POPULATION PHARMACOKINETICS OF CEFTAZIDIME AFTER A SINGLE SUBCUTANEOUS INJECTION AND NORMAL ORAL AND CLOACAL BACTERIAL FLORA SURVEY IN EASTERN HELLBENDERS (CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS)

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POPULATION PHARMACOKINETICS OF CEFTAZIDIME AFTER A SINGLE SUBCUTANEOUS INJECTION AND NORMAL ORAL AND CLOACAL BACTERIAL FLORA SURVEY IN EASTERN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*)

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Abstract: Population pharmacokinetics utilizing sparse sampling were used to determine pharmacokinetics of ceftazidime in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) due to their slow growth rate and the limited number of appropriately sized individuals in the zoo-housed population. Twenty-five eastern hellbenders received a single subcutaneous injection of ceftazidime at 20 mg/kg. Each animal had blood samples collected up to four times between 0 and 192 hr postinjection. Plasma samples were analyzed by high-pressure liquid chromatography. A nonlinear mixed-effects model was fitted to the data to determine typical values for population parameters, an ideal method due to the sampling limitation of each hellbender. Results indicate an elimination half-life of 36.63 hr and volume of distribution of 0.31 L/kg. Antibiotic concentrations were above a minimum inhibitory concentration (MIC) value of 8 μg/ml for 120 hr. Prior to antibiotic administration, six hellbenders had oral and six other individuals had cloacal swabs taken for aerobic culture. Fifty-five bacterial isolates were obtained (24 cloacal, 31 oral) with 10/12 (83%) individuals growing three or more different isolates and 11/12 (92%) growing *Shewanella putrefaciens*. Twelve isolates had susceptibility testing performed and all were susceptible to ceftazidime. These results indicate that ceftazidime is an appropriate choice of antibiotic in hellbenders and when given at a dosage of 20 mg/kg subcutaneously, maintains concentrations above the MIC of susceptible bacteria for up to 5 days.

INTRODUCTION

The eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is a subspecies of large aquatic salamander native to the eastern United States, with populations ranging from New York to Georgia and extending west to the Ozarks. Wild populations are currently decreasing due to habitat destruction and emerging disease threats, including chytridiomycosis (*Batrachochytridium dendrobatidis*) and *Ranavirus*.[1,8,14,15] Another significant source of morbidity and mortality is traumatic injury, frequently caused by predation or intraspecific aggression. Wild hellbenders are frequently found missing digits or limbs as a result of these injuries.[1] Some distal appendage wounds resist healing, and local infection of tissues may progress to exposed bone.[7,13] Resolution of such infections often requires the use of an antibiotic in addition to potential surgical debridement.

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Antibiotic therapy in eastern hellbenders is frequently performed empirically, with little species-specific scientific data available. The need for prompt treatment generally precludes bacterial culture and susceptibility testing in individual cases. The challenges of interpreting cultures in an aquatic animal also limit their use. Ceftazidime is a third-generation cephalosporin commonly used in reptiles and amphibians for a variety of clinical presentations. It is favored for its long duration of action in these species, which is attributed to slow renal clearance and a long half-life. These properties allow for infrequent handling of individuals for administration every 2 to 5 days. Dosing regimens in amphibians are based on pharmacokinetic data in reptiles, particularly chelonians, with most dosage regimens based on extrapolations from a 1999 study in eight juvenile loggerhead sea turtles (*Caretta caretta*), and a more recent 2018 study in wild turtles.[2,6,16] Variations in physiology, size, diet, and environment likely contribute to differences in pharmacokinetics between amphibians and chelonians. Ceftazidime (20 mg/kg SQ q72–96 hr) is frequently used empirically in eastern hellbenders with traumatic injuries or infections.[7]

Population pharmacokinetics utilizing sparse sampling have been used to determine pharma-

cokinetics in some reptile species and amphibian species.[2] This method was used for this study because of the slow growth rate of hellbenders and the limited number of appropriately sized individuals in the zoo-housed population. The sparse-sampling protocols and application of nonlinear mixed-effects modeling (NLME) used in population pharmacokinetics are ideal for these studies in which limited blood samples are possible.

