0 + \frac{E_{max} \times C_e^N}{EC_{50}^N + C_e^N}$$

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 (absence of MB) compared with the initial inoculum $\log_{10}$ cfu/mL, E_{max} is the maximum antibacterial effect determined as difference in $\log_{10}$ cfu/mL in samples incubated with MB between time 0 and 24 h when the detection limit (10 cfu/mL) is reached, C_e is the $AUC_{24\,h}$/MIC in the effect compartment (*ex vivo* site), EC_{50} is the $AUC_{24\,h}$/MIC value producing a 50% reduction in bacterial count from the initial $\log_{10}$ cfu/mL, and N is the Hill coefficient that describes the steepness of the $AUC_{24\,h}$/MIC-effect curve. These PD parameters were calculated using the nonlinear WinNonlin regression programme (Pharsight Corporation).

The antibacterial effect of MB was quantified for three levels of growth inhibition by calculation of $AUC_{24\,h}$/MIC for bacteriostatic action, bactericidal action and bacterial eradication. $AUC_{24\,h}$/MIC values for bacteriostatic and bactericidal actions were defined as those values that produced $E = 0$ (no change in bacterial count after 24-h incubation) and $AUC_{24\,h}$/MIC = −3 (a 3 log or 99.9% reduction of the original inoculum count after 24-h incubation), respectively. $AUC_{24\,h}$/MIC for bacterial eradication was defined as the lowest $AUC_{24\,h}$/MIC that provided a 4-log reduction in bacterial count of original inoculum.

Statistical analyses

All data are presented as mean ± SEM. Differences between animals, times, sequence and cross-over periods and their associated two-factor interactions were analysed by analysis of variance analysis (ANOVA). For PD measurements, the significance of differences at each time point between treatment groups and between pretreatment and post-treatment values was further investigated using Fisher's multiple comparisons test. For PK variables, the significance of differences between MB and MB + TA was assessed using the Mann–Whitney test. All tests of significance were carried out at a 5% probability level.

RESULTS

Pharmacokinetics of MB

Serum. Serum concentration–time data for MB and MB + TA are presented in Figs 1 and 2, respectively. The data provided a best fit to a mono-compartment model with a first-order absorption phase in 13 of 16 calves. The data from three
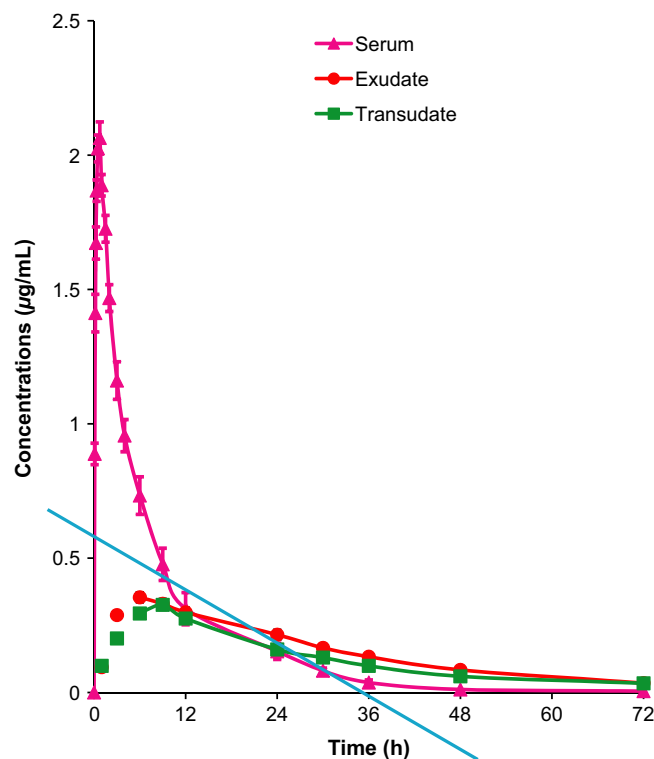

Fig. 1. Arithmetic plot of marbofloxacin (MB) concentration in serum (▲), exudate (●) and transudate (■) after intramuscular administration of MB. Values are mean ± SEM ($n = 8$).
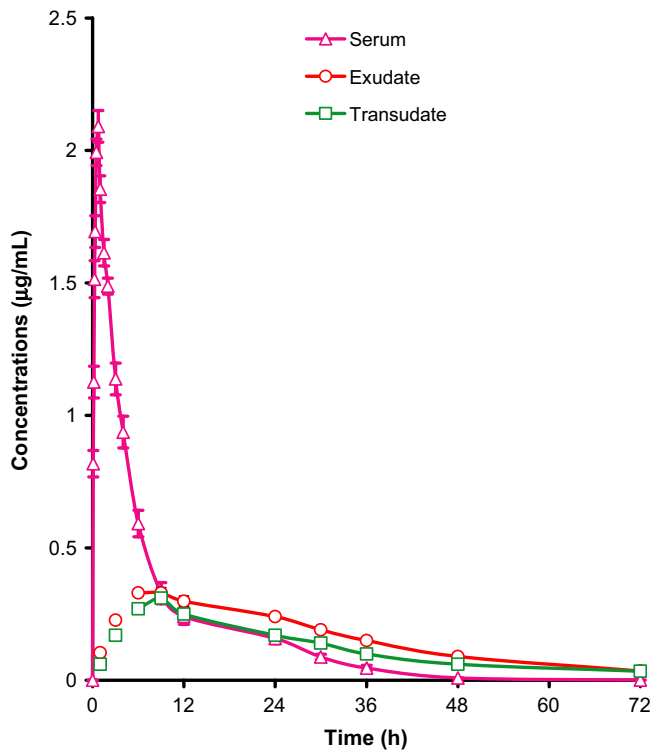
Fig. 2. Arithmetic plot of marbofloxacin (MB) concentration in serum (△), exudate (○) and transudate (□) after intramuscular administration of MB + TA. Values are mean ± SEM ($n = 8$).

