Research in Veterinary Science 95 (2013) 1114–1124

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Mutant prevention concentration and PK–PD relationships of enrofloxacin for *Pasteurella multocida* in buffalo calves

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ARTICLE INFO

Article history:
Received 25 July 2012
Accepted 16 July 2013

Keywords:
Buffalo calves
Enrofloxacin
Mutant prevention concentration
PK–PD modeling
Plasma protein binding

ABSTRACT

This study validated the use of mutant prevention concentration (MPC) and pharmacokinetic and pharmacodynamic (PK–PD) modeling approach for optimization of dose regimen of enrofloxacin to contain the emergence of *Pasteurella multocida* resistance. The PK and PD characteristics of enrofloxacin were investigated in buffalo calves after intramuscular administration at a dose rate of 12 mg/kg. The concentration of enrofloxacin and ciprofloxacin in serum were determined by high-performance liquid chromatography. The serum peak concentration (C_{max}), terminal half-life ($t_{1/2}K_{10}$), volume of distribution ($Vd_{(area)}/F$) and mean residence time (MRT) of enrofloxacin were 1.89 ± 0.35 μg/ml, 5.14 ± 0.66 h, 5.59 ± 0.99 l/kg/h and 8.52 ± 1.29 h, respectively. The percent metabolite conversion ratio of ciprofloxacin to enrofloxacin was 79. The binding of enrofloxacin to plasma proteins was 11%. The MIC, MBC and MPC for enrofloxacin against *P. multocida* were 0.05, 0.06 μg/ml and 1.50 μg/ml.*In vitro* and *ex-vivo* bactericidal activity of enrofloxacin was concentration dependent. Modeling of *ex-vivo* growth inhibition data to the sigmoid E_{max} equation provided AUC_{24h}/MIC values to produce bacteriostatic (19 h), bactericidal (43 h) and bacterial eradication (64 h). PK–PD data in conjunction with MPC and MIC_{90} data predicted dosage schedules for enrofloxacin that may achieve optimum efficacy in respect of bacteriological and clinical cure and minimize the risk of emergence of resistance.

1. Introduction

There is a growing need to optimize the use of old and new antimicrobials to treat infectious diseases effectively due to increasing emergence of drug resistance and lack of discoveries of these agents (Toutain et al., 2002; Drusano, 2004; Lees et al., 2006; Mouton et al., 2011). More recently, it has been suggested that optimization of dosing regimen doesn't only involves maximizing therapeutic outcome but also includes minimizing the risk of resistance emerging during therapy (Toutain and Lees, 2004; Ambrose et al., 2007; Mouton et al., 2011). Therefore, the clinical decisions should be based on exposure–response relationships for both outcomes. Using pharmacokinetic (PK) and pharmacodynamic (PD) principles is a widely accepted approach for optimization of antimicrobial therapy (Aliabadi and Lees, 2000; MacGowan and Bowker, 2002; Hickey, 2007; Mouton et al., 2011). It has been demonstrated in a number of studies that integration and modeling of PK–PD relationships quantify the potency and efficacy of antimicrobials against target pathogens and provide a basis to dose optimization (Aliabadi and Lees, 2000; Drusano, 2004; Mouton et al., 2005; Toutain, 2009).

Pharmacodynamic parameters used to establish antimicrobial activity include: minimum inhibitory concentration (MIC-the lowest *in vitro* concentration which inhibits visible growth under standardized conditions; minimum bactericidal concentration (MBC- the lowest concentration of antimicrobial drug that reduces the microbial population size by 99.9%) and time-kill curves, which establish the reduction in bacterial count over time (Giguere, 2007). For antimicrobials, three PK–PD indices (C_{max}/MIC, AUC_{24h}/MIC, T > MIC) are most often used as a basis for optimum dosage determination by describing and quantifying the killing actions of antimicrobial drugs on pathogenic microorganisms. A C_{max}/MIC is the ratio of the peak serum concentration measured *in vivo* (C_{max}) to the MIC determined *in vitro*; AUC_{24h}/MIC is the ratio of the area under the serum concentration curve over 24 h (AUC) to MIC. These are the most significant predictors of efficacy for fluoroquinolones. T > MIC is the proportion of the dose interval (expressed as percentage), for which the serum concentration exceeds MIC and it correlates best with outcome for time dependent drugs (Mouton et al., 2002; Mouton, 2003; McKellar et al., 2004).

