Pharmacokinetics of cefovecin sodium after subcutaneous administration to Hermann’s tortoises (*Testudo hermanni*)

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Objective—To determine the pharmacokinetics of cefovecin sodium after SC administration to Hermann’s tortoises (*Testudo hermanni*).

Animals—23 healthy adult Hermann’s tortoises (15 males and 8 females).

Procedures—Cefovecin (8.0 mg/kg) was injected once in the subcutis of the neck region of Hermann’s tortoises, and blood samples were obtained at predetermined time points. Plasma cefovecin concentrations were measured via ultraperformance liquid chromatography coupled to tandem mass spectrometry, and pharmacokinetic parameters were calculated with a noncompartmental model. Plasma protein concentration was quantified, and the percentage of cefovecin bound to protein was estimated with a centrifugation technique.

Results—Cefovecin was absorbed rapidly, reaching maximum plasma concentrations between 35 minutes and 2 hours after administration, with the exception of 1 group, in which it was reached after 4 hours. The mean ± SD time to maximum concentration was 1.22 ± 1.14 hours; area under the concentration-time curve was 220.35 ± 36.18 h·µg/mL. The mean protein-bound fraction of cefovecin ranged from 41.3% to 47.5%. No adverse effects were observed.

Conclusions and Clinical Relevance—Administration of a single dose of cefovecin SC appeared to be well-tolerated in this population of tortoises. Results of pharmacokinetic analysis indicated that the 2-week dosing interval suggested for dogs and cats cannot be considered effective in tortoises; however, further research is needed to determine therapeutic concentrations of the drug and appropriate dose ranges. (Am J Vet Res 2014;75:918–923)

Herrmann’s tortoises (*Testudo hermanni*) are frequently kept as pets. The small size (carapace rarely > 210 mm in length in wild populations),1 herbivorous habits, and resilience make these tortoises an exceptional garden inhabitant. Although Herrmann’s tortoises are listed by the International Union for Conservation of Nature2 as a near-threatened species, although Herrmann’s tortoises are listed by the International Union for Conservation of Nature2 as a near-threatened species, the number of such tortoises in the wild continues to decrease.

Antimicrobial treatment is often necessary in chelonian medicine,3 but only a few studies have been performed to investigate the pharmacokinetics and pharmacodynamics of antimicrobials in these animals. Most studies have been performed with marine turtles,4–12 with few focusing on freshwater13,14 and terrestrial chelonians.15–17 Consequently, to establish antimicrobial treatment protocols, veterinary practitioners often have to rely on anecdotal reports18 for information instead of proper pharmacokinetics studies.

Cefovecin sodium is a semisynthetic third-generation cephalosporin registered for veterinary use in dogs and cats in the United States and in Europe. Cephalosporins act by interfering with bacterial cell wall synthesis and are often used in treatment of reptiles.19,20 In

<table>
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<th>Abbreviations</th>
<th>Definition</th>
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<tr>
<td>AUC0–last</td>
<td>Area under the concentration-time curve from time 0 to last measurable drug concentration</td>
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<tr>
<td>AUMC0–last</td>
<td>Area under the first moment curve from time 0 to last measurable drug concentration</td>
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<td>Cmax</td>
<td>Maximum (peak) drug concentration</td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>Tmax</td>
<td>Time to reach maximum (peak) drug concentration</td>
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dogs and cats, cefovecin is a long-acting antimicrobial, and a single SC injection can provide up to 14 days of treatment. In dogs, cefovecin (8.0 mg/kg) had a mean elimination half-life of 5.5 days after SC administration, whereas that in cats (following administration with the same dose and route) was 6.9 days. Following SC or IV administration, cefovecin has been described in a noncompartmental model characterized by a rapid initial distribution phase, followed by a slower elimination phase.

The use of a long-acting, broad-spectrum antimicrobial would be of particular interest for clinicians working with chelonians. The metabolism of reptiles differs from that of mammals in several aspects. In addition to variations in energy requirements, drug efficacy and metabolism may be substantially different from those in mammals. Therefore, it may be inappropriate to prescribe medical treatment for reptiles by extrapolating doses from research performed in other animal species.

The purpose of the study reported here was to determine the pharmacokinetics of cefovecin in healthy Hermann’s tortoises and to assess whether the therapeutic protocol used in dogs and cats could be applicable to chelonians.