Table 1. Pharmacokinetic parameters for marbofloxacin (MB) in serum after intramuscular administration of MB and MB + TA at the dose of 2 mg/kg (mean and SEM, $n = 8$): compartmental modelling

Parameter (units)	MB Arithmetic mean	SEM	MB + TA Arithmetic mean	SEM
k_{abs} (per h)	8.44	0.61	7.06	0.47
k_{el} (per h)	0.15	0.02	0.12	0.01
$t_{\frac{1}{2}abs}$ (h)	0.14	0.05	0.10	0.01
$t_{\frac{1}{2}el}$ (h)	5.24	0.59	5.69	0.23
MRT_{0-last} (h)	8.61	0.06	9.17	0.27
C_{max} (μg/mL)	1.84	0.11	1.70	0.05
T_{max} (h)	0.63	12	0.60	0.03
$AUC_{0-\infty}$ (μg·h/mL)	15.1	1.3	15.1	1.0
AUC_{0-24} (μg·h/mL)	14.0	0.06	12.6	0.7
$V(d)_{area}/F$ (L/kg)	0.99	0.011	1.10	0.030
Cl_B/F (L·kg/h)	0.138		0.136	0.008

Pharmacokinetic parameters were calculated using a one-compartment model with first-order input and output: k_{abs} = absorption rate constant; k_{el} = elimination rate constant; $t_{\frac{1}{2}abs}$ = absorption half-life; $t_{\frac{1}{2}el}$ = elimination half-life; C_{max} = maximum concentration; T_{max} = time to achieve maximum concentration; $AUC_{0-\infty}$ = area under plasma concentration–time curve to infinity; AUC_{0-24} = area under plasma concentration–time curve to sampling time of 24 h; $V(d)_{area}/F$ = volume of distribution scaled to bioavailability; Cl_B/F = clearance scaled to bioavailability. There were no significant differences between MB and MB + TA.

animals (one calf after MB and two calves after MB + TA) gave best fit to a two-compartment model. PK parameters were calculated using a one-compartment model for consistency. Following MB administration, the absorption of MB was rapid ($t_{\frac{1}{2}abs} = 0.14$ h). Mean elimination half-life was 5.24 h. Other variables are presented in Table 1. In calves receiving MB + TA, there were no significant differences from MB administration (Table 1).

Tissue cage fluids. Exudate and transudate drug concentration–time profiles are illustrated in Fig. 1 for MB and Fig. 2 for MB + TA. For both MB and MB + TA administrations, exudate and transudate MB concentrations exceeded LLOQ at 1 h and were lower than serum levels up to 12 h but then exceeded those in serum from 12 to 72 h (Figs 1 & 2). MB concentrations exceeded LLOQ up to 72 h. The data fitted a one-compartment model.

Penetration of MB into exudate was relatively rapid but somewhat slower for transudate, as indicated by the values of $t_{1/2pen}$ of 2.11 and 3.33 h, respectively (Table 2). Mean AUC values for exudate (12.1 μg·h/mL) and transudate (9.6 μg·h/mL) for MB were similar but somewhat lower than serum (15.1 μg·h/mL). The slower elimination of MB from tissue cage fluids compared with serum was indicated by mean $t_{1/2el}$ values of 18.1–19.1 h for exudate and 13.7–14.5 h for transudate, compared with 5.24–5.69 h for serum ($P < 0.01$). Longer persistence of drug in exudate and transudate compared with

serum ($P < 0.01$) was further indicated by MRT values (Tables 1 and 2). There were no significant differences between MB and MB + TA for either exudate or transudate.

Pharmacodynamics, PK–PD integration and PK–PD modelling

In vitro MIC and MBC and time–kill data and integration with in vivo PK data. The MICs of MB against *M. haemolytica* 3575 for the four fluids, MHB, serum, exudate and transudate, are presented in Table 3, together with integrated PK–PD indices derived from *in vivo* PK data and *in vitro* MIC values. MBC values were only moderately greater than MIC values. Both C_{max}/MIC and $AUC_{24 h}$/MIC ratios indicated that serum concentrations of MB would be expected to have a high level of activity against this strain of *M. haemolytica*. The *in vitro* time–kill curves in serum for eight multiples of MIC (0.25–8) indicate a concentration-dependent killing action of MB (Fig. 3). At $2 \times$ MIC bacterial count was reduced <10 cfu/mL at 4 h and at $3 \times$ MIC and higher, the count was reduced to <10 cfu/mL at 2 h. The initial bacterial count ranged from 6.0 to 6.9×10^6 cfu/mL.

As mean exudate and transudate MB concentrations exceeded the MIC concentrations of 0.045 and 0.040 μg/mL for 48 h, respectively, and in some animals at 72 h, it is relevant to consider average concentrations (C_{av}) over each of the time periods 0–24, 24–48 and 48–72 h, relative to MICs (Table 4). For both exudate and transudate, and MB and MB + TA, C_{av}/MIC was >1 over each of the periods 0–24, 24–48 and 48–72 h.

