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Population pharmacokinetics of ceftazidime after a single intramuscular injection in wild turtles

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Ceftazidime, a third-generation cephalosporin, is important for treating opportunistic bacterial infections in turtles. Antibacterial dosage regimens are not well established for wild turtles and are often extrapolated from other reptiles or mammals. This investigation used a population pharmacokinetic approach to study ceftazidime in wild turtles presented for rehabilitation. Ceftazidime was administered to 24 wild turtles presented to the Turtle Rescue Team at North Carolina State University. A sparse blood sampling protocol was used to collect samples from 0 to 120 hr with three samples per individual after injection. Plasma samples were analyzed by high-pressure liquid chromatography (HPLC). A nonlinear mixed-effects model (NLME) was fitted to the data to determine typical values for population parameters. We identified a long half-life (T½) of approximately 35 hr and volume of distribution (V_{SS}) of 0.26 L/kg. We concluded that this long T½ will allow for a dose of 20 mg/kg injected IM to maintain concentrations above the MIC of most wild-type bacteria for 5 days. Because of long intervals between injections, stability of stored formulations was measured and showed that 90% strength was maintained for 120 hr when stored in the refrigerator and for 25 days when stored in the freezer.

1 | INTRODUCTION

Chelonian populations are experiencing precipitous population declines worldwide because of habitat destruction, population disintegration, infectious disease, pollution, collection from the wild for the pet trade, and trauma (Allender et al., 2011; Hausmann et al., 2015; Jacobson, 1997; Moll & Moll, 2004; Swarth & Hagood, 2005). Trauma caused by vehicles, horticultural equipment, and animal attacks is some of the most common presentations for Eastern box turtles (*Terrapene carolina carolina*) admitted to wildlife rehabilitation clinics throughout the United States (Schenk & Souza, 2014; Schrader, Allender, & Odoi, 2010; Stranahan, Lewbart, Alpi, Passingham, & Kosmerick, 2016). In particular, at the Turtle Rescue Team (TRT) at North Carolina State University College of Veterinary Medicine (NC State CVM), vehicular and other types of traumatic injury are the most common presenting complaints followed by infection, aural abscessation, and nutritional disorders (Stranahan et al., 2016).

In reptiles, empiric antibiotic selection may be necessary in critical cases whereby the nature of the traumatic injury precludes

culture and susceptibility testing. Successful treatment of bacterial infections depends on the appropriate selection of an antimicrobial agent, including the dose, frequency, and duration of administration.

In turtle rescue facilities, gram-negative bacteria are associated with the high morbidity rates in cases involving traumatic injury (Gibbons, 2014; Norton, 2005; Stewart, 1990). Therefore, antibiotics that are active against these pathogens are important to a successful outcome. Ceftazidime is a third-generation cephalosporin approved for people that is commonly used prophylactically in reptiles after traumatic injury or to treat an infection. The spectrum is ideal for this use because it includes opportunistic gram-positive cocci, gram-negative bacilli (Enterobacteriaceae), and gram-negative nonfermenting bacilli (*Pseudomonas aeruginosa)* (Papich, 2018). Ceftazidime is used for management of traumatic injuries in turtles that may acquire secondary infections. The use in turtles has been documented by other veterinarians who treat infections in turtles (Allender et al., 2006; Gibbons & Steffes, 2013; Sim et al., 2016).

Dosage regimens for chelonians are based on extrapolations from a 1999 study conducted in eight juvenile loggerhead sea

turtles (*Caretta caretta*) (Stamper et al., 1999). However, differences in size, diet, and environment exist which may contribute to differences in pharmacokinetics between sea turtles and the freshwater species that are the subject of this study. Therefore, this study collected plasma pharmacokinetic data to be used for calculating a dose for administration of ceftazidime in other species of chelonians. Furthermore, to evaluate the stability of opened injection vials, we evaluated the strength of ceftazidime solutions after reconstitution according to manufacturer's instructions and storage in the refrigerator and freezer in a manner often used in turtle treatment hospitals.

2 | MATERIALS AND METHODS

2.1 | Animals

Turtles were entered into the ceftazidime treatment protocol and subsequently sampled for this study if they met inclusion criteria. Turtles met the criteria if the TRT staff deemed these cases sufficiently serious to warrant the administration of ceftazidime to prevent secondary infections, and blood samples could be collected without undue stress or discomfort. The turtles were not receiving any other medications during the study. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University.

