Population pharmacokinetics of enrofloxacin in purple sea stars (*Pisaster ochraceus*) following an intracoelomic injection or extended immersion

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OBJECTIVE
To determine population pharmacokinetics of enrofloxacin in purple sea stars (*Pisaster ochraceus*) administered an intracoelomic injection of enrofloxacin (5 mg/kg) or immersed in an enrofloxacin solution (5 mg/L) for 6 hours.

ANIMALS
28 sea stars of undetermined age and sex.

PROCEDURES
The study had 2 phases. Twelve sea stars received an intracoelomic injection of enrofloxacin (5 mg/kg) or were immersed in an enrofloxacin solution (5 mg/L) for 6 hours during the injection and immersion phases, respectively. Two untreated sea stars were housed with the treated animals following enrofloxacin administration during both phases. Water vascular system fluid samples were collected from 4 sea stars and all controls at predetermined times during and after enrofloxacin administration. The enrofloxacin concentration in those samples was determined by high-performance liquid chromatography. For each phase, noncompartmental analysis of naïve averaged pooled samples was used to obtain initial parameter estimates; then, population pharmacokinetic analysis was performed that accounted for the sparse sampling technique used.

RESULTS
Injection phase data were best fit with a 2-compartment model; elimination half-life, peak concentration, area under the curve, and volume of distribution were 42.8 hours, 18.9 μg/mL, 353.8 μg•h/mL, and 0.25 L/kg, respectively. Immersion phase data were best fit with a 1-compartment model; elimination half-life, peak concentration, and area under the curve were 56 hours, 36.3 μg•h/mL, and 0.39 μg/mL, respectively.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested that the described enrofloxacin administration resulted in water vascular system fluid drug concentrations expected to exceed the minimum inhibitory concentration for many bacterial pathogens. (*Am J Vet Res* 2016;77:1266–1275)

An ongoing mass mortality event has resulted in the loss of millions of wild and captive sea stars along the western coast of North America.[1] It is suspected that the sea stars are dying from a disease termed sea star wasting disease. Although a densovirus has been associated with the disease, the definitive etiology of sea star wasting disease has not been identified.[2] Numerous bacterial organisms have been isolated from aquatic invertebrates including *Pseudomonas* spp, *Flavobacterium* spp, *Aeromonas* spp, and *Vibrio* spp.[3] Previous sea star mortality events were believed to be caused by *Vibrio* spp[4]; however, the extent to which microbial agents are associated with the deaths of sea stars is currently unknown.

Anecdotal reports indicate that antimicrobials have varying efficacy for the treatment of clinically ill sea stars in aquarium collections. Enrofloxacin is one of the most commonly used antimicrobials because of its broad spectrum of activity. Like other fluoroquinolones, enrofloxacin is a synthetic bactericidal agent with favorable pharmacokinetic properties.[5,6] Although the pharmacokinetic parameters of enrofloxacin have been determined in other aquatic invertebrates,[7–13] data regarding the pharmacokinetics of enrofloxacin in echinoderms are lacking.

ABBREVIATIONS

AUC	Area under the drug concentration–time curve
CL/f	Clearance per fraction absorbed
C_{max}	Peak concentration
HPLC	High-performance liquid chromatography
MIC	Minimum inhibitory concentration
NLME	Nonlinear mixed-effects modeling
STS	Standard 2 stage
$t_{1/2}$	Terminal half-life
t_{max}	Time to peak concentration
V_{ss}/f	Volume of distribution at steady-state per fraction absorbed
WVSF	Water vascular system fluid

The objectives for the study reported here were to determine pharmacokinetic parameters of enrofloxacin in purple sea stars (*Pisaster ochraceus*) following an intracoelomic injection or extended immersion. Purple sea stars were selected for the study because they are the predominant intertidal species commonly found in aquaria, and the species population has been greatly reduced by the ongoing epizootic.[4,14]

Materials and Methods

Animals and housing

Twenty-eight clinically normal purple sea stars of undetermined age and sex were used in the study. The sea stars were collected from the coastal waters of British Columbia, Canada, in November 2014, in accordance with license XR 1 2014 granted by the Department of Fisheries and Oceans Canada. They were allowed to acclimate for at least 3 weeks before the study was initiated.

The sea stars had a mean body weight of 281 ± 168 g (range, 98 to 625 g). They were housed at the Vancouver Aquarium's research laboratory in two 190-L tanks with a flow-through, nonrecirculating seawater system. The sea stars used for the intracoelomic injection phase of the study were housed in 1 tank, and those used for the immersion phase of the study were housed in the other. The water-quality parameters (temperature, 10° to 12°C; pH, 7.7 to 7.8; salinity, 27.7 to 28.3 parts per thousand; nitrate concentration, < 10 mg/L; and phosphate concentration, < 24 mg/L) for the seawater system were standardized and maintained for the duration of the study. The sea stars were fed frozen Manila clams (*Ruditapes philippinarum*) twice per week.

Experimental design

All study procedures were approved by the Vancouver Aquarium Animal Care Committee. Within 24 hours prior to study initiation, each sea star was weighed and underwent a physical examination. From each sea star, WVSF samples were collected from the aboral aspect of the limbs approximately 1 cm from the distal tip with a 27-gauge needle and 1-mL syringe. Only sea stars that appeared clinically normal on the basis of the results of the physical examination were enrolled in the study. Sea stars were purposely allocated to either the injection or immersion phase of the study in a manner that allowed for individual sea star identification on the basis of physical characteristics such as size, coloration, and number of limbs and ensured each group consisted of an adequate number of animals with an even distribution of body weight. Each phase (injection and immersion) of the study consisted of 12 sea stars that received the designated treatment and 2 sea stars that served as untreated controls to determine whether treated sea stars excreted enrofloxacin or ciprofloxacin (an active metabolite of enrofloxacin) in a quantity sufficient for absorption and measurement in untreated cohorts.