Table 2. Pharmacokinetic parameters for marbofloxacin (MB) in exudate and transudate after intramuscular administration of MB and MB + TA at the dose of 2 mg/kg (mean and SEM, $n = 8$): compartmental modelling

	Exudate				Transudate			
	MB		MB + TA		MB		MB + TA	
Parameters (units)	Arithmetic mean	SEM	Arithmetic mean	SEM	Arithmetic mean	SEM	Arithmetic mean	SEM
$AUC_{0-\infty}$ (μg·h/mL)	12.1	0.9	13.0	0.5	9.6	0.9	9.2	0.5
C_{max} (μg/mL)	0.35	0.01	0.34	0.02	0.31	0.02	0.28	0.01
T_{max} (h)	7.24	0.82	9.37	0.71	8.82	0.82	9.25	0.49
$t_{\frac{1}{2}pen}$ (h)	2.11	0.38	3.05	0.45	3.33	0.48	3.40	0.37
$t_{\frac{1}{2}el}$ (h)	18.1	1.6	19.1	0.6	13.7	1.6	14.5	1.2
$MRT_{(0-last)}$ (h)	23.6	0.7	24.8	0.3	22.6	1.2	23.5	0.7

Pharmacokinetic parameters were calculated using a one-compartment model with first-order input and output: $t_{\frac{1}{2}pen}$ = penetration half-life; $t_{\frac{1}{2}el}$ = elimination half-life; C_{max} = maximum concentration; T_{max} = time to achieve maximum concentration; $AUC_{0-\infty}$ = area under exudate/transudate concentration–time curve; MRT_{0-last} = mean residence time to last sampling time. There were no statistically significant differences between exudate and transudate and no significant differences between MB and MB + TA.
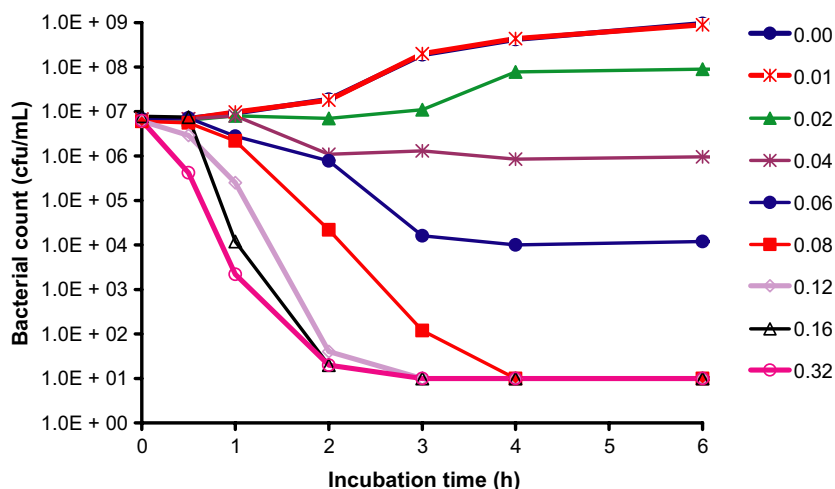
Table 3. MIC and MBC values* in four matrices and surrogate markers derived by PK-PD integration[†] for *Mannheimia haemolytica* strain 3575

			C_{max}/MIC		$AUC_{24 h}$/MIC (h)	
Matrix	MIC (μg/mL)	MBC (μg/mL)	MB	MB + TA	MB	MB + TA
MHB	0.040	0.055	–	–	–	–
Serum	0.040	0.060	46.1:1	42.6:1	350	316
Exudate	0.045	0.060	7.83:1	7.45:1	142	140
Transudate	0.040	0.060	7.85:1	7.09:1	140	129

MHB, Mueller–Hinton broth.
*MIC and MBC determined *in vitro*; [†]C_{max} and $AUC_{24 h}$ determined *in vivo*.

Ex vivo *antibacterial activity of MB in serum.* The *ex vivo* antibacterial time–kill curves for MB in serum against *M. haemolytica* 3575 are illustrated in Fig. 4a (MB) and Fig. 4b (MB + TA). Samples collected up to 9–12 h after drug administration reduced bacterial counts to <10 cfu/mL after 6-h incubation. The 24 h samples were bactericidal and 36-h samples were bacteriostatic. There was no regrowth between 6 and 24 h incubation times.

Ex vivo *antibacterial activity of MB in exudate and transudate.* The *ex vivo* antibacterial time–kill curves for MB in exudate and transudate are illustrated in Figs 5 and 6, respectively. In all exudate samples collected up to 24 h after dosing with MB (Fig. 5a) and 30 h after MB + TA (Fig. 5b), bacterial count was reduced to <10 cfu/mL after 6 h incubation. For both MB and MB + TA, 36 h samples produced a 2-log reduction in bacterial count. There was no regrowth between 6 and 24 h incubation times. Growth inhibition in transudate samples was similar to that in exudate samples (Fig. 6a, b), except that the lower concentrations of MB at late sampling times (30, 36 and 48 h) produced correspondingly less growth inhibition.

