POPULATION PHARMACOKINETICS OF ENROFLOXACIN AND ITS METABOLITE CIPROFLOXACIN IN THE GREEN SEA URCHIN (*STRONGYLOCENTROTUS DROEBACHIENSIS*) FOLLOWING INTRACOELOMIC AND IMMERSION ADMINISTRATION

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Source: Journal of Zoo and Wildlife Medicine, 47(1):175-186.
Published By: American Association of Zoo Veterinarians
DOI: http://dx.doi.org/10.1638/2015-0110.1
URL: http://www.bioone.org/doi/full/10.1638/2015-0110.1

Journal of Zoo and Wildlife Medicine 47(1): 175–186, 2016

POPULATION PHARMACOKINETICS OF ENROFLOXACIN AND ITS METABOLITE CIPROFLOXACIN IN THE GREEN SEA URCHIN (*STRONGYLOCENTROTUS DROEBACHIENSIS*) FOLLOWING INTRACOELOMIC AND IMMERSION ADMINISTRATION

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Abstract: Sea urchin mass mortality events have been attributed to both infectious and noninfectious etiologies. Bacteria, including *Vibrio* spp. and *Pseudoalteromonas* spp., have been isolated during specific mortality events. Aquarium collection sea urchins are also subject to bacterial infections and could benefit from antimicrobial treatment, but pharmacokinetic studies have been lacking for this invertebrate group until recently. This study evaluated the pharmacokinetics of enrofloxacin and its active metabolite ciprofloxacin in the green sea urchin (*Strongylocentrotus droebachiensis*) after intracoelomic injection and medicated bath immersion administration. The utility of a population pharmacokinetic method using nonlinear mixed effects modeling (NLME) was also evaluated. Thirty sea urchins were assigned to either the injection or immersion group. Twelve study animals and three untreated controls were utilized for each administration method: enrofloxacin 10 mg/kg intracoelomic injection or a 6-hr enrofloxacin 10 mg/L immersion. Each animal was sampled four times from 0 to 120 hr. Water samples were collected during immersion treatment and posttreatment time points in both groups. Hemolymph and water sample drug concentrations were analyzed using high-performance liquid chromatography, and pharmacokinetic parameters were determined using an NLME population pharmacokinetic method. Enrofloxacin concentrations were fit to a two-compartment model with first-order input for the intracoelomic injection group. The enrofloxacin elimination half-life ($t½$), peak hemolymph concentration (C_{MAX}), and area under the curve (AUC) were 38.82 hr, 90.92 μg/ml, and 1,199 hr·μg/ml, respectively. Enrofloxacin was modeled to a one-compartment model with first-order input for the immersion treatment. The enrofloxacin $t½$, C_{MAX}, and AUC were 33.46 hr, 0.48 μg/ml, and 32.88 hr·μg/ml, respectively. Ciprofloxacin was detected in trace concentrations in all hemolymph samples, indicating minimal production of this metabolite. The concentrations of enrofloxacin achieved far exceeded minimum inhibitory concentrations reported for teleost pathogens. No adverse effects were associated with enrofloxacin administration by either treatment method or from hemolymph sampling.

Key words: Ciprofloxacin, echinoderms, enrofloxacin, green sea urchin, pharmacokinetics, *Strongylocentrotus droebachiensis.*

INTRODUCTION

Sea urchins are found in a wide range of climates and play an important role in marine ecosystems. These echinoderms feed mostly on algae and provide a food source for predator species including sea stars, teleosts, and sea otters. Fluctuations in sea urchin populations can have a major effect on the marine ecosystem. Marked population increases in *Strongylocentrotus franciscanus* and *Strongylocentrotus purpuratus* in the waters of Alaska, California, and British Columbia associated with sea otter population declines resulted in destructive grazing of regional kelp forests.[6,25] In contrast, epizootic events of sea urchins cause ecosystem shifts that have resulted in algae overgrowth.[6]

Disease outbreak and mass mortality events of sea urchins have been attributed to a variety of causes. The black sea urchin plague, or the 1980's

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mass mortality event of black sea urchins (*Diadema antillarum*) in the Caribbean, did not have a specific etiologic agent identified.[8] A recurring epizootic event on the Atlantic coast of Nova Scotia affecting green sea urchins (*Strongylocentrotus droebachiensis*) is caused by the parasite *Paramoeba invadens*.[5] Other mortality events have yielded cultures of bacterial species ubiquitous in the marine environment. These include isolation of various *Vibrio* spp., *Pseudoalteromonas tertradonis*, and *Shewanella aquimarina* from *Strongylocentrotus intermedius* mortality in North China.[24] An investigation of *Diadema africanum* mass mortality yielded isolates of *Vibrio alginolyticus*.[4] Other sea urchin epizootic events were associated with environmental changes, harmful algal blooms, and weather events.[6]

A current echinoderm mass mortality event affecting multiple sea star species on the Pacific coast of the United States and Canada is called sea star wasting disease.[9] Clinical signs include progressive epidermal erosions, limb dissolution, and acute death. Although associated with a densovirus, a secondary bacterial infection may be involved in sea star wasting disease. Anecdotal reports indicate that the fluoroquinolone antimicrobial enrofloxacin administered to sea stars and sea urchins with erosive lesions either slowed lesion progression or resolved clinical signs (M. Haulena, pers. obs.). This suggests that enrofloxacin may be an effective antimicrobial agent in echinoderms in some situations, although no clear beneficial effect was observed for sea stars with wasting syndrome treated by enrofloxacin intracoelomically at 5 mg/kg every 4 days.[23]

