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PHARMACOKINETICS OF CEFTIOFUR CRYSTALLINE-FREE ACID (EXCEDE STERILE SUSPENSION®) ADMINISTERED VIA INTRAMUSCULAR INJECTION IN WILD CALIFORNIA SEA LIONS (*ZALOPHUS CALIFORNIANUS*)

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?1 *Abstract:* The pharmacokinetics of ceftiofur crystalline-free acid (EXCEDE Sterile Suspension, 200 mg
?2 ceftiofur equivalents/ml) were determined for the California sea lion (*Zalophus californianus*). A single dose of EXCEDE was administered intramuscularly at 6.6 mg/kg to 12 wild California sea lions during rehabilitation. The first 10 animals were each assigned to two blood collection time points, with a total of 10 time points at: 6, 12, 24, 48, 72, 96, 120, 144, 168, and 192 hr after administration of the drug. An additional two animals were sampled 1, 2, 3, 4, 5, and 6 hr postinjection. Plasma was separated within 10 min of blood collection and stored at –20°C until analysis. Plasma concentrations of ceftiofur, desfuroylceftiofur, and related metabolites, were determined using liquid chromatography with tandem MS. Maximum plasma concentrations of ceftiofur and related metabolites were observed 24 hr postdosing with a mean concentration of 3.6 μg/ml. The half life (60 hr) and area under the curve (270 μg·hr/ml) were also determined. These data indicate that a single dose of EXCEDE at 6.6 mg/kg i.m. would likely maintain a mean plasma drug level >0.6 μg/ml for 5 days and >0.5 μg/ml for 8 days.

Key words: Antibiotic, sea lion, ceftiofur, EXCEDE, pharmacokinetics, *Zalophus californianus.*

INTRODUCTION

EXCEDE® Sterile Suspension (200 mg ceftiofur equivalents/ml), developed for use in veterinary medicine, is a third-generation cephalosporin formulation with extended release properties (EXCEDE; Zoetis Inc., Madison, New Jersey 07940, USA). The sustained-release properties of EXCEDE are the result of the placement of the crystallization form of ceftiofur (ceftiofur crystalline-free acid, CCFA) into an oil-based formulation composed of high-purity caprylic–capric
?3 triglyceride (Miglyol® 812) and cottonseed oil. This formulation creates a depot of CCFA after administration. The depot at the injection site slowly releases ceftiofur, and this slow absorption rate results in a longer half-life and prolonged therapeutic concentrations of ceftiofur and its primary active metabolite, desfuroylceftiofur (DFC).[4,5,20]

Third-generation cephalosporins have broad spectrum activity against gram-positive and gram-negative bacteria, including β-lactamase–producing strains.[18] Like other cephalosporins, ceftiofur is bactericidal, due to disruption of cell wall synthesis, which occurs in the peptidoglycan layer of the bacterial cell wall, which maintains cell rigidity. β-Lactam agents bind to penicillin-binding proteins, enzymes that are involved in the synthesis of peptidoglycan precursors, inhibiting cell wall synthesis and ultimately causing cell lysis.[18] Peptidoglycan synthesis occurs on a continuing basis; therefore, a therapeutic level of antimicrobial must be maintained for an extended period until bacterial cell death occurs. Thus, EXCEDE has a time-dependent, rather than concentration-dependent, mode of action. The time above the minimum inhibitory concentration (MIC) is the best predictor of efficacy; therefore, the goal of therapy is to maximize the duration of time above the MIC.[18,20]

EXCEDE is approved for subcutaneous use in beef and dairy cattle and for intramuscular administration in swine and horses in the United States and Canada. It is also approved in Europe under the trade name Naxcel®. Investigations have also evaluated the use of the drug in other domestic species, including domestic goats (*Capra*

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aegagrus hircus) and alpacas (*Vicugna pacos*), both of which have demonstrated pharmacokinetic parameters similar to those documented in cattle.[7,9]