PK–PD *modelling of* ex vivo *data.* PK–PD modelled data for serum are presented in Table 5. Values of $AUC_{24 h}$/MIC for bacteriostatic action were 18.3 and 20.1 h for MB and MB + TA, respectively. Higher exposure levels were required for bactericidal and eradication levels of activity. The data in Table 6 indicate that the multiples of MIC required for bacteriostatic, bactericidal and eradication levels were 0.76, 3.8 and 5.8,

Fig. 3. *In vitro* time–kill curves for marbofloxacin (concentration range 0.0–0.32 μg/mL) against *Mannheimia haemolytica* 3575 in calf serum.

Table 4. C_{av}/MIC* ratios for serum, exudate and transudate over three 24-h time periods for marbofloxacin (MB) and MB + TA against *Mannheimia haemolytica* strain 3575

| | C_{av}/MIC | | | | | |
| | 0–24 h | | 24–48 h | | 48–72 h | |
Matrix	MB	MB + TA	MB	MB + TA	MB	MB + TA
Serum	13.5:1	12.2:1	1.24:1	1.51:1	0.08:1	0.11:1
Exudate	5.90:1	5.80:1	3.00:1	3.52:1	1.28:1	1.40:1
Transudate	5.79:1	5.40:1	2.44:1	2.60:1	1.15:1	1.12:1

*C_{av} = average 24-h concentration measured *in vivo* and MIC values determined *in vitro*.

respectively, for MB and 0.84, 2.9 and 4.4, respectively, for MB + TA.

Values of AUC$_{24 h}$/MIC for bacteriostasis, bactericidal action and eradication were similar for MB alone and MB + TA for exudate (Table 6; Fig. 7). For transudate also, AUC$_{24 h}$/MIC mean values were similar for MB and MB + TA. Moreover,

transudate and exudate AUC$_{24 h}$/MIC values were very similar. For both MB and MB + TA, the concentration–effect relationships were steeper for exudate and transudate than for serum.

DISCUSSION

Pharmacokinetics

The PK of MB administered i.m. or i.v in calves and adult cows has been investigated by previous workers (Thomas *et al.*, 1994a,b; Schneider *et al.*,1996; Shem-Tov *et al.*, 1997; Aliabadi & Lees, 2002; Ismail & El-Kattan, 2007). The latter workers compared healthy calves with those naturally infected with *M. haemolytica*, demonstrating significantly longer elimination $t_{1/2}$ and MRT in diseased calves. In the present study in healthy male calves of the Holstein Friesian breed, PK indices ($t_{1/2abs}$, $t_{1/2el}$, AUC MRT and C_{max}) were similar to those previously reported in healthy male Friesian calves (Aliabadi & Lees, 2002) and in cross-breed Simmental calves, gender not stated (Ismail &
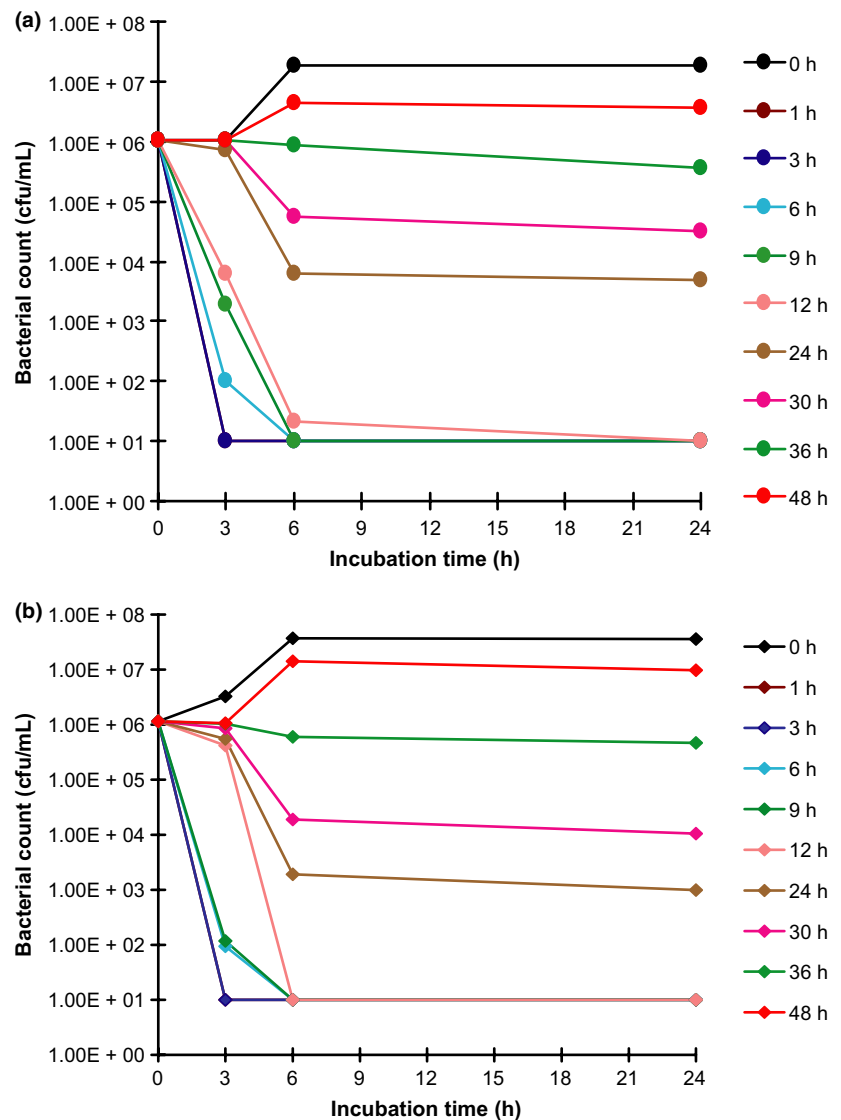