A wide variety of bacterial organisms can be cultured from captive amphibians and their environments; however, the role of these organisms in disease can be difficult to determine. Commensal organisms may provide a host with resistance to potentially pathogenic invaders, or have the potential to become pathogenic, particularly in an individual with a compromised immune system. Ceftazidime was selected because of potential favorable pharmacokinetics (cited above), and because it is unique among third-generation cephalosporins with antimicrobial activity against published potential pathogens of interest.

The objectives of this study were two-fold: to determine the pharmacokinetic characteristics of a commonly used antibiotic, ceftazidime, in order to establish appropriate dosage and treatment intervals in this threatened amphibian species and, in addition, to determine the normal oral and cloacal bacterial flora and their antibiotic susceptibilities of eastern hellbenders in a zoo-housed population, with the aim of confirming ceftazidime as an appropriate first-choice antibiotic in this species.

MATERIALS AND METHODS

Animals

This study was approved by the Saint Louis Zoo Institutional Animal Care and Use Committee as well as the Missouri Department of Conservation, who have oversight for this species. Eastern hellbenders ($n = 25$) of unknown sex from the same clutch were selected. All individuals had a normal physical exam prior to inclusion in the study and weighed between 264 and 408 g at the time of antibiotic administration.

Study animals were brought in as eggs from the Gasconade River on 28 September 2015 and hatched from 17 October to 08 November 2015. They were housed independently in 114-L (30-gallon) tanks as part of an indoor aquatic 41-tank system that shared water with other eastern hellbenders and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*). Each tank had its own flow and drained into a common sump area where it was distributed to the filtration of ultraviolet sterilization, activated carbon filters, and granular ferric oxide reactors. Throughout the study period the room and water were both temperature controlled and maintained between 15 and 17°C (59–62°F). The water pH was 7.8–8.2 and the total dissolved solids were 130–160 mg/L. The photoperiod was from 08:00 to 18:00 CST. Their diet consisted of thawed frozen krill, thawed frozen chopped smelt, and live night crawlers fed throughout the week, with continuous access to live golden shiner fish.

Experimental design

Pilot study: Five eastern hellbenders were used for a pilot study to ensure appropriate timing of sample collection. Body weight was obtained immediately prior to drug administration to ensure accurate dosing for each individual. Ceftazidime (Tazicef®, 1 g ceftazidime for injection reconstituted to 100 mg/ml, Hospira, Inc, Lake Forest, IL 60045, USA) vials for injection were reconstituted according to the manufacturer's directions and administered within 1 hr. The injections were delivered subcutaneously in the right forelimb at 20 mg/kg. Blood samples (0.6 ml/sample) were collected from the ventral tail vein at 2, 4, 8, 12, 24, 48, 72, 96, and 120 hr postadministration using a 1-ml syringe with a 23- to 27-ga needle that was preflushed with heparin. Using a sparse sampling method, no individual hellbender had blood drawn at more than four time points during the study period and total volume obtained did not exceed 1% of their body weight. Sparse sampling was performed on the subjects with the goal of minimizing the number of collections from each animal to reduce stress from handling and volume loss from blood sample collection.

Samples were immediately placed into lithium heparin microtubes (BD Microtainer, Becton, Dickinson and Company, Franklin Lakes, NJ 07417, USA), centrifuged at 2,800 × g for 10 min within 2 hr of collection, and the plasma was pipetted into a cryovial for storage at 80°C until analysis. The five individuals that received ceftazidime in this study were not used in the main study. Two additional hellbenders did not receive ceftazidime. These individuals had blood drawn and processed in the same fashion as previously listed to act as blank samples for quality control and calibration. These individuals were utilized for blank samples in both the pilot and main study.

Main study: Eighteen eastern hellbenders were manually restrained and weighed immediately prior to drug administration to ensure accurate dosing for each individual. Prior to antibiotic administration, six hellbenders had swabs of their oral cavity collected and six individuals had swabs of their cloaca collected for aerobic culture and salmonella polymerase chain reaction (PCR). Ceftazidime was administered at the same dose, and in the same fashion as in the pilot study.

Blood samples (0.6 ml/sample) were collected from the ventral tail vein and processed as described in the pilot study except sampling was done at 2, 12, 24, 28, 72, 96, 120, 144, 168, and 192 hr postadministration.

