European Journal of Pharmaceutics and Biopharmaceutics 142 (2019) 240–246

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Activity and pharmacokinetics of a praziquantel crystalline polymorph in the *Schistosoma mansoni* mouse model

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ARTICLE INFO

Keywords:
Schistosoma mansoni
Praziquantel
Polymorph B
Pharmacokinetics
Activity

ABSTRACT

Schistosomiasis is a global disease of significant public health relevance. Only one racemic drug, praziquantel, characterized by low bioavailability, low water solubility and extensive first pass metabolism, is currently available. We studied a new praziquantel formulation (polymorph B), which is based on a racemic praziquantel crystalline polymorph (TELCEU01). Its *in vitro* activity was tested on newly transformed schistosomula (NTS) and adult *Schistosoma mansoni*. *In vivo* studies were conducted in mice harboring chronic *S. mansoni* infections. Pharmacokinetic (PK) profiles of R- and S-praziquantel and R- and S- polymorph B following oral administration with both formulations were generated by sampling mice at 30, 60, 240 min and 24 h post-treatment, followed by LC-MS/MS analysis. PK parameters were calculated using a non-compartmental analysis with a linear trapezoidal model. *In vitro,* commercial praziquantel and the polymorph B performed similarly on both NTS (IC_{50} = 2.58 and 2.40 μg/mL at 72 h) and adults (IC_{50} = 0.05 and 0.07 μg/mL at 72 h). Praziquantel showed higher *in vivo* efficacy with an ED_{50} of 58.75 mg/kg compared to an ED_{50} of 122.61 mg/kg for the polymorph B. The PK profiles of the two drugs exhibited differences: R-praziquantel showed an overall 40% higher area under the plasma drug concentration–time curve ($AUC_{0\rightarrow 24}$) (R-praziquantel = 3.42; R-polymorph B = 2.05 h*μg/mL) and an overall 30% lower apparent clearance (Cl/F) (R-praziquantel = 70.68 and R-polymorph B = 97.63 (mg)/(μg/mL)/h). Despite the lack of improved activity and PK properties of polymorph B against *S. mansoni*, here presented; research on pharmaceutical polymorphism remains a valid and cost-effective option for the development of new praziquantel formulations with enhanced properties such as increased solubility and/or dissolution.

1. Introduction

Schistosomiasis is a global disease, which predominantly affects countries in Sub-Saharan Africa and some parts of Asia and South America [1,2]. An estimated 779 million people are at risk of infection, of which more than 50% are children [3]. More than 200 million people are infected [3,4], with *Schistosoma haematobium*, *S. mansoni* and *S. japonicum* being responsible for the bulk of infections [1]. There is no vaccine available and the only drug marketed for treatment against *Schistosoma* spp. infections is praziquantel. Alarmingly, the drug pipeline against schistosomiasis is empty. Praziquantel is commonly used in mass drug administration (MDA), so-called preventive chemotherapy [1,5]. For example, in 2016 alone, about 89 million doses of praziquantel were distributed in Sub-Saharan Africa [6,7]. However, it is not a perfect drug, primarily because of its low efficacy against juvenile stages of *Schistosoma* spp., high inter-individual variability of effects and poor compliance [8-11]. Praziquantel is a racemic compound, composed by 50% of R-praziquantel and 50% of S-praziquantel. Of these two enantiomers, R-praziquantel is the main active form. S-praziquantel shows less activity, while being responsible for the bitter taste [12,13].

According to the biopharmaceutical classification system (BCS), praziquantel belongs to the class II drug category, because of its high permeability and low solubility (0.4 mg/mL) [14,15]. Praziquantel also has a high first pass metabolism (1–3 h), which converts the active R-praziquantel into inactive metabolites very rapidly [10,16]. To overcome the low bioavailability, a crystalline polymorph of racemic praziquantel was prepared. The novel polymorph, polymorph B, as well as commercial praziquantel, consists of a mixture of R- and S- enantiomers.