Fig. 4. *Ex vivo* inhibition of bacterial growth in serum before and after i.m. administration of (a) marbofloxacin (MB) and (b) MB + TA (sampling times of 1, 3, 6, 9, 12, 24, 30, 36 and 48 h). Values are means (*n* = 8). SEM bars not included for clarity.
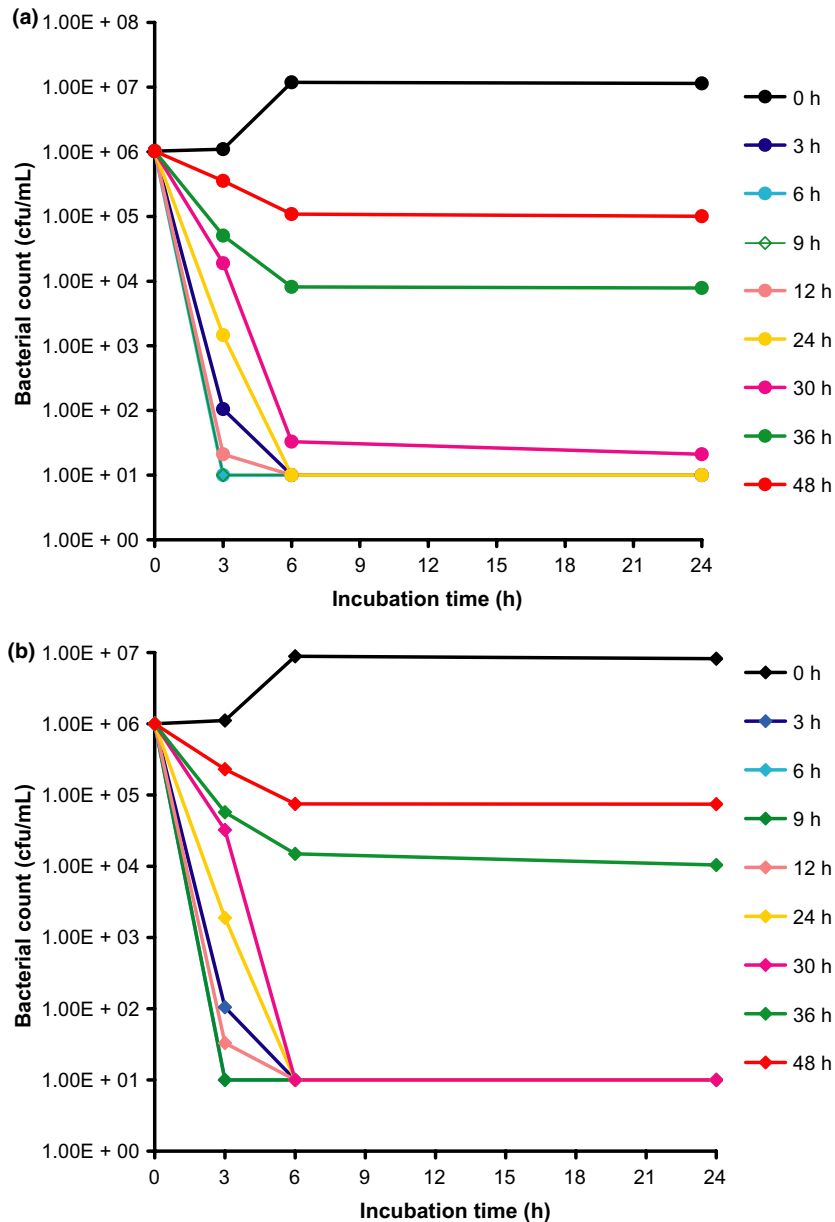
Fig. 5. *Ex vivo* inhibition of bacterial growth in exudate before and after i.m. administration of (a) marbofloxacin (MB) and (b) MB + TA (sampling times of 1, 3, 6, 9, 12, 24, 30, 36 and 48 h). Values are mean (*n* = 8). SEM bars not included for clarity.

El-Kattan, 2007). Similar findings were reported for MB in adult lactating cattle (Shem-Tov *et al.*, 1997).

In the present study the PK of MB after i.m. administration of MB and MB + TA was best described by a one-compartment model with absorption phase. Moreover, all derived PK variables for MB were similar for administration of the drug alone and in combination with TA. This absence of influence of co-administered TA on the PK profile of MB is in contrast to the effect of MB on the PK of TA. Sidhu *et al.* (2005) reported in a calf study decreased C_{max} and AUC of TA in serum and exudate and decreased AUC in transudate in the presence of MB.