However, there is limited correlation between the values of C_{max}/MIC, AUC_{24h}/MIC and the probability of drug resistance

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http://dx.doi.org/10.1016/j.rvsc.2013.07.019

development. This is due to the fact that MIC defines efficacy and potency for the entire pathogen population and not for sub-populations, with variable susceptibilities to a given drug. Recently, it has been suggested that mutant prevention concentration (MPC); the lowest drug concentration that prevents the growth of the least susceptible first-step resistant mutants should be determined (Drlica, 2003). Previous workers have demonstrated that AUC_{24h}/MPC ratio could serve as an indicator of drug exposure that prevents the selection of drug-resistant mutants (Allen et al., 2004; Olofsson et al., 2006). The MPC of enrofloxacin for the *Pasteurella multocida* causing pasteurellosis in bovines was not known. Therefore, it was important to determine AUC_{24h}/MPC values for enrofloxacin against*P. multocida* in buffalo calves.

Enrofloxacin, a bactericidal and broad spectrum fluoroquinolone is used exclusively in veterinary medicine for the treatment of septicaemia, respiratory tract, urinary tract, skin, soft tissues, bone and joint infections (McKellar et al., 1999; Sanjib et al., 2005). Although, the PK profile of enrofloxacin has been described in calves, cattle, horses, goats, sheep and pigs using low dose of 2.5–5 mg/kg body weight (Kaartinen et al., 1995; Mengozzi et al., 1996; Steeve et al., 1996; Anadon et al., 1999; Mckellar et al., 1999; Elmas et al., 2001) but there are no major reports linking the PK and PD of enrofloxacin in calves within a single investigation. Moreover, in buffalo species there are only two investigations describing the PK disposition of enrofloxacin despite its common use in this species (Verma et al., 1999; Kumar and Jayachandran, 2008). Recently, enrofloxacin has been approved for the treatment and control of bovine respiratory disease associated with *P. multocida and Mannheimia haemolytica* at the high dose rate of 10–12.5 mg/kg body weight as a single shot administration. Short durations of treatment and decreased dosing frequency of antimicrobials may increase drug compliance, lower costs and handling stress of animals and decrease adverse effects. The present investigation therefore, extended previous studies by: (a) determination of serum concentration–time profile and obtaining PK data in buffalo calves for enrofloxacin after intramuscular administration at the dose rate of 12 mg/kg (b) establishing MIC, MBC in two matrices; serum and broth in two strains of *P. multocida* (c) determining MPC of enrofloxacin against two strains of *P. multocida* to address the issue of inter-strain variability (d) establishing *ex-vivo* time-kill curves of enrofloxacin to obtain detail information about the speed and duration of bacterial killing (e) modeling PK–PD data for enrofloxacin AUC_{24h}/MIC ratios for three levels of bacterial kill in buffalo species.

2. Materials and methods

2.1. Animals

The study was conducted in six healthy male buffalo calves of 6–8 months age and weight between 100–140 kg. The animals were procured from department of Animal Genetics and Breeding of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The animals were acclimatized in the experimental shed under uniform conditions for 3 weeks before the start of experiment. During this period the animals were subjected to deworming and regular clinical examination. Green fodder mixed with wheat straw and water *ad libidum* was provided. The experimental protocol followed the ethical guidelines on the proper care and use of animals and was approved by the Institutional Animal Ethics Committee of the university.