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https://doi.org/10.1016/j.ejpb.2019.06.029
Received 22 February 2019; Received in revised form 3 June 2019; Accepted 28 June 2019
Available online 29 June 2019
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For the preparation of the polymorph B, standard praziquantel was milled by neat grinding in a vibrational mill [17]. During the milling process in suitable conditions, in absence of solvents, the standard praziquantel turns into a new polymorphic anhydrous crystalline form, polymorph B [17], indexed as TELCEU01 in the Cambridge Structural Database [18]. As widely known, a polymorphic variety has different physical properties with respect to the standard crystal form, due to the different crystalline lattice [19–22]. In this case, polymorph B, forming a monotropic pair with commercial praziquantel crystal form, is characterized by double water solubility, and doubled intrinsic dissolution rate in comparison to the starting solid form [17]. In addition, it is a promising product because it is physically stable at the solid state for at least 1 year at ambient temperature [17]. Moreover, the polymorph B showed promising results in preliminary *in vitro* and *in vivo* studies on adult *S. mansoni* [17].

The aim of the present study was to thoroughly evaluate whether polymorph B would offer benefits over standard praziquantel such as increased efficacy and improved pharmacokinetic (PK) parameters. We conducted an in-depth side by side comparison of praziquantel and the polymorph B formulation (as aqueous suspensions). Both *in vitro* and *in vivo* studies as well as a PK analysis of the praziquantel polymorph B and standard praziquantel were performed. *In vitro* studies were conducted on the larval and adult stages of *S. mansoni*. ED_{50} values were determined in mice harboring a chronic *S. mansoni* infection. Finally, we determined PK parameters of R- and S-praziquantel and R- and S-polymorph B following treatment with both formulations in mice using a validated liquid chromatography (LC) tandem mass spectrometry (MS-MS) method (LC-MS/MS).

2. Materials and methods

2.1. Reagents

Ammonium formate (cat. no 70221-25G-F), formic acid (cat. no 5.33002.0050), ammonium acetate (cat. no. 73594-25G-F), methanol (cat. no. 1.06035.2500), acetonitrile (cat. no. 1.00029.2500), and 2-propanol (cat. no. 1.02781.1000) were MS grade. All MS reagents were purchased from Sigma-Aldrich (Buchs, Switzerland). Dimethylsulfoxide (DMSO) was the product of Sigma-Aldrich, Buchs, Switzerland, (cat. no. 276855-2L). Ultrapure water was filtered using a Millipore MilliQ water purification system (Merck Millipore, MA, USA). Human blood was supplied in lithium heparin-coated vacutainer tubes (Becton Dickinson (BD), Allschwil, Switzerland, cat. no. 367962) by the local blood donation center (Basel, Switzerland). Internal standard (ISTD) praziquantel d11 was purchased from Toronto research (Toronto, Canada, cat. no. P702097). Praziquantel as racemic powder was purchased from Sigma Aldrich (Buchs, Switzerland, cat. no. P4668-5G).

Penicillin/Streptomycin 10′000 U/mL (Sigma-Aldrich, Buchs, Switzerland, cat. no. P4333-100ML) and inactivated fetal calf serum (iFCS, Bioconcept AG, Allschwil, cat. no. 2-01F30-I) were purchased from Bioconcept AG (Allschwil, Switzerland). M199 medium and RPMI 1640 were obtained from Gibco (Waltham, USA cat. no. 22340-020). All media were filter sterilized using a 0.22 μm filter bottle (Corning Stericup 500 mL, Vitaris AG, Allschwil, Switzerland, cat. no. 431097-COR).

2.2. Praziquantel polymorph B

The preparation of the crystalline polymorph B was realized via a neat grinding process of standard racemic praziquantel in a vibrational mill as described elsewhere [17]. Briefly, this treatment resulted in the high yield and low costs production of a racemic crystalline polymorph B, physically stable for at least one year. The crystalline form of the praziquantel underwent chemical analysis by HPLC, NMR and polarimetry, confirming that the chemical entity of the praziquantel remained the same. Further analysis reported different physical properties of the polymorph B compared to standard praziquantel, such as increased water solubility and lower melting point [17].