The prolonged duration of action, because of its extended release properties, is attractive for use in wildlife species requiring less frequent injections to minimizing handling and stress. Use of EXCEDE in nondomestic species is increasing, and pharmacokinetic analysis and safety have been reported in Asian elephants (*Elephas maximus*), ball pythons (*Python regius*), helmeted guinea fowl (*Numida meleagris*), and American black ducks (*Anas rubripes*).[1,2,15,22]

Currently, EXCEDE is used for treatment of bacterial infections in various pinniped and cetacean species and was recently administered to free-ranging live humpback whales (*Megaptera novaeangliae*).[10] Although the use of EXCEDE in marine mammals is increasing, pharmacokinetics for the drug have not been reported for any species, and dosing and frequency have been extrapolated from domestic species. The objective of this study was to determine the pharmacokinetics of EXCEDE in the California sea lion (*Zalophus californianus*) after a single intramuscular injection.

MATERIALS AND METHODS

Study animals

Animals used in this study stranded along the coast of California and presented to The Marine Mammal Center (TMMC) in Sausalito, California, for rehabilitation under a Letter of Authorization from the National Marine Fisheries Service to TMMC. Twelve California sea lions (seven males and five females) were included in the clinical study. The animals ranged in weight from 18 to 62 kg.

All animals required antimicrobial therapy for various minor wounds or presumed bacterial infections. For the first 10 animals, a single dose of EXCEDE (200 mg ceftiofur equivalents/ml, 6.6 mg/kg i.m.) was administered in the right or left pelvic limb muscles. Blood samples were collected postadministration from either the right or left caudal gluteal vein at the following times: 6, 12, 24, 48, 72, 96, 120, 144, 168, and 192 hr after administration of the drug. Each animal was assigned to two blood collection time points (Table 1). An additional two animals, maintained under general anesthesia for other reasons, were administered EXCEDE at the same dose and route as previously described, and blood samples were collected at time points 1, 2, 3, 4, 5, and 6 hr postinjection (Table 1).

Blood was collected into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) from all animals. Samples were immediately centrifuged for 10 min, and the EDTA plasma was removed and frozen at –20°C until analysis.

Analysis

The plasma concentration of ceftiofur and ceftiofur-related metabolites was determined following solid phase extraction methods and ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) detection as previously described.[5] Analysis was completed following Pfizer Animal Health standard operating procedures and met the accuracy and precision requirements outlined in the Guidance for Industry, Bioanalytical Method Validation (US Department of Health and Human Services, FDA, CDER, CVM, May 2001). Briefly, samples were analyzed for concentrations of ceftiofur, DFC, and related metabolites after a reduction step using 1,4-dithioerythritol solution (20 mg/ml in 0.1 M ammonium acetate [NH_4OAc], pH 8.9) containing the internal standard, 200 ng/ml stable labeled ceftiofur (ceftiofur-d_3, MW +3). The reduced samples were loaded on to 96-well plates (Isolute-96, 100-mg C18 Fixed Well SPE plates, Biotage, Uppsala, Sweden) and derivatized on-column to desfuroylceftiofur acetamide (DCA) after the addition of iodoacetamide solution (40 mg/ml in 0.1 M NH_4OAc). Samples were eluted with 200 µl 30:70 acetonitrile:0.01 M NH_4OAc with 0.1% trifluoroacetic acid (TFA) into a 500-µl 96-well polypropylene plate containing an equal volume of 0.01 M NH_4OAc with 0.1% TFA. The resulting samples were injected on to an UPLC column (Waters Acquity UPLC™ BEH C18 1.7 µm 2.1 × 50 mm column; Waters Corporation, Milford, Massachusetts 01757, USA) and resolved after a 1.5-min gradient method from 5% to 45% organic mobile phase (0.3% formic acid in acetonitrile). Aqueous mobile phase consisted of 5 mM ammonium formate with 0.3% formic acid. The column was flushed with 95% organic mobile phase and equilibrated for approximately 1 min between sample injections. This gradient method results in improved chromatography compared with the isocratic method described previously.

The bioanalytical method for analysis of DCA was completed in two analytical runs with calibration standards, quality control samples, and

Table 1. Time points for blood collection assigned per animal after administration of ceftiofur crystalline-free acid.

