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Praziquantel depletion from muscle plus skin tissue of gilthead sea bream (*Sparus aurata*)

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Abstract

This study determined the depletion pattern of praziquantel (PZQ) from gilthead sea bream (*Sparus aurata*) muscle plus skin tissue. Fish averaging 100.2 ± 17.7 g and kept at 25°C received a PZQ-dosing of 150 mg/kg fish for 3 days. Muscle plus skin tissue of ten fish were sampled on days 1, 2, 3, 4, and 6 days post-treatment. Depletion of PZQ from edible gilthead sea bream tissues was rapid, as PZQ concentrations decreased to 0.04 μg/g as early as 24 h post-treatment, while it was undetectable at 72 h. This information confirms the fact that PZQ is depleted fast from farmed animals, including fish such as the gilthead sea bream, and its levels in edible tissues fell below the detection limit in approximately 75 dd.

Keywords: Praziquantel; depletion; gilthead sea bream; *Sparus aurata.*

Introduction

Praziquantel (PZQ) is a synthetic drug that has received early approval as human medicine (Andrews *et al.*, 1983). It is effective against a broad range of internal and external human and animal parasites (Eom *et al*., 1988), but registration as a fish medicine is limited. The compound affects the integumental parasitic membrane by disrupting regulatory processes and inducing spastic muscular paralysis (Staudt *et al*., 1992).

A wide and comprehensive review of dietary administered PZQ to control platyhelminth parasites of fish has been published recently (Bader *et al*., 2019). Praziquantel has been proven to be a very effective antiparasitic against monogeneans (Hirazawa *et al.*, 2004; Sharp *et al*., 2004) and digeneans of fish (Bader *et al*., 2019). Thus, being a promising dietary fish anthelmintic, PZQ is an alternative to bath-administered formalin, which currently seems to be the only compound registered to confront fish parasites in most European countries, the United States, and elsewhere. PZQ could be used in aquaculture as a non-registered fish therapeutic method, within the 'off-label' framework described as the cascade principle (Council Directive 90/676/EEC, Directive 2001/82/EC, Commission Regulation 37/2010). In such cases, a standard withdrawal time (WT) of 500 dd is imposed to ensure consumer safety, although a maximum residue level (MRL) has not been established for farmed animals (EMEA, 1988). Information on the kinetic profile of the therapeutics in targeted organisms during and post-treatment is useful for adjusting recommended dosing regimens and estimating withdrawal from the body of treated animals.

While the withdrawal profile of PZQ has been studied in some farmed fish species including rockfish (*Sebastes schlegeli*) (Kim *et al*., 2001; 2003), rice field eel (*Monopterus albus*) (Xu *et al*., 2006) and rainbow trout (*Oncorhynchus mykiss*) (Björklund & Bylund 1987; Soukupova-Markova *et al*., 2016), limited information exists for gilthead sea bream (*Sparus aurata*) (Baralla *et al*., 2020), an important commercialized Mediterranean farmed finfish species. Gilthead sea bream suffers from severe gill infections due to the monogenean *Sparicotyle chrysophrii* (Sitjà-Bobadilla *et al*., 2010) and PZQ could potentially be used to combat this ectoparasite, provided that a rapid removal from the fish body compartment will be beneficial for its use as aquatic medicine. The aim of this study was to determine the depletion profile of dietary administered PZQ in gilthead sea bream following multiple dosing administration. The results can be used to determine the appropriate withdrawal time in PZQ-treated fish and evaluate the enforced cascade principle wherever the compound is not registered.

Materials and Methods

Experimental fish

Two hundred clinically healthy *S. aurata* averaging 100.2 ± 17.7 g were obtained from a local fish farm. One hundred fish were distributed in 1 m^3 cages, located within a 50 m^3 cement tank. Water was supplied by open flow and oxygen was provided continuously by bubbling air. Water temperature and salinity were 25°C and 38‰, respectively. The fish were allowed to acclimate prior to experimentation and fed a drug-free commercial diet at 2% B.W. Management of experimental animals followed EU legislation "on the protection of animals used for scientific purposes", according to Directive 2010/63/EU of the European Parliament and of the Council (EU, 2010).

Medicated feed and drug administration

Fish received a commercial feed (BioMar, Denmark) (Table 1) with oil-coated PZQ (Bayer Ltd.), aiming to simulate an *in situ* preparation of a medicated diet. One batch of experimental diet was prepared by mixing appropriate amounts of feed, PZQ (1 kg of the diet with 7.5 g active PZQ), and 100 mL fish oil for several minutes. During the trial, the experimental diet was stored at 4°C and was left to reach ambient temperature before delivery. The fish were fed the medicated diet by hand once per day for 3 consecutive days at a daily rate of 2% B.W, thus aiming for a dose of 150 mg/kg fish per day.