2.3. In vitro studies with newly transformed schistosomula (NTS)

Biomphalaria glabrata snails infected with a Liberian strain of *S. mansoni* were placed under a neon light to allow cercarial shedding for 3–4 h. The cercarial suspension was then transformed using a technique based on the mechanical transformation proposed by Milligan & Jolly [23]. The obtained NTS were incubated at 37 °C with 5% CO_2 overnight. The NTS were resuspended at 2 NTS/μL in M199 medium supplemented with 1% v/v penicillin/streptomycin, 5% v/v iFCS. The proper volume of medium was added to each well of a 96-well-plate and supplemented with the appropriate volume of drug. A volume of 50 μL of NTS solution (100 NTS) was dispensed into each well [24]. Both praziquantel formulations were first suspended in DMSO to a working concentration of 10 mg/mL. The drug aliquots were kept at −20 °C until use. Polymorph B and praziquantel concentrations evaluated ranged from 100 to 0.78 μg/mL (320–2.50 μM). For the negative controls (in triplicates), DMSO was used at a final volume of 1% v/v. Each assay was evaluated every 24 h [25–27]. The parasites were given a score between 0 and 3, depending on their viability. On this scale, 0 represents dead parasites and 3 indicates alive and undamaged parasites [28,29]. The scores are then used to compute the IC_{50} curves with Compusyn® [24,30].

2.4. In vitro studies with adult S. mansoni

Schistosoma mansoni adult worms were recovered from female NMRI mice 7 weeks post infection. Mice were dissected after CO_2 euthanasia and cervical dislocation. The intestines and the liver were excised and analyzed with a dissection microscope. The adult worms were recovered from the organs by manual picking, using flat-tip tweezers. Alive worms were recovered and incubated in a Petri dish in supplemented RPMI 1640 at 37 °C with 5% CO_2, for up to 3 days. For the IC_{50} calculation, 2 worm pairs or 3 single worms were randomly chosen from the Petri dishes. The worms were then placed in 24 well plates at a final volume of 1.6 mL in fully supplemented RPMI 1640. Duplicates or triplicates, depending on the worm availability, were performed for every condition. Negative control wells consisted of medium with 1% v/v DMSO. The polymorph B and the standard praziquantel were tested using a concentration range of 0.45–0.05 μg/mL (1.44–0.16 μM). The drug effect was evaluated every 24 h by visual scoring with a light microscope, as described above, using a magnification of 4-10X. Similarly to the NTS, the adult *S. mansoni* were given a score from 0 to 3, depending on their viability. On this scale, 0 represents dead parasites and 3 indicates alive and undamaged individuals [24]. As for the NTS procedure, the IC_{50} values were calculated using Compusyn® [30].

2.5. In-vivo studies

All animals were ordered from Charles-River (Sulzfeld, Germany). Animal experiments were conducted in accordance with the local cantonal veterinary guidelines, license number 2070. A total of 66 three-week-old female NMRI mice were used for this study. Upon arrival, the animals were left for one week for acclimatization. The animals were housed at 25 °C in a controlled environment (temperature ~ 25 °C; humidity ~ 70%; 12-hour light and 12-hour dark cycle) with free access to water and rodent diet. The mice were infected subcutaneously with 100 *S. mansoni* cercaria in the neck area. Seven weeks post-infection, mice were treated with 400, 300, 200, 100, 50 mg/kg of the polymorph B or praziquantel. The oral suspensions were freshly prepared in a vehicle, composed of 90% v/v tap water and 10% v/v ethanol-Tween 80 (7% v/v Tween 80 and 3% v/v absolute ethanol). The administered volume was calculated for each mouse weight, as described elsewhere [27]. Each treatment arm had four mice, randomly

chosen within the same infected batch. After treatment, the mice were monitored daily, for the next 21 days, before undergoing CO_2 euthanasia. The mice were then dissected and their livers were excised. Adult worms were picked, sexed and counted as described above. The worm burden reduction (WBR) was calculated by comparing the average number of recovered worms from each treatment arm to the control arm. The detailed *in vivo* procedure is described elsewhere [24]. The following formula was used for the evaluation of the WBR [27], where WB represents the average worm burden.

$$WBR(\%) = 100 - \left(\left(\frac{100}{WB_{Average_{Control}}}\right) * WB_{Average_{Test}}\right)$$