Ceftazidime analysis

Ceftazidime in plasma was analyzed with high-pressure liquid chromatography. The method used was identical to the method cited in a previous publication from our laboratory.[2]

We performed a partial validation by fortifying blank (control) hellbender plasma with nominal concentrations of the ceftazidime analytical reference standard to prepare calibration curves and quality control (QC) standards. Blanks also were analyzed to measure background noise and detect presence of interfering peaks in the chromatograms. Calibration standards for the calibration curve ranged from 0.05 to 100 μg/ml. Fresh calibration standards were prepared for each day's analysis. The calibration curve and QC sample results met our acceptance criteria for the assay.[2]

Pharmacokinetic analysis

A naïve pooled data analysis using a one- and two-compartment model was used to obtain initial estimates (results not shown). From these initial estimates, each model was tested using NLME modeling fitted to these data (Phoenix NLME™ version 8.1, Certara Inc., St. Louis, MO, USA).

Different pharmacokinetic models were tested to determine which one was the best fit to these data. The models were run with the first-order conditional estimation–extended least-squares engine in Phoenix. Model selection was based on goodness-of-fit plots, diagnostic plots of residuals, scatter plots of predicted vs observed values, and statistical significance between models using 2LL (twice the negative log likelihood), Akaike information criterion, obtained in Phoenix, and CV% of parameter estimates. A one-compartment open model with bolus injection was the model selected for these data.

The modeling provided the typical value (fixed effect) for the population estimate of the parameter of interest, and random effect for the interindividual (between subjects) differences of the parameter of interest. A multiplicative model described the residual random variability of the data.

Culture analysis

Culture and susceptibility testing were performed by the Microbiology Section of the University of Illinois Veterinary Diagnostic Laboratory and are briefly described below.

Aerobic culture: Specimens were plated on 5% sheep blood agar, blood agar with colistin and naladixic acid and MacConkey's agar (Remel Microbiological Labs™, Thermo Fisher™, Lenexa, KS, USA); one set was incubated at 37°C and one set at 25°C and then examined at 24 and 48 hr. Organisms recovered were isolated and identified using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) Biotyper™ Version 3.4 (Bruker Daltronics, Billerica, MA 01821, USA).

Salmonella PCR: The specimens were also screened for *Salmonella* by selective enrichment in tetrationate broth (TTB) incubated overnight at 37°C followed by extraction and PCR amplification using the BAX™ Kit (Hygiena LLC, Mississauga, Ontario, Canada L5N 6M5) according to the manufacturer's directions. *Salmonella*-positive TTBs were plated on XLD4 and brilliant green agars (Remel) incubated overnight at 37°C. Suspect colonies were verified as *Salmonella* by MALDI-TOF MS or triple-sugar iron and lysine iron agar tube tests (Remel) and sero-grouped using Difco (BD Diagnostics, Sparks, MD, USA) typing sera. Serotypes were determined by the National Veterinary Services Laboratories.

Antimicrobial susceptibilities: Isolates with moderate or heavy growth had susceptibility testing performed using Thermo Scientific Sensititre™ Companion Animal 1F panels according to the manufacturer's directions. Results were reported as minimal inhibitory concentrations (MIC in μg/ml) for 22 antibiotics frequently used in veterinary medicine. While there are no interpretive categories for interpretation of MIC data for amphibians, Clinical Laboratory Standards Institute (CLSI) breakpoints for similar classes of organisms were used for interpretations where possible.[11]

Table 1. Population pharmacokinetic data of ceftazidime following a single-dose administration of 20 mg/kg SQ to eastern hellbenders, using nonlinear mixed-effects modeling.

Parameter[a]	Value	Units
θVD	0.31	L/kg
θKe	0.019	1/hr
AUC	3,565.22	μg × hr/ml
C0	67.47	μg/ml
Cl	0.0059	L/kg/hr
MRT	52.85	hr
Half-life	36.63	hr

[a] θVD indicates volume of distribution; θKe, elimination rate; AUC, area under the curve; C0, maximum plasma concentration; Cl, clearance; MRT, mean residence time. (Theta notation [θ] is used to show that this is the "true value" for the population).