The tissue cage model used in this study was developed by Higgins *et al.* (1984) and Sidhu *et al.* (2003). It is important to note that it is not a model which is the predictive of penetration of drugs into tissue interstitial fluid. However, it does give an indication of rates of drug penetration into and persistence in poorly vascularized tissues (Clarke, 1989). The shape and size of tissue cages affects the kinetics of drug disposition into and removal from cages, being dependent on surface area/volume ratio. Nevertheless, the model does allow comparisons to be made of penetration into inflamed and noninflamed sites. Peak MB concentrations in exudate and transudate in this study were similar and significantly lower than the peak serum concentration. Mean exudate/serum AUC ratios were 0.80:1 for MB and 0.87:1 for MB + TA. Corresponding transudate/serum ratios were 0.62:1 and 0.61:1, respectively. Values of MRT indicated significantly longer persistence of MB in tissue cage fluids than in serum. Similar findings for penetration of MB into tissue cage fluids of goats and sheep were reported previously by Sidhu *et al.* (2010a,b).
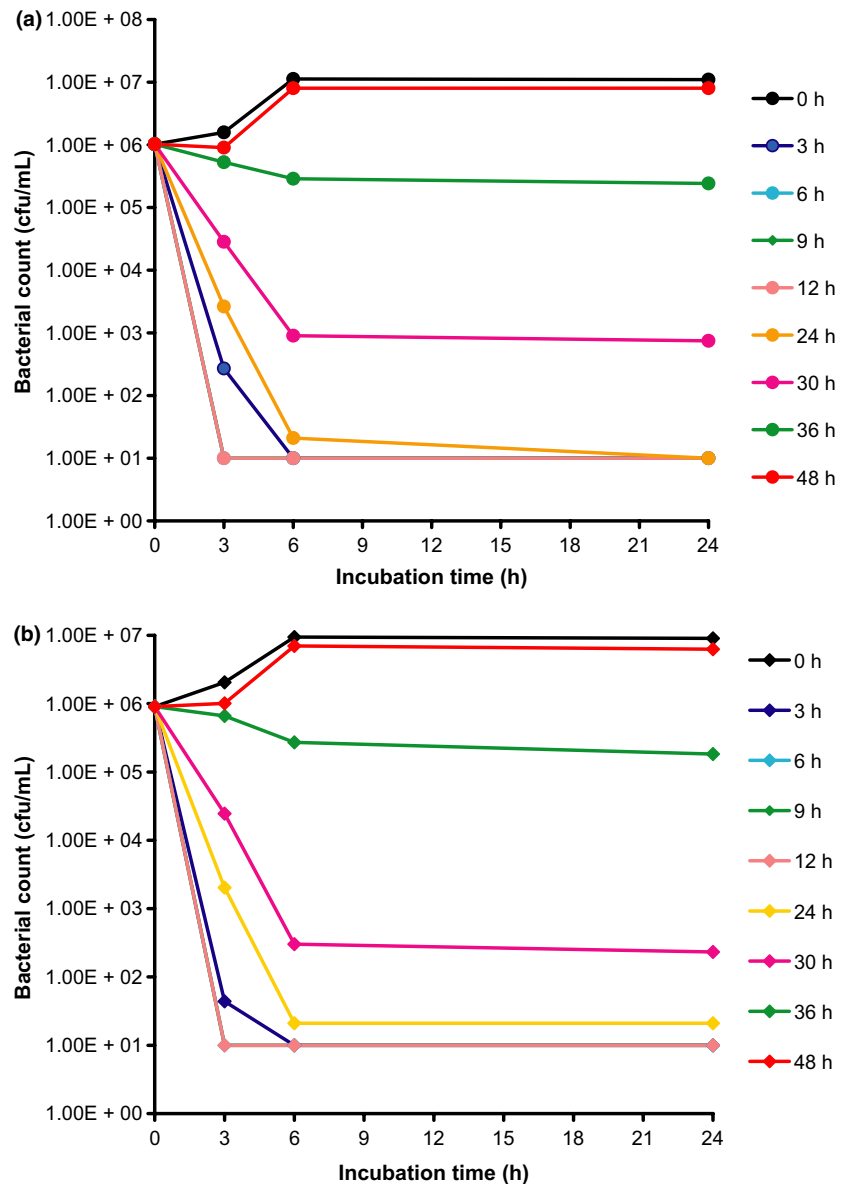
Fig. 6. *Ex vivo* inhibition of bacterial growth in transudate before and after i.m. administration of (a) marbofloxacin (MB) and (b) MB + TA (sampling times of 1, 3, 6, 9, 12, 24, 30, 36 and 48 h). Values are means (*n* = 8). SEM bars not included for clarity.

Table 5. PK-PD modelling of *ex vivo* serum marbofloxacin data (mean ± SEM, *n* = 8) after intramuscular administration of marbofloxacin (MB) and MB + TA

Parameter (units)	MB		MB + TA	
	Mean	SEM	Mean	SEM
Log E_o (cfu/mL)	1.05	0.03	0.24	0.06
Log E_{max} (cfu/mL)	−5.13	0.10	−5.25	0.07
$AUC_{24 h}$/MIC for bacteriostatic action (h)	18.3	3.0	20.1	1.5
$AUC_{24 h}$/MIC for bactericidal action (h)	92	14	69	4
$AUC_{24 h}$/MIC for bacterial eradication (h)	139	25	106	11
Slope (N)	3.4	1.2	3.2	1.1

E_o = difference in number of bacteria (cfu/mL) in control sample in absence of drugs between time 0 and 24 h. E_{max} = difference in number of bacteria (cfu/mL) in sample incubated with MB or MB + TA between time 0 and 24 h. $AUC_{24 h}$/MIC values for bacteriostatic, bactericidal and eradication responses derived from the sigmoid E_{max} curve. N = slope of $AUC_{24 h}$/MIC-response curve.