Intracoelomic injection

The 12 sea stars designated for intracoelomic injection of enrofloxacin (injected sea stars) had a mean ± SD body weight of 260 ± 24 g (range, 98 to 554 g), and the 2 control sea stars had body weights of 148 and 440 g, respectively. Each injected sea star received 1 injection of enrofloxacin[a] (5 mg/kg) intracoelomically. Within 20 minutes before injection, the calculated dose was diluted with sterile water to attain a solution with a concentration of 10 mg of enrofloxacin/mL The injection was administered via a 27-gauge needle on the aboral surface at the base of a limb, near its junction with the central disc. Following injection, the injected sea stars were randomly allocated into 3 sampling groups (groups 1 through 3) of 4 sea stars and placed back in their water tank. Plastic mesh partitions were placed in the tank to keep each sampling group and the control group separate from each other. Water vascular system fluid samples (approx 0.3 mL) were collected as previously described at 0.01, 2, 12, and 72 hours after the enrofloxacin injection from each sea star in group 1; 0.5, 4, 24, and 96 hours after the enrofloxacin injection from each sea star in group 2; and 1, 8, 48, and 120 hours after the enrofloxacin injection from each sea star in group 3. Thus, 4 samples were obtained at each sample acquisition time, and 4 samples (total volume, 1.2 mL) were collected from each injected sea star, which represented 0.5% of the mean body weight for those animals. Water vascular system fluid samples (approx 0.3 mL) were collected from each of the 2 control sea stars at each time when samples were obtained from the injected sea stars. Therefore, 12 samples (total volume, 3.6 mL) were collected from each control sea star, which represented 2.4% of the body weight for the smallest control sea star. A 1-mL sample of the tank water was collected at 0.01, 24, 48, and 120 hours after the treated sea stars were injected with enrofloxacin.

Extended immersion

The 12 sea stars designated for extended immersion in enrofloxacin (immersion sea stars) had a mean ± SD body weight of 305 ± 32 g (range, 98 to 625 g), and the 2 control sea stars had body weights of 155 and 336 g, respectively. An immersion bath was created by mixing 60 L of sea water with 300 mg of enrofloxacin,[b] which resulted in a solution with a concentration of 5 mg of enrofloxacin/L. The immersion sea stars were immersed in the bath for 6 hours, during which time the water was aerated through an air stone and the water temperature ranged from 10.2° to 13.3°C. Following the immersion treatment, the sea stars were randomly allocated into 3 sampling groups (groups 1 through 3) of 4 sea stars and placed back in their water tank. Plastic mesh partitions were placed in the tank to keep each sampling group and the control group separate from each other. Water vascular fluid samples (approx 0.3 mL) were collected as previously described from group 1 at 0.01, 3, and 6

hours during immersion (6 hours during immersion was considered equivalent to 0.01 hours after immersion) and at 2, 12, and 72 hours after immersion; from group 2 at 1 and 4 hours during immersion and at 0.5, 4, 24, and 96 hours after immersion; and from group 3 at 2 and 5 hours during immersion and at 1, 8, 48, and 120 hours after immersion. Thus, 4 samples were obtained at each sample acquisition time, and 6 samples (total volume, 1.8 mL) were collected from each immersion sea star, which represented 0.6% of the mean body weight for those animals. Water vascular system fluid samples (approx 0.3 mL) were collected from each of the 2 control sea stars at 0.01, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, and 120 hours after the end of the immersion treatment for the immersion sea stars. Therefore, 12 samples (total volume, 3.6 mL) were collected from each control sea star, which represented 2.3% of the body weight for the smallest control sea star. A 1-mL sample of the tank water was collected at 0.01, 24, 48, and 120 hours after the immersion sea stars were removed from the enrofloxacin solution.

Sample processing

Blank (control) WVSF samples were collected from untreated sea stars prior to study initiation for use in assay calibration, quality control measurements, and analysis of assay background noise. Cytologic evaluation indicated that those samples had low cellularity. Therefore, those samples as well as the WVSF samples obtained during the injection and immersion phases of the study were not centrifuged following collection and were placed directly into cryovials. Water samples collected from the tanks were also placed directly into cryovials. All samples were stored frozen at –70°C until analysis.

The concentrations of enrofloxacin and its metabolite ciprofloxacin were determined for the WVSF samples collected from the study sea stars and water samples collected from the holding tanks by use of an HPLC method adapted from other studies conducted in our laboratory. The HPLC system used consisted of a quaternary solvent delivery system (flow rate, 1 mL/min), autosampler,[c] and UV detector[d] set at a wavelength of 279 nm. Chromatograms were integrated with a computer program.[e] The analytic column[f] was a reverse-phase C8 column that was maintained at a constant temperature (40°C). The mobile phase consisted of 75% distilled water and 25% acetonitrile. A 0.1% solution of trifluoroacetic acid was added to the mobile phase as a pH modifier.

The reference standard of ciprofloxacin[g] was used to prepare a stock solution for fortifying a blank sample matrix. The reference standard for enrofloxacin was supplied by the manufacturer[h]; no internal standard was used because no volumes were transferred. Stock solutions were sealed and stored in the dark in a refrigerator. The calibration curve for ciprofloxacin consisted of 8 standard solutions with concentrations that ranged between 0.05 and 10 µg/mL and included a blank sample (ciprofloxacin concentration, 0 µg/mL). The blank sample was used to detect interfering peaks that elute into the window of the chromatographic peak of interest and to measure background interference. Blank samples from untreated sea stars were fortified (spiked) with a range of concentrations of enrofloxacin and ciprofloxacin and compared with blank PBS solution samples. Results indicated almost 100% agreement between the measurements at each concentration studied. Therefore, the calibration curve samples and quality control samples were prepared with PBS solution as the matrix. Calibration curves were constructed by fortifying the matrix with ciprofloxacin at 7 concentrations ranging from 0.01 to 4 µg/mL. The calibration curve for enrofloxacin consisted of 9 samples with concentrations ranging from 0.05 to 40 µg/mL. A blank sample (enrofloxacin concentration, 0 µg/mL) was also included to check for the presence of interfering peaks. The calibration curve was accepted if the linear coefficient of determination (R^2) was ≥ 0.99 and the calibration curve concentrations could be back-calculated to ≤ 15% of the true concentration of the standard. Fresh calibration curves were prepared for each day's run.