2.2 | Procedure

A grid was prepared to instruct the TRT staff for timing and frequency of sparse sampling for each patient admitted. The sparse sampling protocol reduced the stress and frequency of blood collection. Ceftazidime (Tazidime, 1 gram, Eli Lilly, Indianapolis, IN) was reconstituted with 10 ml sterile water according to the manufacturer's instructions to produce a solution of 100 mg/ml. Turtles were housed individually in 2- to 20-L containers with adequate access to water. They were fed and cleaned at least every other day. A controlled temperature (22.9°C–23.8°C) was maintained in the housing area, and the minimum and maximum temperatures were recorded daily. Turtles were given ceftazidime (20 mg/kg) as a single IM injection into the left triceps muscle.

2.3 | Collection of blood samples

Blood was collected from the right brachial vein at 0 (predose sample), 2, 4, 8, 12, 24, 48, 72, 96, and 120 hr after injection. Approximately 0.4 ml of blood was collected at each time point using a 1-ml tuberculin syringe with a 25-ga needle. The syringe and needle interiors were rinsed before use with 0.1 ml of 1,000 IU/ml sodium heparin solution (McKesson Medical-Surgical Inc., Jacksonville, Florida 32216, USA) as an anticoagulant. Blood was placed into amber polyethylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, Pennsylvania 15219, USA), which were capped and immediately submerged in ice water. The blood was then centrifuged (2350 × *g*) to harvest approximately 0.3 ml of plasma, which was placed into amber polyethylene microcentrifuge tubes via micropipette. The tubes were capped and stored at −70°C until high-pressure liquid chromatography (HPLC) analysis.

2.4 | Analysis of ceftazidime concentrations

Ceftazidime in plasma was quantified using HPLC. Ceftazidime was eluted on a C-18 reverse-phase column (Zorbax SB-C18, Agilent) with the detection set at 260 nm and a flow rate of 1.0 ml/min. The mobile phase consisted of 88% double-distilled and filtered water, 12% acetonitrile, and 0.10% trifluoroacetic acid.

Blank plasma pooled from untreated turtles was fortified with ceftazidime and used for quality control samples and calibration standards. 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 was linear with a R^2 value of at least .99. Blank samples (plasma collected prior to drug administration) from most turtles prior to drug administration were analyzed to ensure that there were no interfering peaks in the chromatogram.

2.5 | Pharmacokinetic analysis

A naïve pooled analysis using a two-compartment model was used to determine initial estimates (results not shown). From these initial estimates, a two-compartment pharmacokinetic model and nonlinear mixed-effects modeling (NLME) were fitted to these data (Phoenix NLME™ version 7.0, Certara Inc., St. Louis, Missouri).

Compartmental analysis of the data from the ceftazidime injection was calculated using a two-compartment model according to the following formula:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \tag{1}$$

Where C is the ceftazidime concentration, A is the distribution phase y-axis intercept, e is the base of the natural logarithm, t is time after injection, α is the distribution rate constant, B is the elimination phase y-axis intercept, and β is the elimination phase rate constant (terminal phase). Secondary parameters calculated include distribution (α) and elimination (β) half-lives (T½), microdistribution rate constants, area under the curve (AUC), apparent volume of distribution at steady-state (V_{SS}), systemic clearance (CL), and mean residence time (MRT).

Sparse sampling was performed on the subjects with the goal of determining three samples per individual turtle, plus a sample collected prior to drug administration for some animals. Various models were tested with different error structures to determine the best fit base model. The models were parameterized as described above after testing other models. The models were run with the first-order conditional estimation—extended least squares (FOCE-ELS) 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 (AIC), collected

TABLE 1 Results of pharmacokinetic analysis with nonlinear mixed-effects modeling using the Phoenix® NLME™ software (Certara, St. Louis, MO)

Parameter	Value	Units	Eta	CV%
θ A	114.82	µg/ml	1.13	144.8
θ Alpha	0.81	1/hr	0.92	122.1
θ B	71.11	µg/ml	0.040	20.17
θ Beta	0.02	1/hr	0.20	47.3
AUC	3708.92	µg hr ml^{-1}	–	–
Cl	0.01	L kg^{-1} hr^{-1}	–	–
MRT	48.30	hr	–	–
V_{SS}	0.26	L/kg	–	–
Distribution T½	13.83	hr	–	–
Elimination T½	34.77	hr	–	–