**Materials and Methods**

**Animals**—Twenty-three healthy adult Hermann’s tortoises (15 males and 8 females; body weight, 0.735 to 2.300 kg) were included in the study. The tortoises were obtained from the Parco Natura Viva Garda Zoological Park, Bussolengo, Verona, Italy, and were identified by writing a unique number on each carapace with an aqueous-based marker. Health status was determined on the basis of clinical history, physical examination, and results of a CBC and serum biochemical analysis for each animal. The tortoises were divided in 8 same-sex groups (7 groups of 3 tortoises and 1 group of 2 tortoises). Each group was placed in a vivarium and acclimatized for 7 days prior to the start of the study. Each vivarium was equipped with a fluorescent UVB-emitting lamp and an infrared lamp as heat source. The fluorescent bulb was oriented diagonally with the end of the longitudinal axis 21 cm above the floor of the vivarium. The infrared lamp was oriented so that it heated the same portion of the vivarium that was exposed to UVB radiation via the fluorescent lamp. For each vivarium, light and heat were provided for 12 continuous hours each day (8:00 AM to 8:00 PM). Shelters were provided in each vivarium so that tortoises could easily regulate their exposure to UVB radiation and heat. Environmental temperature was maintained at 22 ± 1°C during the day (8:00 AM to 8:00 PM) and at 19 ± 1°C during the night (8:00 PM to 8:00 AM). A thermostat was used in basking zones to prevent temperatures from exceeding 35°C. Substrates in the enclosures consisted of natural soil and a layer of fir bark and coconut fiber a few centimeters thick. Relative humidity was recorded by an analogical hygrometer and ranged between 65% and 75%. The tortoises were fed a mixed diet composed of vegetables suitable for human consumption and spontaneously growing vegetation. Greens and water were provided to the tortoises ad libitum.

Tortoises’ clinical conditions were monitored throughout the experiment, including possible variations in the amount of food consumed and feces produced. Experimental procedures were approved by the Italian Ministry of Health, in accordance with European regulations.

**Injection and sample collection**—The day before beginning the experiment, an IV catheter was aseptically placed in a jugular vein of each tortoise. On day 0, a commercially available cefovecin sodium formulation was reconstituted with the associated solvent (to a concentration of 80 mg/mL) and delivered by a single 0.1 mL/kg (8.0 mg of cefovecin as the sodium salt/kg) injection with a 20-gauge needle in the subcutis of the pectoral region contralateral to the catheter site.

Blood samples were collected into lithium heparin–containing tubes prior to administration (baseline), at 35 minutes, 1, 2, 4, 8, 12, and 24 hours during day 1 (the first day after treatment), and then every 24 hours on days 3, 4, 5, 6, 8, 10, 12, and 14. Catheters were flushed with 0.5 mL of a calcium heparin solution after sample collection. To create a pool of approximately 1 mL of blood for each group, 0.3 to 0.4 mL/tortoise/time point was obtained. Samples were centrifuged within 30 minutes after collection. Plasma was collected and stored in microtubes at –20°C until assayed.

**Sample preparation and testing**—For optimization and validation of the analytical method as well as preparation of matrix-matched calibration curves during each day of analysis, a pure cefovecin standard was used. Cephalexin was used as an internal standard. Standard stock solutions of cefovecin and cephalexin were each prepared at a concentration of 2 mg/mL by dissolving the pure substances in flasks with ultrapure water. For spiking purposes, cefovecin standard solutions were prepared at different concentrations, obtaining a specific dilution for each point of the calibration curve; this allowed adding a fixed amount of standard solution to each sample. An internal standard working solution was obtained by diluting cephalexin stock solution in ultrapure water at a concentration of 0.01 mg/mL, and 10 µL of the working solution was added to each sample. All standard solutions were stored in glassware at 5 ± 3°C in darkness until use.

Plasma samples were thawed at room temperature (approx 23°C) and mixed by vortexing. A 100-µL aliquot was transferred into a self-capping 1-mL microcentrifuge tube, and 10 µL of internal standard solution (cephalexin-water solution at a concentration of 10 µg/mL) and 10 µL of water were added. Samples were mixed, 400 µL of acetonitrile was added, and the mixture was vortexed again for 10 seconds and centrifuged for 10 minutes at 3,400 x g. After centrifugation, 200 µL of supernatant was transferred into another microcentrifuge tube containing 800 µL of 0.3% formic acid solution and 2mM ammonium acetate in water and vortexed again. The contents were transferred into a glass vial, and 5 µL was injected in the ultraperformance liquid chromatography–tandem mass spectrometry system. Similarly, to prepare the calibration curves, 10 µL of the specific standard solution was added to 100 µL of plasma to obtain the follow-
ing cefovecin concentrations; 0, 0.01, 0.05, 0.25, 1, 5, 10, 25, and 50 μg/mL. Quality control samples were prepared at cefovecin concentrations of 0.05, 1 and 25 μg/mL. After adding cefovecin and cephalixin working solutions, samples were vortexed for 10 seconds and allowed to stand for 10 minutes at room temperature before extraction.