All calibration, quality control, blank, and incurred samples collected from the sea stars were prepared in an identical manner. Each sample was centrifuged at 500 × *g* for 10 minutes to remove any particulate matter or cells. The sample was then decanted into an HPLC injection vial; 30 µL of the sample was used for injection into the HPLC system.

The retention times for ciprofloxacin and enrofloxacin were 2.6 to 2.8 and 3.1 to 3.3 minutes, respectively. The limit of quantification was 0.01 µg/mL for ciprofloxacin and 0.05 µg/mL for enrofloxacin. Those limits were determined from the lowest point on the respective linear calibration curves that yielded an acceptable accuracy and were within accepted International Conference on Harmonization[15] and United States Pharmacopeia[16] guidelines for the signal-to-noise ratio. Signal-to-noise ratios of 3 and 10 were used for the limits of detection and quantification, respectively. The mean accuracy (deviation from the true value) of the method was 3.75% across all concentrations on the calibration curve.

Pharmacokinetic analysis

A pharmacokinetic software program[i] was used to calculate initial pharmacokinetic parameter estimates by means of a naïve averaged pooled sample method. Models with various error structures were evaluated to identify the error structure that provided the best fit for the data; that error structure was then used in the base model for all subsequent analyses. The model for the injection phase data was parameterized by first-order input and a 2-compartment structure and was run with the quasi-random parametric expectation maximization engine, whereas the model for the immersion phase data was pa-

rameterized by first-order input and a 1-compartment structure and was run with the first-order conditional estimation–Linstrom Bates engine. For each phase, the best model for subsequent analyses was selected on the basis of visual evaluation of goodness-of-fit plots, comparison of the –2 log likelihood ratios, and Akaike information criterion values (a goodness-of-fit measure that is based on the log likelihood ratio, which has been adjusted for the number of parameters in the model [ie, degrees of freedom]) calculated by the pharmacokinetic software program[j] and the coefficient of variation for parameter estimates.

For the injection phase, analysis was performed by use of a 2-compartment model with first-order absorption of the injected enrofloxacin in accordance with the following equation:

$$C_{enro} = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-k01}$$

where C_{enro} is the enrofloxacin 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, β is the elimination rate constant (terminal phase), C is –(A + B), and k_{01} is the drug absorption rate. Secondary parameters calculated included distribution (α) and elimination (β) half-lives, microdistribution rate constants, AUC, V_{SS}/f, CL/f, C_{max}, and t_{max}.

For the immersion phase, analysis was performed by use of a 1-compartment model with first-order absorption of enrofloxacin in accordance with the following equation:

$$C_{enro} = (k_{01} \times D)/V(k_{01} - k_{10}) \times (e^{-k10t} - e^{-k01t}),$$

where C_{enro} is the enrofloxacin concentration, t is sample acquisition time, k_{01} is the first-order absorption rate, k_{10} is the elimination rate constant, V is the apparent volume of distribution, and D is the dose. Secondary parameters calculated from the model included C_{max}, t_{max}, AUC, absorption half-life, and $t_{1/2}$.

Population pharmacokinetics

Because WVSF samples were not acquired from all enrofloxacin-treated sea stars at each sample acquisition time, a population pharmacokinetic analysis was performed with NLME models by use of pharmacokinetic software[j] so that all subjects within a particular phase (injection or immersion) could be analyzed together. The parameters calculated by the naïve averaged pooled sample method were used as the initial estimates for the NLME analysis. Various models were evaluated as previously described to identify the model that best fit the data. Interindividual (between subject) variability was expressed by use of an exponential error model, which was parameterized as follows: $P_i = \theta P \times \exp(\eta_i P)$, where P is the pharmacokinetic parameter of interest for individual i, θP is the fixed effect for the population estimate of the parameter of interest, and ηiP is the random effect for the parameter of interest for individual i. The random effects for the population were assumed to be independent and have a normal distribution with a mean of 0 and variance of ω2. A multiplicative model was used to describe the residual random intrasubject variability (ε) of the data for once-daily dosing; ε had a mean of 0 and a variance of σ2 in accordance with the following equation:

$$C_{obs} = C_{pred} \times (1 + \varepsilon)$$

where C_{obs} is the observed concentration for an individual and C_{pred} is the model-predicted concentration for that individual.

Once the final population model was obtained for each phase, the covariates were examined to determine whether any factors explained the variability in the primary covariates for the respective models previously described in the pharmacokinetic analysis section. In particular, the covariate for sampling group was evaluated to determine whether that factor could have been the source of error. Box plots were constructed to visually assess whether the interindividual variability for each parameter differed significantly among the sampling groups. Additionally, the effect of sampling group on the other covariates in the NLME model was assessed by comparing the –2 log likelihood ratios for models with and without a fixed effect for sampling group. Sampling group was considered an important source of variability only if the –2 log likeli-