RESULTS

Ceftazidime pharmacokinetics

Twenty-five eastern hellbenders were sampled and remained clinically healthy throughout the pilot study and main study, and for the 6 mo following. No adverse effects of changes in behavior, activity level, or appetite were noted throughout the study. Twenty-three of the hellbenders received ceftazidime at 20.03 mg/kg (±0.82) and 54 blood samples were obtained from these individuals for pharmacokinetic analysis. Two individuals did not receive ceftazidime and served as blank control animals for calibration standards and quality control.

Ceftazidime was present in all samples obtained after drug administration. The results of the NLME analysis including the geometric mean and ranges of the pharmacokinetic study are summarized in Table 1. There was a long half-life and concentrations were detected throughout the duration of the sampling. The mean plasma concentrations of ceftazidime vs time postadministration are presented in Figure 1. There was an observed improvement in the model after accounting for interindividual (between-subject) population variability.

Oral and cloacal bacterial flora and antimicrobial susceptibility

All oral and cloacal samples were negative for *Salmonella* on PCR. All oral samples and five of six cloacal samples were positive for one or more isolates on aerobic culture. The organisms cultured are listed in Table 2. Fifty-five isolates were obtained (24 cloacal, 31 oral) with 10/12 (83%) of individuals growing three or more different isolates. The most consistent isolate among individuals was *Shewanella putrefaciens*, which was isolated from 11/12 (92%) animals. The most prolific genera of bacteria cultured was *Aeromonas* spp. with 17/55 (31%) of isolates from 10/12 individuals. Isolates classified with moderate growth or higher (12/55) had susceptibility panels performed (Table 3). All isolates that had suscep-

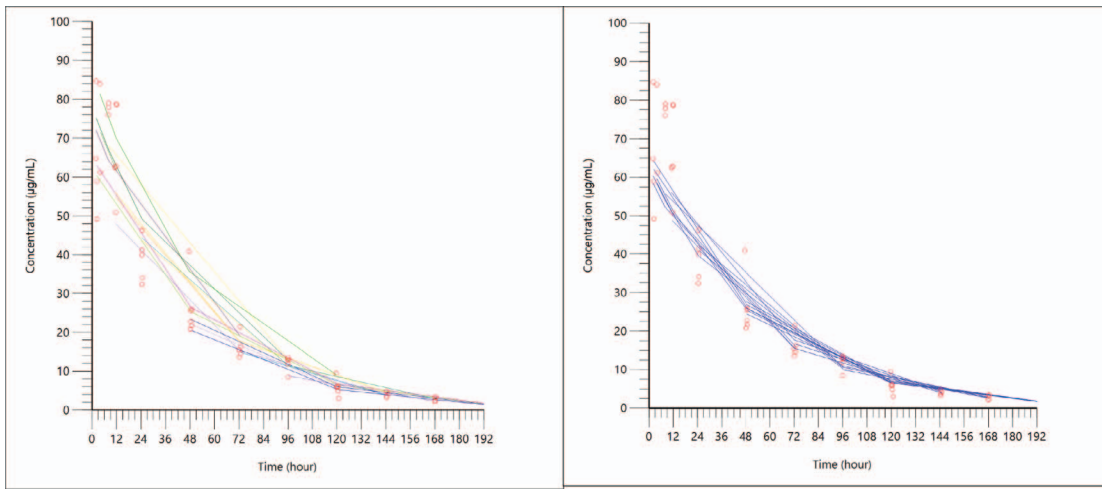

Figure 1. Population pharmacokinetic model plots (spaghetti plots) using nonlinear mixed-effects modeling. The fitted curves are shown for all individual animals (solid line) with observed data points (open circles) after administration of ceftazidime at 20 mg/kg. Left panel shows individual eastern hellbenders fitted to model. Right panel shows individual eastern hellbenders fitted to population model, accounting for interindividual variability (random effect). minimum inhibitory concentration cutoff of 8 μg/ml is shown (dashed line).

Table 2. Bacteria isolated from oral and cloacal swabs of eastern hellbenders.

