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Enrofloxacin Pharmacokinetics and Sampling Techniques in California Sea Hares (*Aplysia californica*)

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This pharmacokinetic study was designed to determine the pharmacokinetics of enrofloxacin at 5 mg/kg when given to sea hares in their hemolymph. Enrofloxacin is a commonly used antimicrobial in veterinary medicine and potentially could be used to treat sea hares exposed to susceptible bacterial species. We individually identified 8 juvenile *Aplysia californica* and group housed them in an open seawater flow system at 14 to 18 °C; 2 served as untreated controls. The remaining 6 animals were injected into the hemocoel with 0.030 mL of 22.7 mg/mL enrofloxacin (average dose, 5 to 6 mg/kg). At each time point, 300 µL hemolymph was collected from the pedal hemolymph sinus and HPLC-analyzed for enrofloxacin and ciprofloxacin levels. Enrofloxacin was detected in all dosed animals, at an average peak concentration of 3 µg/mL in hemolymph, and remained in the body for 20.3 h with an average clearance of 0.19 µg×h/mL. No ciprofloxacin was detected in any *Aplysia* in this study. Hemocoel injection appears to be an effective way to administer enrofloxacin to *Aplysia* and reach clinically relevant concentrations. Enrofloxacin reached therapeutic target concentrations in *A. californica* when dosed according to the regimen described in the current report.

Abbreviation: MIC, minimal inhibitory concentration

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Sea hares (*Aplysia* spp.) are opistobranch gastropods that have a reduced, vestigial shell and are found in temperate and tropical coastal waters worldwide.[3] Specifically, *Aplysia californica* is found in the coastal, temperate waters from the Humboldt Bay in Northern California to the Gulf of California, Baja California, Mexico.[12] Because of their simple, well-mapped nervous systems and large nerve cell bodies, these marine invertebrates are widely used for studies of neurobiology, particularly for investigation into the cellular basis of memory and learning.[1,8]

One of the most commonly encountered microbial infections of marine gastropod mollusks is *Vibrio* spp., which often live in warm coastal waters. Many *Vibrio* spp. are zoonotic, affecting both invertebrate as well as vertebrate animals, including humans.[21] Vibriosis and other microbial infections could have a devastating effect on a laboratory population of animals as well as pose a threat of infection to human caretakers. However, no drugs have been approved in the United States for use in *Aplysia* or other mollusks, and minimal research has been done to determine the safety and appropriateness of available antibiotics should illness occur in a colony or aquatic collection. Both fluoroquinolones and tetracyclines are used to treat human vibrosis; because the fluoroquinolone enrofloxacin has a lower prevalence of antibiotic resistance in *Vibrio* spp., we considered it for this study.[10,22,24]

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Therefore, this preliminary study was conducted in 8 *A. californica* to assess the safety and pharmacokinetics of enrofloxacin.

Materials and Methods

Animal housing and care. Eight juvenile sea hares from the same egg batch (no. 301; weight, 120 to 153 g) were housed at the National Resource for *Aplysia* at the University of Miami, Florida, in compliance with USDA regulations. Animals were group housed in plastic bins with open water flow through perforations in the bottom of the bin to allow for the use of filtered, chilled water from a nearby bay, according to facility standard procedures. Seawater temperatures were 14 to 18 °C, and seawater pH ranged from 7.7 to 7.8. *Aplysia* were fed a free-choice diet of red algae (*Gracilaria ferox*).

Animal study design. Individual sea hares were identified according to rhinophore length and by unique colored beads attached to a parapodium by using a single simple-interrupted 4-0 silk suture (Cutting FS1 Suture, Ethicon, Cornelia, GA; Figure 1 A).[2] Individual rhinophores (Figure 1 A) were characterized as missing, short, or long, and unique combinations of rhinophore categorization (for example, right missing and left long) identified each animal. Six animals were injected with 0.03 mL of 22.7 mg/mL enrofloxacin, yielding an average dose of 5 to 6 mg/kg. This dose is commonly used as the initial dose for enrofloxacin in mammalian and exotic species to assess safety and minimize neurotoxicity.[23] An additional 2 animals were untreated controls and were kept in a separate bin positioned 0.6 m upstream of the treated sea hares in the continuously emptying water table to avoid enrofloxacin exposure of control animals. During each procedure, all sea hares were handled gently to minimize the release of ink; after manipulation, they