Fluoroquinolones are synthetic antimicrobials used in veterinary and human medicine for their broad spectrum of activity and favorable chemical properties, including solubility, lipophilicity, and stability in a range of environments and vehicles. This class of antimicrobials is bactericidal and active against both gram positive and gram negative bacteria.[17] Enrofloxacin is approved for use in some domestic mammals but also is widely used in nondomestic species.[17,19] Several studies have evaluated enrofloxacin pharmacokinetics and have shown it to be highly variable in teleost and aquatic invertebrate species.[3,7,14,16,20] Enrofloxacin is metabolized in most animals, including some teleost and aquatic invertebrates, to the active metabolite ciprofloxacin.[12,26] Ciprofloxacin is more water soluble and less lipophilic but more active against many bacteria, particularly *Pseudomonas aeruginosa* and related aquatic gram negative nonfermenters. The degree of enrofloxacin metabolism in teleost and invertebrate species can be varied by administration route and aquatic environmental parameters.[7,15,20]

The anatomy of echinoderms poses certain challenges to pharmacokinetic studies. The external surface of the sea urchin is composed of fused ossicles, creating the test, and is lined by epidermal tissue and spines that cover the majority of the test surface.[8] Drug administration routes are limited to intracoelomic injection, medicated feeds, or immersion in a medicated bath. Similar to other invertebrates, the circulatory fluid of sea urchins is hemolymph, a mixture of interstitial fluid and hemocytes. Hemolymph has reportedly been collected from sea urchin species, and although not detailed, access through the peristomial membrane has been generally described.[8,13] Hemolymph has been used to study pharmacokinetics in other aquatic invertebrates.[3,7,18,26]

This study investigated the pharmacokinetics of enrofloxacin administered to green sea urchins after intracoelomic injection and immersion techniques. It was hypothesized that enrofloxacin administered intracoelomically would produce a higher peak concentration (C_{MAX}) and higher exposure (AUC, or area under the curve) than a medicated bath immersion. Both treatments were evaluated for their ability to produce sufficient exposure (AUC), critical for the antibacterial efficacy of fluoroquinolones, as well as the extent of enrofloxacin metabolism to ciprofloxacin.[19] Additionally, the study evaluated the utility of a population pharmacokinetic method using nonlinear mixed effects modeling (NLME), which was recently used in a study of enrofloxacin pharmacokinetics in purple sea stars (*Pisaster ochraceus*).[20] The NLME approach allows for an estimate of theta (θ), the typical value for a population pharmacokinetic parameter, and eta (η), the interindividual (between-subject) variation in the population. The mixed effects modeling approach allows for an examination of the source of variability in the population (covariates). Because sample volumes must be limited in small invertebrates such as sea urchins, this analysis allows for a sparse sampling technique to yield robust pharmacokinetic values.

MATERIALS AND METHODS

Animals and housing

A total of 30 green sea urchins was selected from the Vancouver Aquarium's education wet lab population. These animals were in the collection for at least 6 mo and were previously

collected locally in Vancouver, British Columbia, Canada, in accordance with a license granted by the Department of Fisheries and Oceans Canada. This project was approved by the Vancouver Aquarium's Animal Care Committee. Urchins were housed in 100-L flow-through, nonrecirculating tanks. They were fed 3 days/wk and diet consisted of leafy green vegetables, squid (*Loligo opalescens*), and krill (*Euphausia pacifica*).

Physical examinations and body weights were obtained approximately 24-48 hr before the study period. Body weight ranged from 77 to 161 g, with a median body weight of 98 g. Animals were selected for inclusion in the study based on displaying adequate appetite and lack of clinical signs, such as epidermal erosions or ulcerations, drooping or shed spines, and decreased feeding response. Each animal was assigned a study identification number, individually marked, and transferred to a labeled basket in 190-L flow-through, nonrecirculating tanks. Water quality parameters during the study period ranged as follows; pH 7.67–7.68, nitrate 6.0–7.0 mg/L, salinity 28.2–28.8 ppt, and temperature 10.4–11.5°C.

Twelve animals were arbitrarily assigned to the intracoelomic group and 12 to the immersion dose group. Six additional animals, three per dose group, were used as untreated controls that did not receive enrofloxacin. Enrofloxacin 50 mg/ml injectable solution (Baytril®, Bayer Inc., Toronto, Ontario M9W 1G6, Canada) was used in this study. During the study, all animals were monitored for any adverse effects daily, which included droopy or loss of spines, loss of feeding response, discoloration or erosion of the peristomial membrane, or death.

Intracoelomic administration

Twelve green sea urchins were administered enrofloxacin 10 mg/kg intracoelomically using a 100-unit insulin syringe with a 28-ga, 1.3-cm straight needle (Terumo Medical Products, Somerset, New Jersey 08873, USA). Because of the small dose volume required, enrofloxacin was diluted to a 5 mg/ml injectable solution using sterile saline approximately 30 min before administration. Each animal was manually restrained in aboral recumbency for injection, and the enrofloxacin dose was administered by inserting the needle perpendicularly through the peristomial membrane lateral to the mouth at the centermost edge of the test. After enrofloxacin administration, animals were returned to designated plastic baskets attached to the tank wall based on time group. The three untreated control animals were placed in labeled baskets in the same system. Hemolymph was collected using an insulin syringe as described above through the peristomial membrane on the opposite side of the mouth from where the injection was administered. Each animal was assigned to one of three hemolymph collection time series. A sparse sampling strategy was used so that four treated and one untreated control animal was in each time series group: 0 (before enrofloxacin administration), 2, 12, and 72 hr; 0.5, 3, 24, and 96 hr; and 1, 6, 48, and 120 hr. Tank water samples (0.3 ml) were obtained at 0, 24, 48, and 120 hr. A total volume of 1.2 ml of the hemolymph was sampled from each animal, corresponding to 1.2% of median body weight.