θ A, distribution intercept; θ Alpha, distribution rate constant; θ B, elimination intercept; θ Beta, elimination rate constant; (θ, theta, is used to indicate that these are the typical values, *tv*, for the population); C_{MAX}, AUC, area under the drug concentration curve; Cl, systemic clearance; MRT, mean residence time; V_{SS}, apparent volume of distribution at steady-state; Distribution T½ and Elimination T½ are the distribution and elimination half-lives, respectively. Eta (η) indicates differences between subjects (random effects) and the associated CV%.

from Phoenix, and CV% of parameter estimates. Interindividual (between subject) variability (variance of a parameter among different subjects) was expressed using an exponential error model according to the equation:

$$Pi = \theta P \times \exp\ (\eta Pi) \tag{2}$$

Where *P* is the pharmacokinetic parameter of interest for the individual *i*, θ *P* is θ (theta), or the typical value (fixed effect) for the population estimate of the parameter of interest, and η*Pi* is the η (eta, random effect) for the interindividual (between subject) differences in the parameter of interest. The η values were assumed to be independent and have a normal distribution with a mean of zero and variance of ω^2. A multiplicative model was used to describe the residual random variability (ε) of the data for once daily dosing, where ε is the residual intrasubject (within subject) variability with a mean of zero and a variance of σ^2, according to the equation:

$$Cobs = Cpred \times (1 + \varepsilon,) \tag{3}$$

Where Cobs is the observed concentration for the individual and Cpred is the model predicted concentration plus the error value (ε).

2.6 | Stability during storage

Stability of ceftazidime during refrigeration and freezer storage was also evaluated because there are no data published on the effects of freezer storage in plastic syringes or original container vials stored in the refrigerator for longer than 3 days. Three vials of ceftazidime (Tazidime, 100 mg/ml, Eli Lilly, Indianapolis, IN) were prepared with sterile water according to manufacturer's instructions (concentrations of 100 mg/ml). An initial analysis of the reconstituted vials was performed in triplicate via HPLC to verify the strength of the

ceftazidime solution. Twenty 1-ml tuberculin syringes were filled with 0.2 ml of ceftazidime from each vial, then labeled and placed into the freezer at −18°C. At day 3, 5, 10, 15, 20, and 25, one syringe from each of the three vials was removed from the freezer, thawed, and HPLC analysis was performed. The remainder of the vials was stored in the dark in the refrigerator at 4°C. Aliquots of the vials were analyzed at day 3, 5, 10, 15, 20, and 25 days after reconstitution, and the strength of the ceftazidime solution was measured with HPLC.

3 | RESULTS

3.1 | Population

A total of ten Eastern box turtles (*T. carolina carolina*), nine yellow-bellied sliders (*Trachemys scripta scripta*), and five river cooters (*Pseudemys concinna*) were completed in this study. The turtles (*n* = 24) weighed 1.28 ± 1.03 (mean ± *SD*). All turtles presented to the NCSU TRT for injuries. Seventeen turtles were presented for vehicular trauma, three for suspected upper respiratory tract infection, one for aural abscessation, one for fish hook trauma, one for animal attack trauma, and one for unknown trauma.

3.2 | Pharmacokinetics

Ceftazidime was detected in all samples from all treated animals. The population-based pharmacokinetic parameters determined by the NLME are summarized in Table 1. Plasma drug concentrations are shown for each sampling time point and the average for the entire group in Figure 1. The pharmacokinetic fit to these data is shown in Figure 2, with the model fitted to each individual in Panel A of Figure 2, and the model fitted after accounting for interindividual variability shown in Panel B. There was an observed improvement in the model after accounting for between-subject variability.

Diagnostic plots are shown in Figures 3 and 4. The plots in Figure 3 show the predicted points vs dependent variables for the population (PRED) and for individuals (IPRED). Except for a few points in the PRED vs DV plot, there is general symmetry with equal number of points above and below the line of unity. The IPRED vs DV plot shows the individual-specific predicted values vs DV. This plot (right side of Figure 3) shows that after the between-subject differences are accounted for, there is large improvement compared to the PRED vs DV plot. In a perfect model fit, all points would fall on a line with unit slope indicting perfect correspondence.

In Figure 4, we have presented the plots for the conditional weighted residuals (CWRES) vs time (hours) and vs predicted values (PRED). Note the equal distribution of our points above and below the line of CWRES = 0, which illustrates a good-fitting model.