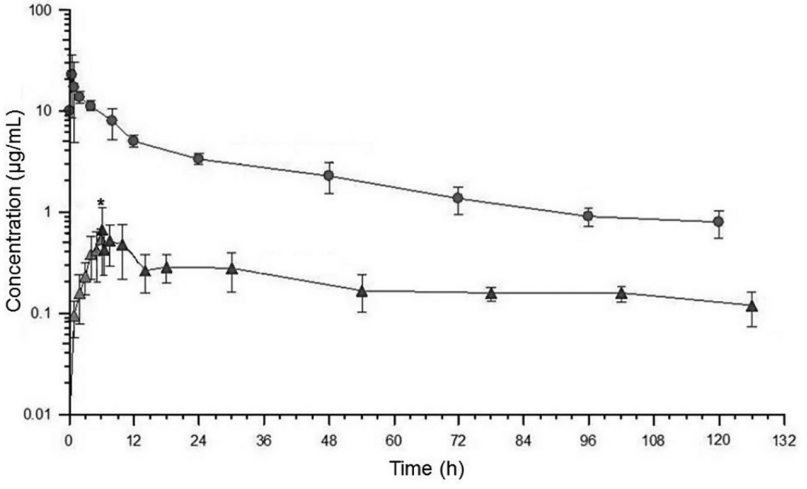

Figure 1—Mean ± SD enrofloxacin concentration over time in WVSF samples collected from purple sea stars (*Pisaster ochraceus*) following an intracoelomic injection of enrofloxacin (5 mg/kg; circles; n = 12) or during (gray triangles) and after (black triangles) immersion in an enrofloxacin solution (5 mg/L) for 6 hours (12). Samples were collected from only 4 sea stars at each sample acquisition time; therefore, each symbol represents the mean for 4 sea stars. Enrofloxacin injection and placement of the sea stars in the enrofloxacin solution were designated time = 0 hours. *Indicates return of the sea stars to their normal aquarium environment following immersion in the enrofloxacin solution.

hood ratio for the model without that covariate was less than that for the model with that covariate and if that comparison had a $P < 0.01$.

Results

Sea stars

No adverse effects were observed in any of the sea stars following enrofloxacin administration or serial collection of WVSF samples. Enrofloxacin was not detected in either of the untreated control sea stars during the injection or immersion phases. Enrofloxacin was not detected in any of the tank water samples collected during the injection phase. During the immersion phase, the enrofloxacin concentration in the immersion solution slowly decreased from 6.57 to 6.21 µg/mL during the 6-hour administration period, and enrofloxacin was not detected in any of the tank water samples collected after the administration period.

The mean enrofloxacin concentration in WVSF samples over time following both routes of administration was summarized **(Figure 1)**. Enrofloxacin concentrations greater than the limit of quantification for the HPLC assay used (0.05 µg/mL) were detected up to 120 and 126 hours after initiation of enrofloxacin administration (last sample acquisition time) during the injection and immersion phases, respectively. Ciprofloxacin concentrations greater than the limit of quantification for the HPLC assay used (0.01 µg/mL) were detected up to 96 hours after the enrofloxacin injection and were detected only twice (at 5 hours during immersion and at 8 hours after immersion) during and after immersion in the enrofloxacin solution. During the injection phase of the study, low concentrations (0.01 to 0.1 µg/mL) of ciprofloxacin were detected in most WVSF samples evaluated up to 96 hours after enrofloxacin administration. Although enrofloxacin and ciprofloxacin can have additive effects against bacteria, the concentrations of ciprofloxacin detected in the sea stars of this study were so low that it was considered clinically irrelevant; therefore, pharmacokinetic analyses were not performed for the ciprofloxacin data.

Table 1—Naïve averaged pooled-sample pharmacokinetic parameters for enrofloxacin in purple sea stars (*Pisaster ochraceus*) following a single intracoelomic injection of enrofloxacin (5 mg/kg; n = 12) or immersion in an enrofloxacin solution (5 mg/L) for 6 hours (12).

	Administration method	
Parameter	**Injection**	**Immersion**
$t_{1/2}$ (h)	43.7	92.8
t_{max} (h)	0.5	6.5
C_{max} (µg/mL)	22.1	0.67
AUC (h•µg/mL)	370.1	39.7
V_d/f (L/kg)	0.85	—
CL/f (mL/h/kg)	13.5	—

— = Not calculated. V_d/f = Volume of distribution of drug per fraction absorbed.

Pharmacokinetic analyses

The initial pharmacokinetic parameters for enrofloxacin determined by the naïve averaged pooled sample method were summarized **(Table 1)**, and the population-based pharmacokinetic parameters determined by the NLME population pharmacokinetic

Table 2—Population pharmacokinetic parameters for enrofloxacin in 12 purple sea stars following a single intracoelomic injection of enrofloxacin (5 mg/kg).

Parameter	**Value**	**SE**	**CV**
θk_a (1/h)	48.7	5.2×10^9	1.1×10^{10}
θA (µg/mL)	14.8	4.83	32.7
θα (1/h)	0.21	0.08	38.7
θB (µg/mL)	4.61	1.04	22.5
θβ (1/h)	0.02	0.002	14.2
k_{21} (1/h)	0.06	1.5×10^4	2.4×10^7
k_{10} (1/h)	0.06	1.3×10^4	2.4×10^7
k_{12} (1/h)	0.11	2.0×10^3	1.8×10^6
V_{ss}/f (L/kg)	0.71	1.4×10^5	4.6×10^7
AUC (h•µg/mL)	353.9	4.2×10^7	1.2×10^7
CL/f (mL/kg/h)	0.01	1.7×10^3	1.2×10^7
t_{max} (h)	0.12	1.0×10^7	8.8×10^9
C_{max} (µg/mL)	18.9	3.9×10^7	2.1×10^8
$k_a t_{1/2}$ (h)	0.01	1.5×10^6	1.1×10^{10}
$\alpha t_{1/2}$ (h)	3.28	1.27	38.7
$\beta t_{1/2}$ (h)	42.8	6.06	14.2

$\alpha t_{1/2}$ = Terminal half-life for the first-order distribution rate constant. $\beta t_{1/2}$ = Terminal half-life for the first-order elimination rate constant. CV = Coefficient of variation. k_{10} = Microdilution elimination rate constant. k_{12} = Microdilution rate constant for the transfer of the drug from the central compartment to the peripheral compartment. k_{21} = Microdilution rate constant for the transfer of the drug from the peripheral compartment to the central compartment. $k_a t_{1/2}$ = Terminal half-life for the absorption rate constant. θA = Fixed effect for the distribution intercept. θα = Fixed effect for the first-order distribution rate constant. θB = Fixed effect for the elimination intercept. θβ = Fixed effect for the first-order elimination rate constant. θk_a = Fixed effect for the absorption rate constant.