Sampling

Fish sampling was performed at predetermined time points post-treatment. The fish was anaesthetized with clove oil (40 ppm) and then killed by a blow on the head before taking tissue samples. Muscle plus skin tissue (approximately 5 g) was obtained from the anterior dorsal region of ten fish collected on days 1, 2, 3, 4, and 6 post-treatment. All prepared tissue samples were immediately frozen and stored at -20°C until analysis.

Table 1. Composition of the experimental diet.

Proximate composition	g/100g
Protein	44
Lipid	20
NFE	22
Fibre	3
Ash	9
Total phosphorus	1.25
PZQ	0.75

Chemicals and reagents

Praziquantel analytical standard was obtained from Sigma-Aldrich (USA). High performance liquid chromatography (HPLC) grade ethyl acetate, hexane, acetone, diethyl ether, and HPLC gradient grade acetonitrile were purchased from Fisher Scientific (USA). Other solvents and reagents of analytical grade were supplied by Fisher Scientific (USA), while heparin (5000 U.I/mL) was obtained from Merck KGaA (Germany). The stock solution of 100 μg/mL PZQ was prepared by dissolving PZQ in acetonitrile and it was stored at -20°C, while the working solution (10 μg/mL) was prepared before use with acetonitrile:water (35:65 v/v). The working solution was further diluted with acetonitrile:water (35:65 v/v) for the calibration curve.

Sample preparation

Muscle plus skin tissue samples of *S. aurata* were prepared according to Tubbs & Tingle (2006). Briefly, tissue sample was sheared, and subsequently 1 g of ground sample was placed in a 50 mL centrifuge tube. Six mL of ethyl acetate were added and the mixture was homogenized with an IKA Ultra-Turrax T25 Disperser (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 30 s at 16,000 rpm/min. The mixture was shaken for 10 min and then centrifuged at 10,000 *g* for 10 min at 10°C. The supernatant was transferred to a 15 mL tube and liquid extraction was repeated with 4 mL ethyl acetate before being subjected to vortex mixing. The combined extract (10 mL) was then evaporated to dryness at 45°C under a nitrogen stream. The dried residue was resuspended in 5 mL hexane and loaded onto an activated silica column (Isolute 500 mg SI-IST, UK), which was rinsed with 5 mL of 15% v/v diethyl ether-hexane where drug elution was performed with 5 mL 70% acetone in hexane. Finally, the solvent was evaporated to dryness at 45°C under a nitrogen stream and the dry residue was reconstituted by 1 mL of mobile phase solution, filtered using 0.22 μm nylon filter and injected (200 μL) into the HPLC apparatus.

Chromatographic conditions

Chromatographic separation of PZQ was carried out in an HPLC apparatus combining a Waters 600 Pump and a 600 Pump system Controller (Milford, MA, USA, a Waters 717 Plus Autosampler (Milford, MA, USA) set at 10°C injection temperature, a 150 mm × 4.6 mm Luna-C18 column packed with 5 μm particle size equipped with a 4mm × 3.0 mm C18 security guard cartridge (both from Phenomenex, USA), a 2487 UV detector set at 210 and Empower Chromatography Software (both from Waters, Milford, MA, USA). An isocratic mixture of 35:65 v/v acetonitrile:water was used as a mobile phase. The flow rate was constantly maintained at 1.0 mL/min, column temperature was maintained at 30°C, and was rinsed for 20 min with 100% acetonitrile between injections. The retention time of PZQ was 18.9 min.

Calibration curves and recovery rates

To establish the calibration curves for quantification of PZQ concentration in muscle plus skin tissue samples, PZQ standards were spiked into blank *S. aurata* tissues at final concentrations of 0.01, 0.05, 0.1, 0.25, 0.5 1, 2.5, 5, 10 μg/g. To determine the drug from the spiked samples, the extraction procedure and HPLC method described above were used. To evaluate the recovery rates of PZQ and the intra- and inter-day Relative standard deviation (RSD), three replicates of spiked samples containing different concentrations of the substances (0.25-5 μg/g) were examined for two days. For quantification, the peak area measurements were used. The limits of quantification (LOQ) were set to 0.04 μg/g in muscle plus skin. The recovery of the method was calculated by comparing the determined concentration of spiked samples with those of standard solutions (Table 2). The limits of detection (LOD) and the limits of quantification (LOQ) were estimated to be 3.3*σ/S and 10*σ/S, respectively (σ = standard deviation of the y-intercept of the regression line; S = slope of the calibration curve) and indicate good sensitivity of the method.