Bacteria	Cloacal	Oral	Total
Acinetobacter	2	6	8
Aeromonas	11	6	17
Chromobacterium	1	2	3
Citrobacter	2	1	3
Elizabethkingia	0	4	4
Enterobacteriaceae	1	4	5
Pseudomonas	2	2	4
Shewanella	5	6	11
Total	24	31	55

tibility panels performed were reported susceptible to ceftazidime using CLSI breakpoints from other animal species (Table 4).[12]

DISCUSSION

In this study of eastern hellbenders, the ceftazidime pharmacokinetic population parameters at a dose of 20 mg/kg SQ contributes information not previously available for this species and amphibians as a whole. This study showed that plasma concentrations of ceftazidime could be maintained in eastern hellbenders for 120 hr after a dose of 20 mg/kg (Fig. 1).

Ceftazidime is a third-generation cephalosporin with a broad-spectrum gram-negative activity, particularly with *Pseudomonas aeruginosa*, and other pathogens normally found in aquatic environments.[3,12] It is a time-dependent antibiotic, meaning that its inhibitory effect is due to the plasma concentration exceeding the MIC of the bacteria for as long as possible during a dose interval. In this study a target MIC of 8 μg/ml was chosen because it is the MIC value for susceptible *Pseudomonas aeruginosa* as determined by CLSI; bacteria of the Enterobacterales (e.g., *Escherichia coli, Klebsiella* spp.) have a lower breakpoint for susceptible organisms of 4 μg/ml.[3,11]

The half-life of subcutaneously administered ceftazidime in eastern hellbenders was shown to be 36.63 hr. This is longer than previously described when given intramuscularly in reptiles such as, wild turtles, loggerhead sea turtles and snakes, which were 34.77, 19.05, and 24 hr, respectively.[2,6,9] No studies documenting ceftazidime pharmacokinetics in amphibians have been published. Other antibiotics have been studied in anurans, particularly fluoroquinolones and aminoglycosides.[4–6,10,17–19] However, these drugs have different antibacterial activity and pharmacokinetic–pharmacodynamic properties and those results cannot be extrapolated to the use of ceftazidime in hellbenders.

Aeromonas spp. were the most commonly isolated bacteria making up 31% (17/55) of the isolates. This is consistent with a previous study on microorganisms in Ozark hellbenders, which isolated common environmental flora in both abnormal or injured limbs as well as uninjured limbs. The most common organisms identified included *Aeromonas hydrophila*, *Granulicatella adiacens*, *Stenotrophomonas malophilia*, and a variety of pseudomonads.[13] This study was done with a small sample size of free-ranging individuals of a different subspecies and antibiotic susceptibility was not conducted. *Aeromonas* spp. are gram-negative nonfermenting bacilli often found in water and routinely cultured as part of normal bacterial flora in healthy captive amphibians.[20] They can be opportunists with potentially extensive resistance profiles.[20] This is consistent with the findings in this study, as all *Aeromonas* spp. isolates showed resistance to both amoxicillin and cefazolin, as well as a high level of resistance (83%) to cephalexin. Additional antibiotic resistance concerns were present with other bacterial

Table 3. In vitro antimicrobial susceptibility of isolated oral and cloacal bacterial flora of healthy eastern hellbenders.

		No. of antimicrobial-resistant isolates (resistance rate)[11]										
Bacteria	*n*[a]	AMK	AMX	CPX	CFZ	CFV	CAZ	CHL	DOX	ENO	TET	TMS
Acinetobacter[b]	2	0%	0%	100%	100%	50%	0%	NI	0%	NI	NI	50%
Aeromonas	6	0%	100%	83.3%	100%	16.7%	0%	0%	NI	NI	33.3%	0%
Citrobacter	2	0%	100%	100%	0%	0%	0%	0%	0%	NI	0%	0%
Enterobacter	1	0%	0%	100%	0%	0%	0%	0%	0%	NI	0%	0%
Shewanella	1	0%	0%	NI	0%	0%	0%	0%	0%	NI	NI	0%

[a] *n* indicates number of isolates; AMK, amikacin; AMX, amoxicillin; CPX, cephalexin; CFZ, cefazolin; CFV, cefovecin; CAZ, ceftazidime; CHL, chloramphenicol; DOX, doxycycline; ENO, enrofloxacin; TET, tetracycline; TMS, trimethoprim/sulfa; NI, no interpretation possible.

