PHARMACOKINETICS OF DISODIUM FOSFOMYCIN IN THE SHRIMP *Litopenaeus vannamei*

Pharmacokinetics of fosfomycin in shrimp

Ocampo L. and Sumano H.

Departamento de Fisiología y Farmacología, Facultad de Medicina Veterinaria y Zootecnia. Universidad Nacional Autónoma de México. Av. Universidad 3000, Coyoacán, México City 04510. México.

*Corresponding author:
Héctor Sumano DVM, MD, PhD
Tel.: + 52 55 56-22-59-80, Fax: + 52 55 56-22-59-80, E-mail: sumano@servidor.unam.mx

ABSTRACT

This study examined the pharmacokinetics of a single and three times a day dose of disodium fosfomycin (DF) in shrimp, *Litopenaeus vannamei*, under controlled laboratory conditions while attempting to reproduce pond conditions. Brackish-like water (salinity 40 g/L) was used at a constant temperature of 26-28°C with water turnover and aeration provided. DF was administered either as a single in-feed dose in the morning at a dose of 250 ppm or three times a day (*tid*), also at a rate of 250 ppm on each dosing. Feed intake was calculated as 3% of biomass and dose rate was calculated as 75 mg/kg of biomass/day or 225 mg/kg/day when administered *tid*. Peak haemolymph concentration (C_{max}), time to reach peak (T_{max}), and elimination half-life ($T½_{\beta}$) for a single dose of DF were: 1.8 ± 0.5µg/mL; 2.8 ± 0.4 h, and 0.35 ± 0.3 h, respectively. After dosing three times per day of DF the same variables were: C_{max} = 1.7 ± 0.6 µg/mL; T_{max} = 3.0 ± 1.2 h; and $T½_{\beta}$ = 0.8 ± 0.2 h, respectively. Muscle and hepato-pancreatic concentrations of DF were only assessed in the shrimp doses three times a day: values reached a maximum concentration of 0.38 µg/g in muscle and 1.73 µg/g in hepato-pancreas at 6.2 and 7.5 hours after the first dose. Values in these tissues decreased with a tissue elimination half-life value of 0.21 hours for muscle and 0.783 hours for hepato-pancreas, respectively. The results show that a customized pharmaceutical design is required for this species. Only very sensitive microorganisms could be destroyed with the concentrations of DF shown above and that rapid elimination of DF requires a higher dose at intervals of DF at three times a day dosing.

Key Words: disodium-fosfomycin, *Litopenaeus vannamei,* pharmacokinetics, shrimp

INTRODUCTION

Organisms that cause shrimp diseases include viruses, parasites, fungi and bacteria. Bacteria that affect shrimp include numerous species of *Vibrio* such as *V. harveyi, V. vulnificus, V. parahemolyticus* and *V. anginolyticus*. Other microorganisms like Rickettsia and *Mycobacterium fortuitum,* responsible for necrotizing hepato-pancreatitis (NHP), can also cause high morbidity and mortality in cultured shrimp. In Mexico, intensive shrimp farming has rapidly developed in the last decade. The greater part of the country's production is exported and trade is growing. Hence the shrimp industry demands antibacterial agents in the hope of limiting or preventing outbreaks and spread of bacterial diseases. A limited number of chemotherapeutic agents, as medicated feeds, have been approved for use in Mexico[1]. Due to the broad antibacterial spectrum and high potency of disodium fosfomycin (DF), it has been approved as antibiotic resource to combat bacterial infections in fish farming in Mexico. It has been empirically incorporated in farm-raised shrimp feed for the treatment of vibriosis and necrotizing hepatopancreatitis infections. Yet, this drug lacks formal pharmacokinetic (PK) analysis and is usually added to pelleted feed in concentrations that fluctuate around 250 mg/kg, for feeding shrimps three times a day (*tid*). Fosfomycin (*cis*-1,2-epoxyphosphonic acid) is a broad-spectrum antibacterial drug discovered in 1961. Some PK data has been produced in chickens (Aramayona et al., 1887), rabbits (Fernandez et al., 1987), cattle (Sumano et al., 2007), dogs (Gutiérrez et al., 2007) and horses (Zozaya et al., 2008), but an effective single or multiple dose schedule has not been established for fosfomycin in shrimp species. Furthermore, for DF there is no information on its PK in aquatic species, nor has a published therapeutic experiment been proposed to assess the usefulness of a given dose for susceptible bacterial infections in shrimp, In view of the above, the aim of this study was to define the basic PK variables of DF after a single dose and

[1]NOM-0064 Secretaría de Agricultura, Ganadería, Recursos Forestales y Pesca. Mexico. www.google.com.mx/search?hl=es&rlz=1W1SKPB_es&q=Nom+antibioticos+pesca&btnG=Buscar&meta=&aq=f&oq=

a *tid* dose as an in feed medication in *Litopenaeus vannamei* shrimp.