Results and Discussion

The calculated calibration curves presented herein reflect a successful linear relationship for PZQ over the range of 0.01-10 μg/mL for *S. aurata* muscle plus skin with coefficients of correlation greater than 0.999. The average recovery rates of PZQ were estimated to be 84.5% in the spike tissues, an indication that the analytical protocol used for PZQ detection was also sufficient. Previously, the recoveries of PZQ in muscle were estimated to be 100% in *S. aurata* (Baralla *et al*., 2020), 79- 100% in *O. mykiss* (Rogstad *et al*., 1987; Hormazábal & Yndestad, 1995), 82.7% in *S. schlegeli* (Kim *et al*., 2001), 93- 100% in *M. albus* (Xu *et al*., 2016) and 95.2% in Pacific bluefin tuna (*Thunnus orientalis*) (Ishimaru *et al*., 2013).

Measured PZQ concentrations in muscle plus skin samples of gilthead sea bream fed a PZQ-dosing of 150 mg/kg for 3 days are presented in Table 3. Depletion of PZQ from muscle plus skin was very rapid in the tested fish, given that concentrations decreased to 0.04 μg/g as early as 24 h post-treatment, while they were not detectable after 72 h. This information confirms the fact that PZQ is rapidly removed and does not accumulate in farmed animals (EMEA, 1998), including fish such as *S. aurata*. Importantly, the drug was undetectable 3 days after treatment completion at 25°C or in approximately 75 dd.

In other farmed fish species, removal of PZQ has also exhibited a rapid profile. Specifically, in *O. mykiss* kept at 10.5°C and administered a single dose of 50 mg/kg fish, using, the compound was not detectable in muscle at almost 224 dd (Soukupova-Markova *et al*., 2016). Earlier, in *O. mykiss* kept at 12 or 18°C and force-administered a PZQ-dosing of 500 mg/kg fish (tube), it was demonstrated that PZQ in muscle among other tissues, decreased rapidly at both temperatures tested. In particular, 32 h after administration, 67-96% of the maximum quantities had been excreted (Björklund & Bylund, 1987). In *M. albus* fed 10 mg PZQ/kg fish for 3 consecutive days at 22°C, PZQ was not detected on the 3rd and 4th day after completion of the treatment in muscle and skin, respectively (Xu *et al*., 2006). Comparably, in *S. schlegeli* kept at 19 - 20°C and fed 200 mg/kg fish for 3 days, PZQ was detectable in muscle and skin tissue until 1 and 3 days post-treatment, respectively (Kim *et al*., 2003). In the same study, a higher dose of 400 mg/kg fish caused a delay in PZQ removal from the analyzed tissues; drug concentrations in muscle or skin 5 and 6 days after therapy were measured. Lastly, in *T. orientalis* following a single dietary administration of 15 mg PZQ/kg fish, PZQ was undetectable after 24 h in all examined tissues including muscle (Ishimaru *et al*., 2013).

Concerning PZQ excretion, it has been suggested that

Table 3. Muscle plus skin concentrations of PZQ in gilthead sea bream receiving a dose of 150 mg/kg/day for 3 consecutive days at 25°C (mean ± st.dev.), n=10.

Sampling time (days after treatment)	Muscle (μg/g)
1	0.04 ± 0.006
2	< LOQ
3	n.d
4	n.d
5	n.d
7	n.d

n.d: not detected

Table 2. Recovery rate and RSD (%) of PZQ in spiked gilthead sea bream muscle plus skin samples (n=3).

PZQ added (μg/g)	Recovery (%)	Intra-day RSD (%)	Inter-day RSD (%)
0.25	98.5 ± 1.9	2.0	1.9
0.5	78.1 ± 1.7	2.1	3.3
1	82.8 ± 2.2	2.6	2.7
5	78.6 ± 1.3	1.7	2.4
Average	84.5		

PZQ is mainly excreted with bile and partly through the kidneys since large amounts of the drug were found in the bile fluid and hind kidneys of *O. mykiss* (Björklund & Bylund, 1987). However, the physiological differences among fresh and marine fish should be taken into consideration in drug excretion. PZQ is also subjected to metabolic processes in fish and appears to be metabolized into hydroxylated derivatives in kingfish (*Seriola lalandi*) (Tubbs *et al*., 2008). The factors affecting the metabolism of PZQ in fish such as *S. aurata* require further investigation although the estimated parent PZQ concentration in edible *S. aurata* tissues will not be affected.

In conclusion, available data on the removal of PZQ from edible fish tissues signifies that PZQ can be used safely in *S. aurata*. The anthelmintic efficacy of PZQ against gill parasites of *S. aurata*, a subject of parallel investigation at the Institute of Marine Biology, Biotechnology and Aquaculture, will provide added value to the results presented herein.

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