Analysis was performed with an ultraperformance liquid chromatography binary pump equipped with a 50 × 2.1-mm (1.7 μm particle size) C18 reversed-phase column and interfaced with a tandem mass spectrometer. The mobile phase consisted of 0.3% formic acid and 2mM of ammonium acetate in water (solvent A) and 0.3% formic acid in acetonitrile (solvent B), at a flow rate of 0.5 mL/min. The gradient used was 95% solvent A and 5% solvent B from time 0 to 0.5 minutes, switched to 90% solvent B over 1.0 minute and held for 0.5 minutes, then returned to 95% solvent A and 5% solvent B over 1.0 minute and held for 1.0 additional minute to equilibrate the column before sample injection. The mass spectrometer interface was operated in positive electrospray ionization mode with capillary voltage at 4.00 kV. Analysis was performed in multiple reaction monitoring mode, observing 2 transitions for cefovecin (cone voltage = 24 V; m/z, 453.6→240.9 at 14 eV of collision energy and 453.6→125.1 at 55 eV) and 2 transitions for cephalixin (cone voltage = 15 V; m/z, 347.7→137.9 at 9 eV and 347.70→173.9 at 14 eV).

Method validation—The developed method was validated in accordance with current European guidelines by evaluation of specificity, linearity, trueness, and precision. Plasma samples collected from tortoises that had not been treated with cefovecin or cephalixin were used for the validation. Specificity was evaluated by injection of 10 blank plasma samples extracted and analyzed following the described procedure, to assess presence or absence of potential interfering compounds with the same retention times as cefovecin and cephalixin. A 9-point matrix-matched calibration curve was prepared each day of analysis with blank plasma samples fortified at various concentrations (0 to 50 μg/mL) to assess the method’s linearity; peak area ratios between cefovecin and the internal standard were plotted against their concentrations, and the resultant coefficient of determination (R²) was always > 0.99. The calibration curve was also used to calculate the limit of quantification of cefovecin in plasma, defined as the concentration providing a chromatographic signal with a signal-to-noise ratio of 10, which was 0.015 μg/mL. Because no certified reference material was available, accuracy (the combination of trueness and precision) was evaluated through the injection of 3 replicates of plasma samples spiked at 3 concentrations of cefovecin (0.05, 1.0, and 25.0 μg/mL) prepared on 2 days. Trueness, expressed as relative difference between the mean value measured and the spiked concentration, fell within the range set in the European guidelines. Precision was acceptable, with results always within the range of accepted values and with a maximum relative SD to the mean (coefficient of variation) of 15%.

Pharmacokinetic analysis—Pharmacokinetics parameters were deduced from plasma concentration-time data by use of software that allowed both compartmental and noncompartmental analyses of experimental data. All data points were weighted by the inverse square of the fitted value. A noncompartmental analysis was carried out on cefovecin plasma concentrations. The terminal elimination half-life was calculated as ln2/λ, where λ is the first-order rate constant of the terminal portion of the curve. Harmonic means and pseudo-SDs were calculated for half-life with a jackknife technique. The AUMC₀–last and AUC₀–last were calculated via the trapezoidal method. Mean residence time to the last detectable drug concentration (MRTlast) was determined from the following equation: MRTlast = AUMC₀–last /AUC₀–last

The Cmax and Tmax of cefovecin in plasma were obtained from the experimentally observed data.

Plasma protein binding analysis—After measuring total protein and albumin concentrations in samples before spiking with cefovecin, estimates of plasma protein binding were determined as follows: aliquots (450 μL) of a pool of tortoise plasma thawed at room temperature were fortified with 50 μL of cefovecin stock solutions at various concentrations to obtain final concentrations of 1, 5, and 10 μg/mL, prepared in triplicate (9 samples in total); 3 additional blank aliquots (plasma without cefovecin added) were also prepared. The samples were vortexed and incubated at 30°C for 60 minutes, then transferred to centrifugal filter units and centrifuged at 14,000 x g for 10 minutes. For each sample, 100 μL of the ultrafiltrates was collected and, after spiking each blank to obtain 1, 5, or 10 μg of cefovecin/mL, processed as previously described for cefovecin extraction and quantification. The bound fraction was then calculated at each concentration as follows:

% protein binding = ([total concentration – unbound concentration] / total concentration) × 100%

where total concentration is the amount of drug measured in the blank sample fortified after centrifugation and the unbound concentration is the mean of the drug concentrations found in the 3 samples spiked at the beginning of the experiment.

Results

No evidence of change in food consumption or fecal production was observed in any tortoises during the study. All animals appeared to be in good clinical condition, with stable activity levels and good adaption to group housing and to the temperature gradients of the vivaria. No gross changes were observed at the cefovecin injection sites.