Immersion administration

Twelve green sea urchins were exposed to a medicated water bath containing enrofloxacin at a concentration of 10 mg/L via immersion for 6 hr. Solubility of enrofloxacin in fresh water ranges from 1,969 mg/L to 3,397 mg/L (www.chemspider.com). Saltwater may affect enrofloxacin solubility because of higher pH, salinity, and hardness, and the drug may adsorb to surfaces or undergo microbial degradation; therefore, treatment water samples were obtained throughout the immersion treatment to verify concentration achieved. Immersion trials were conducted in 8-L buckets containing 4 L each of water from the exhibit. An air stone was placed in each bucket, and the drug was added to each bath system. Two study animals were placed into each medicated bath system and remained there for 6 hr. Animals were removed from the treatment buckets at the completion of immersion and returned to their labeled basket housing. The three untreated control animals had been placed into these housing baskets at the start of the immersion treatment. Hemolymph was collected as described above. Each animal was assigned to one of the following hemolymph collection time series so that four treated and one untreated control animal were in each time series group: 0 (before enrofloxacin immersion), 2, 12, and 72 hr; 0.5, 3, 24, and 96 hr; and 1, 6, 48, and 120 hr. During the enrofloxacin immersion, water samples (0.3 ml) were collected at 0, 0.5, 1, 2, 3, and 6 hr so that one water sample was obtained from each bath during the immersion period (sample points shown in Fig. 1). After bath administration, tank water samples (0.3 ml) were obtained at 0, 24, 48, and 120 hr. A total volume of 1.2 ml of hemolymph was sampled from each animal.

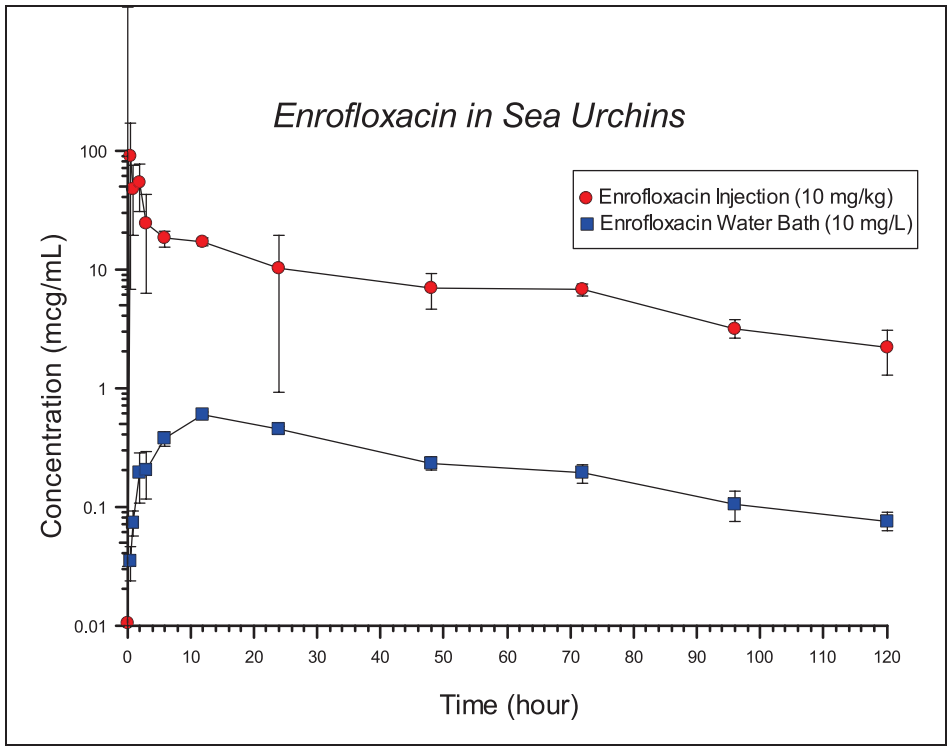

Figure 1. Enrofloxacin concentrations in the green sea urchin (*S. droebachiensis*) after injection of enrofloxacin 10 mg/kg ($n = 12$) or 6-hr immersion in medicated water bath of 10 mg/L ($n = 12$). Solid points represent mean values ± SD (four points per sample time). Ciprofloxacin was detected in samples at low concentrations but not shown.

Sample preparation

Hemolymph samples were placed into individually labeled 1.5-ml microcentrifuge tubes (Jorgensen Laboratories Inc., Loveland, Colorado 80538, USA). Water samples were placed directly into cryogenic vials (Thermo Fisher Scientific, Rochester, New York 14625, USA) and labeled with dose group (injection vs. immersion), time of collection, and date. Hemolymph samples were centrifuged at 11,000 *g* for 8 min to exclude a small hemocyte pellet, and the supernatant was transferred using a glass pipette (Canadawide Scientific, Ottawa, Ontario K1G 6B1, Canada) into a cryogenic vial labeled with the animal identification, date, dose route, and time of collection. All samples were transferred to a −70°C freezer within 30 min of initial collection. Samples remained in this freezer until they were shipped to the North Carolina State University College of Veterinary Medicine Clinical Pharmacology Laboratory (Raleigh, North Carolina) for drug analysis and pharmacokinetic evaluation.

Enrofloxacin and ciprofloxacin analysis

High-performance liquid chromatography (HPLC) was used to determine the concentrations of enrofloxacin and its active metabolite in the hemolymph and water samples. Hemolymph samples collected from untreated green sea urchins were used as blank samples before study sample analysis to evaluate for potential interference. All hemolymph and water samples from the study were evaluated separately.

