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RESEARCH ARTICLE

Pharmacokinetics of Enrofloxacin and Its Metabolite Ciprofloxacin after Single Intramuscular Administration in South American Rattlesnake (*Crotalus Durissus Terrificus*)

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A B S T R A C T

Gram-negative organisms are implicated in serious infectious diseases of reptile species, which play an important role as causes of disease and death in captive snakes. Enrofloxacin represents a good alternative to treat these bacterial infections. In previous studies, significant pharmacokinetic differences with clinical implications have been observed in the only two species of snakes studied. The pharmacokinetic behavior of enrofloxacin was assessed in six South American rattlesnakes (*Crotalus durissus terrificus*), following intramuscular injections of 10mg/kg. High-performance liquid chromatography was used to measure the plasma concentrations of enrofloxacin and its active metabolite, ciprofloxacin. In rattlesnakes, enrofloxacin presented a slow absorption (Tmax=7.61±3.92h) with peak plasma concentration of 5.49±2.42µg/mL and a long elimination half-life ($T_{1/2\lambda}$=20.20±4.40h). Ciprofloxacin showed a high peak plasma concentration of 1.57±0.72µg/mL at 33.63h and the fraction of enrofloxacin metabolized to ciprofloxacin was around 45%. The long persistence (MRTt=57.71±15.78h; $T_{1/2\lambda}$= 33.86±11.97 h) and the high values of Cmax and AUC observed for ciprofloxacin in the *Crotalus* genus could indicate that the active metabolite might possess a high influence in the antimicrobial effect in this species. We consider the administration of 10mg/kg of enrofloxacin by the IM route to be a good choice in rattlesnakes against infections caused by microorganisms with MIC values ≤2.31µg/ml.

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INTRODUCTION

The extralabel use of enrofloxacin in non-traditional species is widely performed in veterinary medicine. This could be because of its good pharmacokinetic and pharmacodynamic properties, such as a large volume of distribution, a rapid and a concentration-dependent bactericidal effect with activity against gram-negative organisms implicated in serious infectious diseases of reptile and, in general, low minimum inhibitory concentrations (except for *Pseudomonas sp*.) (Mitchell, 2006; Prados *et al.*, 2011; Schumacher, 2013; Subramani *et al.*, 2013; Foti *et al*., 2013; Schink *et al.*, 2013). Also, fluoroquinolones are an important alternative to aminoglycosides, the most frequently used antibiotics against gram-negative bacteria in reptiles, as fluoroquinolones are not as nephrotoxic as aminoglycosides (López-Cadenas *et al.*, 2013; Wargo and Edwards, 2014).

Previously, only two pharmacokinetic studies have been performed in snake species (Young *et al.*, 1997; Waxman *et al.*, 2013). The fact that metabolic scaling for drug dose calculation might not always be a good approach and the large pharmacokinetic differences in the behavior of fluoroquinolones among reptile species, remark the importance of conducting studies for individual species rather than extrapolating doses and dosing intervals from data generated in other species, even within a taxonomic group (Jacobson, 1996; Maxwell and Jacobson, 2008). The purpose of this study was to determine the pharmacokinetic behavior of enrofloxacin in rattlesnakes (*Crotalus durissus*

terrificus) after intramuscular administration, in order to estimate pharmacokinetic/pharmacodynamic integration for the optimization of dosage schedules in this species. The intramuscular route was chosen as it is considered as the most practical and widely used in vipers.

MATERIALS AND METHODS

Six adult snakes (*Crotalus durissus terrificus*) (1.45±0.19kg) were used. The animals were housed at the National Institute for Biologics Production INPB-ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina. Snakes were acclimated at 27-29º for at least 6 months prior to the study and maintained at this temperature during the period of sample collection. Animals were allocated individually in plastic containers (50x45x25 cm) at 27-29ºC, relative humidity oscillated between 70-75% and fed in an appropriate manner for the species (4-6 mice every 20 days). No drugs were administered for at least two months prior to the start of the study. The criteria for selection of clinically healthy animals were the routine acceptance of meals, maintenance of body weight, hematological control and complete physical examination. The injection site was monitored every time the animals were taken out of the cages for sample collection during the sample collection period, once daily during the second week and one month after the intramuscular administration of enrofloxacin. The study was approved by the Institutional Animal Care and Use Committee, Veterinary Sciences School, University of Buenos Aires.