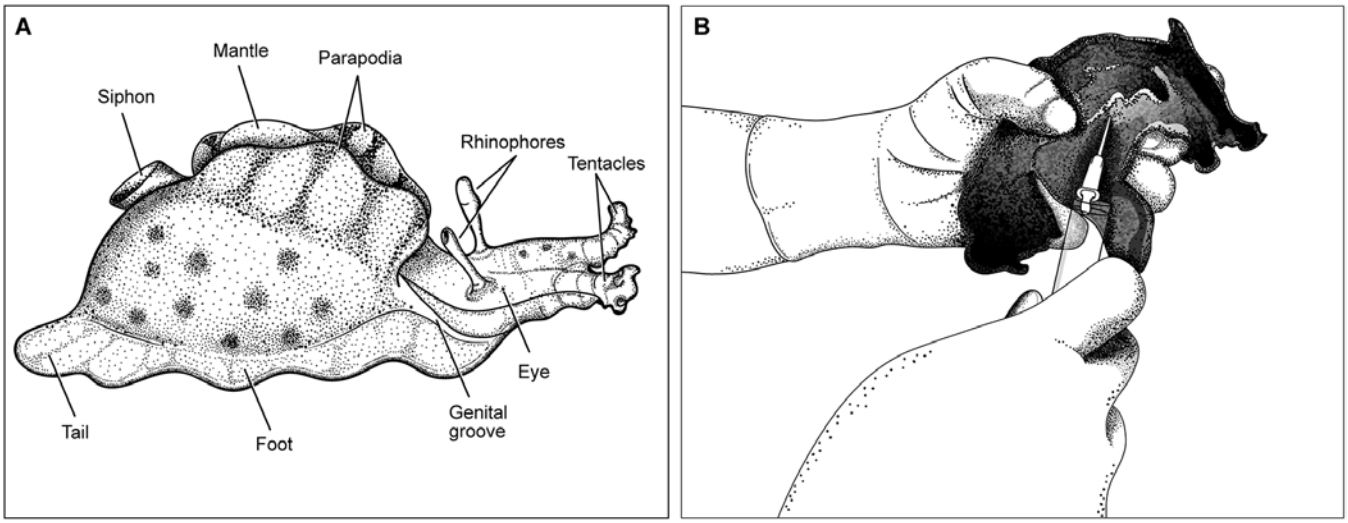

Figure 1. *Aplysia* illustrations. (A) External anatomy of *Aplysia californica*. Original drawing by Mal Hoover based on photographs taken by AS Davis and review of drawings in reference 9. (B) Hemolymph collection technique. The needle for hemolymph collection is placed at a 30° angle directly below the genital groove on the animal's right side. Hemolymph drips from the needle hub into a sterile collection tube.

were individually assessed for normal behavior through direct observation and testing of their righting reflex.

Hemolymph collection. Prior to each hemolymph collection, the sea hares were weighed. The landmark for hemolymph collection from the pedal hemolymph sinus is the midneck, below the genital groove, on the animal's right side (Figure 1 A). The needle was brought in caudally at an approximately 30° angle parallel to the genital groove (Figure 1 B). The pedal hemolymph sinus was accessed by using a 20-gauge needle, and 5 or 6 hemolymph drops were collected into a sterile 1.5-mL microfuge tube. All tubes were immediately placed on ice and centrifuged at 5180 × *g* for 10 min within 20 min of collection; 300 µL of supernatant were collected from each and stored at –80 °C until further processing. Samples for enrofloxacin analysis were collected at baseline and at 1, 2, 4, 8, 12, 24, and 72 h after injection.

HPLC analysis. Hemolymph samples were analyzed by using a previously published HPLC method.[7] In brief, hemolymph underwent solid-phase extraction (Oasis HLB cartridges, Waters, Milford, MA) after preconditioning with 1.0 mL of methanol and then 1.0 mL of distilled water. Samples of hemolymph (200 µL) were extracted under vacuum pressure and washed with 95% double-distilled water–5% methanol. The samples were eluted with 1.0 mL methanol and then dried under nitrogen flow (20 psi) at 45 °C for 15 min. The sample was reconstituted with 100 µL mobile phase composed of 15% methanol–85% double-distilled water, and 200 µL trifluoroacetic acid per 1 L. Sample (25 µL) was injected into the HPLC system of autosampler, quaternary pump, and UV detector (1000 series, Agilent Technologies, Wilmington, DE). The reverse-phase column (Zorbax C8 column, Agilent Technologies) was heated to 40 °C, with a mobile phase flow rate of 1 mL/min and UV detector set at 279 nm.