	Hours														
Animal	1	2	3	4	5	6	12	24	48	72	96	120	144	168	192
1	X	X	X	X	X	X									
2	X	X	X	X											
3						X					X				
4							X				X				
5								X					X		
6									X			X			
7										X			X		
8						X								X	
9							X					X			
10								X			X				X
11									X					X	
12										X					X

control blanks prepared in untreated sea lion plasma. Each analytical run contained seven calibration standards ranging from 0.01 to 10 μg/ml and replicate quality control samples at 0.03, 0.75, and 7.5 μg/ml. For detection of DCA via MS, a mass spectrometer (Applied Biosystems MDS Sciex API4000; Applied Biosystems, Foster City, California 94404, USA) was set to operate in positive ion mode using an electrospray ionization source (TurboIonSpray® probe; Applied Biosystems). Positive ions were monitored in the multiple-reaction monitoring mode with precursor → product ion pairs of 487 → 241 for DCA and 490 → 244 for DCA-d3. Integration of chromatograms and standard regression using peak area ratios was performed using Analyst® Software (Analyst Software v1.4.2, Applied Biosystems).

Pharmacokinetic calculations

The plasma concentration data were analyzed in WinNonlin® (version 5.2, Pharsight, Sunnyvale, California 94086, USA) using compartmental modeling. A naïve pool approach was used with a first-order one-compartment model with no lag time and first-order elimination. Weighting of the data using the inverse of the predicted concentration resulted in the best fit as determined by visual examination.

The following pharmacokinetic parameters: area under the plasma concentration time curve from time 0 to infinity ($AUC_{0\text{-}\infty}$) and terminal half-life ($t_{1/2}$) were calculated from the model. The pharmacokinetic parameters observed maximum plasma concentration (C_{max}) and time to maximum concentration (t_{max}) were reported directly from the data.

The equation for the model is shown here as Eq. 1, where C_t is plasma concentration at time t, D is dose, K_a is apparent absorption rate, K_e is apparent elimination rate, F is bioavailabilty, and V is volume of distribution.

$$C_t = \frac{DFK_a}{V(K_a - K_e)} \times e^{-K_e t} - e^{-K_a t} \qquad (1)$$

RESULTS

The animals showed no adverse reactions such as swelling at the injection sites or anaphylaxis.

The analytical method used to determine plasma concentrations converts all ceftiofur-related substances containing an intact β-lactam ring (and thus possessing antimicrobial activity) into DCA, which is subsequently quantified. Theoretical values for standards ranged from 96.3% to 103%, including samples at the lower limit of quantitation (0.01 μg/ml), and quality control values ranged from 91.8% to 111% with a coefficient of variation of <10%.

The observed plasma concentration data and the model predicted profile is shown graphically in Figure 1. The pharmacokinetic parameters, including individual values and associated statistics for C_{max}, t_{max}, and $AUC_{0\text{-}\infty}$, are listed in Table 2. The maximum plasma concentration was observed at 24 hr postdose with a mean concentration of 3.6 μg/ml. These data indicate that a dose of 6.6 mg/kg would likely maintain a mean plasma drug level >0.6 μg/ml for 5 days and >0.5 μg/ml for 8 days.