ED_{50} values were determined using the WBR and the doses using CompuSyn.[1]

2.6. Blood micro sampling for establishing pharmacokinetic profiles in mice

The day of drug administration of the polymorph B or praziquantel, plasma samples were collected from the infected mice by tail microsampling using 75 mm sodium hepatized microhaematocrit capillary tubes (Paul Marienfeld, cat: TX79.1, Lauda-Königshofen, Germany) at 30, 60, 240 min, and 24 h post-drug-administration. Volumes of 50 µL of whole blood were collected into capillary tubes. Each capillary tube was sealed on one side with one cm of wax by pressing them gently on a wax plate (Paul Marienfeld, Lauda-Königshofen, Germany, cat. no. 2960409). The tubes were placed in a microcentrifuge (Sigma-Zentrifugen, 1-16 special edition, Osterode am Harz, Germany) and centrifuged for 5 min at 5000 rpm at room temperature. The plasma was pipetted into a previously labelled 1.5 mL tube (Eppendorf, Hamburg, Germany). Finally, the tubes were stored at −80 °C until further analysis.

2.7. Preparation of the standards for LC-MS/MS

Quality controls (QCs) and calibration line (CL) samples were freshly prepared before every experiment. 2 µL of blank mouse plasma were mixed with 6 µL of blank human plasma. The blank mouse plasma was obtained from the control mice by heart puncture. Blank human plasma was obtained from the local blood donation centre (Basel, Switzerland). The plasma mixture was spiked with 2 µL of the appropriate drug concentration. The spiking solution was prepared in 10% v/v methanol and 90% v/v milliQ water. The CL covered the following drug concentrations: 0.01, 0.025, 0.05, 0.1, 0.25, 1.0, 2.0, 4.0 µg/mL. The QCs were prepared by adding the appropriate volume to obtain the following drug concentrations: 0.01, 0.1, 0.5, 2.0 µg/mL. Each validation set contained the 4 quality control (QCs) concentrations in 6 replicates each, plus a calibration line with 9 points. The QCs covered the lower limit of quantification (LLOQ), low, middle and high concentrations as recommended by the Food and Drug Administration (FDA) guideline for industry for bioanalytical method validation [31]. Each set was accepted within the partial validation criteria described below. Praziquantel was dissolved in 100% methanol to a final concentration of 5 mg/mL. This solution was used for preparing the QCs and CLs. The stock of internal standard (ISTD) was resuspended at a concentration of 1.25 mg/mL in methanol. The ISTD working solution was prepared in 20% v/v milliQ water, 80% acetonitrile and 400 ng/mL d11-PZQ and it was used as extraction solution for the samples. The spiking solution used was in 10% methanol v/v and 90% milliQ water v/v.

2.8. Validation of the LC-MS method

The quantification of the praziquantel enantiomers in this study was adapted from an already validated method for human plasma using a lower sample volume and a slightly changed matrix [32]. The adapted matrix for this study was a mixture of human and mice plasma at a ratio 3:1. A partial validation was performed by running 3 complete validation sets on three different days. Inter-day precision, accuracy, matrix-effect and recovery were evaluated.

The accuracy was calculated as the percentage of the measured concentration compared to the nominal spiked concentration. The precision was calculated as the percentage of the standard deviation of multiples compared to their average value. The recovery was evaluated as the calculated spiked sample's concentration, compared to the nominal one in solution without matrix. We followed the US FDA-guidelines [31]. The guidelines recommend limits for the quantification of analytes of high, middle, low concentration to be within ± 15% of their nominal spiked concentration, or ± 20% for the LLOQ. The matrix effect was tested by comparing the normalized area under the curve (AUC) of the spiked plasma samples to the AUC of the extracted samples of blank plasma, which were added to extraction solvent.