Calibration curves were prepared daily through the addition of known amounts of enrofloxacin reference standard (Bayer Animal Health, Shawnee Mission, KS) to untreated hemolymph. The lower limit of detection was 0.02 µg/mL. The method produced quantifiable and reproducible results over a range of 0.05 to 7.5 µg/mL, with a retention time of 5.5 min for enrofloxacin. No interfering compounds in hemolymph were identified that corresponded to the elution of the enrofloxacin peak. Reference standards of ciprofloxacin (United States Pharmacopeia, Twinbrook Parkway, Rockville, MD, USA) were also added and analyzed because ciprofloxacin is a metabolite of enrofloxacin in mammals, birds, fish, and some reptiles.[4,6,11,13,15,18]

Pharmacokinetic analysis. Noncompartmental analysis was performed by using pharmacokinetic software (WinNonlin Phoenix Software, St Louis, MO). Analysis included enrofloxacin and ciprofloxacin concentrations in the hemolymph collected from 6 sea hares. Pharmacokinetic parameters that were calculated included AUC, mean residence time (area under the first moment curve divided by AUC), total body clearance (dose divided by AUC), volume of distribution at steady state (clearance multiplied by mean residence time), and the peak concentration; values for peak concentration divided by minimal inhibitory concentration (MIC) and AUC divided by minimal inhibitory concentration were calculated also.

Results

All 6 treated sea hares recovered the righting reflex and exhibited normal behavior, including active feeding, immediately after manipulation. Release of ink was rare. All animals in both the control (n = 2) and experimental (n =6) groups gained weight during the experimental period.

Enrofloxacin was detected in all 6 of the treated sea hares and in neither of the 2 control animals. No ciprofloxacin was detected in the hemolymph of any of the sea hares. Pharmacokinetic parameters are shown in Table 1. In particular, the mean residence time of enrofloxacin was 26.6 h, with a corresponding mean half-life of 20.3 h.

Concentration–time plots of the data for individual treated *Aplysia* (Figure 2) show that 4 of the treated animals had similar profiles, whereas the remaining 2 sea hares had lower concentrations throughout the observation period.

Discussion

Pharmacokinetic parameters (Table 1) were consistent with data in some invertebrate species but not others.[7,9,19,20] Enrofloxacin pharmacokinetic studies have been performed in cuttlefish, sea stars, mud crab, horseshoe crabs, and sea urchins.[5,7,9,19,20] Data from a pharmacokinetic analysis of cuttlefish (a mollusk species like A*plysia*, but from a different taxonomic class and a species with a closed rather than open circulatory system)

Table 1. Pharmacokinetics of enrofloxacin in *Aplysia* by using a noncompartmental approach

Animal	MRT (h)	AUC (µg×h/kg)	Volume of distribution (L/kg)	Clearance (µg×h/mL)	Elimination half-life (h)	Peak concentration (µg /mL)	Dose (mg/kg)
B1	25.8	58.7	2.2	0.1	19.7	6.0	5.0
B2	30.7	60.2	2.9	0.1	22.4	3.2	5.7
B3	18.1	34.4	3.4	0.2	19.7	4.2	6.4
B4	16.5	64.1	1.3	0.1	14.6	3.3	5.1
B5	35.6	13.8	13.8	0.4	23.6	0.4	5.3
B6	32.9	18.1	9.4	0.3	21.8	1.0	5.2
Mean	26.6	41.6	5.5	0.2	20.3	3.0	5.5
1 SD	7.9	22.5	5.0	0.1	3.2	2.1	0.5

shows discrepancies in enrofloxacin elimination half-life (1.8 h for cuttlefish, 20.3 h for *Aplysia*) and AUC (26.7 µg×h/mL in cuttlefish, 41.6 µg×h/mL in *Aplysia*).[7] A study in sea urchins used a higher dose of enrofloxacin (10 mg/kg) than that used here (5 to 6 mg/kg) and reported a longer elimination half-life (38.3 h). The peak enrofloxacin concentration in hemolymph (90.9 µg/mL) and AUC (1199 h×µg/mL) that we obtained for sea hares are higher than reported for sea urchin.[19] The elimination half-life in sea stars given 5 mg/kg enrofloxacin is considerably longer than in similarly dosed sea hares (42.6 h compared with 20.3 h).[20] In one species of crab, the AUC for a 10-mg/kg dose was reported to be 636 h×mg/L,[5] which is much higher than in the current report. In contrast, horseshoe crabs given a single 5-mg/kg dose of enrofloxacin in the dorsal cardiac sinus had a similar elimination half-life (27.9 h) to that of *Aplysia* (20.3 h). In addition, although the average peak concentration and AUC of enrofloxacin in horseshoe crabs (9.0 µg/mL and 367.4 h×µg/mL, respectively) were higher than those of *Aplysia* (3.0 µg/mL and 41.6 h×µg/mL, respectively), they were closer than those for sea urchins.[9,19] Expanding the comparison to include many species of fish,[4,5,13,14,17] the elimination half-life and volume of distribution of enrofloxacin in A*plysia* are consistent with those of other invertebrate and marine vertebrate species.