The HPLC system consisted of a quaternary solvent delivery system (flow rate, 1 ml/min), an autosampler (Agilent 1200 Series solvent delivery system, Agilent Technologies, Wilmington, Delaware 19808, USA), and an ultraviolet detector (Agilent 1200 Series Variable Wavelength Detector, Agilent Technologies, Wilmington, Delaware 19808, USA) set at a wavelength of 279 nm. Chromatograms were integrated with a computer program (Agilent OpenLAB software, Agilent Technologies). The Eclipse XDB-C8 analytic column (Agilent Technologies) was a reversed-phase C8 column 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 (ciprofloxacin analytical reference standard, U.S. Pharmacopeial Convention (USP), Rockville, Maryland 20851, USA) was used to prepare a stock solution to fortify a blank sample matrix. The enrofloxacin reference standard was supplied by the manufacturer (Bayer Animal Health, Shawnee Mission, Kansas 66216, USA). Stock solutions were sealed and stored in the dark at 4°C. Blank samples from untreated green sea urchins were fortified (spiked) with a range of concentrations of enrofloxacin and ciprofloxacin and compared with blank phosphate-buffered saline (PBS) samples. The results showed almost 100% agreement between the measurements at each concentration studied. Therefore, the calibration curve samples and quality control samples were prepared using PBS as the matrix. Calibration curves were constructed by fortifying the matrix with ciprofloxacin at seven concentrations ranging from 0.01 to 4 μg/ml. The calibration curve for enrofloxacin consisted of nine concentrations ranging from 0.05 to 40 μg/ml. A blank (zero concentration) sample also was added to check for presence of interfering peaks. The calibration curve was accepted if the linear coefficient of determination (R^2) was $\geq$0.99 and if the calibration curve concentrations could be back-calculated to $\leq$15% of the true concentration of the standard. Fresh calibration curves were prepared for each day's analysis.

All hemolymph, calibration, quality control, and blank hemolymph samples were prepared in an identical manner. After centrifugation to remove any particulate matter or cells, the samples were transferred to a HPLC injection vial. Thirty microliters of each sample was used for injection into the HPLC system.

Retention time for ciprofloxacin and enrofloxacin was 2.6–2.8 and 3.1–3.3 min, respectively. Limit of quantification was 0.01 μg/ml and 0.05 μg/ml for ciprofloxacin and enrofloxacin, respectively, which was determined from the lowest point on a linear calibration curve that yielded an acceptable accuracy and within accepted guidelines for signal-to-noise ratio.[10,22]

Pharmacokinetic analysis

The hemolymph concentrations were analyzed using standard pharmacokinetic techniques. A pharmacokinetic computer program (Phoenix® NLME™, Pharsight Corporation, Certara, St. Louis, Missouri 63101, USA) was used to calculate the pharmacokinetic parameters. Hemolymph concentrations of enrofloxacin and ciprofloxacin were plotted on a semilogarithmic graph to assess potential pharmacokinetic models for analysis (Fig. 1).

Initial estimates of parameters were obtained using naïve averaged samples (pooled samples) in which a pharmacokinetic model was fit to the average concentration at each time point. This model determines the best initial estimate for primary pharmacokinetic parameters to be used for the population pharmacokinetic method. Small invertebrates such as these sea urchins cannot be sampled as frequently as large vertebrate animals in which traditional standard two-stage (STS) pharmacokinetic methods are used. The sparse sampling strategy was used so that each animal in a group was sampled four times to cover a wide range of time points from 0 to 120 hr. Population pharmacokinetics (Pop-PK) was performed by fitting the concentrations to a model using the Phoenix NLME software. This analysis allowed for a population-based approach in which the primary pharmacokinetic parameters for the population were considered fixed effects and the interindividual (between-subject) variability was modeled as a random effect. Remaining differences between predicted concentrations and measurements were accounted for by residual errors (within-subject variation). The NLME approach also allowed for an analysis of other factors (covariates) in the study that may account for variation in the parameters.

The models were parameterized with compartmental analysis of the data from intracoelomic injection of 10 mg/kg using a two-compartment with first-order absorption from the injection and the formula (Model 13 in Phoenix)

$$C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} + C \cdot e^{-\mathrm{K01} \cdot t}, \qquad (1)$$

where C is the hemolymph 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 phase rate constant (terminal phase), C is $-(A + B)$, and K01 is the absorption rate from the injection. Secondary parameters calculated include distribution (α) and elimination (β) half-lives ($t½$), microdistribution rate constants, AUC, volume of distribution at steady state (Vss), clearance

(CL), C_{MAX}, and time to peak concentration (T_{MAX}).

Compartmental analysis of the data from the 10 mg/L immersion administration was calculated using a one-compartment model with first-order absorption according to the formula (Model 3 in Phoenix)

$$C = (D \cdot \mathrm{K01})/(V \cdot [\mathrm{K01} - \mathrm{K10}] \cdot (e^{-\mathrm{K10} \cdot t} - e^{-\mathrm{K01} \cdot t}), \quad (2)$$

where C is the hemolymph concentration; t is time; K01 is the absorption rate, assuming first-order absorption; K10 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, and K01 and K10 corresponding half-lives ($t½$). The models were run with the first-order conditional estimation–Lindstrom Bates (FOCE LB) engine. Model selection was based on goodness-of-fit plots, statistical significance between models using improvements in –2LL (twice the negative log likelihood), Akaike information Criterion (AIC, a goodness-of-fit measure based on the log likelihood adjusted for the number of parameters [degrees of freedom] in the fit) obtained in Phoenix, and the coefficient of variation 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

$$P_i = \theta_P \cdot \exp(\eta_{iP}), \quad (3)$$

where P_i is the pharmacokinetic parameter of interest for individual i, θ_P is θ or the typical value (fixed effect) for the population estimate of the parameter of interest, and η_{iP} is the η (random effect) for the interindividual (between-subject) differences of the parameter of interest. The η values were assumed to be independent and have a log 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

$$C_{obs} = C_{pred}\, x(1 + \varepsilon), \quad (4)$$

where C_{obs} is the observed concentration for the individual and C_{pred} is the model predicted concentration plus the error value (ε).

