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The pharmacokinetic disposition of Enrofloxacin in zebrafish by aquarium water exposure, oral and intraperitoneal administration

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Abstract
Zebrafish (*Danio rerio*) is an emerging alternative animal model used for screening pharmacological effects and safety studies. One of the bottlenecks for the lack of PK data in zebrafish is the inability to collect the volume of blood required for bioanalysis. To make zebrafish a suitable alternative animal model to mammalian species to study the PK profile of a drug, we have used the skeletal muscles as an alternative to blood for bioanalysis based on the assumption that they may exhibit an equilibrium with the concentration of drug in blood and it is easy to collect muscle after euthanasia. Hence the current study was undertaken to assess the comparative pharmacokinetic profile of enrofloxacin administered by oral route, intraperitoneal (IP) route, and mixed in aquarium water. The fishes were euthanized and the skeletal muscle was collected at various time points. An assay of Enrofloxacin in skeletal muscle was done using a validated HPLC method. The AUC (µg/g.h) values for oral, intraperitoneal and exposed water routes are 2.82, 2.91 and 4.34 respectively, Cmax (µg/g) values are 0.71, 0.64 and 0.30 respectively. The results revealed that the oral and intraperitoneal (IP) route showed a better PK profile than mixed in water for enrofloxacin. The results show that the PK of enrofloxacin administered by oral and intraperitoneal route showed the typical absorption and elimination phase, whereas enrofloxacin mixed in water did not show the absorption and elimination phase. Hence it is reasonable to conclude that zebrafish can be used as an alternative model to mammals for understanding the PK of a drug. However further studies are required to ascertain their utility as an alternative to studying PK or TK of drugs.

Keywords: Zebrafish, enrofloxacin, pharmacokinetics, HPLC

1. Introduction
Zebrafish (*Danio rerio*) is an emerging alternative front-loading animal model used for screening pharmacological effects and safety studies for the NCEs. Commonly, the zebrafish is exposed to a test substance by mixing it in water where the adult or larval zebrafish are maintained. Pharmacokinetics studies were limited in zebrafish as it is commonly done in mammalian animal models. One of the major reasons for the lack of PK data in zebrafish is the quantity of blood required for bioanalysis is too high to be obtained from adult zebrafish. Moreover, zebrafish are freshwater fish and they do not drink water continuously as in marine fishes (Wilson, 2004). Hence, the drug absorption from the water is either through gills or dermal routes but not through the gastrointestinal tract. This makes the zebrafish an unsuitable model to replace the mammalian species for the PK study of drugs if they are mixed in water.
To make zebrafish a suitable alternative animal model for mammalian species, we have attempted to study PK of enrofloxacin by oral or intraperitoneal route of administration and compared with the traditional way of adding drug into the aquarium water. For bioanalysis, the skeletal muscle was collected by euthanizing the fish at various time points, as an alternative to blood.

2. Materials and Methods
The analytical standard Enrofloxacin (100%) was procured from Sigma Aldrich private limited, India. Enrofloxacin 10% oral solution and Enrofloxacin 10% injection were prepared from enrofloxacin standard (Sigma Aldrich private limited) mixed in sterile distilled water. Methanol, Acetonitrile, Orthophosphoric acid, Triethylamine, and perchloric acid were obtained from Merck Specialities Private Limited, Mumbai. Adult zebra fishes from the colony maintained in the Department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Chennai, with an average weight of 0.3 to 0.5 g were used in the study.

All the fishes were fasted and acclimatized for 24 hrs. Fishes were divided into 3 groups for three different routes of administration *viz.* oral, intraperitoneal (IP), and exposed through aquarium water.

2.1 Experimental design

Zebrafishes were divided into three groups (33 zebrafishes/group) *viz.* Group I (oral route), Group II (intraperitoneal route- IP), and Group III (aquarium water) respectively. For oral and intraperitoneal (IP) routes, fishes were further separated into 11-time points for skeletal muscle collection after enrofloxacin drug administration *viz.* 0 min, 5 min, 10 min, 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs, and 24 hrs with 3 fishes at each time point in the separate glass container. The volume of the formulation administered by oral and IP route was 10 µl/fish and 25µl/fish (Dose@10mg/kg b.wt) respectively. Oral administration of the formulation was done using a micropipette to dispense 10µl as shown in Figure 1 and the intraperitoneal administration was done using a 26G insulin syringe as shown in Figure 2. Enrofloxacin was mixed in water where fishes were kept in the same tank at the concentration of 10mg/L of aquarium water as shown in Figure 3 and euthanized at different time points as shown in Figure 4 as like the other two routes to collect the skeletal muscle. Fishes were euthanized by rapid chilling in ice water, all visceral organs were removed and only skeletal muscle was collected and weighed before processing. The skeletal muscle was frozen and stored at -20 °C until analysis.