A 10 mg/kg single dose of an enrofloxacin 5 % injectable solution (Baytril®, Spain) was administered to each snake into the dorsal muscles of the cranial half of the animal. Blood samples (0.6 mL at each time point) were collected from the ventral coccygeal veins with a 22G needle attached to a 1 mL heparinized syringe at 0.5, 1, 2, 4, 6, 9, 12, 24, 36, 48, 60, 72, 84, 96, 108, 168 and 180 h. No other drugs (anesthetics or tranquilizers) were administered for sample collection. Animals had free access to water all over the study.

Plasma concentrations of enrofloxacin and its active metabolite, ciprofloxacin, were simultaneously quantified in all samples using high performance liquid chromatography (HPLC) with an UV detector, according to a method previously described (de Lucas *et al.*, 2013). Ofloxacin, used as internal standard (5µg/mL), enrofloxacin and ciprofloxacin were used for the preparation of calibration standards (Sigma-Aldrich Química, S.L., Spain). The quantification limit (LOQ) of the assay method was 0.025 µg/mL and 0.05 µg/mL for enrofloxacin and ciprofloxacin, respectively. The standard curves were linear to 10µg/mL for enrofloxacin, and its active metabolite ciprofloxacin (R^2>0.98). Intraday precision was <9%, interday precision was <11% and accuracy oscillated between 88 and 120%.

The data are expressed as arithmetic mean±SD. The statistical analysis was performed using the SPSS 19.0 software package. Pharmacokinetic parameters were determined by means of non-compartmental analysis (PCNONLIN version 4.0 program; SCI Software, USA). Values calculated following the intramuscular administration were: area under the plasma concentration vs time curve (AUC), mean residence time (MRT, where MRT = AUMC/AUC), terminal rate constant (λ, calculated as the slope of the terminal phase of the plasma concentration curve that included a minimum of four points) and terminal half-life ($t_{1/2\lambda}$, where $t_{1/2\lambda}$= 0.693/λ). The AUC was calculated using trapezoidal rule with extrapolation to infinite (∞). The extrapolated area did not exceed 3% of the total area for enrofloxacin; however, the extrapolated area obtained to ciprofloxacin were <12% in five snakes but it reached a 20% in one animal due to a maintenance of metabolite concentration in the last sampling times.

Also, the following PK/PD indices to predict clinical success and the development of resistant mutants were determined, Cmax/MIC and AUC/MIC. These indices were calculated on the basis of the minimum inhibitory concentration values (MICs) of 0.12, 0.2, 0.5 and 1µg/ml according to previously published works in reptile species (Young *et al.*, 1997; Martelli *et al.*, 2009; Waxman 2014). Ciprofloxacin is described to have more potent antimicrobial effect than the parent drug for many veterinary pathogens (Grobbel *et al.*, 2007). For this reason, it would be more appropriate to utilize the AUCe+c and Cmaxe+c values obtained by adding AUC and Cmax values of enrofloxacin and ciprofloxacin, in computing pharmacodynamic variables. Normality of data was evaluated using the Saphiro-Wilk test. A non-parametric Mann-Whitney U test was used to compare the plasma concentrations of drugs and the pharmacokinetic parameters between our results and those previously published in *Bothrops alternatus* (Waxman *et al*., 2014). The level of significance was P<0.05.

RESULTS

All animals remained in good health throughout the acclimatization and study periods. No alterations were observed in clinical evaluation, including review of food intake, responses to stimuli and physical observation of all animals during the experiment and for the following two months. No signs of pain or any adverse reaction at the site of injection were observed. Enrofloxacin and ciprofloxacin plasma concentrations *vs* time curves after intramuscular administration of enrofloxacin are shown in Fig. 1 (arithmetic mean±SD). The pharmacokinetic parameters obtained for enrofloxacin and its active metabolite are presented in Table 1 and PK/PD indices obtained using different MIC values are present in Table 2. Ciprofloxacin was detected at the first sampling time (0.5h) in two animals and at 1h post-administration in the other four snakes. In *C. durissus terrificus*, ciprofloxacin AUC comprised 45±15% of the total fluoroquinolone (enrofloxacin and ciprofloxacin) AUCe+c. If we compare our results with those obtained in urutu pit vipers (Waxman *et al*., 2014), significant differences were found between *Crotalus durissus terrificus* and *Bothrops alternatus* on plasma concentration of enrofloxacin and ciprofloxacin in most of the sample times that could be compared (that fall between 2 and 24 h for enrofloxacin and between 4 and 72 h for ciprofloxacin). Also, statistical differences in AUC between both snake species for enrofloxacin (P=0.046) and ciprofloxacin (P=0.01) were found.