Pharmacodynamics

Plasma protein binding prevents the antimicrobial actions of antimicrobial drugs, and this was specifically demonstrated for the fluoroquinolones, moxifloxacin and trovafloxacin, in the investigation of Zeitlinger *et al.* (2008). However, in the present investigation MIC values for MB were the same in serum, transudate and MHB and only slightly higher in exudate. This is likely to reflect the low degree of protein binding of MB in calf serum. Ismail and El-Kattan (2007) reported percentage binding of MB to plasma protein of 29 and 27, respectively, in healthy and diseased calves, although a higher range of 58–69% was reported in adult cattle (Shem-Tov *et al.*, 1997). However, there are other differences in composition (as well as protein content) between MHB and biological fluids, such as ion concentrations and pH. It is therefore strongly preferable, as highlighted by Zeitlinger *et al.* (2008), to determine MICs, as the standard index

Table 6. PK-PD modelling of *ex vivo* marbofloxacin data (mean ± SEM, *n* = 8) for exudate and transudate after intramuscular administration of marbofloxacin (MB) and MB + TA

Parameter (units)	Exudate				Transudate			
	MB		MB + TA		MB		MB + TA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
$AUC_{24\,h}$/MIC for bacteriostatic action (h)	44	4	55	8	58	11	48	5.0
$AUC_{24\,h}$/MIC for bactericidal action (h)	77	4	69	8	77	10	66	5.0
$AUC_{24\,h}$/MIC for eradication (h)	91	4	74	7	86	9	75	5.0
Slope (N)	7.4	1.4	19.4	4.3	10.4	2.5	11.5	4.47

$AUC_{24\,h}$/MIC values for bacteriostatic, bactericidal and eradication responses derived from the sigmoid E_{max} curve. N = slope of $AUC_{24\,h}$/MIC–response curve.

of drug efficacy and potency, in biological fluid matrices, when the objective of the study is the prediction of an effective dose for clinical use.

There was no regrowth when organisms were exposed to low MB concentrations either *in vitro* or *ex vivo*. Brown (1996) proposed that maximum concentrations of fluoroquinolones at the site of infection should be >4–8 times the *in vitro* determined MIC to minimize resistance development. The present *in vitro* data in serum and *ex vivo* data in serum, exudate and transudate demonstrated the virtual eradication of organisms by MB (reduced cfu/mL to <10) at concentrations 4 times MIC and greater after 3 to 6 h exposure. Our data confirm previous reports that fluoroquinolones in general and MB in particular exert concentration-dependent killing of bacteria (Spreng *et al.*, 1995; Aliabadi & Lees, 2001, 2002; Sarasola *et al.*, 2002; Aliabadi *et al.*, 2003). The present study further demonstrates no interference in the antimicrobial action of MB in the presence of TA.

PK–PD integration of MB

Optimizing dosage schedules of antimicrobial drugs is crucial in ensuring bacteriological and clinical cures and minimizing the emergence of resistance (Drusano, 2003; Lees *et al.*, 2006; Fabrega *et al.*, 2008). In veterinary medicine, approaches based
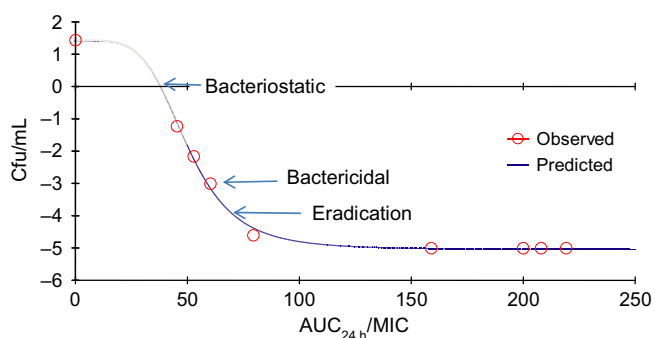

Fig. 7. A specimen plot of *ex vivo* $AUC_{24\,h}$/MIC versus bacterial count (log cfu/mL) for *Mannheimia haemolytica* 3575 in calf exudate. Each point represents individual animal values. The curve is the line of best fit based on the Sigmoid *E*-max equation.

on PK–PD integration and PK–PD modelling, using surrogate markers of clinical efficacy, have been applied to dosage determination for subsequent evaluation in clinical trials (Aliabadi & Lees, 2001, 2002; Toutain, 2002, 2003a,b; Toutain & Lees, 2004; Dova *et al.*, 2006; Haritova *et al.*, 2006). The surrogates most commonly used for fluoroquinolones relate maximum concentration in serum/plasma (C_{max}) and area under plasma/serum concentration–time curve (AUC) measured *in vivo* to MIC against pathogens determined *in vitro*, using the ratios C_{max}/MIC and AUC/MIC.

In early studies on ciprofloxacin, it was suggested that a C_{max}/MIC ratio of 10:1 and AUC/MIC ratio of 125 h or greater predict a successful clinical outcome (Forrest *et al.*, 1993; Sullivan *et al.*, 1993). Similar or higher values have been proposed for all fluoroquinolones for avoidance of the emergence of resistance. In this study, C_{max}/MIC and $AUC_{24\,h}$/MIC ratios for MB were 46.1:1 and 350 h, respectively, for serum. Similar values were reported in a previous study on MB in calves (Aliabadi & Lees, 2002). However, Drugeon *et al.* (1997) reported MIC_{50} and MIC_{90} values of 0.024 and 0.17 μg/mL for MB against 120 bovine strains of *M. haemolytica*. For MIC_{90}, the integrated C_{max}/MIC and $AUC_{24\,h}$/MIC ratios are 10.8:1 and 82 h, respectively. On these data, MB and MB + TA are predicted to provide effective serum concentrations when MB is administered i.m. once daily at a dose of 2 mg/kg.