Once the final model was obtained for the population, an examination of covariates was performed to determine whether there were factors that may explain the variability in the primary parameters listed in Equations 1 and 2. The covariate examined to explore the possibility that the sampling group could be a source of error was the group (housing basket) in which they were kept. Box plots were constructed to examine the change in η (between-subject variability) for each parameter by group. The effect of grouping was tested in a simple stepwise approach with forward inclusion and backward elimination. The effects of the covariate on a parameter was evaluated based on changes in the –2LL (equivalent to the objective function value [OFV] in NONMEM), and results were considered statistically significant if the decrease was significant with $P < 0.01$.

For this class of drugs, the C_{MAX} or AUC, compared with the minimum inhibitory concentration (MIC) of the organism, is the most favorable predictor of antibacterial success. MIC values for sea urchin bacterial pathogens have not been reported. We compared enrofloxacin C_{MAX} and AUC in the green sea urchin to published enrofloxacin MIC of teleost pathogens, including *Vibrio* spp. and *Pseudomonas* spp.[1,11]

RESULTS

All animals, including untreated controls, survived the study. No adverse effects were observed after administration of enrofloxacin or hemolymph sampling. Two untreated control animals developed droopy spines during the study period but maintained an adequate appetite and activity level.

The hemolymph profiles for enrofloxacin administered via intracoelomic injection and medicated immersion are shown in Figure 1. Pharmacokinetic results for the initial naïve pooled analysis are presented in Table 1. The pharmacokinetic results for the NLME population pharmacokinetic analysis are shown in Tables 2 and 3 for the injection and immersion treatments, respectively. The fitted curves using NLME population analysis are shown in Figures 2 and 3 for injection and bath immersion, respectively.

Ciprofloxacin, the active metabolite, was detected above the level of quantification after both injection and immersion treatments (data not shown). In most samples, ciprofloxacin concentrations were <0.1 μg/ml and were inconsistent and low for all samples. Although ciprofloxacin and enrofloxacin can produce additive effects

Table 1. Naïve pooled analysis of pharmacokinetic parameters for enrofloxacin after single-dose intracoelomic injection of 10 mg/kg and 6-hr medicated immersion at 10 mg/L in the green sea urchin (*S. droebachiensis*). A two-compartment model with first-order input and multiplicative error term was used.

Parameter[a]	Value	SE	CV%
Ka (/hr)	27.14	779.22	2871
A (μg/m)	129.08	30.62	23.72
α (/hr)	0.76	0.15	20.2
B (μg/ml)	18.74	2.26	12.06
β (/hr)	0.02	1.69×10^{-3}	9.25
K21 (/hr)	0.11	0.07	61.92
K10 (/hr)	0.12	0.08	62.26
K12 (/hr)	0.54	0.13	23.55
Vss (/kg)	0.40	0.11	93.10
AUC (hr·μg/ml)	1,192.97	173.09	14.51
CL (L/kg/hr)	0.0084	0.0012	14.51
T_{MAX} (hr)	0.14	3.07	2,186.98
C_{MAX} (μg/ml)	131.42	358.16	272.53
Ka *t*½ (hr)	0.03	0.73	2,870.99
α *t*½ (hr)	0.91	0.18	20.28
β *t*½ (hr)	38.06	3.52	9.25

[a] CV% indicates coefficient of variation; Ka, absorption rate; *A*, distribution intercept; α, distribution rate constant; *B*, elimination intercept; β, elimination rate constant; K21, K10, and K12, microdistribution rate constants; Vss, volume of distribution at steady state; AUC, area under the curve; CL, clearance; T_{MAX}, time to peak concentration; C_{MAX}, peak concentration. Ka *t*½, α *t*½, and β *t*½ are the absorption, distribution, and elimination rate half-lives, respectively.

Table 2. Population pharmacokinetic parameters using nonlinear mixed effects modeling of enrofloxacin after single-dose intracoelomic injection of 10 mg/kg in the green sea urchin (*S. droebachiensis*) ($n = 12$).

Parameter[a]	Value	SE	CV%
θ Ka (/hr)	20.45	178.80	874.12
θ *A* (μg/ml)	84.71	24.08	28.43
θ α (/hr)	0.74	0.13	17.78
θ *B* (μg/ml)	19.46	1.59	8.16
θ β (/hr)	0.02	1.12×10^{-3}	6.27
K21 (/hr)	0.16	0.05	32.65
K10 (/hr)	0.08	0.03	31.12
K12 (/hr)	0.52	0.11	21.19
Vss (/kg)	0.45	0.06	43.41
AUC (hr·μg/ml)	1,199.06	87.02	7.26
CL (L/kg/hr)	0.0083	0.0006	7.26
T_{MAX} (hr)	0.18	1.18	660.34
C_{MAX} (μg/ml)	90.92	88.28	97.10
Ka *t*½ (hr)	0.03	0.30	874.13
α *t*½ (hr)	0.94	0.17	17.78
β *t*½ (hr)	38.82	2.43	6.27

[a] CV% indicates coefficient of variation; θ Ka, theta (typical value) for absorption rate; θ *A*, theta for distribution intercept; θ α, theta for distribution rate constant; θ *B*, theta for elimination intercept; θ β, theta for elimination rate constant; K21, K10, and K12, microdistribution rate constants; Vss, volume of distribution at steady state; AUC, area under the curve; CL, clearance; T_{MAX}, time to peak concentration, C_{MAX}, peak concentration. Ka *t*½, α *t*½, and β *t*½ are the absorption, distribution, and elimination rate half-lives, respectively.

against bacteria, ciprofloxacin concentrations were very low, and therefore not included in the figures or the pharmacokinetic analysis.