Ceftazidime from IM injection produced a high-peak concentration followed by rapid distribution and an elimination half-life of 34.77 hr. We assessed the duration of the plasma drug
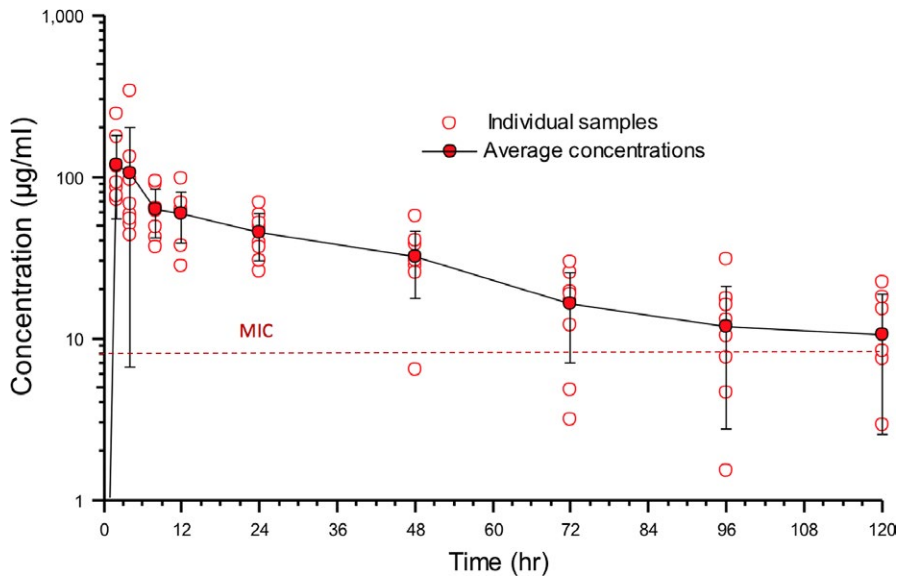
FIGURE 1 Plasma concentrations from 0 to 120 hr of ceftazidime after administration at a dose of 20 mg/kg IM in 24 turtles. The dashed line at a MIC of 8 μg/ml shows the value for wild-type strains of *Pseudomonas aeruginosa* [Colour figure can be viewed at wileyonlinelibrary. com]
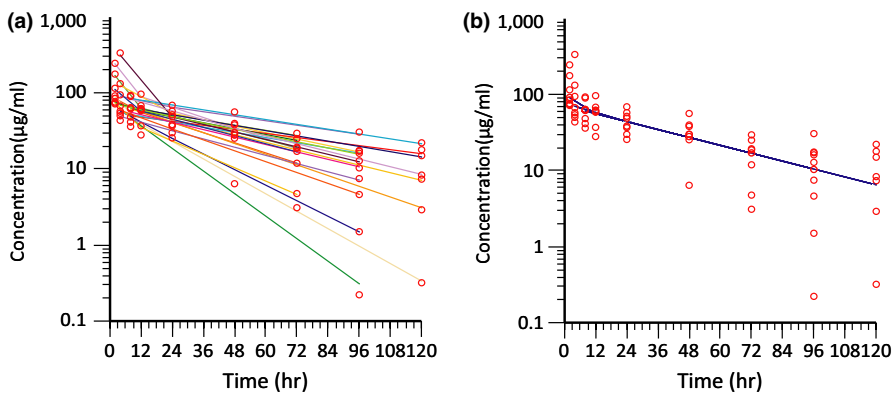

FIGURE 2 Population pharmacokinetic model plots (spaghetti plots) of fitted curves for all individual turtles (solid line) with observed data points (open circles) after intramuscular administration of ceftazidime at 20 mg/kg. (a) individual turtles fitted to model. (b) individual turtles fitted to population model, accounting for interindividual variability (random effect). Note improvement in fit in Panel (b), using population model [Colour figure can be viewed at wileyonlinelibrary.com]
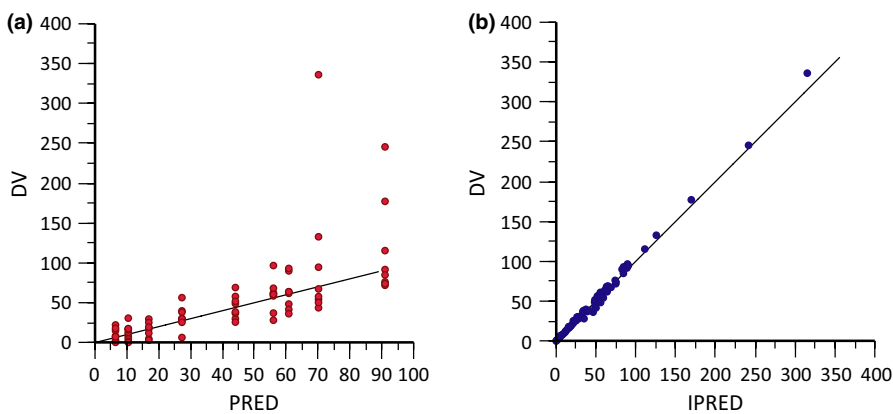