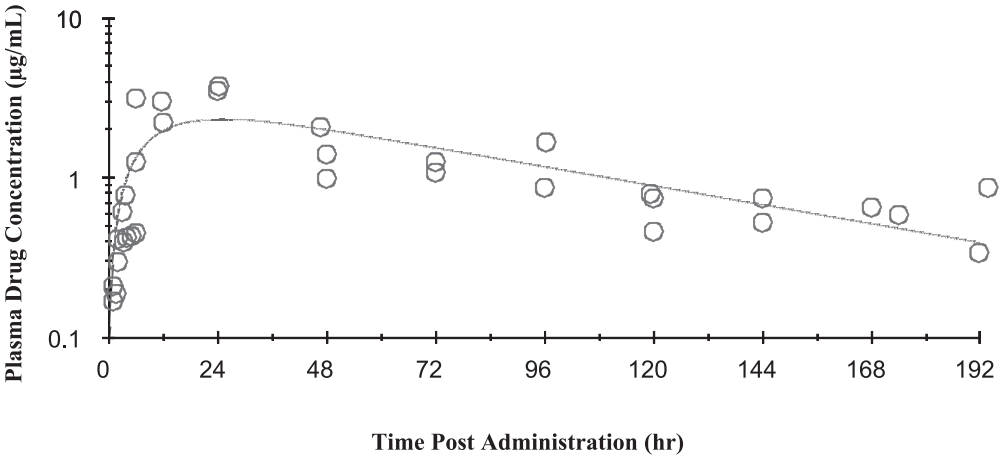

Figure 1. Plasma concentration time profile for ceftiofur and related metabolites measured as desfuroylceftiofur acetamide (DCA) after a single 6.6 mg/kg i.m. administration of ceftiofur crystalline-free acid (CCFA) to California sea lions. The circles represent measured levels from individual animals, and the line represents the model predicted values.

DISCUSSION

The drug levels measured in this study after a single administration of EXCEDE at a dose of 6.6 mg/kg i.m. in the California sea lion demonstrate the extended-release characteristics, typical of the formulation. The pharmacokinetic data obtained from this study were similar to previously documented data described in other mammalian species. The C_{max} (µg/ml) measured in the California sea lion (3.6) was higher than equine (0.78 ± 0.19) and the Asian elephant (1.36 ± 0.74), lower than beef cattle (6.39 ± 1.79) and dairy cattle (4.44 ± 1.65), and most comparable to levels measured in alpaca (2.7 ± 0.9) and domestic goats (2.3 ± 1.1)[2,5,7,9,12] (supplemental NADA 141–209 2006, 2009; EXCEDE package insert, 2009). The t_{max} (hr) measured in the sea lion (24.0) was most similar to domestic goats (26.7) and equine (21.6), later than beef cattle (19.8 ± 5.81) and dairy cattle (19.0 ± 8.02), and earlier than alpacas (36.0) and the Asian elephant (47.18 ± 31.30)[2,5,7,9,12] (supplemental NADA 141–209 2006, 2009; EXCEDE package insert, 2009). The terminal half life ($t_{1/2}$, hr) in the California sea lion (60.8) was longer than goats (36.9), beef cattle (40.7 ± 11.2), dairy cattle (43.92 ± 9.84), and alpaca (44.7) and earlier when compared with the Asian elephant (83.36 ± 30.01) and equine (81.0)[2,5,7,9,12] (supplemental NADA 141–209 2006, 2009; EXCEDE package insert, 2009).

In cattle, a single dose of EXCEDE has been documented to provide plasma drug levels >0.2 µg/ml in order to target common respiratory

Table 2. Pharmacokinetic results after a single intramuscular dose at 6.6 mg/kg of ceftiofur crystalline-free acid administered to California sea lions. The measured pharmacokinetic parameters included are $C_{max(obs)}$, observed maximum plasma concentration; $t_{max(obs)}$, observed time to reach maximum plasma concentration. The model fit parameters include $AUC_{0-\infty}$, area under the plasma concentration time curve from time 0 to infinity; $t_{1/2}$, terminal half life; K_a, apparent absorption rate; K_e, apparent elimination rate; and V/F, apparent volume of distribution.

Pharmacokinetic parameter	Estimate	Standard error[a]	% CV
$C_{max(obs)}$ (µg/ml)	3.6	NC	NC
$i_{max(obs)}$ (hr)	24.0	NC	NC
$AUC_{0-\infty}$ (µg·hr/ml)	267	34.0	12.7
$t_{1/2}$ (hr)	60.8	15.4	25.3
K_a (1/hr)	0.0974	0.0333	34.2
K_e (1/hr)	0.0114	0.00289	25.3
V/F (mL/kg)	2,163	471	21.8

[a] NC: not calculated.

bacterial pathogens for approximately 7 days (EXCEDE package insert, supplemental NADA 141–209, 2006). Drug levels measured in alpacas suggest that a single dose of EXCEDE will maintain a concentration >0.25 μg/ml for 6 days.[7] A single dose administered to the Asian elephant would likely maintain a concentration >0.25 μg/ml for 7–10 days.[2] The plasma concentrations measured in the current study indicate that a single dose of 6.6 mg/kg administered to the California sea lion would likely maintain a mean plasma drug level >0.6 μg/ml for 5 days and >0.5 μg/ml for 8 days.