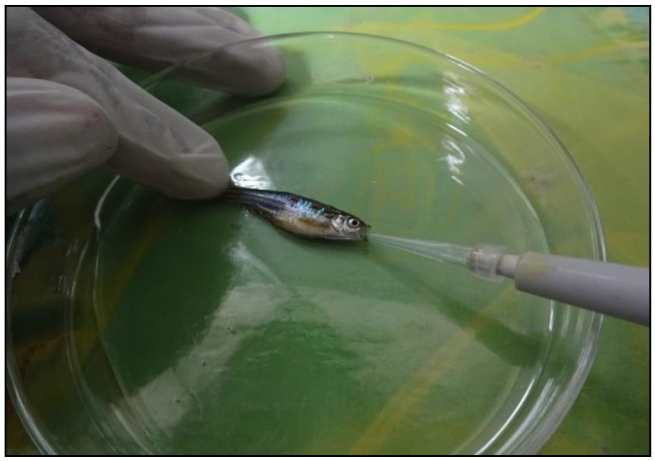

Fig 1: Enrofloxacin administration by oral route (10 µl/fish)

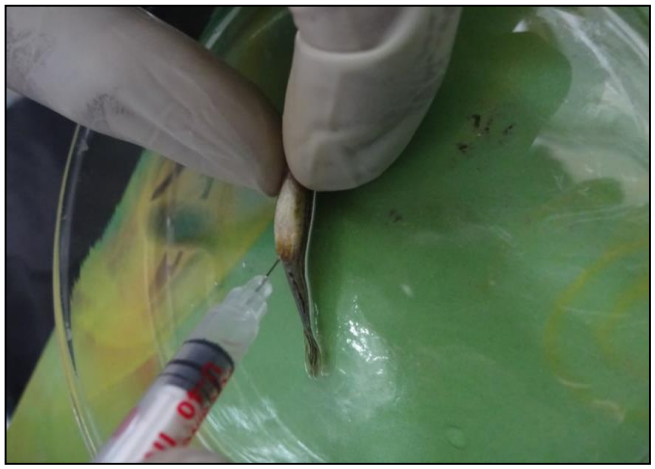

Fig 2: Enrofloxacin administration by intraperitoneal route (25µl/fish)

Fig 3: Enrofloxacin administration by aquarium water (10mg/L)

Fig 4: Euthanization by the rapid chilling method

2.2 Analytical method

The concentration of enrofloxacin in skeletal muscle was determined using a validated High-Performance Liquid Chromatography (HPLC) Method. The HPLC system consisted of a pump (Prominence, LC 20 AD), Photo Diode Array detector (Prominence, SPD-M20A), auto-sampler (Prominence, SIL-20 AC HT), column oven (Shimadzu, CTO-10AS VP), and LC Solution software for data analysis. A reverse phase C18 column (Syncronis, particle size 5μm, 4.6 x 250 mm, Thermo-scientific, USA) as stationary phase. and the mobile phase was Orthophosphoric acid (80%) + Acetonitrile (17%) + Methanol (3%), and the pH was adjusted to 2.5 with triethylamine. Limit of detection (LOD) and Limit of quantification for enrofloxacin is 0.03µg.ml^{-1} and 0.05µg.ml^{-1} respectively.

2.3 Bioanalytical method

The homogenized tissue samples were extracted by liquid-liquid extraction. To 450 µl of skeletal muscle sample, 50µl of perchloric acid was added and vortexed for 30 seconds and centrifuged at 1000X for 5 min. The clear supernatant obtained was then filtered through a 0.2 µ HNN membrane filter. 10 µl was the volume was injected by the autosampler that is used for analysis. The flow rate was 1.2 mL/min and the chromatogram was analyzed at a wavelength of 287 nm with the column temperature maintained at 30°C. Drug-free zebrafish homogenized skeletal muscle sample was spiked with the known concentrations of enrofloxacin ranging from

0.01 to 5 µg/ml for bioanalytical method standardization and a calibration curve was plotted.