FIGURE 3 Diagnostic plots for nonlinear mixed-effects modeling of ceftazidime in turtles. The population predicted values (PRED) are plotted against the dependent variable (DV) on the left panel (a), and the individual prediction (IPRED) is shown plotted against the DV on the right panel (b). The individual prediction (right panel) is improved by incorporating the sources of variability. Each point on the graphs represents an observed concentration [Colour figure can be viewed at wileyonlinelibrary.com]

concentration (time above MIC) to maintain the concentration above a MIC value of 8 μg/ml for *Pseudomonas aeruginaosa*. This value was used as our therapeutic target because it is the epidemiological cutoff value (wild-type cutoff) established by the European Committee on Antimicrobial Susceptibility Testing, EUCAST, https://mic.eucast.org/ for this organism. Plasma concentrations were maintained above the MIC for *P. aeruginaosa* for 120 hr (Figure 1).

3.3 | Stability during storage

The ceftazidime solution stored in the dark in the original vial at 4°C maintained greater than 90% strength of solution for 120 hr (Figure 5). This was followed by a steady decline in ceftazidime strength over the remainder of the 25-day period. Ceftazidime solution stored in 20 tuberculin syringes in the freezer (−18°C) maintained a strength of above 90% throughout the 25 days

FIGURE 4 Diagnostic plots of conditional weighted residuals (CWRES) for nonlinear mixed-effects modeling of ceftazidime in turtles. The left panel (a) shows the independent variable (time) vs the CWRES. The right panel (b) shows the predicted value concentrations (PRED) vs the CWRES. Each point on the graphs represents an observed concentration [Colour figure can be viewed at wileyonlinelibrary.com]
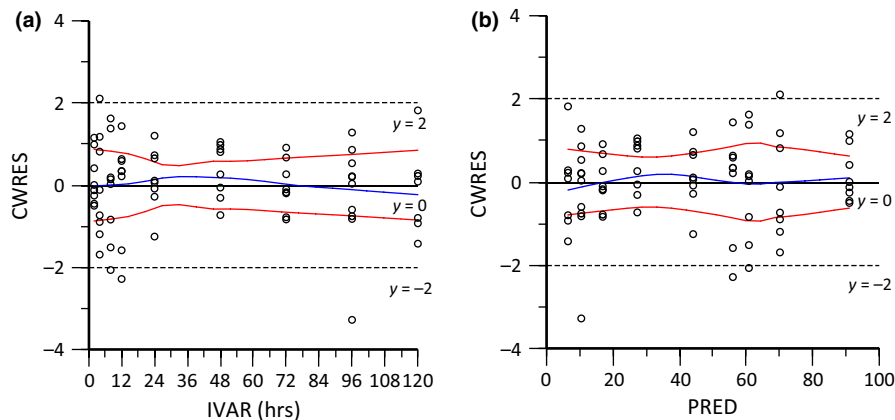
tested during this study (Figure 5). We used a 90% threshold for determining strength because this is the value that meets acceptance criteria for the United States Pharmacopeia (USP, www.USP. org). The USP states that to meet the compendial standard, each vial should contain not less than 90% of the labeled amount of ceftazidime.

4 | DISCUSSION

We showed that plasma drug concentrations can be maintained in a population of turtles presented for rehabilitation for 120 hr after a dose of 20 mg/kg IM. Our pharmacokinetic analysis used NLME, which is ideal for small reptiles in which only sparse sampling (four samples per animal) is practical to avoid undue stress and discomfort to the animals. Furthermore, the NLME analysis improved pharmacokinetic model fit by accounting for interindividual variation

(random effect). Our diagnostic plots (Figures 3 and 4) show that we achieved a good-fitting model.

Because ceftazidime is a time-dependent antibiotic, a long time above MIC (T>MIC) is important for optimum efficacy. Therefore, the dosage regimen should maintain the plasma drug concentration above the MIC as long as possible. The half-life of 34.77 hr is longer than loggerhead sea turtles (19.05 hr). A 20 mg/kg dose of ceftazidime given IM produced concentrations above 8 μg/ml for 5 days in the turtles in our population. A long-dosing interval for this clinical dosage regimen will maintain adequate concentrations for bacteria resistant to other agents with minimal distress to the patient.

