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7. Development of a strategy for the administration of praziquantel to the terrestrial edible snail *Cornu aspersum* parasitized by *Brachylaima* sp. metacercariae

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Abstract. *Cornu aspersum* is a terrestrial edible snail, often parasitized by *Brachylaima* (Trematoda) metacercariae. Ingestion of undercooked snails by humans allows metacercariae to develop to adult in the intestine causing brachylaimiasis (expected mortality rate 5-10%). The treatment of these snails with praziquantel in the farms where they are reared would be a tool to control this food-borne disease. In the present work, a strategy has been proposed to administer the drug to the snails including quality control of the manufacturing of the feeding stuff supplemented with praziquantel and its acceptance by the snails.

Introduction

Cornu aspersum (= syn. *Helix aspersa*) (Müller, 1774) (Gastropoda: Helicidae) is a terrestrial edible snail of commercial interest. *Brachylaima*

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(Trematoda: Brachylaimidae) metacercariae often parasitize the kidney of the gastropod in nature and marketplaces [1, 2], where the prevalence of parasitization can reach 85.4%. Ingestion of undercooked snails by humans allows metacercariae to develop to adult in the intestine causing brachylaimiasis [3, 4]. The main presenting symptoms are diarrhoea (100%), abdominal pain (58%), anorexia (58%), and weight loss or poor weight gain (58%) due to the hematophagous nature of *Brachylaima* sp. intestinal adults [5, 6]. In Australia up to 13 human cases have been recorded [3, 4, 5]. Additionally, brachylaimiasis could reach a medical concern considering that in some trematode infection, eggs can enter the systemic circulation and reach other tissues, as cardiac one, and other organs producing pathological lesions [7, 8, 9]. In this sense, two of the human brachylaimid cases in Australia had cardiac conditions in which systemic trematode infection could not be entirely discarded [3]. Meerburg et al. [10] classified brachylaimiasis as pathology with an expected mortality rate in humans (without treatment) between 5 and 10% and claimed livestock and food products as one of the most important via to spreading this snail-borne disease to human. In Australia all recorded cases were produced due to accidental ingestion of snails. However, in countries such as China, France, Spain, Italy, Romania, etc., the consumption of snails is not accidental but usual, because snails are commonly consumed being part of the human diet [11, 12]. The Iberian Peninsula is the geographic area where the consumption of *C. aspersum* is of the most importance [13]. The countries which imported the greatest amount of snails in 2015 according to data retrieved from COMTRADE from United Nations [14] are: Spain (11,636,006 kg), France (2,793,846 kg), Portugal (1,896,886 kg), Greece (1,435,169 kg), Bosnia Herzegovina (1,067,479 kg), Romania (956,272 kg), Czech Republic (839,391 kg), Italy (807,872 kg), Turkey (674,197 kg), Hungary (387,782 kg), Malaysia (338,737 kg), Lithuania (278,841 kg), Hong Kong (276,153 kg), China (258,269 kg), and Serbia (241,844 kg).

This scenario implies a higher risk of human parasitization, and its consequent acquisition of brachylaimiasis, a disease which is actually underdiagnosed for several reasons. Firstly, the observation of eggs in human feces is difficult due to the small size of the eggs (25-30 μm length), secondly, the diagnosis is not under of the regular clinical practices in areas where the snail consumption is usual, and thirdly, the clinical signs are largely unspecific. Up to the date, snails are not subject to any parasitological control prior its distribution in public marketplaces. In this context, the development of a pharmacological treatment of farmed snails would be a potential tool to control brachylaimiasis.

1. *Cornu aspersum* and heliciculture farms facilities

C. aspersum presents a big cone-globulus shell with a high spiral, hard and opaque, being its height between 18-40mm and its maximum diameter 20-45 mm. Its color varies from brown to grey, including five darkened lines. Its shell aperture is wide, obliquus and rounded. The peristome is thick, reflected and whitish [15, 16].

Heliciculture farms (Fig. 1) are focused on the rearing of snails, the most under semi-intensive conditions (temperature is not controlled and humidity is managed through sprinklers). These farms are delimited by fences and covered by a net to protect snail from invaders such as small mammals or birds. The emplacement is divided through rails containing herbage, where snails are located. The snail food is placed on a plastic feeding located over a metallic frame supporting mesh pieces vertically disposed. (Fig. 2) [15].

A 100 m2 snail farm could allow about 17,000 adults. According to Chevallier [15] the maximum density of *C. aspersum* adults (9g) would be 170 snails/m2, and for young specimens the maximum density are 300 snails/m2 (5g) and 750 snails/m2 (2g).