MATERIAL AND METHODS

Disodium fosfomycin, as a standard, was purchased from SIGMA (St Louis, MO, USA). Approximately one thousand healthy shrimps (*Litopenaeus vannamei*), weighing 19.7 ± 2.8 g, as assessed with a 100 shrimp sample, were obtained from a shrimp farm in Hermosillo, State of Sonora, Mexico. Before the inclusion in this trial twenty shrimps were selected at random and analysed as pooled samples for haemolymph, muscle and hepatopancreas to confirm the absence of DF and other possible bacterial growth-inhibitor drugs. Shrimps were maintained in 1000 L tanks with continuous flow of brackish water at an approximate rate of 10 L/h. Temperature was kept at 23-25°C using a thermostat (LED 200 watts Dymax); pH was approximately 7.6-7.8 (Aqualytic, Germany), and continuous aeration was provided at 6.79-6.56 mL/min. Animals were fed *ad libitum* with commercial shrimp drug-free pellets (Camaronina Purina®, Sonora, México), containing a minimum of 35% protein, 9% fat calculated on a 3% feed intake per day with respect to the biomass as established by Nutcharnart (2005). Lack of ecdysis in shrimp was ensured before initiation of the trials.

Commercial 5% disodium fosfomycin (DF) premix, (Magnamix® Avimex, Mexico City) was obtained from a retailer, and added to antibacterial-free feed ingredients of Camaronina® at a rate of 250 mg/kg of feed. The concentration of fosfomycin in feed was assessed after mixing it with the powdered feed and only then was this mixture pelleted. Quantification of fosfomycin in pelleted food was determined in 6 replicates. Disodium fosfomycin was administered to shrimp either once at 7:00 am or three times a day at 7:00 am, 12:00 and 5 pm, also at a rate of 250 ppm each time. Hence two dose schemes were tested, 75 mg/kg of biomass/day, and 225 mg/kg of biomass/ day respectively.

Disodium fosfomycin concentrations in feed, in haemolymph and in shrimp tissues were determined by the agar diffusion analysis as described by Bennett *et al*. (1966), using *Bacillus cereus* (ATCC 11778) grown on Müeller-Hinton agar (Bioxon® México City) as the test organism. Log-transformed values of drug concentrations were determined using linear regression analysis to compare diameters of inhibition zones with those of various dilutions of the standard prepared in distilled sterile water. The intra-assay coefficient error was < 4.8. The analytical assay was linear over a concentration range of 0.2 µg/mL to 18 µg/mL, with a percent recovery of 82 % and a correlation coefficient of 0.985.

Haemolymph was sampled from the ventral sinus cavity using a 2.5 mL syringe containing sodium citrate (Sigma), as anticoagulant. Muscle and hepatopancreas were collected from each shrimp and stored in Eppendorf tubes and fully identified. All samples were kept frozen at -40°C. Sampling times were 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10, 11, 12 and 24 h. Twelve shrimp were included in each sampling time and samples were pooled in sets of three so as to end up with four sets of samples. Pharmacokinetic analysis was performed by the computer program WinNonlin (version 1.1; Scientific Consulting, Apex, NC, USA) assuming a first order kinetics. Models were selected in accordance with Akaikes information criterion. The area under the concentration-time curve (AUC) was calculated using the trapezoid rule, including the terminal portion.

RESULTS

The analysis of DF in feed samples revealed only a 3.8% reduction from the expected drug concentration (250 ppm *vs* 240 ± 8.5 ppm). Haemolymph concentration profiles of fosfomycin were obtained after a single dose of DF as an in-feed medication and after three doses during the day (7:00 am, 12:00 and 5:00 pm), at a rate of 250 ppm in both instances (see Figure 1). Neither the once-a-day, nor the three times-a-day haemolymph concentrations *vs* time profile, could be fitted by the nonlinear least square method using either one or two compartment models with first order absorption. Therefore, the profile was analyzed by a non-compartmental analysis using WinNonlin and the variables obtained are listed in Table 1.