2.2. Drug administration and sample collection

Enrofloxacin (Bayrocin 10%, Bayer Pharmaceuticals, India) was administered in thigh muscle of calves by deep intramuscular route at the dose rate of 12 mg/kg body weight. Blood samples (10 ml) were collected from jugular vein into sterilized glass tubes before and after administration of the drug at time intervals *viz.* 0, 5, 10, 15, 20, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 9, 12, 24, 30, 36 and 48 h. The blood samples were immediately kept on ice for 1 h after the collection. After this tubes were placed in incubator at 37 °C for clot formation and retraction of clot for 2 h. Serum was separated by centrifugation at 2000*g* for 10 min and stored in sterile plastic vials at −20 °C till further analysis. Additional blood samples were collected at times of 0, 1, 3, 6, 9, 12, 24, 30, 36 and 48 h after drug administration and serum separated for determination of *ex-vivo* antibacterial activity of enrofloxacin against *P. multocida*.

2.3. Drug analysis

2.3.1. Chemicals and reagents

Enrofloxacin (VETRANAL, 99.1%) for preparation of standard curve was purchased from Sigma–Aldrich. Ciprofloxacin Hydrochloride was purchased from MP Biomedicals, LLC (India). De-ionised water for chromatography was purified by Milli-*Q* water purification system (Millipore, Bedford, MA, USA) and other chemicals viz., acetonitrile (HPLC Grade, Merck), 85% Ortho-Phosphoric acid (Fluka), 70% Perchloric acid (Merck), 40% Tetrabutylammonium Hydroxide (Fluka), Triethylamine (Sigma–Aldrich) were purchased from the local dealers of the respective companies.

2.3.2. Chromatographic system

The concentrations of enrofloxacin and ciprofloxacin in serum were determined simultaneously using reverse-phase High Pressure Liquid Chromatography (HPLC) with UV detector, according to the method described previously (Kung et al., 1993; Dimitrova et al., 2007). Briefly, 300 μl of thawed serum samples were added with 10 μl of 85% phosphoric acid and 40 μl of 70% perchloric acid. After mixing with vortex-mixer for 20 s the mixture was centrifuged at 14,100*g* for 15 min and 30 μl aliquot of the supernatant was injected into the HPLC system. High Performance Liquid Chromatography system (Perkin Elmer) consists of a single pump (mode series 200) and auto-sampler injector with 200 μl loop, dual wavelength UV detector (model 200 series) and Total Chrome software® for analysis. A reverse phase C_{18} column (SunFire™ Particle size 5 μ, and 4.6 × 250 mm, Waters USA) was used as a stationary phase. The mobile phase consisted of water (HPLC grade): acetonitrile in the ratio (80:20, v/v) containing 0.4% v/v ortho-phosphoric acid, 0.5% v/v triethylamine and 5 mM of 40% tetrabutylammonium hydroxide. The mobile phase was pumped at rate of 0.75 ml/min. The detection of enrofloxacin and ciprofloxacin were performed by UV detection at the wavelength of 278 nm at an ambient temperature of 20 °C ± 5 °C. The retention time of ciprofloxacin and enrofloxacin in spiked serum was 6.0 ± 0.5 min and 7.0 ± 0.5 min, respectively. The retention time of enrofloxacin and ciprofloxacin in extracted serum samples were found to match with peak of respective standards (Fig. 1). The serum from control buffalo calves was spiked with concentration range of 0.010–5.0 μg/ml of enrofloxacin and ciprofloxacin for preparation of calibration curve. The standard curves of enrofloxacin and ciprofloxacin were linear in the range of 0.02–5.0 μg/ml with regression coefficient (r^2) of 0.999. The limit of quantification of enrofloxacin and ciprofloxacin were 0.025 and 0.01 μg/ml, respectively. The precision and accuracy of the method for enrofloxacin and ciprofloxacin were evaluated by repetitive analysis of serum samples (n = 16) spiked with 0.0125, 0.025, 0.05, 0.5, 1 and 2 μg/ml. The repeatability and reproducibility were 1.92% and 1.92%, respectively. The accuracy and coefficient of variation were 96.7% and 1.97%, respectively. The percentage of recovery for enrofloxacin and ciprofloxacin were 91.33% and 87.78%, respectively.