2.4 Pharmacokinetic analysis

The pharmacokinetic parameters such as peak plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under curve (AUC), area under moment curve (AUMC), mean residence time (AUMC/AUC), clearance (Cl_B/F=Dose/AUC), volume of distribution ($Vd_{(area)}$/F= Dose/AUC*β), and elimination half- life ($t_{1/2}$ = 0.693/β) were calculated using PK solver 2.0.

3. Results

The HPLC method was validated for enrofloxacin in zebrafish skeletal muscle. The calibration curves were linear over a broad range from 0.05 to 5 µg/ml with an R^2 value of 0.999 shown in Figure.5. The limit of quantification obtained in the present study was 0.02 µg/ml and the analytical recovery was 100.66%. Hence, the HPLC method for enrofloxacin in this study was highly reliable.

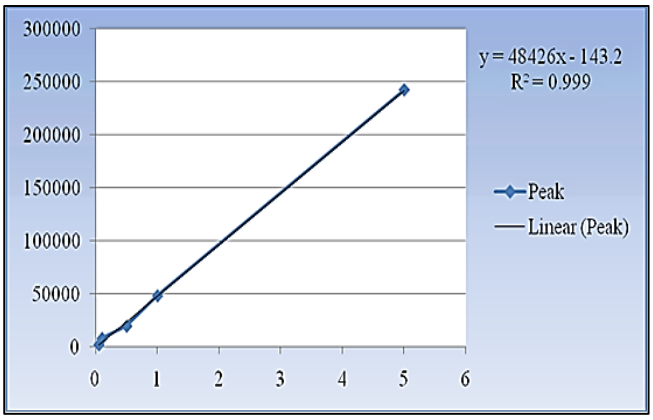

Fig 5: Calibration curve of enrofloxacin spiked in the skeletal muscle of zebrafish at concentrations range between 0.05 and 5 µg/ml

Following oral and intraperitoneal (IP) administration, the initial detectable tissue concentration of enrofloxacin was at 0.08 h which suggests a better absorption rate of the drug. Following 'aquarium water' administration, initial detectable tissue concentration of enrofloxacin was found at 0.5 hrs which may be due to delayed absorption when compared to direct administration by oral or IP route. In all three routes, the concentration of enrofloxacin was detected up to 24 h (Table 1). Comparative chart of enrofloxacin *vs* time concentration for all the three routes shown in Figure 6.

Table 1: Skeletal muscle concentrations of enrofloxacin after administration through three different routes (oral, IP, water).

Time (h)	Enrofloxacin concentration in tissue (µg.g^{-1})		
	Oral	IP	Water
0.08	0.05	0.37	BDL*
0.5	0.09	0.32	0.17
1	0.71	0.64	0.13
2	0.27	0.15	0.05
4	0.23	0.15	0.1
8	0.12	0.06	0.19
12	0.06	0.11	0.3
24	0.03	0.07	0.11

*BDL – Below Detectable Limit

Data in the table is the mean concentration of enrofloxacin in skeletal muscle at each time point

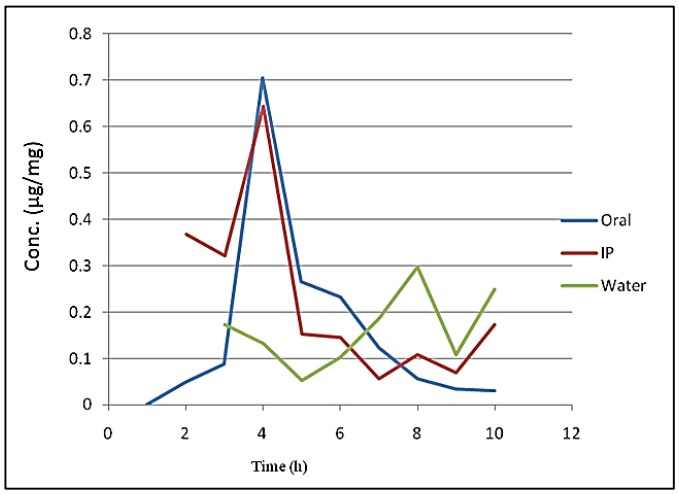

Fig 6: Comparative chart of time *vs* enrofloxacin concentrations of the three groups

The estimated pharmacokinetic parameters of enrofloxacin administered by all three routes are given in Table 2.