The elimination *t*½ for enrofloxacin after intracoelomic injection was slightly longer, at 38.82 hr, compared with enrofloxacin immersion terminal *t*½, which was 33.46 hr. Enrofloxacin intracoelomic AUC (zero to infinity) was 1,199 hr·μg/ml, and immersion AUC (zero to infinity) was 32.88 hr·μg/ml, resulting in only 2.7% relative exposure from the bath immersion. The C_{MAX} and T_{MAX} were 90.92 μg/ml and 0.18 hr, respectively, after intracoelomic injection. The enrofloxacin immersion C_{MAX} and T_{MAX} were 0.48 μg/ml and 16.60 hr, respectively. Enrofloxacin clearance following intracoelomic injection was 0.008 L/kg/hr (8.34 mL/kg/hr) and the volume of distribution at steady state (Vss) was 0.45 L/kg. Clearance and volume of distribution were not calculated from the immersion treatment because the exact dose absorbed is not known.

During the 6-hr immersion, enrofloxacin concentrations ranged from 13.32 to 14.31 μg/ml in the water samples. Enrofloxacin was not detected in any of the water samples from the intracoelomic injection or immersion groups at 24, 48, and 120 hr or in the hemolymph samples collected from the untreated control animals throughout the study period.

The AUC of enrofloxacin after intracoelomic administration at 10 mg/kg, 1,199 hr·μg/ml, exceeded the MIC for teleost *Vibrio* spp. (0.08 μg/ml) up to 120 hr. The AUC of enrofloxacin after immersion administration at 10 mg/L (32.88 hr·μg/ml) exceeded the MIC for teleost *Vibrio* spp. up to 66 hr.[1,11] The AUC / MIC ratio was 14,987 for the intracoelomic injection and 411 for the immersion.

Variability among subjects is shown in Figures 2 and 3, as well as in Tables 2 and 3. The change in η (between-subject variability) examined using box plots, did not show any obvious source of variability that could be attributed to the grouping. The covariate effect on the parameter was not considered significant and was not used in the model Thus, the grouping was not considered a source of variation for either drug application.

Table 3. Population pharmacokinetic parameters using nonlinear mixed effects modeling of enrofloxacin after 6-hr medicated bath immersion in enrofloxacin (10 mg/L) in the green sea urchin (*S. droebachiensis*) (n = 12).

Parameter[a]	Value	SE	CV%
θ Ka (/hr)	0.13	0.02	13.66
θ Ke (/hr)	0.02	1.35×10^{-3}	6.53
T_{MAX} (hr)	16.60	1.24	7.49
AUC (hr·μg/ml)	32.88	1.30	3.96
C_{MAX} (μg/ml)	0.48	0.03	5.40
Ka $t½$ (hr)	5.23	0.71	13.66
Ke $t½$ (hr)	33.46	2.19	6.53

[a] CV% indicates coefficient of variation; θ Ka, theta (typical value) for absorption rate; θ Ke, theta for elimination rate constant; AUC, area under the curve; T_{MAX}, time to peak concentration; C_{MAX}, peak concentration. Ka $t½$ and Ke $t½$ are the absorption and elimination rate half-lives, respectively.

DISCUSSION

The pharmacokinetics of enrofloxacin and its active metabolite ciprofloxacin were investigated using two different routes of administration in the green sea urchin. Following either intracoelomic injection or immersion, none of the animals exhibited any adverse effects to enrofloxacin treatment or hemolymph sampling. Additionally, this study successfully used hemolymph obtained from green sea urchins for pharmacokinetic analyses.

The study design and analysis were novel among invertebrate species antimicrobial pharmacokinetic studies, in that a population pharmacokinetic approach (NLME) was used to allow estimation of θ, the typical value for population pharmacokinetic values in the study (fixed effects), and η, the interindividual (between-subject) variation in the population (random effects). A sparse sampling strategy from four animals at each predetermined time point was used (sample times shown in Fig. 1). The challenges of sampling many zoo or exotic species make this an ideal approach to yield valid population pharmacokinetic estimates from these populations when limitations to sampling prevent the traditional STS analysis, wherein single animals can be sampled more frequently.

Following intracoelomic injection with enrofloxacin, the green sea urchin displayed a rapid rise in hemolymph enrofloxacin concentration with an absorption $t½$ of 0.03 hr. The C_{MAX} of 90.92 μg/ml was reached at 0.18 hr (T_{MAX}), likely because of the open circulatory system of the green sea urchin and enrofloxacin diffusion within the hemolymph in the coelomic cavity. Slower absorption occurred after enrofloxacin immersion treatment, with an absorption $t½$ of 5.23 hr and a T_{MAX} of 16.60 hr. The relative exposure of bath immersion compared with injection (determined by AUC ratios) was 2.7%. The route of enroflox-