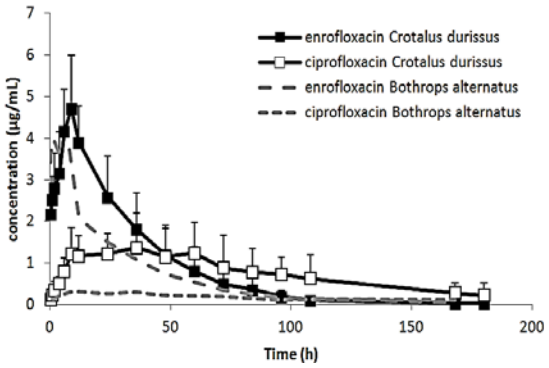

Fig. 1: Plasma concentrations (mean±SD) of enrofloxacin and its metabolite, ciprofloxacin, versus time (h) in *Crotalus durissus terrificus* (n=6), following administration of a single intramuscular 10 mg/kg dose. The grey lines represent the temporal evolution of plasma concentration of enrofloxacin and ciprofloxacin in *Bothrops alternatus* after intramuscular administration of 10 mg enrofloxacin/kg (Waxman *et al.,* 2014).

DISCUSSION

In our study no adverse reactions were observed, the absence of local pain could possibly be related to the low volume administered (0.25-0.34 mL). The venopuncture was preferred over cardiocentesis as the method for sample collection in awake and manually restrained snakes, because it causes the least amount of pain and suffering (Johnson, 2011; Wilkinson, 2014). Also, the possible influence of the reptile renal-portal system on enrofloxacin's pharmacokinetics was avoided by injecting the drug into the cranial muscles of the snake (Sladky and Mans, 2012). Evidence exists to support administration of most parenteral drugs in the cranial half of the body, when possible, to avoid the first-pass effect of drugs that are eliminated via renal tubular excretion or hepatic metabolism. Venous blood from the caudal half of the body enters the caudal vena cava through either the renal portal system and peritubular capillaries or the hepatic portal system from the abdominal or mesenteric veins and hepatocellular parenchyma. Giving therapeutic agents in the caudal half of the body is acceptable for a few specific products and may be considered for other drugs when the cranial half is not available (Gibbon, 2014).

The reptile patient represents a novel challenge to the clinician and enrofloxacin is considered an important therapeutic tool. This drug has been widely used to treat bacterial infections in reptiles, because it is active against most of the gram-positive and gram-negative bacterial pathogens isolated from these species (Mitchell, 2006; Latney and Wellehan, 2013). However, pharmacokinetic studies to evaluate the pharmacokinetic behavior in snakes are very limited. In previous works, differences between urutu pit vipers (*Bothrops alternatus*) and burmese pythons (*Python molurus bivittatus*) have been observed (Young *et al.*, 1997; Waxman *et al*., 2014). These snakes belong to Family Viperidae and Phytonidae, respectively. However, our study shows that, even species belonging to a same family (*Viperidae*) as *Bothrops alternatus* and *Crotalus durissus terrificus* have significant differences on the pharmacokinetic behavior of enrofloxacin, especially regarding its biotransformation rate into ciprofloxacin.

In ectothermic species, the room temperature possesses a high influence on the pharmacokinetic behavior of drugs and this could have some influence on the differences observed among species. In most cases, before therapeutic agents are administered, the body temperature of a reptile patient must match that of the subjects in a given pharmacokinetic study, if available (Gibbons, 2014). Our experiment was conducted at a room temperature of 27-29ºC, which is within the optimum temperature range of *Crotalus sp*. (27-32°C) and this could be taken into account when this drug is used. This temperature range is the same as that used for urutu pit vipers (Waxman *et al.*, 2014) and similar to the reported for pythons (30ºC) (Young *et al.*, 1997).

If enrofloxacin relative bioavailability was calculated taking *Crotalus sp*. as 100%, the values obtained for urutu pit vipers and pythons would be 73 and 28%, respectively. The higher AUC observed in rattlesnakes compared to urutu pit vipers could be related to a lower Vd/F, because both possess a similar Cl/F. This represents an important clinical advantage for rattlesnakes, as enrofloxacin is a concentration-dependent antimicrobial. The terminal half-life of both Viperidae snakes (rattlesnake: 20 h; urutu pit viper: 27.91h) was longer than that described for pythons (6.37h). If Cl/F and Vd/F of enrofloxacin were extrapolated using the values of AUC, slope and dose published by Young *et al.* (1997) for python snakes, values of 0.22 L·h/kg and 2.07 L/Kg are obtained. While similar Vd/F values are obtained for pythons and rattlesnakes, higher Cl/F values are observed in pythons. The high value of apparent clearance obtained in pythons could justify its short elimination half-life.