In cattle, horses, domestic goats, and alpaca, the crystalline-free acid extended-release formulation of ceftiofur (EXCEDE) has a longer half-life compared with the immediate-release ceftiofur formulations with sodium and hydrochloride salts.[4,5,7,9] This longer terminal half-life and prolonged efficacious concentration of CCFA is due to "flip-flop kinetics," wherein the rate of elimination is dependent on the slower absorption rate.[19] Because of the lack of published intravenous data for immediate-release ceftiofur formulations in the sea lion, which is needed to determine the intrinsic elimination rate of ceftiofur, it is unknown whether the observed pharmacokinetic profile after CCFA administration exhibits flip-flop kinetics.[19] However, it is reasonable to conclude that sea lions also exhibit flip-flop kinetics on the basis of the long half-life demonstrated in the current study. Therefore, the apparent calculated elimination rate is likely an overestimation of the intrinsic elimination rate because it is likely confounded by the absorption.

One possible explanation for the variation in pharmacokinetic parameters when comparing the data obtained from the California sea lion to other mammalian species might be the difference in the route of administration, as well as the difference in anatomical locations of injections. An intramuscular route of administration was chosen in the current study because of the logistical ease of administering the drug to wild sea lions in a rehabilitation facility. A recent study compared subcutaneous compared with intramuscular administration in horses and found that drug levels remained above the therapeutic threshold regardless of the route of administration.[11] In general, the pharmacokinetic data in the California sea lion is most similar to the data reported in alpaca and domestic goats, in which the drug is administered subcutaneously. Therefore the differences noted among the various mammalian domestic and nondomestic species evaluated are less likely due to route of administration and more likely due to species variation in drug absorption and rates of drug metabolism.

Investigations in avian and reptilian species have used higher doses (10–15 mg/kg i.m.) with a higher dosing frequency needed to achieve the desired MICs for bacterial isolates specific to these species. These studies have revealed a higher C_{max} (μg/ml) in the helmeted guineafowl (5.26), ball python (7.096 ±1.95), and American black duck (13.1) compared with that measured in the California sea lion.[1,15,22]

Common bacterial organisms isolated from pinnipeds include *Leptospira* spp., *Escherichia coli*, *Staphylococcus* spp., *Streptococcus* spp., and *Salmonella*, among others.[8,10,16,17,21] Ceftiofur has been shown to be an effective antibiotic against these organisms.[13,16,20] Published in vitro MIC data for ceftiofur against these bacteria are: *E. coli*, 0.13–1.0 μg/ml; *Salmonella* spp., 0.06–2.0 μg/ml; and *Staphylococcus* spp., 0.13–1.0 μg/ml.[20] Although MIC data was not found for ceftiofur specifically against *Leptospira* spp., ceftiofur has been documented to be an effective treatment for cattle infected with *Leptospira borgpetersenii* serovar *hardjo*.[3,6,13] The potential for EXCEDE to be a possible treatment for leptospirosis in the California sea lion should be further investigated.

CONCLUSIONS

This study demonstrated EXCEDE to be a safe antibiotic for use in the California sea lion. The plasma drug levels measured after single-dose administration reached similar levels to those seen in domestic mammals and would likely maintain a mean plasma drug level >0.6 μg/ml for 5 days and >0.5 μg/ml for 8 days; therefore, it should be an effective treatment for infections caused by bacteria with MICs for ceftiofur ≤ 0.5 μg/ml. The MIC for each bacterial isolate can vary, and when possible, obtaining current antibiotic sensitivity data is ideal for determining the optimum dosing frequency. Furthermore, microbiology MIC data are warranted to evaluate the efficacy of EXCEDE against various bacterial organisms common to pinnipeds. Additional research is needed to determine the optimal dosing interval with multiple-dose therapy. This antibiotic is appealing for treatment of various bacterial diseases in the California sea lion because of its extended release properties and therefore the need for less frequent injections.

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