Figure 1. Heliciculture farm in Spain. Detail of rails containing herbage where the snails are reared.

Figure 2. Heliciculture farm in Spain. Detail of mesh pieces vertically disposed supported by a metallic frame where snails rest.

2. Strategies of the administration of Praziquantel in the veterinary field

Praziquantel (PZQ) is an anthelmintic drug extensively used in the laboratory, as well as in the field, mainly against nematodes and trematodes. In the laboratory, PZQ has been administrated as subcutaneous injection single dose in hamsters parasitized by *Schistosoma magrebowiei* [17], or by oral administration single dose through gastric tube or syringe in mice parasitized by *Schistosoma japonicum*, in hamsters parasitized by *Echinostoma paraensei*, and in albin rats parasitized by *Fibricola seoulensis* [18, 19, 20]. In farms or natural reserves, oral administration is the widest used, rarely as single dose through syringe assistance, for instance the case of goats parasitized by *Schistosoma bovis* [21], and mostly as medicated feeding stuff. This last way is the most common strategy not only intended for PZQ [22, 23], but as well for other drugs [24, 25, 26, 27, 28]. Notwithstanding, heliciculture farms are distinguished from common farms or natural reserves, because of the extremely high number of specimens reared in (See section 1) [15]. Hereby, the only economically viable strategy to treat snails would be to administer it

through medicated feeding stuff in the usual feeding system of the heliciculture farm.

The most recent works on gastropods treated with PZQ, involve species such as the aquatic snail *Lymnaea stagnalis* (Gastropoda: Lymnaeidae) parasitized by *Echinoparyphium aconatium*, treated with a bath of PZQ (10 mg/L) [29], and *Biomphalaria glabrata* infected with *Schistosoma mansoni* treatead with PZQ over a 72 h period, in this case in the food (20-30 micrograms/g body weight including shell weight) [30].

3. Chemistry of Praziquantel

PZQ is a lipophilic compound with a molecular weight of 312.40, slightly soluble in water, and easily soluble in alcohols and chloroform. It is a white crystalline powder, not odorous, with a bitter taste (Fig. 3).

Nowadays, the mechanism of action of PZQ remains unknown. PZQ produces a disruption of Ca2+ homeostasis, initiating a cascade of events, such as a sustained contraction of the worm musculature, and vacuolization and disruption of the parasite tegument, which leads to the elimination of parasites from the host [31].

Despite of the unknown mechanism of action, PZQ remains being the drug of choice for the treatment of trematodosis. Its effectiveness has been

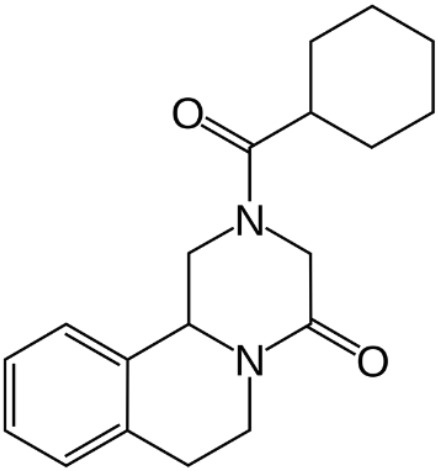

Figure 3. Chemical structure of praziquantel.

demonstrated in fish, goat and ovine species intended for human consumption [21, 22, 32]. In regards of trematodes, PZQ has been used against *Clonorchis sinensis* [33], *Echinostoma paraensei* [19] and *Schistosoma mansoni* [30, 34], *Schistosoma japonicum, Dicrocoelium dendriticum, Metagonimus yokogawai, Opisthorchis viverrini, Paragonimus westermani,* and *Neodiplostomum seoulense* [35].

4. Dosage design and snail food consumption by *C. aspersum*

Data on snail food consumption by *C. aspersum* were provided by heliciculture professionals in farms: 150 g of snail food per 4.14 kg snails without shell each day.