Figure 2 shows both, muscle and hepatopancreas concentration *vs* time profile curves of DF achieved with the three times a day dosing. Under this dosing scheme concentrations peaked in muscle (0.38µg/g) at 3.3 hours after the first administration. Hepatopancreas concentrations of DF showed a peak value of 3.2µg/mL at 3.5 hours, also after the first administration. From this point onwards, concentrations decreased constantly to fall below the detection limit at 8 hours after first dosing, and at the end of the second dose, with tissue elimination half-lives of 0.21 h and 0.733 h for muscle and hepatopancreas, respectively. No further increments in these concentrations were detected in spite of being dosed for the third time.

DISCUSSION

Pharmacokinetic values for DF have been described in chickens (Aramayona et al., 1887), rabbits (Fernandez et al., 1987), cattle (Sumano et al., 2007), dogs (Gutiérrez et al., 2007) and horses (Zozaya et al., 2008); yet at present there is no documented information in shrimp or aquatic species. With such background information it is not possible to attempt any comparison of PK values obtained in this trial; which, to the best of our knowledge, is the first attempt to describe the PK of DF in shrimp. Under the conditions described, a C_{max} of 1.7µg/mL was achieved 3.0 hours after the first oral administration of 250 ppm of the drug. This data is almost identical for the dosing schedules of one or three doses per day of the drug as in-feed medication. Hence, drug accumulation seems unlikely. Yet, as expected, AUC, AUMC and MRT values were much higher in shrimps dosed *tid*. After peaking in haemolymph, DF seems to concentrate rather rapidly in hepatopancreas reaching the highest concentration found in this study (3.2µg/g), even

higher than the Cmax value for haemolymph. This feature may be a useful finding, considering that many bacterial diseases target this tissue.

Disodium fosfomycin has a very rapid elimination half-life under both dose schemes, but particularly so in the group dosed only once in the morning ($T½_{\beta}$ = 0.35 h). The drug disappears from all haemolymph samples by the fourth hour post-administration. Considering that the *in vitro* MIC´s for DF against *Vibrio* spp., isolated from diseased shrimps has been reported to range from 2 to 32µg/Ml (Reparaz, 1997), it is reasonable to assume that if the drug is only administered in the morning, little or no clinical efficacy should be expected. If DF is administered three times a day as in-feed medication at 250 ppm then, some clinical efficacy could be achieved, but only in sensitive *Vibrio* species. Pharmacokinetic/pharmacodynamic simulations in mammals have raised the speculation that optimal microbial killing by fosfomycin in tissues is time-dependant and minimal inhibitory concentration must be achieved during most of the dose intervals (Mckellar et al., 2004). According to these results, if an increased dose interval is attempted, *i.e*. twice a day medication with DF, a considerable reduction in clinical efficacy should occur. On the other hand, higher doses to achieve greater serum concentrations in humans have shown to add little benefit to the clinical outcome (Pfausler et al., 2004). This finding should be assessed in shrimp in further trials to attempt a customized pharmaceutical design; for example with stomodeum-retentive pharmaceutical preparations to increase bioavailability and MRT through an extended gastrointestinal transit time.

Although no data on the PK of DF in shrimp is available for comparison, Cmax and AUC values seem rather small, as compared to other antibacterial drugs; for example oxytetracycline with a Cmax = 21 µg/mL and AUC = 459 µg/mL/h when administered at a dose of 50 mg/kg (Uno, 2004). These differences may include low bioavailabiity *per se* but may also reflect, methodological differences and perhaps considerable lixiviation [2] of DF in brackish water, even greater than that observed for oxytetracycline.

In spite of the above, DF is widely used in shrimp farms, with claimed good clinical outcomes. If good clinical outcomes indeed occur, it is possible that part of the apparent beneficial effects derived from its empirical use may be mediated through a post-antibiotic effect, based on the proposed immunomodulatory effects of fosfomycin observed in humans (Perez et al., 1995), (Paape et al., 1991) To date, this is only a speculation that requires research into function of haemocytes. Based on these results, it is feasible to conclude that with the referred dose scheme, based on the drug haemolymph concentrations achieved, and because of the short-lived $T½_{\beta}$ found in haemolymph and hepatopancreas, the use of fosfomycin and/or its pharmaceutical design for shrimp species, require a profound revision.