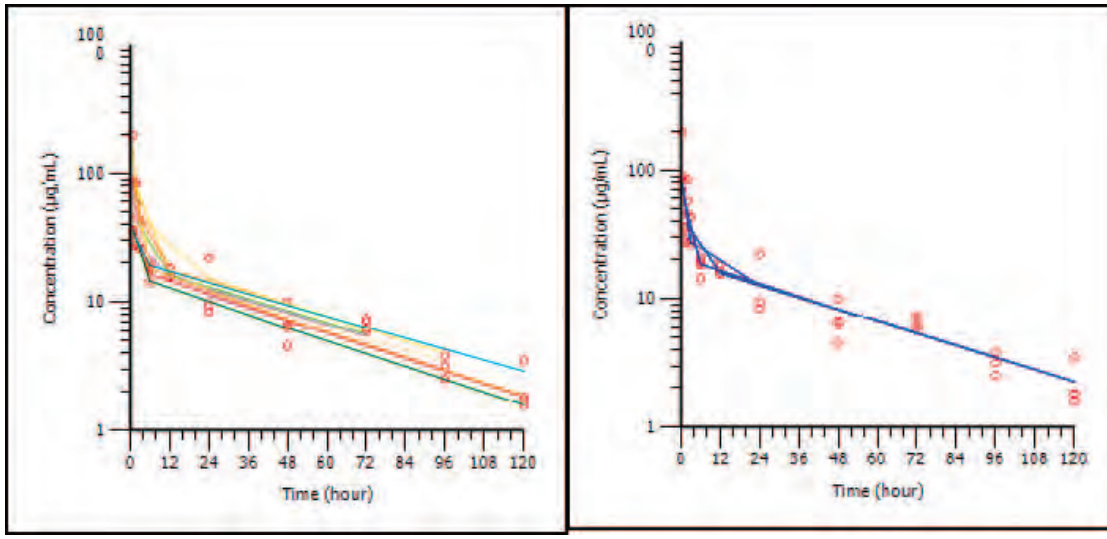

Figure 2. Hemolymph concentration (μg/ml) of enrofloxacin 10 mg/kg administered intracoelomically in the green sea urchin (*S. droebachiensis*). Open circles represent observed points. The solid line is a fitted line to the two-compartment model. The left panel is the individual subjects with the model fitted to each animal (spaghetti plots). The right panel is population model fitted to the data to account for interindividual (between-subject) variability. Note the improvement in fit when variability is accounted for in the right panel.

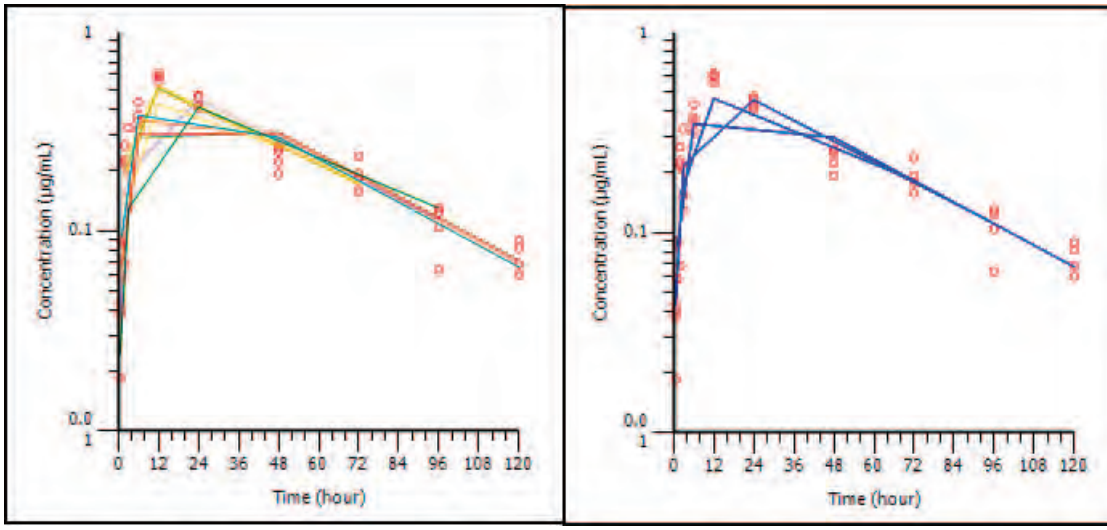

Figure 3. Hemolymph concentration (µg/ml) of enrofloxacin administered via 6-hr immersion at 10 mg/L in the green sea urchin (*S. droebachiensis*). Open circles represent observed points. The solid line is a fitted line to a two-compartment model. Left panel is the individual subjects with the model fitted to each animal (spaghetti plots). The right panel is a population model fitted to the data to account for interindividual (between-subject) variability. Note the improvement in fit when variability is accounted for in the right panel.

acin absorption via immersion in the green sea urchin is unknown but may occur via the peristomial gills, ingestion of the medicated water into the gastrointestinal tract, or the water vascular system through the madreporite located on the aboral surface. Similarly, the mechanism and tissue of enrofloxacin excretion is not known. Further research would be required to determine the site of enrofloxacin absorption, metabolism, and clearance in the green sea urchin by measuring tissue concentrations of enrofloxacin after immersion treatment.

A similar rapid absorption occurred after enrofloxacin intracoelomic injection in purple sea stars and intramuscular injection in Chinese mitten-handed crabs (*Eriocheir sinensis*).[20,26] The C_{MAX} occurred closer to the time of enrofloxacin administration in the green sea urchin compared with intramuscular injection, intracoelomic injection, or both of enrofloxacin in the red pacu (*Colossoma brachypomum*) and Atlantic salmon (*Salmo salar*).[14,21] After enrofloxacin immersion, C_{MAX} in the green sea urchin occurred at 16.60 hr, >9 hr after the completion of the immersion treatment. The concentration of enrofloxacin may eventually equilibrate with the concentration of the medicated bath if provided a sufficient time period, which could be explored in the future. Future studies could explore variation in time of immersion and drug concentration in the medicated bath as experimental factors that may affect drug concentrations.

Variability in the terminal half-lives of enrofloxacin after intracoelomic, intramuscular, and immersion administration routes in aquatic invertebrate and teleost species is extensive. The intracoelomic terminal $t½$ in the green sea urchin was only slightly longer than the immersion $t½$: 38.8 and 34.5 hr, respectively. In contrast, the purple sea star enrofloxacin immersion $t½$ (56.0 hr) exceeded the $t½$ of the intracoelomic injection (42.8 hr).[20] The intracoelomic and immersion terminal $t½$ in the green sea urchin exceeded the terminal $t½$ reported in red pacu after intramuscular administration (28.9 hr), the $t½$ reported in manila clams (*Ruditapes philippinarum*) (6.2–17.5 hr depending on temperature and salinity) and European cuttlefish (*Sepia officinalis*) (1.8 hr intravenous, 1.0 hr immersion).[3,7,14] The intracoelomic and immersion terminal $t½$ in the green sea urchin were comparable to the immersion terminal $t½$ reported in ridgetail white prawns (*Exopalaemon carinicauda*) (1.2–2.3 days, depending on tissue) and shorter than the Chinese mitten-handed crab intramuscular terminal half-life, 52.4 hr.[16,26] Continued pharmacokinetic study of enrofloxacin in invertebrate species is warranted based on the variability reported. When reviewing $t½$ data from animals, it is important to recognize that $t½$ is a dependent pharmacokinetic parameter. It is dependent on the relation-

ship of: k = CL/*V*, where CL is clearance and *V* is volume of distribution. Half-life is related to k by: $t½ = \ln(0.5)/k$. Therefore, differences in $t½$ can be attributed to changes in clearance, volume of distribution, or both.