It was decided to prepare an initial dosage form considering 400 PZQmg/snail kg, with the purpose of developing an optimal dosage form mixing PZQ with snail food. Being calculations as follow:

$$\frac{400\ PZQmg}{1\ kg\ snails} \times \frac{4.14\ kg\ snails}{150\ g\ snail\ food} = \frac{1656\ PZQmg}{150\ g\ snail\ food} = \frac{1.656\ PZQg}{0.150\ kg\ snail\ food} = \frac{11.04\ PZQg}{1\ kg\ snail\ food}$$

Taking into account that ulterior experiments could involve a design with boxes of 10 specimens each, it must be calculated as well the adequate amount of snail food to place in each box to cover the nutritional needs of the snails. An adult specimen in markets without shell is about 4-5 g. Thus, a 10 adult specimen group would have a total weight of 50 g without shell. Considering what stated in the previous paragraph it is possible to calculate the group consumption as follows:

$$0.050\ kg\ snails \times \frac{150\ g\ snail\ food}{4.14\ kg\ snails} = 1.81 g\ snail\ food$$

5. Manufacturing the snail food supplemented with Praziquantel

According to the European Pharmacopoeia 9.2 [36] veterinary oral powders are preparations consisting of slid, loose, dry particles of varying degrees of fineness. They contain one or more active substances, with or without excipients. They are generally administered in or with water or another suitable liquid, as well as they may be swallowed directly. And premixes for medicated feeding stuffs for veterinary use are mixtures of one or more active substances, usually in suitable bases, that are prepared to facilitate feeding the active substances to animals. Premixes occur in granulated, powdered, semi-solid or liquid form. When used as powders or

granules, they are free-flowing and homogeneous; any aggregates break apart during normal handling. The particle size and other properties are such as to ensure uniform distribution of the active substance in the final feed. Unless otherwise justified and authorized, the instructions for use state that the concentration of a premix in granulated or powdered form is at least 0.5% in the medicated feeding stuff [36].

Taking into account the statements from the Pharmacopoeia 9.2, a first lot of 0.150 kg of snail food supplemented with PZQ was manufactured containing 11.04 PZQg/kg snail food. The amount of PZQ to be incorporated was calculated as follows:

$$\frac{11.04\ PZQg}{1\ kg\ snail\ food} \times 0.150kg = 1.656g\ PZQ$$

PZQ (Lot L11060258, 99.52% pure) amount was weighted (1.656 g) as well as the snail food (148.34 g) and manually mixed through mortar, firstly incorporating the total amount of PZQ and, adding small amounts of snail food in three times until adding the total amount. The total mixing time was controlled by a timer being it 20 minutes. A sample of 10 g of the mixture was taken to be analyzed through HPLC-MS/MS.

6. Quality control of the snail food supplemented with Praziquantel

Due to the characteristics of the mixture and the total amount of the lot, an uniformity of mass test was performed to assess homogeneity of the mixture, being the acceptance value of 15%. For this purpose, it was used a HPLC-MS/MS method to determine PZQ in the mixture: 50 mg of supplemented snail food (11.04 PZQmg/g snail food) were diluted in 10 ml of methanol (HPLC-grade), the supernatant was filtered through a Durapore PVDF 0.45 Mm Ø Millipore Millex-HV syringe driven unit and disposed in a vial and diluted 1:10 prior to injection in HLPC-MS/MS.

Theoretical concentration of the solution for injection was calculated as follows:

$$50\ mg\ of\ PZQ\ suplemented\ snail\ food \times \frac{11.04\ PZQmg}{1000\ mg\ supplemented\ snail\ food} \times \frac{1}{10\ ml\ methanol} \times \frac{1000\ PZQ\ \mu g}{1\ PZQ\ mg} \times \frac{1}{10\ ml\ methanol} = 5.52\ \mu gPZQ/ml$$

The HPLC-MS/MS conditions comprehended an Acquity UPLC system, equipped with a binary pump and DAD detector (Waters, Milford MA, USA) and coupled to an API 3000 triple quadrupole mass spectrometer (Sciex, Concord, Ont., Canada). The reversed stationary phase employed was a Luna C18 column 50 x 2.0 mm id., 5 µm; Phenomenex, Torrance, CA, USA). The mobile phase was acetonitrile as eluent A and 0.1% acetic acid in Milli-Q water as eluent B. The elution started at 25% of A and was increased linearly up to 65% of A in 2.5 min and kept isocratic for 0.5 min. It was returned to the initial conditions in 0.1 min and the reequilibration time was 1.9 min. The flow rate was 1.00 ml/min. All the samples were filtered through 0.22 µm filters before the chromatographic analyses and the injection volume was 10 µl.