Table 3—Population pharmacokinetic parameters for enrofloxacin in 12 purple sea stars following immersion in an enrofloxacin solution (5 mg/L) for 6 hours.

Parameter	Value	SE	CV
θk_a (1/h)	0.31	0.05	16.2
θk_e (1/h)	0.01	0.001	8.5
t_{max} (h)	10.8	1.16	10.7
AUC (h•µg/mL)	36.3	4.07	11.2
C_{max} (µg/mL)	0.39	0.04	10.6
$k_a t_{1/2}$ (h)	2.23	0.36	16.2
$k_e t_{1/2}$ (h)	56.0	4.76	8.49

$k_a t_{1/2}$ = Terminal half-life for the absorption rate constant. $k_e t_{1/2}$ = Terminal half-life for the elimination rate constant. μk_a = Fixed effect for the absorption rate constant. θk_e = Fixed effect for the elimination rate constant.

See Table 2 for remainder of key.

approach for the injection **(Table 2)** and immersion **(Table 3)** phases were likewise summarized. The data for the injection phase were best fit with a 2-compartment model with first-order absorption, whereas the data for the immersion phase were best fit with a 1-compartment model with first-order absorption.

During the injection phase, enrofloxacin was rapidly absorbed (absorption half-life, 0.01 hours). The distribution and elimination half-lives were 3.3 and > 42 hours, respectively, and the AUC was 353.9 h•µg/mL. The apparent V_{ss}/f was only 0.26 L/kg, and the CL/f (0.01 mL/kg/h) was slow.

During the immersion phase, enrofloxacin was absorbed into the WVSF much more slowly (absorption half-life, 2.23 hours) than following injection; however, the elimination half-life (56 hours) was fairly long. The AUC (36.3 h•µg/mL) and C_{max} (0.39 µg/mL) following immersion in the enrofloxacin solution were much lower than those following intracoelomic injection of enrofloxacin. Thus, the exposure of sea stars to enrofloxacin following administration by immersion was 10% of that following intracoelomic injection (calculated by dividing the AUC for the immersion phase by the AUC for the injection phase).

The enrofloxacin concentrations over time for individual sea stars and as determined by the fitted curves derived from the NLME population pharmacokinetic analysis in which interindividual variability was controlled following intracoelomic injection **(Figure 2)** or immersion **(Figure 3)** were provided. Results suggested that the enrofloxacin concentration varied substantially among sea stars in both the injection and immersion phases. Additional analyses indicated that sampling group did not represent a significant source of variability in either the injection or immersion phases.

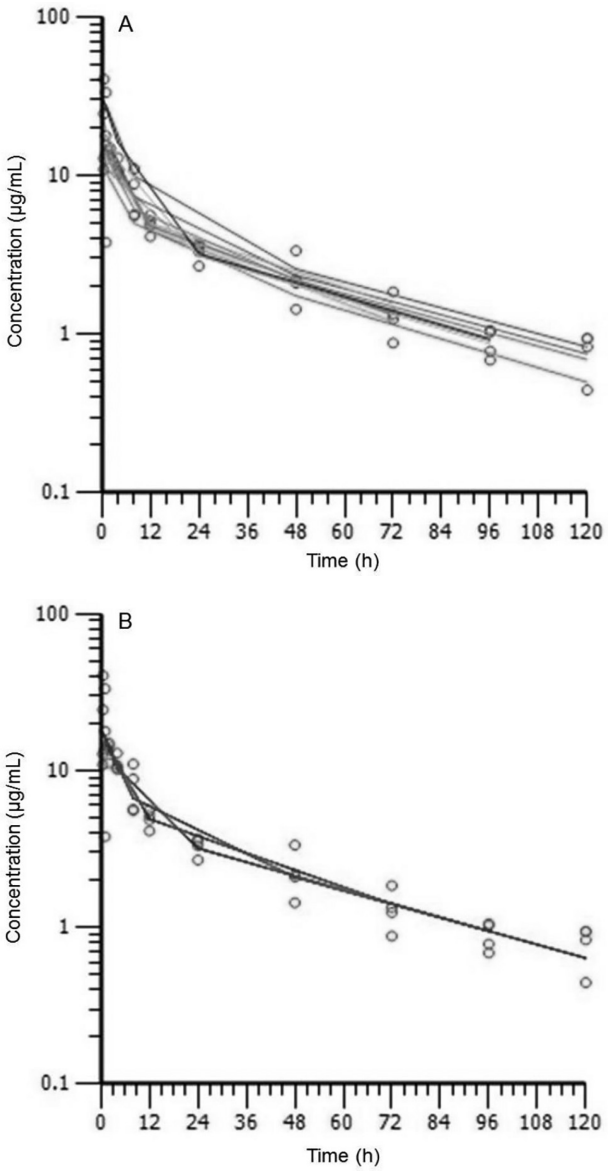

Figure 2—Enrofloxacin concentrations over time in WVSF samples collected from 12 purple sea stars following an intracoelomic injection of enrofloxacin (5 mg/kg; time = 0 hours). A—Spaghetti plots of enrofloxacin concentrations for individual sea stars fitted by a population pharmacokinetic model. B—Graphic presentation of the results for a fitted 2-compartment model with first-order adsorption in which interindividual variability was controlled. White circles represent measurements for individual sea stars. Samples were collected from only 4 sea stars at each sample acquisition time. Notice that the model in panel B fit the data better than did the spaghetti plots in panel A.