After intracoelomic administration, a two-compartment model was the best fit to the enrofloxacin concentrations in the green sea urchin. The shape of the curve (Figs. 1, 2) clearly shows a bi-exponential decline in concentrations typical of a two-compartment model and indicates initial unequal distribution among tissues. Enrofloxacin has also demonstrated a two-compartment distribution in teleosts, including Atlantic salmon, yet the anatomy of the sea urchin differs greatly from teleost species.[21] Echinoids are composed of a test that encases an open system of tissue and hemolymph. It also contains a water vascular system, a gastrointestinal system, muscle, and gonadal tissue.[8] A study that evaluated the effect and residence time of oxytetracycline administered orally in food pellets to the sea urchin *Psammechinus miliaris* reported oxytetracycline detected in the gonadal tissue up to 70 days postadministration, indicating that antimicrobials may be detected in other tissues after oral administration.[2] Evaluation of tissue concentrations of enrofloxacin in the green sea urchin are necessary to understand the two-compartment distribution of enrofloxacin in this species.

Ciprofloxacin was detected after intramuscular and immersion administration of enrofloxacin in the red pacu and after intramuscular injection in the Chinese mitten-handed crab.[14,26] Similar to purple sea stars and European cuttlefish, however, the green sea urchin did not have appreciable concentrations of ciprofloxacin in hemolymph samples after intracoelomic and immersion administration.[7,20] Environmental parameters may affect the degree of enrofloxacin metabolism to ciprofloxacin in aquatic species. For example, turbot (*Scophthalmus maximus*) kept at lower water temperatures had reduced metabolism of enrofloxacin to ciprofloxacin than when kept at higher temperatures.[15] The low concentration of ciprofloxacin detected in green sea urchin hemolymph samples may reflect the effects of the cold water (10.4–11.5°C) on enrofloxacin metabolism or that metabolic pathways for converting enrofloxacin to ciprofloxacin are poorly developed in this particular species.

In mammalian species, an AUC:MIC ratio >100 is sufficient for antibacterial efficacy. The optimum AUC:MIC ratio in marine invertebrates is not known. Nevertheless, at the enrofloxacin doses administered in this study (10 mg/kg intracoelomic injection and 10 mg/L immersion), an AUC:MIC ratio >100 would be attained for bacteria with MIC values <12 μg/ml for the injection route. This is considerably higher than the MIC of bacteria considered susceptible to enrofloxacin in other animals (breakpoint for susceptibility is <0.5 μg/ml), suggesting that even some bacteria considered "resistant" could be treated with this dose. The immersion route produced an AUC:MIC ratio sufficient for bacteria, with MIC < 0.32 μg/ml, which includes many bacteria from a wild-type population distribution.

The C_{MAX} of enrofloxacin in sea urchins was compared with the MIC of enrofloxacin to common teleost pathogens. The intracoelomic injection of 10 mg/kg enrofloxacin in the green sea urchin achieved hemolymph concentrations exceeding the enrofloxacin MIC for >24 different teleost pathogens with MIC ranges of 0.005–4.0 μg/ml.[1,11] The immersion treatment of 10 mg/L enrofloxacin achieved hemolymph concentrations exceeding the MIC for enrofloxacin to 18 different teleost pathogens, which included MIC ranges of 0.005–0.16 μg/ml.[1,11] Both treatment techniques and the dosages used exceeded the MIC for *Vibrio* and *Pseudomonas* spp. To address the efficacy of antimicrobials in the treatment of bacterial infection in the green sea urchin, determining the MIC of bacterial pathogens isolated from this species as well as clinical trials of enrofloxacin in cases of bacterial disease is required.

CONCLUSIONS

Enrofloxacin administration to the green sea urchin via intracoelomic injection and immersion was well tolerated by all of the study animals. The $t½$ was comparable to other aquatic invertebrate and teleost species, but great species variability exists. With a $t½$ exceeding 30 hr for both treatments and C_{MAX} exceeding teleost pathogen MIC for 120 hr after intracoelomic injection and 66 hr for immersion, it may be possible to administer enrofloxacin at 10 mg/kg intracoelomic injection or at 10 mg/L immersion for 6 hr once every 5 and 3 days, respectively, and achieve a microbiologic cure. Based on anecdotal reports, enrofloxacin may prove to be an effective antimicrobial treatment of erosive lesions in sea urchins. Evaluating additional doses and dosing protocols with enrofloxacin and other microbials in echinoids with respect to species-specific pathogens would be useful.

Acknowledgments: Funding for the pharmacokinetic analysis was provided by the American Association of Zoological Veterinarians Wild Animal Health Fund, and funding for supplies was provided by the Vancouver Aquarium. The authors thank Shannon Balfry, Dave Carlson, Boaz Hung, and Dana Turner of the Vancouver Aquarium for their assistance with the housing and care of the animals used in this study. The authors also thank Danny Kent and the BC Waters Aquarists at the Vancouver Aquarium for acquiring the animals under Department of Fisheries and Oceans Canada permit XR 1 2014. From the North Carolina State University College of Veterinary Medicine pharmacology lab, we greatly appreciate the work of Delta Dise in the sample analysis.

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Received for publication 19 May 2015