The active metabolite reached a highest concentration and remained longer in *Crotalus durissus terrificus*. Ciprofloxacin is an active metabolite of enrofloxacin, which is more active than enrofloxacin against gram-negative pathogen microorganisms, for this reason, it may have an additive antimicrobial effect on the concurrent enrofloxacin plasma levels. The biotransformation into ciprofloxacin suffers a wide variation among species. Unlike rattlesnakes that biotransforms a high proportion of enrofloxacin into ciprofloxacin (Fig. 1), low concentrations of this metabolite are observed in urutu pit vipers after enrofloxacin administration of the same dose (0.39µg/mL at 13.45h, AUCt=20µg·h/mL, 10mg/kg; Waxman *et al.*, 2014). In pythons, a value of 0.35µg/mL at 13h and AUCt extrapolated around 14µg·h/mL was found using a dose of 5mg/kg (Young *et al.*, 1997). In Crocodilus porosus, the biotransformation never exceeded 3.2% of the parent drug (Martelly *et al.*, 2009). When the mean AUC of enrofloxacin and ciprofloxacin for the interval of 24h is compared between the three snake species (*Python*: 18.41 and 5.75µg·h/mL; *Bothrops*: 65.63 and 5.79µg·h/mL; *Crotalus*: 83.49 and 23.38µg·h/mL for AUC24 of enrofloxacin and ciprofloxacin, respectively) the fraction of enrofloxacin metabolized into ciprofloxacin is similar between pythons (24%) and rattlesnakes (22%), but much lower in urutu pit vipers (7.8%). If the comparison is established from time 0 to last quantifiable time (AUCt), rattlesnakes showed the

Table 1: Pharmacokinetic parameters of enrofloxacin and its active metabolite ciprofloxacin obtained after intramuscular administration of enrofloxacin (10 mg/kg) in *Crotalus durissus terrificus*

Pharmacokinetic parameters	arithmetic mean	SD	Median	min	Max	geometric mean
Enrofloxacin						
Tmax (h)	7.61	3.92	9.00	0.58	12.00	5.60
Cmax (µg/mL)	5.49	2.42	4.93	2.62	9.79	5.08
Clast (µg/mL)	0.0379	0.0174	0.0308	0.0252	0.0685	0.0351
Tlast (h)	142.15	41.86	144.14	96.17	180.27	136.87
λ (h^{-1})	0.0361	0.0097	0.0336	0.0282	0.0544	0.0351
$T_{1/2\lambda}$(h)*	20.20	4.40	20.79	12.75	24.61	19.23
AUC_t (µg·h/mL)	161.64	60.08	163.08	59.57	239.46	149.15
AUC_∞ (µg·h/mL)	163.14	60.78	163.88	60.18	240.85	150.50
Vz/F (L/kg)	2.12	1.22	1.67	1.16	4.42	1.89
Cl/F (L/h·kg)	0.0745	0.0459	0.0611	0.0415	0.1662	0.0664
MRT_t (h)*	28.04	7.11	28.16	18.54	38.95	26.52
MRT_∞ (h)*	29.16	7.30	28.90	19.72	39.97	27.63
Ciprofloxacin						
Tmax (h)	33.63	22.79	30.10	9.00	60.40	26.43
Cmax (µg/mL)	1.5742	0.7166	1.4820	0.8959	2.8983	1.4593
Tlast (h)	148.07	41.33	168.00	84.00	180.23	142.35
Clast (µg/mL)	0.2674	0.1939	0.2726	0.0541	0.5686	0.1963
λ (h^{-1})	0.0226	0.0074	0.0221	0.0131	0.0314	0.0215
$T_{1/2\lambda}$(h)*	33.86	11.97	31.68	22.05	52.78	30.68
AUC_t(µg·h/mL)	127.56	85.28	94.51	74.82	296.89	111.61
AUC_∞ (µg·h/mL)	142.34	97.31	107.70	84.19	337.33	124.23
MRTt (h)*	57.71	15.78	63.54	34.83	71.78	55.65
MRT_∞ (h)*	71.61	22.69	66.60	43.96	103.53	68.66
Enrofloxacin and Ciprofloxacin						
Tmax (h)	9.50	1.22	9.00	9.00	12.00	9.44
$Cmax_{e+c}$ (µg/mL)	6.05	1.66	5.95	3.60	8.67	5.85
$AUCt_{e+c}$(µg·h/mL)	289.20	112.42	262.40	154.21	465.16	271.40
$AUC_{\infty e+c}$(µg·h/mL)	305.48	123.29	281.92	157.27	506.16	285.30