The package insert for human use states that ceftazidime "when constituted as directed with sterile water for injection, maintains satisfactory potency for 12 hr at room temperature or for 3 days under refrigeration. Solutions in sterile water for injection that are frozen immediately after constitution in the original container are stable for 3 months when stored at −20°C." Turtles do not require
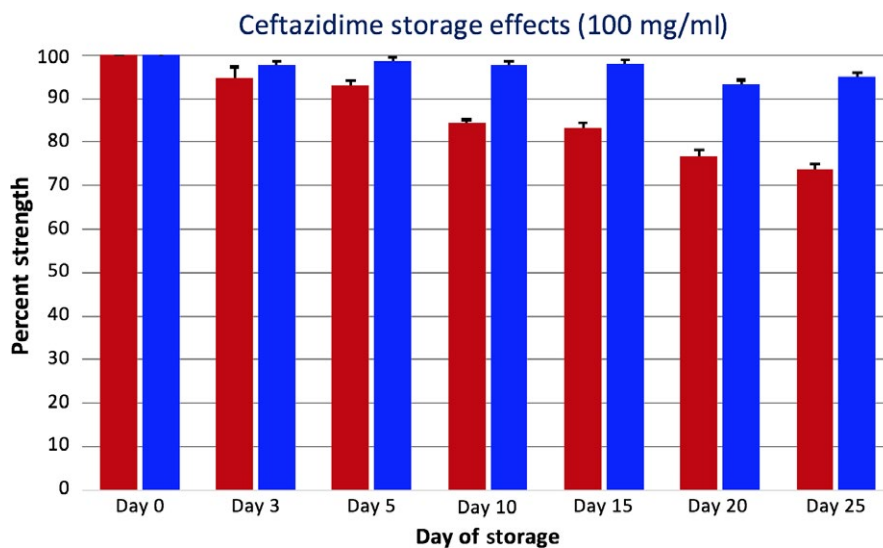

FIGURE 5 Percent strength of ceftazidime solution analyzed at 0, 3, 5, 10, 15, 20, and 25 days after reconstitution. Red bars indicate refrigerated samples stored in the original vials reconstituted according to manufacturer's directions and stored in a controlled temperature refrigerator. Blue bars indicate frozen samples stored in individual aliquots (doses) in 1 ml tuberculin syringes. Analysis performed on each syringe after removing from freezer and thawing. After day 5, strength drops below 90% for vials (red bars). Frozen samples remain above 90% strength for 25 days (blue bars). Each bar in the figure represents the mean (+ standard deviation) from three replicates [Colour figure can be viewed at wileyonlinelibrary.com]

an entire vial (1000 mg) for each dose, and stability data for refrigerated and frozen solutions were needed to guide the clinical staff who treat turtles. Because of long intervals between injections, stability of stored formulations is important to the success of treatment. Individual doses for small reptiles are often stored in 1-ml plastic syringes in the freezer for long periods; therefore, testing was necessary to confirm that the solutions retain their strength during these storage conditions. We confirmed that 90% strength of the ceftazidime solution is maintained for 120 hr when ceftazidime is stored in the refrigerator (4° C) which is slightly longer than the duration listed on the manufacturer's insert (5 days vs 3 days). When stored in the freezer (−18° C) in individual plastic tuberculin syringes equivalent to one dose per patient (0.2 ml), it remained stable throughout the 25 days tested in this study.

In the present study, through the use of a unique sparse sampling technique and NLME population model, we determined reliable estimates for pharmacokinetic parameters in a wild turtle population presented for treatment. This approach provided a robust fit to the data and produced fixed-effect estimates for the pharmacokinetic parameters by incorporating interindividual variability in the analysis. This method allowed us to successfully establish a clinical dosage regimen that is predicted to maintain adequate concentrations for bacteria resistant to other agents with minimal distress to the patient.

CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

AUTHOR CONTRIBUTION

AJC read and approved the final manuscript and contributed to designing study, collecting, samples, drug analysis, and manuscript preparation and review. GAL read and approved the final manuscript and contributed to designing study, results analysis, and manuscript preparation and review. DRD read and approved the final manuscript and contributed to designing study, collecting, samples, drug analysis, and manuscript review. MGP read and approved the final manuscript and contributed to designing study, supervised the study, designed the drug analysis protocol, data analysis, and manuscript preparation and review.

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