Discussion

Results of the present study indicated that purple sea stars administered an intracoelomic injection of enrofloxacin (5 mg/kg) or immersed in an enrofloxacin solution (5 mg/L) for 6 hours developed WVSF enrofloxacin concentrations that exceeded the therapeutic target enrofloxacin concentration for vertebrates. This study also described a method for obtaining reliable population pharmacokinetic estimates in the presence of sampling limitations. To our knowledge, the present study was the first to estimate the pharmacokinetic parameters for an antimicrobial in an invertebrate species by use a population pharmacokinetic (NLME) approach, which included fixed effects for pharmacokinetic parameters and a random effect to control for interindividual

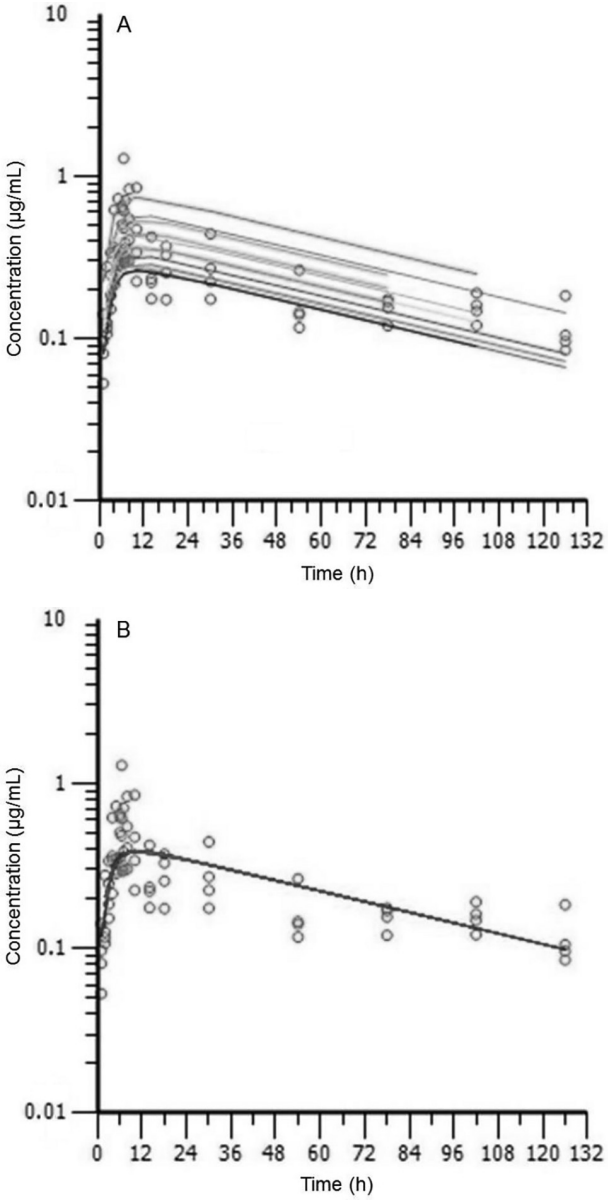

Figure 3—Enrofloxacin concentrations over time in WVSF samples collected from 12 purple sea stars during and after immersion in an enrofloxacin solution (5 mg/L) for 6 hours. A—Spaghetti plots of enrofloxacin concentrations for individual sea stars fitted by a population pharmacokinetic model. B—Graphic presentation of the results for a fitted 1-compartment model with first-order adsorption in which interindividual variability was controlled. Sea stars were placed in the enrofloxacin solution at 0 hours. **See** Figure 2 for remainder of key.

variation. Compared with more traditional STS methods, the NLME approach is preferred when circumstances restrict the number of samples that can be collected. The STS method, which involves intensive sampling of each study animal, is acceptable for studies involving domestic species, but is less practical for species that are small or difficult to sample, such as invertebrates, exotics, and zoo animals. By combining concentration-time data derived from a small number of samples collected from many individuals, the stress and burden (number and volume of samples collected) of sample collection on each individual are reduced. The small size of sea stars of the present study precluded the collection of WVSF samples from each individual at each predetermined sample acquisition time; thus, a sparse sampling strategy was implemented so only 4 sea stars were sampled at each sample acquisition time (ie, only 4 to 6 samples were collected from each sea star).

The NLME approach allowed us to subdivide the study population for each phase into sampling groups. Water vascular system fluid samples were collected from all sea stars within each sampling group at various times during the observation period. Mixed-effects models can evaluate data sets for populations that underwent a sparse sampling strategy because it treats within- and between-subject variability separately. A population approach such as NLME represents an extension of traditional individual-subject models in that it accounts for the magnitude, and sometimes the sources, of interindividual variability in the model parameters. Consequently, it allows investigators to derive useful pharmacokinetic data from study populations that require the implementation of a sparse sampling strategy, which precludes traditional STS analysis.