For fluoroquinolones as a group, the values of integrated PK–PD surrogates, C_{max}/MIC and $AUC_{24\,h}$/MIC, for successful bacteriological outcome, of 10:1 and 125 h (or greater), respectively, have been generally accepted by the scientific community. However, they are based on both the results of experimental studies in laboratory animals (which in many studies were immunosuppressed) and human clinical trial data (predominantly patients with severe lung infections). Moreover, the outcome of antimicrobial therapy, including the emergence of resistance, depends on a wide range of factors, including disease severity, causative microbial species and strain, pathogen load and immunocompetence (Lees *et al.*, 2006). Ferran *et al.* (2007) demonstrated in an *in vitro* pharmacodynamic model that the likelihood of selection of *E. coli* mutants resistant to MB was increased when the initial inoculum size was high. The same group reached a similar conclusion when MB was investigated in a rat lung infection model based on *Klebsiella*

pneumoniae and a mouse thigh infection model based on *E. coli* (Ferran *et al.*, 2009; Kesteman *et al.*, 2009). Indeed, the murine model indicated that only the time within the mutant selection window (and not AUC/MIC or C_{max}/MIC) was a good predictor for prevention of emergence of resistance (Ferran *et al.*, 2009). Mutant selection window is that period for which plasma concentrations are greater than MIC but less than mutant prevention concentration (MPC). The MPC is the lowest drug concentration that prevents the growth of the least susceptible first-step resistant mutants. In the rat lung infection model, prevention of resistance required an AUC/MIC ratio of 189 h for a low (10^5 cfu) inoculum, while for a high (10^9 cfu) inoculum, resistant subpopulation enrichment occurred for AUC/MIC ratios up to 756 h (Kesteman *et al.*, 2009).

In summary, success in killing bacteria can be correlated with one or more of the indices, $AUC_{24\ h}$/MIC, C_{max}/MIC and $T >$ MIC. However, they may be less appropriate to ensure that resistance does not emerge. This is because MIC defines efficacy and potency for the whole population and not for sub-populations, with varying susceptibilities. Olofsson *et al.* (2007) suggested that $AUC_{24\ h}$/MPC could be used as an indicator of the exposure required to prevent the selection of resistant mutants. They reported that an $AUC_{24\ h}$/MPC ratio $\geq$22 h prevented the resistance development in *E. coli*. The MPC of strains of *M. haemolytica* isolated from calves with BRD is not known but will be the focus of future studies.

PK–PD modelling of MB

As an alternative to using the widely quoted ratios of 10:1 for C_{max}/MIC and 125 h for $AUC_{24\ h}$/MIC for fluoroquinolones, it is desirable to determine for individual drugs acting against specific pathogens, the actual ratios required to provide differing levels of growth inhibition. In this study, the PK–PD modelling approach was applied to MB against a pathogenic strain of *M. haemolytica*. Based on exposure over a 24 h period, the data indicated that numerically lower values of $AUC_{24\ h}$/MIC were required to achieve both 3 and 4-log reductions in bacterial count with MB + TA compared with MB alone for all three matrices (serum, exudate and transudate) investigated. However, the differences were not statistically significant, so that greater bacterial kill of MB in the presence of TA cannot be claimed.

For MB, the modelled $AUC_{24\ h}$/MIC ratios providing a 3-log reduction in bacterial count were 92 h (serum), 77 h (exudate) and 77 h (transudate), giving a mean of 82 h for the three fluids. Corresponding modelled values for MB + TA were 69 h (serum), 69 h (exudate) and 66 h (transudate), giving a mean of 68 h. Based on the relationship Dose = Cl/F × $AUC_{24\ h}$/MIC × MIC_{90}, these data indicate a MB once daily dosage of 0.44 mg/kg for the strain of organism used in this study (serum MIC = 0.040 μg/mL). The corresponding estimated dose for MB + TA = 0.36 mg/kg. However, Meunier *et al.* (2004) reported MIC_{90} values for bovine *M. haemolytica* isolates which varied considerable from year to year (between 1994/1995 and 2001) from 0.108 μg/mL(1998) to 0.420 μg/mL (2001). The mean value over 8 years was 0.240 μg/mL, whereas previously

Drugeon *et al.* (1997) reported an MIC_{90} of 0.17 μg/mL. Based on the 0.24 μg/mL value, the calculated dose for bactericidal activity is 2.65 mg/kg for MB and 2.19 mg/kg for MB + TA. Overall, therefore, the present findings support the currently recommended daily dose of MB of 2 mg/kg.

CONCLUSIONS

This study established no difference in the PK profile of MB when administered in combination with TA. *Ex vivo* findings demonstrated the ability of MB to eradicate a pathogenic strain of *M. haemolytica* in serum, exudate and transudate samples. The latter fluids are in the extracellular compartment, and most bacteria in most infections are present in these spaces (Kissane, 1997). The data suggest that MB, either alone or in combination with TA, should be therapeutically successful in infectious diseases of calves caused by *M. haemolytica* at the dosage of 2 mg/kg administered every 24 h based on a MIC_{90} value of 0.240 μg/mL.

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