[b] *Acinetobacter* isolates were from oral cultures, all other isolates were from cloacal cultures.

Table 4. In vitro antimicrobial susceptibility and minimum inhibitory concentration data of isolated oral and cloacal bacterial flora of healthy eastern hellbenders.

	Susceptibility and minimum inhibitory concentration of isolates (μg/ml)[11]										
Bacteria	AMK[a]	AMX	CPX	CFZ	CFV	CAZ	CHL	DOX	ENO	TET	TMS
Acinetobacter[b]	4	1	16[c]	>32[R]	4[I]	4	8	0.25	0.12[e]	4[e]	1
Acinetobacter	4	4	>16[c]	>32[c]	8[c]	4	8	0.12	0.12[e]	>16[e]	0.5
Aeromonas	4	>8[c]	8[c]	16[c]	0.25	4	2	2[e]	0.25[e]	16[c]	0.5
Aeromonas	4	>8[c]	8[c]	16[c]	0.25	4	2	0.25[e]	0.12[e]	4	0.5
Aeromonas	4	>8[c]	4[d]	8[c]	0.25	4	2	2[e]	0.12[e]	16[c]	0.5
Aeromonas	4	4[c]	>16[c]	32[c]	8[c]	4	2	0.5[e]	0.12[e]	4	0.5
Aeromonas	4	>8[c]	>16[c]	>32[c]	4[d]	4	2	0.5[e]	0.12[e]	4	0.5
Aeromonas	4	>8[c]	>16[c]	>32[c]	0.25	4	2	0.25[e]	0.12[e]	4	0.5
Citrobacter	4	>8[c]	>16[c]	>32[c]	1	4	8	2	0.12[e]	4	0.5
Citrobacter	4	>8	>16[c]	>32[c]	1	4	8	2	0.12[e]	16	0.5
Enterobacter	4	4	8[c]	2[e]	0.5	4	2	0.5	0.12[e]	4	0.5
Shewanella	4	0.5	2[e]	8[c]	0.25	4	2	8[d]	0.12[e]	>16[e]	0.5

[a] AMK indicates amikacin; AMX, amoxicillin; CPX, cephalexin; CFZ, cefazolin; CFV, cefovecin; CAZ, ceftazidime; CHL, chloramphenicol; DOX, doxycycline; ENO, enrofloxacin; TET, tetracycline; TMS, trimethoprim/sulfa; NI, no interpretation possible.

[b] *Acinetobacter* isolates were from oral cultures, all other isolates were from cloacal cultures.

[c] Isolates classified as resistant.

[d] Isolates classified as intermediate susceptibility.

[e] Isolates with no interpretation possible.

isolates, particularly with cephalexin, cefazolin, and amoxicillin. Immunosuppression caused by poor water quality, inappropriate housing, toxins, or fungal or parasitic pathogens can allow *Aeromonas* spp. to cause disease. It is the most common genus isolated in published cases of clinical bacterial disease in amphibians.[18] *Aeromonas hydrophila* has historically been associated with dermatosepticemia in amphibians.[20] Their tendency towards opportunistic infections, association with aquatic environments and frequent culturing on hellbenders makes them primed for secondary infections when the skin barrier is broken either by trauma, or by other pathogens, such as *Chytridiomycosis dendrobatidis*.

Ordinarily, the small study size and limited sampling opportunities would prevent a robust pharmacokinetic analysis. Nevertheless, a strength of this study was the large population that was included because of a sparse-sampling strategy. Furthermore, the use of NLME provided a population estimate for the true value (fixed effect) of important parameters that guide dosing. A limitation of the current study is that it included only healthy animals. It is undetermined if these pharmacokinetic values will be similar in a diseased population. Further studies are needed to test the dose regimen used here in clinical treatment of a population of hellbenders with active infections.

CONCLUSIONS

Ceftazidime demonstrated a long half-life in these hellbenders after a subcutaneously administered dose of 20 mg/kg. Based on the population pharmacokinetic analysis (Figure 1), concentrations were maintained above 8 μg/ml, which is the MIC value for susceptible bacteria in other species as determined by CLSI for approximately 120 hr after a single dose.[3,11]

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