Although the impetus for conducting the present study was sea star wasting disease, the study was not designed to investigate the treatment or prevention of that disease. Instead, the objective of this study was to determine the pharmacokinetics of enrofloxacin for sea stars in the hope that the data would provide justification or guidance for anecdotal treatment regimens. Fluoroquinolones, particularly enrofloxacin, are commonly used for the treatment of aquatic species because of their primarily aerobic gram-negative spectrum of activity and efficacy against many aquatic bacterial organisms.[6] Fluoroquinolones are typically well absorbed and have a large volume of distribution in most animals.[5]

The results of the present study should be interpreted cautiously because they were generated by use of a pharmacokinetic model for enrofloxacin instead of being derived from empirical measurements of enrofloxacin concentration in sea stars. Consequently, parameters such as the absorption half-life and t_{max} were predicted values and not measured directly. The primary purpose of this study was to determine the pharmacokinetics of enrofloxacin in sea stars so that current dosing regimens could be evaluated. Evaluation of dosing regimens relies more on the C_{max}, elimination half-life, and AUC than on absorption parameters. To obtain empirical measurements for enrofloxacin absorption, WVSF samples would need to be collected from the sea stars more frequently during the period immediately after enrofloxacin administration than was done in the present study.

The short estimated absorption half-life (0.01 hours) and t_{max} (0.12 hours) of enrofloxacin following

intracoelomic injection suggested that the drug was rapidly absorbed in purple sea stars. Rapid absorption of a drug is indicative of extravascular administration. In sea stars, although the coelomic fluid, WVSF, and hemal fluid are suspected to interact at the primitive heart-like structure, the body cavities are believed to be distinct from one another. The supposition that sea stars have distinct body cavities was supported by the fact that the injection phase data best fit a 2-compartment model. As expected, the estimated absorption half-life (2.23 hours) and t_{max} (11 hours) of enrofloxacin during the immersion phase were longer than those following intracoelomic injection. The mean enrofloxacin concentration in the WVSF samples continued to increase during the 6-hour immersion period and began to decrease as soon as the sea stars were removed from the enrofloxacin solution (Figure 1). Additionally, the enrofloxacin concentrations varied substantially among the sea stars throughout the immersion period (Figure 3). It is possible that immersion of the sea stars in the enrofloxacin solution for a longer duration would have resulted in higher drug concentrations in the WVSF samples.

The pharmacokinetic parameters of enrofloxacin in the purple sea stars of the present study were similar to those determined for other invertebrate species,[8,13] which we attributed, at least in part, to the fact that most invertebrates have open circulatory systems rather than closed circulatory systems typical of vertebrates. Although the sea stars absorbed enrofloxacin during the immersion phase, the extent of absorption and C_{max} varied substantially among individual animals. It was difficult to draw conclusions regarding the relative extent of absorption from the immersion solution because the exact dose of enrofloxacin administered to each sea star could not be calculated. We only knew that the enrofloxacin solution had an initial drug concentration of 5 mg/L. In sea stars, the mode of absorption for a drug administered by immersion is unknown, and possibilities include ingestion, diffusion across the skin, or direct flow into the water vascular system through the madreporite, all of which can influence the drug's pharmacokinetics. Likewise, the true bioavailability, or fraction of drug absorbed following extravascular injection, in sea stars is unknown. On the basis of the rapid absorption and short t_{max} determined for enrofloxacin during the injection phase of the present study, we assumed that its bioavailability was equal to 1. However, that assumption might not be correct, and parameters dependent on bioavailability (eg, VD_{ss}/f and CL/f) should be interpreted cautiously.

The V_{SS}/f for enrofloxacin following intracoelomic injection in the present study was 0.71 L/kg, which was less than that reported for vertebrate species, giant freshwater prawns (*Macrobrachium rosenbergii*; 3.4 L/kg),[12] and mud crabs (*Scylla serrata*; 1.6 or 1.1 L/kg, depending on water temperature)[8] but greater than that reported for European cuttlefish (*Sepia officinalis*; 0.385 L/kg).[9] Discrepancies in the pharmacokinetic parameters of enrofloxacin among invertebrate species might be caused by differences in study techniques and the method (eg, naïve pooled sample analysis and noncompartmental and NLME modeling) used for pharmacokinetic analysis. Additional studies to evaluate whether drug concentrations in the WVSF of invertebrates are correlated with those in tissue are warranted and might elucidate the differences in V_{ss}/f observed among studies.

Use of enrofloxacin in aquatic environments is challenging because water temperature and salinity can affect drug absorption and clearance; additionally, excretion of active drug metabolites into the water by treated animals can result in drug reabsorption.[7,8,17,18] For example, alkaline environments (pH > 7.4) increase fluoroquinolone activity against gram-negative bacteria but may decrease the overall bioavailability of the drug,[5,19] and water temperature was negatively associated with the $t_{1/2}$ of enrofloxacin in Manila clams.[7] Water-quality parameters in the present study were kept constant to eliminate the need to adjust or control for environmental factors. Nevertheless, the cold coastal waters of British Columbia, Canada, resulted in the 2 phases of the present study being conducted at lower water temperatures than those used in previous aquatic invertebrate studies. The mean elimination half-life of enrofloxacin after intracoelomic injection (42.8 hours) in the purple sea stars of the present study was consistent with the elimination half-life of enrofloxacin in giant freshwater prawns following oral administration (39.33 hours),[12] but substantially longer than the elimination half-life of enrofloxacin in European cuttlefish (1.81 hours),[9] Manila clams,[7] ridgetail white prawn (*Exopalaemon carinicauda*),[10] and green sea urchins (*Strongylocentrotus droebachiensis*).[20] The mean elimination half-life of enrofloxacin in purple sea stars following immersion (56 hours) was longer than that following intracoelomic injection and the elimination half-life for enrofloxacin in Chinese mitten-handed crabs (*Eriocheir sinensis*)[13] and mud clams following oral administration of 30 mg of enrofloxacin/kg.[8] The variability in the elimination half-life of enrofloxacin in various species indicates that species-specific pharmacokinetic data are needed to guide enrofloxacin dosing in aquatic invertebrates.