Ionization was achieved with a TurboIonSpray interface (ESI) operating in positive mode and data were collected in multiple reaction monitoring mode (MRM). The ionization parameters were: capillary voltage 5000 V, nebulizer gas (N2) 8 (arbitrary units), curtain gas (N2) 8 (arbitrary units), collision gas (N2) 4 (arbitrary units), declustering potential (DP) 100 V, focusing potential (FP) 300 V, entrance potential (EP) 10 V, collision energy (CE) 30 V, and drying gas (N2) heated up to

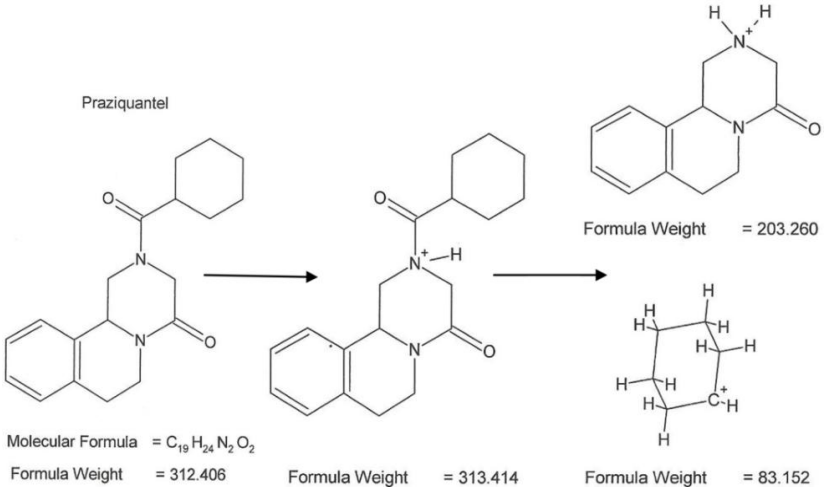

Figure 4. HPLC-MS/MS Praziquantel fragmentation: quantification transition (up) and confirmation transition (down).

350ºC at a flow-rate of 5000 cm3 min–1. MRM acquisition involved recording two transitions, 313.2/203.3 and 313.2/83.2, with a dwell time of 200 ms. First transition was used for quantitation purposes and the second one only for confirmation (Fig. 4), as it was more likely that the

Table 1. Calibration curves: Integrated peak areas per standard, back-calculated concentration and accuracy compliance criteria.

Methanol Standards (µg/ml)	Integrated Peak Area	Back-calculated Concentration (µg/ml)	Accuracy compliance limits ±15%	
			Lower limit	Upper limit
0,8	656225.153	0.774	0.68	1.48
1	952262.966	1.014	0.85	1.85
2	2134266.52	1.975	1.7	3.7
4	4836404.97	4.172	3.4	7.4
6	6805530.36	5.773	5.1	11.1
8	9656052.9	8.091	6.8	14.8

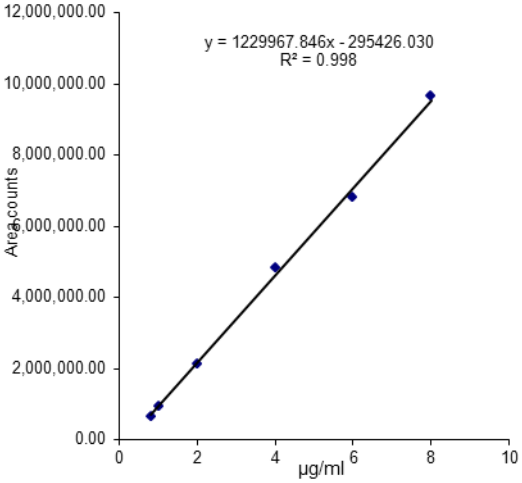

Figure 5. Calibration curve for Praziquantel-Methanol (HPLC grade) determination through HPLC-MS/MS.

Table 2. Praziquantel supplemented snail food quality control through HPLC-MS/MS analysis.

Sample	Integrated Peak Area	Experimental Concentration (µg/ml)	%
Injection 1	7282387.79	6.16	111.61
Injection 2	6924402.25	5.87	106.34
Injection 3	7326523.81	6.20	112.26
Injection 4	6923174.75	5.87	106.32
Injection 5	6786768.99	5.76	104.31
	Mean concentration	5.97	108.17
	Theoretical concentration	5.52	SD: ±0.20

analyzed ions were from PZQ molecules. Analyst® version 1.4 software (Sciex) on a PC was used for data acquisition and processing.

Calibration curves (Table 1 and Fig. 5) were prepared with the following standards: 0.8, 1.0, 2.0, 4.0, 6.0, and 8.0 µg/ml (PZQ/methanol HPLC grade). Each standard was prepared from a mother solution of 4 mg/ml (weighting 40 PZQmg and diluting in 10 ml of methanol). The mother solution was diluted 1:100 to obtain a 40 µg/ml working solution. The linearity was r^2=0.998 and accuracy compliant with all standards. The sample was analyzed through a series of 5 injections.