*: Harmonic mean; Tmax: time of maximal plasma concentration; Cmax: peak drug concentration,; Tlast: time of the last measurable concentration; Clast: last measurable plasma concentration; λ: rate constant for decline in plasma concentration; $T_{1/2\lambda}$: terminal half-life; AUC_t: area under the plasma concentration time curve from time 0 to last quantifiable time, AUC_∞: area under the plasma concentration time curve from time 0 to infinite; Vz/F: apparent volume of distribution during terminal phase after intramuscular administration; Cl/F: apparent total clearance of the drug from plasma after intramuscular administration; MRT_t: mean residence time from time 0 to last quantifiable time; MRT_∞: mean residence time from time 0 to infinite.

Table 2: Efficacy indices (mean±SD) obtained after intramuscular administration of enrofloxacin in *Crotalus durissus terrificus*

MIC_{90} (µg/mL)	$AUCt_{e+c}$/MIC	$AUC_{\infty e+c}$/MIC	$Cmax_{e+c}$/MIC
0.12	2410±936	2546±1027	50.41±13.84
0.25	1157±450	1222±493	24.19±6.65
0.5	578±225	611±246	12.10±3.32
1	289±112	305±123	6.05±1.66

highest value (44%), a slightly lower value was observed in pythons (39%: data extrapolated from the results presented in the publication) and the lower value was obtained in urutu pit vipers (13%).

In pythons (Young *et al*., 1997), the half-life of ciprofloxacin could not be measured from the single dose study, but the accumulation of ciprofloxacin and the evolution of plasma concentrations of the metabolite observed in the multiple dose study induced the authors to suggest that ciprofloxacin might have a longer half-life than enrofloxacin, possibly resulting from continuous formation of ciprofloxacin from enrofloxacin, faster than it was being eliminated. This is in agreement with the results observed in our study, in which ciprofloxacin showed a terminal half-life of 33.84h and enrofloxacin only 20.20h

For concentration-dependent drugs as enrofloxacin, successful treatment of gram-negative bacillary infections requires an AUC/MIC ratio greater than 100–125 or Cmax/MIC ratio greater than 8 to 10. AUC/MIC or Cmax/MIC ratios also can be used to compare the effectiveness of enrofloxacin in different species. When average AUC is known, the pharmacodinamic clinical MIC breakpoint can be calculated by using the following formula for gram-negative bacilli, AUC/125 (Levison and Levison, 2009; Shan *et al*., 2015). Applying these calculations, the respective pharmacodynamic MIC breakpoint for gram-negative bacillary infections would be 2.31, 1.10 and 0.31µg/mL, for rattlesnakes, urutu pit vipers and pythons, respectively.

Little is known about physiological aspects or metabolic routes of rattlesnakes and urutu pit vipers; therefore, it is difficult to determine which could be the cause for the different pharmacokinetic profiles of enrofloxacin. Some differences regarding their habitats and poison composition are documented. Snake venoms may vary biochemically and toxicologically, not only at families, genus and species levels, but also at the individual level. These variations may be related to environmental factors such as climate, geography, according seasons, ontogenetic, food availability and many other causes (Chippaux *et al.*, 1991; Lanari *et al.*, 2010; Costa de Oliveira *et al.*, 2011; Gomes and Almeida-Santos, 2012). However, it is not clear whether these ecological and physiological factors could have influence in the metabolic activity and therefore, in the degree of drug biotransformation.

Conclusion: If we consider enrofloxacin as a concentration-dependent, concretely AUC-dependent antimicrobial agent, the intramuscular administration of 10 mg/kg of enrofloxacin could probably be more efficacious in *Crotalus durissus terrificus* as shown by the higher MIC breakpoint calculated compared to that of the two other species. Also, even when both species are

included into the Viperidae family of venomous snakes, enrofloxacin might show higher efficacy in *Crotalus durissus terrificus* than in *Bothrops alternatus*, possibly due to its higher transformation into ciprofloxacin, a more active metabolite.

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Author's contribution: SW and CR designed the study. PR, VCO and ADR executed the experiment. APP, JJDL and MISA analyzed the plasma samples. CR analyzed the data. CR, SW, JJDL and MISA interpreted the results. CR, SW, PR, VCO and ADR prepared the manuscript. All authors revised the manuscript and approved the final version.

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