Cefovecin was detected at measurable concentrations (> 0.015 μg/mL) in all groups for 3 days, then it was measurable only in some groups in the following 3 days and remained below the limit of quantification in all groups after 1 week (Figure 1). A degree of variability was observed among treatment groups, as confirmed by relative SD at each time point (between 19.1% and
26.9%). The pharmacokinetic parameters are reported (Table 1). After SC administration, cefovecin reached Cmax in plasma between 35 minutes and 2 hours (with the exception of 1 group, in which it was reached after 4 hours), with similar values (mean ± SD, 24.30 ± 6.19 µg/mL at 35 minutes, 24.92 ± 4.88 µg/mL at 1 hour, and 23.00 ± 5.97 µg/mL at 2 hours). The mean Tmax was 1.22 ± 1.14 hours.

Cefovecin was not highly bound to plasma proteins. Mean tortoise plasma total protein and albumin concentrations measured in samples before spiking with cefovecin were 5.3 and 2.1 g/dL, respectively. The mean bound fraction was 47.5% at a cefovecin concentration of 1 µg/mL, 41.3% at 5 µg/mL, and 42.5% at 10 µg/mL, with all values in the range of 37.9% to 50.2%.

**Discussion**

The analytic method used in this study was found to be suitable for pharmacokinetics studies, which require reliable performance with a wide range of drug concentrations. The noncompartmental analysis was suitable to describe the pharmacokinetic properties of cefovecin in Hermann's tortoises, similar to the observations in mammals and in other nonmammalian species. The Cmax of cefovecin (27.37 ± 4.43 µg/mL) was considerably lower than those reported for dogs and cats (121 ± 51 and 141 ± 12 µg/mL, respectively), various species of nonhuman primates (which ranged from 42 ± 9 µg/mL to 93.3 ± 6.61 µg/mL), and ferrets (41.05 ± 3.87 µg/mL) which had all received a single 8.0 mg/kg injection of cefovecin SC (the same dose administered to tortoises in the present study). According to data obtained by Thesuen et al, Cmax values of cefovecin in green iguanas and female Lohmann hens after SC administration at 10 mg/kg were similar or even lower (35 ± 12 µg/mL for iguanas and 6 ± 2 µg/mL for hens) than those found in our study. These observations are supported by differences in AUMC₀–last and AUC₀–last reported for the various species and tortoises in the present study.
were lower than concentrations described for dogs (6.0 to 7.8 and 2.5 to 3.7 g/dL) and cats (6.0 to 7.5 and 2.4 to 3.8 g/dL). It is known that for β-lactam antimicrobials there is not a linear correlation between drug concentration and bactericidal activity. Therefore, for this class of time-dependent antimicrobials, it is more important to consider the time the concentration exceeds the MIC, which has been shown through in vivo studies to be the pharmacokinetic-pharmacodynamic parameter that correlates best with their therapeutic efficacy. To evaluate the concentrations at which cefovecin can be considered effective and the duration of effect, MIC of the drug for common bacterial pathogens of the species of interest must be considered. However, since no tortoise-specific data on the MIC of cefovecin for common pathogens is available, it is not presently possible to suggest a therapeutically effective administration interval.

Currently, only a few reports on the use of cefovecin in exotic animals have been published. This is likely attributable, in part, to the relatively recent introduction of the drug in veterinary medicine. Some reports have highlighted relevant differences in cefovecin pharmacokinetics among various animal species. In dogs and cats, a single-dose SC administration at 8.0 mg/kg can effectively maintain the therapeutic effect on skin and soft tissue (through the maintenance of an effective threshold concentration) for a long period, allowing a 14-day dose interval. This therapeutic approach seems unsuitable for other species that have faster cefovecin elimination; according to the results obtained in other pharmacokinetics studies, daily administration is suggested in nonhuman primates and, similarly, a 2- to 3-day maximum dose interval has been recommended in ferrets, with a 12-hour to 2-day interval suggested for green iguanas.

Clearly, when considered from the practical standpoint and for animal well-being, a long duration of effect requiring less frequent drug administration is beneficial because it contributes to reduction of patient stress and helps to optimize owner compliance at the same time. Especially in nondomestic and wild or feral animals, stress must be considered a very important and limiting factor because these types of animals can be more susceptible to stress caused by repeated handling and restraint. In light of the results of the present pharmacokinetics study, and particularly on the basis of the plasma elimination half-life, it seems that the dosing interval of cefovecin described for dogs and cats (14 days) cannot be considered valid for tortoises.

To our knowledge, our study represents the first research to investigate the pharmacokinetics of cefovecin in Hermann’s tortoises after SC administration at the dose recommended for use in dogs and cats (8.0 mg/kg) and also to evaluate plasma protein binding to cefovecin for this species. With regard to the tolerability of the drug, no apparent adverse reactions (local or systemic) were observed in the study population during the experiments. Finally, it would be interesting, once MIC data for pathogens common to tortoises are available, to evaluate the time the concentration exceeds the relevant MIC values in this species to propose reasonable administration intervals.

References