The elimination of enrofloxacin in invertebrates may vary with water temperature. This effect occurs in crabs, in which a 7 °C increase caused the volume of distribution and elimination half-life to drop appreciably.[5] In addition, the cuttlefish in the previous report[7] were housed at 25 °C; consequently, the elimination pharmacokinetics of the drug might have been much faster than in our analysis, which was done in 14 to 18 °C water. Therefore, in addition to the dose, the water temperature should be considered as a factor when comparing studies.[5,14] Finally, some data suggest temperature changes may influence infection with *Vibrio* spp.[16] Therefore extrapolation of the results reported here to other invertebrate species or different water temperatures is not advised. Rather, further research is warranted to better understand the relationship between infection, pharmacokinetics and treatment of disease.

Regarding the data in Figure 2, a broad peak followed a trough after the first few samples, but the cause of this peak is undetermined. The use of this drug in *A. californica* for the treatment of *Vibrio* infection has not been evaluated previously. However, given the concentrations reached in the study animals, we propose that enrofloxacin may be a viable treatment option for vibriosis in a laboratory colony. The peak concentrations of enrofloxacin achieved a mean of 3.02 µg/mL in the 6 Aplysia used (Table 1). In previous studies on aquatic species, *Vibrio* species (*V. anguillarum* and *V. salmonicida*) have an enrofloxacin MIC_{50} of 0.005 µg/mL.[16] However, some *V. salmonicida* have

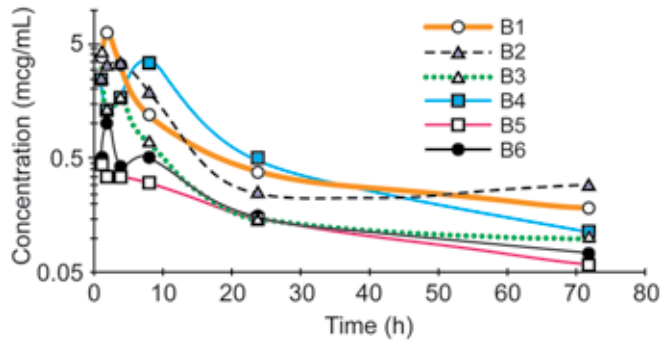

Figure 2. Semilogarithmic graph of data from individual *Aplysia* regarding enrofloxacin concentration relative to time. The *x*-axis crosses the *y*-axis at the highest reported MIC for *Vibrio* spp. and the limit of quantitation for the HPLC assay.

reported MIC of 0.05 µg/mL at 15 °C.[16] In other animals, a peak concentration:MIC ratio of 8 to 10 or higher is associated with therapeutic success and minimizes the emergence of resistance. Therefore, the peak concentration:MIC ratios of 8.6 to 119.8 in the *Aplysia* in this study should be adequate to treat infections caused by these pathogens. Alternatively, AUC:MIC ratios have been used to predict clinical success of fluoroquinolones in animals, where an AUC:MIC ratio of 100 or greater is the desired target. The AUC:MIC ratio for enrofloxacin in our study was 275 to 1282 for *Vibrio* spp. According to these metrics, enrofloxacin administered at approximately 5 mg/kg in *Aplysia* will reach therapeutic targets to treat most *Vibrio* spp. reported in aquatic species. A few papers suggest concentrations in excess of 0.5 µg/mL[24] or on average 0.32 to 0.43 µg/mL (range, 0.18 to 0.63 µg/mL)[22] will control most clinically important *Vibrio* spp. No adverse events were observed during this study; however, only a single injection was administered, and the safety of repeated treatments is undetermined.

Two of the sea hares (B5 and B6 in Figure 2), although treated with the same dosages of enrofloxacin, had significantly lower hemolymph concentrations than other animals in the study. The cause of the low peaks is undetermined but could have been the result of errors from injection or to different drug distribution in these animals. Additional sampling of more time points in our study would have improved the pharmacokinetic modeling for these atypical animals in particular.

The sea hares were not weighed or monitored after the hemolymph collection period; thus no data were collected regarding long-term effects due to hemolymph collection or exposure to enrofloxacin. Finally, the dual identification system was crucial because the bead occasionally detached due to host tissue rejection of the suture material. Although the

rhinophore-based identification system was sufficient for this study, wherein individual *Aplysia* needed to be identified for less than 1 wk, this system was suboptimal in a longer study in which rhinophore growth, including regeneration of missing rhinophores, occurred.

In conclusion, this report showed that hemolymph can be collected from *Aplysia* spp. for pharmacokinetic studies without inducing acute undue harm or stress to the animals. Pharmacokinetics for enrofloxacin were successfully determined from analysis of hemolymph samples. Enrofloxacin was safe for single administration in *Aplysia* at a 5-mg/kg dose. This dose produced concentrations in hemolymph to meet therapeutic targets established for treating other animals. These results apply only to the water temperatures used for this study. If animals are kept at temperatures other than 14 to 18 °C, the changes in pharmacokinetics are unknown. Additional studies are needed to determine the effects of temperature and multiple-dosing regimens on the metabolism of enrofloxacin in *Aplysia* spp.

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