Table 2: Pharmacokinetic parameters of enrofloxacin after administration through three different routes (oral, IP, water).

Parameters	Units	Oral	IP	Water
C_{max}	µg.mg^{-1}	0.71	0.64	0.3
t_{max}	h	1	1	12
$t_{1/2}$	h	7.1	11.0	-
AUC $_{0-t}$	µg.h.mg^{-1}	2.8	2.9	4.3
AUC $_{0-\infty}$	µg.h.mg^{-1}	3.17	4.0	-
MRT $_{0-t}$	h	6.5	8.7	11.9
MRT $_{0-\infty}$	h	9.6	17.3	-
Vd	µg.g^{-1}	32.5	39.5	-
Cl	µg.g^{-1}/h	3.2	2.5	-

Abbreviation: Cmax: maximum plasma concentration; Tmax: time to reach peak plasma concentration; t1/2: half-life; AUC 0-t: Area under the curve from 0 to t hour; AUC 0-": Area under the curve from 0 to infinity; AUMC 0-t: Area under moment curve from 0 to t hour; AUMC 0-": Area under moment curve from 0 to infinity; MRT, mean residence time; ClB/F, Clearance; Vd area/F: volume of distribution.

The Cmax and Tmax were similar for both oral and IV routes of administration whereas in-water administration, the Cmax was half of the other routes of administration. The systemic exposure was similar in both oral and intraperitoneal administration indicating that the absorption of enrofloxacin by IP route was similar to the oral route as seen in mammals (Haritova, 2003) [2]. Though the volume of distribution and clearance are the parameters calculated from the blood concentration, we have calculated the Vd and CL from the tissue concentration and compared them with the Vd and CL of enrofloxacin in laboratory animals. In mice, the Vd and CL for enrofloxacin were 10.5 L/kg and 68.1 ml/min/kg, respectively (Bregante *et al.*,1999) [1], indicating a high volume of distribution and moderate clearance and zebrafish showed a similarly high volume of distribution and moderate clearance when enrofloxacin was administered by oral or IP route. However, when the zebrafish were kept in an enrofloxacin mixed bath, all the parameters of PK could not be calculated due to the instability of the absorption and elimination phase. These data suggest that the skeletal muscle can be used to calculate the PK parameters as an alternative to the blood samples. However, further studies are required to calculate the PK parameters of enrofloxacin by collecting the

blood samples in the zebrafish and compared with the tissue PK. However, this is the first attempt of deviating from the conventional matrix, blood samples, for the PK estimation of any drug.

The tissue concentrations of enrofloxacin were found to be higher than the MIC value of enrofloxacin for major pathogens (0.01 – 0.1 µg.mg^{-1}) (Prescott and Yielding, 1990). The concentration over MIC was maintained for 8 hours indicate that the enrofloxacin could be a good therapeutic agent against the susceptible organism in fish, which is an additional advantage of studying the tissue PK.

4. Conclusion

Based on the results, it was clear that the pharmacokinetics parameters could be calculated for enrofloxacin when administered by oral or intraperitoneal route rather than mixing it in water. Tissue concentration indicates that the enrofloxacin has a high volume of distribution and moderate clearance. Further studies are required to profile the PK of enrofloxacin in zebrafish by collecting the blood following oral or intraperitoneal administration and compared with the tissue concentration pharmacokinetics. If zebrafish is proven to be a suitable model to study and extrapolate to other species and humans, then it will be an excellent replacement model for mammalian species.

5. Acknowledgment

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6. Conflict of interest

The authors declare no conflict of interests

7. Author's contribution

D. Sakthivel and M.R.Srinivasan contributed to design, analysis, drafted the manuscript, critically revised the manuscript, and agreed to be accountable for all aspects of work ensuring integrity and accuracy. P. Karthick Venkatesh contributed to the analysis and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

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