[2]Lixiviation = the process of separating a soluble substance from one that is insoluble, by washing with some solvent, as water; leaching

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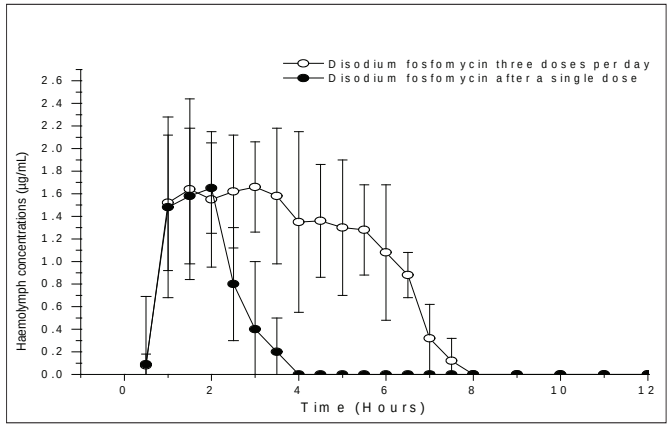

Figure 1:
Mean ± 1 SD haemolymph concentrations of disodium–fosfomycin in *Litopenaeus vanamei* following a single dose or a three times a day dose, at a dose of 250 ppm each feeding time (calculated dose of 75 mg/kg of biomass/day or 225 mg/kg of biomass/day, respectively), when administered as in-feed medication.

Table 1:
Pharmacokinetics values for disodium fosfomycin derived from haemolymph of Litopenaeus vanamei following a single dose or a three times a day dose, at a dose of 250 ppm each feeding time (calculated dose of 75 mg/kg of biomass/day or 225 mg/kg of biomass/day, respectively), when administered as in-feed medication.

PK variable	Value	
	Three times a day	Single dose
Cmax (µg·ml $^{-1}$)	1.7 ± 0.6[a]	1.8 ± 0.5[a]
Tmax (h)	3.0 ± 1.2[a]	2.8 ± 0.4[a]
$T½_{\beta}$ (h)	0.8 ± 0.2[a]	0.35 ± 0.3[b]
AUC (µg/mL/h)	8.74 ± 1.6[a]	2.58 ± 1.4[b]
AUMC (µg/mL/h)	33.28 ± 2.2[a]	12.8 ± 1.8[b]
MRT (h)	4.2 ± 1.2[a]	1.8 ± 0.6[b]

A different letter in a row indicates statistical difference ($P < 0.05$).
Cmax: maximum concentration; t max: time when maximum concentration was obtained; $T ½_{\beta:}$ elimination half – life of the drug; AUC: area under the concentration – time curve
AUMC = area under the first moment of the concentrations curve from zero up to ∞ with extrapolation of the terminal phase; MRT: mean residence time

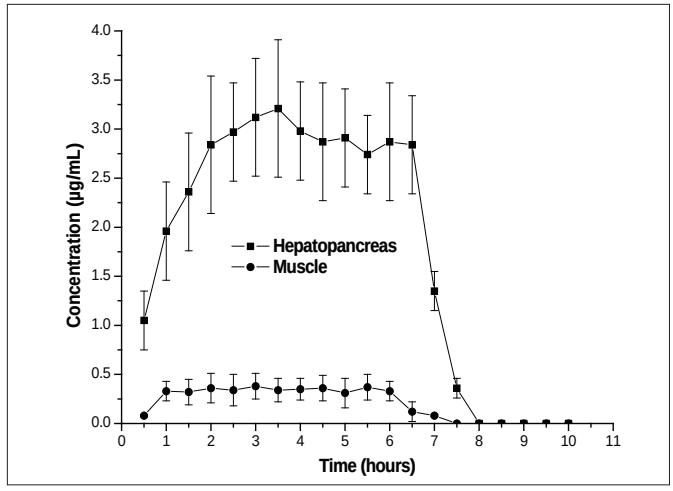

Figure 2:
Mean ± 1 SD concentrations of disodium–fosfomycin in muscle and hepatopancreas of *Litopenaeus vanamei* following a three times a day dose, at a dose of 250 ppm each feeding time (calculated dose of 225 mg/kg of biomass/day), when administered as in-feed medication.