Enrofloxacin is converted to ciprofloxacin, an active bactericidal metabolite, in most mammalian species, but that conversion appears to be species dependent in nonmammal species. Teleosts such as red pacus (*Colossoma brachypomum*),[21] European seabasses (*Dicentrarchus labrax*),[19] turbots (*Scophthalmus maximus*),[18] and Nile tilapia (*Oreochromis niloticus*)[22] convert enrofloxacin into ciprofloxacin, albeit at low levels, whereas seabreams (*Sparus aurata*)[23] do not. Similarly, invertebrates such as Chinese mitten-handed crabs,[13] giant freshwater prawns,[12] ridgetail white prawns,[10] and Chinese shrimp (*Penaeus chinensis*)[22] convert enrofloxacin to ciprofloxacin, whereas European cuttlefish,[9] Ma-

nila clams,[7] and green sea urchins[20] do not. Results of another study[24] suggest that some plants are capable of metabolizing enrofloxacin into ciprofloxacin; however, no plants were housed with the sea stars of the present study and thus were ruled out as a source of the detected ciprofloxacin. Consequently, we concluded that purple sea stars were able to convert enrofloxacin to ciprofloxacin, albeit at low and clinically irrelevant levels.

The primary bacterial pathogens of echinoderms have not been identified. Consequently, the present study did not evaluate the efficacy of enrofloxacin for the treatment of bacterial infections in sea stars. However, preliminary predictions for the treatment efficacy of enrofloxacin in sea stars can be estimated on the basis of data obtained from this study and data reported for teleost pathogens in another study.[25] In the present study, the AUC for enrofloxacin in sea stars was 353.9 h•µg/mL following intracoelomic injection and 36.3 h•µg/mL following immersion. In mammalian species, an AUC/MIC ratio > 100 for a fluoroquinolone is considered sufficient for antimicrobial efficacy and is one of the best indicators for accurate prediction of treatment outcome.[26,27] The optimum AUC/MIC ratio for fluoroquinolones has not been determined for marine invertebrates. In other animals, the MIC breakpoint for bacteria susceptible to enrofloxacin is < 0.5 µg/mL[28] For the sea stars of the present study that were administered enrofloxacin by intracoelomic injection (5 mg/kg) of immersion in a solution with 5 mg or enrofloxacin/L, an AUC/MIC ratio > 100 would be achieved for bacterial isolates with MIC values < 3.5 and < 0.36 µg/mL, respectively. Many bacteria from a wild-type population distribution have an MIC < 0.36 µg/mL for enrofloxacin. When the MICs for bacterial pathogens of teleosts were assessed, it appeared that an intracoelomic injection of enrofloxacin (5 mg/kg) or immersion in an enrofloxacin solution (5 mg/L) for 6 hours would be efficacious against 22 and 18 of those pathogens, respectively, and administration of enrofloxacin by either route would be efficacious against *Vibrio* spp, *Aeromonas* spp, and *Pseudomonas* spp.[25]

In the present study, a unique sparse sampling technique and NLME population model were used to obtain reliable estimates for pharmacokinetic parameters in a small invertebrate marine species. 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.

Purple sea stars are fairly resistant to desiccation and can tolerate fluid losses up to 30% of their body weight.[29] The total volume of the WVSF samples collected from the smallest sea star of the present study was equal to only 2.4% of its body weight. The fact that none of the sea stars developed adverse effects associated with the sampling protocol used in this study suggested that sea stars may be a viable species for further research.

The enrofloxacin concentrations in the WVSF samples steadily increased while the sea stars were immersed in the enrofloxacin solution. Although the mean absorption half-life was fairly long (2.23 hours), the enrofloxacin concentration varied substantially among individual sea stars during the 12 hours immediately after the animals were placed in the enrofloxacin solution. Future studies should focus on the immersion of sea stars in an enrofloxacin solution for a longer duration or with a higher concentration than that used in the present study to ascertain whether higher enrofloxacin concentrations can be attained in WVSF samples. We suspect that enrofloxacin concentrations in WVSF samples will equilibrate with those in the water surrounding the sea stars over time, which may be beneficial for future treatments but might not be clinically relevant given the high concentration of enrofloxacin achieved in the WVSF samples following intracoelomic injection.

In the present study, sea stars administered an intracoelomic injection of enrofloxacin (5 mg/kg) or immersed in an enrofloxacin solution (5 mg/L) attained WVSF drug concentrations sufficient to effectively treat multiple aquatic bacterial pathogens. The long predicted mean elimination half-life of enrofloxacin following intracoelomic injection (42.8 hours) and immersion (56 hours) suggested that the antimicrobial effects of the drug in treated sea stars will persist for an extended period of time. On the basis of these data, we estimate that it should not be necessary to administer enrofloxacin (5 mg/kg) by intracoelomic injection to sea stars more frequently than once every 5 days. Also, immersion of sea stars in a solution with a concentration of 5 mg of enrofloxacin/L on a daily basis may result in cumulative drug concentrations in WVSF that are higher than those attained after a single treatment. Additional research is warranted to isolate bacterial pathogens from sea stars and fully evaluate enrofloxacin dosing recommendations.

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Footnotes

a. Baytril (50 mg/mL), Bayer Inc, Toronto, ON, Canada.
b. Professional Compounding Centers of America, London, ON, Canada.
c. 1200 Series solvent delivery system, Agilent Technologies, Wilmington, Del.
d. 1200 Series variable wavelength detector, Agilent Technologies, Wilmington, Del.
e. OpenLAB software, Agilent Technologies, Wilmington, Del.
f. Eclipse XDB-C8 column (4.6 X 150 mm; 5 µm), Agilent Technologies, Wilmington, Del.
g. Ciprofloxacin analytic reference standard, United States Pharmacopeial Convention (USP), Rockville, Md.
h. Bayer Animal Health, Shawnee Mission, Kan.
i. Phoenix WinNolin, Pharsight Corp, Certara, St Louis, Mo.
j. Phoenix NLME, Pharsight Corp, Certara, St Louis, Mo.

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