Integrated peak areas from analysis injection of the solution, used to extract PZQ from the snail food supplemented (see first paragraph of this section), were used in the calibration curve (Figure 5) in order to calculate the experimental concentration. In basis of the theoretical concentration, it was calculated the percentage of the experimental concentration to assess the uniformity of mass. Results over 100% indicate that the solution was more concentrated, and results under 100% indicate the opposite. If big discrepancies appear among results, the manufacturing procedure must be corrected. According to the results, the prepared lot was compliant with the ±15% acceptance value variation, with a mean value of 5.97 µg/ml, being the standard deviation (SD) ±0.20, and the calculated variation value +8.17, which easily fit the requirement ±15% (Table 2).

7. Acceptance of the snail food supplemented with Praziquantel by *C. aspersum*

PZQ taste is bitter and disgusting for humans [37]. Also, the bitterness of PZQ has been reported as a problem to achieve satisfactory treatment against monogenean parasites in the yellowtail kingfish [38]. The poor acceptance of PZQ has become of economic concern since in several livestock species satisfactory treatment was not achieved. However, poor acceptance of PZQ is not the only concern, especially in snails, were mortality rates were up to 73.4% in *Biomphalaria glabrata* after PZQ treatment [30, 34]. For this reason, the acceptance by *C. aspersum* was tested, as well as potential toxicity prior to any other step.

Taking into account that 4.14 kg of snail without shell consume daily 150 g snail food, the daily amount consumed by one specimen (4-5 g weight) without shell is about 181 mg. For the acceptance test purpose, 10 specimens of *C. aspersum* were individually disposed in plastic boxes (23 cm x 123 cm x 8 cm) covered with a net, maintained at room temperature under natural light-dark cycle, and sprayed with tap water twice in a day. An amount of 450 mg of snail food supplemented with PZQ was disposed on a small plastic plate (8.5 cm x 7.0 cm) inside each box every day along 3 days. Daily food consumption was controlled per specimen, weighting the snail food each 24 hours.

Table 3. Snail food consumption per day and daily mean (mg).

Specimen	Snail food consumption (mg)				
	Day 1	Day 2	Day 3	TOTAL	Daily mean
1	198	0	0	198	66.0
2	0	244.3	0	244.3	81.4
3	219.5	46.8	0	266.3	88.8
4	250.8	55.7	0	306.5	102.2
5	0	253	173.9	426.9	142.3
6	250.5	0	357.8	608.3	202.8
7	199.1	278.7	250.4	728.2	242.7
8	229.9	384.1	137	751	250.3
9	250	299.4	303.6	853	284.3
10	250.3	305.1	348.9	904.3	301.4

Table 4. Temperature registry: maximum, minimum and mean for snail food consumption test.

Day	Máxima (ºC)	Mínima (ºC)	Media (ºC)
Day 1	28.9	21.2	25.1
Day 2	26.8	22.2	24.5
Day 3	24.1	22.7	23.4

The results of the acceptance test are presented in Table 3 and the registry of temperatures in Table 4.

The global daily mean consumption corresponded to 176.2 mg/day/snail being really close to the theoretically calculated 181 mg/day/snail. *C. aspersum,* apparently, did not reveal any toxic effect, since none snail consuming PZQ supplemented snail food died during the test. In regards of acceptability, the global mean consumption value was almost the theoretically calculated, suggesting that the bitter taste is not an issue for *C. aspersum.* Hereby, the mixture 11.04 PZQmg/g snail food appears to be suitable for further experiments.

8. Conclusion

In this work the most suitable way to administer PZQ to the terrestrial edible snail *C. aspersum* in a laboratory or a farm has been assessed. The oral administration of PZQ using PZQ supplemented snail food in *ad libitum* basis has been well tolerated by the snails, taking into account the average consumption of a snail per day. The manufacturing of PZQ supplemented snail food (11.04 PZQmg/g snail food) largely accomplished the acceptance value of 15% through HPLC-MS/MS analysis, assuring homogeneity of the mixture and dosage. The PZQ mixture, 11.04 PZQmg/g snail food, was accepted by *C. aspersum* despite of the bitter taste. Problems described by other species like fishes [37] did not appear. The PZQ supplemented snail food displayed an appropriated toxicological profile since mortality rates were 0% in comparison with other snails like *Biomphalaria glabrata*, which supported a mortality rate of 74.3% [30, 34]. The proposed strategy would be an easy and affordable approach to test drug efficacy in the laboratory and, in the future, in field trials.

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