2.9. LC-MS/MS method LC-MS/MS instrumentation

All measurements were performed using an Agilent 6460 Series triple quadrupole LC–MS/MS. Mass Hunter Workstation (Agilent Technologies, CA, USA, version: B.06.00) was used to operate the instrument and for data analysis. The Agilent triple quadrupole 6460 instrument was coupled with an Agilent 1200 HPLC system. The MS was equipped with an electrospray system (ESI). The HPLC system (Agilent Technologies, CA, USA) consisted of four LC-20AD pumps, a G1367E auto-sampler (Agilent Technologies, CA, USA) and a G1322A degasser (Agilent Technologies, CA, USA). A column-trapping system HALO C–18, 4.6 × 5 mm, (Optimize Technologies, OR, USA) was used before eluting to the main chiral column, a Lux Cellulose-3 column (cellulose tris(3-chloro-4-methylphenylcarbamate) phase), 150 × 4.6 mm, 3 µm (Phenomenex, CA, USA, cat. no. 00F-4456-B0) for the analyte separation.

The elution gradient was defined as follows: 1–3 min A 0–100%; 3–9.5 min, B 0–100%; 9.5–10.5 min A 0–100%. The flow rate was 0.3 mL/min. The six-port switching valve was used to divert the flow from the HPLC columns to the mass spectrometer during 0–3 and 9.5–10.5 min of each sample run.

The mobile phase A consisted of a solution of ammonium acetate 10 mM in milliQ water with 0.015% v/v formic acid. The solution was filtered and degassed using 500 mL 0.22 µm filter bottles. The mobile phase B was based on a mixture of ammonium formate 20 mM in milliQ water 20% v/v and acetonitrile 80% v/v.

The columns were kept at 20 °C. For every sample, 5 µL were injected as sample volume. The product ions were tracked in multiple reactions monitoring (MRM) at 204 and 203 *m/z* for the ISTD and praziquantel, respectively. The gas temperature was set to 400 °C, with a flow rate of 12 L/min.

Carry-over was prevented by rinsing the auto-sampler syringe after each injection with 50% v/v milliQ water and 50% v/v isopropanol.

2.10. Preparation of LC-MS/MS samples

The samples were extracted with 200 µL of extraction solution containing 400 ng/mL d11-PZQ. After extraction, the samples were thermomixed at 20 °C for 20 min at 750 RPM (Eppendorf Themomixer C, Hamburg, Germany). The samples were filtered directly into 96-deep-well plates (500 µL) (Eppendorf, Switzerland, cat. no. 0030501101) by centrifugation (10 min at 2250*g* and 22 °C) of 2 µm PVDF membrane filter 96-well plates (Corning Life Sciences, CA, USA, cat. no. CLS3508-50EA). The 96-deep-well plates were sealed with plastic sealing mats (Eppendorf, Germany, cat. no. 15319247) and stored, for a maximum of 24 h, at 4 °C.

[1] ®.

2.11. Sample analysis

Precision was evaluated using the coefficient of variation (CV) between the replicates, and accuracy was calculated as the percentage ratio of the measured concentration to the nominal concentration. Calibration curves were normalized by the ISTD peak areas and fitted by linear regression. The weighting factor for the linear regression ($1/x^2$) was selected to yield the lowest total error. In every set, a calibration line (CL) ranging from 0.01 to 4 μg/mL was included, with a regression fitting coefficient R^2 above 0.996. Additionally, 6 replicates of QCs LLOQ, low, middle and high concentration were included in each set.

2.12. ISR (incurred sample reanalysis)

10% of the mice plasma samples were randomly chosen for incurred sample reanalysis (ISR). The criterion for ISR acceptance is that two-thirds (67%) of the repeated sample results should be within 20% between the first and the second measurement. The percentage difference of the results is determined with the following equation: ((Repeat – Original) * 100/Mean).

2.13. Statistics and pharmacokinetic parameters

All the data were handled with R version 3.4 and R-studio V 1.1.453 [33]. Statistics were performed with R-studio. Kruskal-Wallis rank sum test was used for the statistical analysis (Supplementary Fig. 1), non-compartmental analysis (NCA) was used to calculate the area under the curve 0-infinity ($AUC_{0-\infty}$), maximum concentration (C_{max}), time to maximum concentration (T_{max}), half-life ($t_{1/2}$), area under the curve 0–24 h (AUC_{0-24h}) and the apparent oral clearance (Cl/F) for both the R- and S- enantiomers of praziquantel and R- and S-enantiomers of polymorph B.

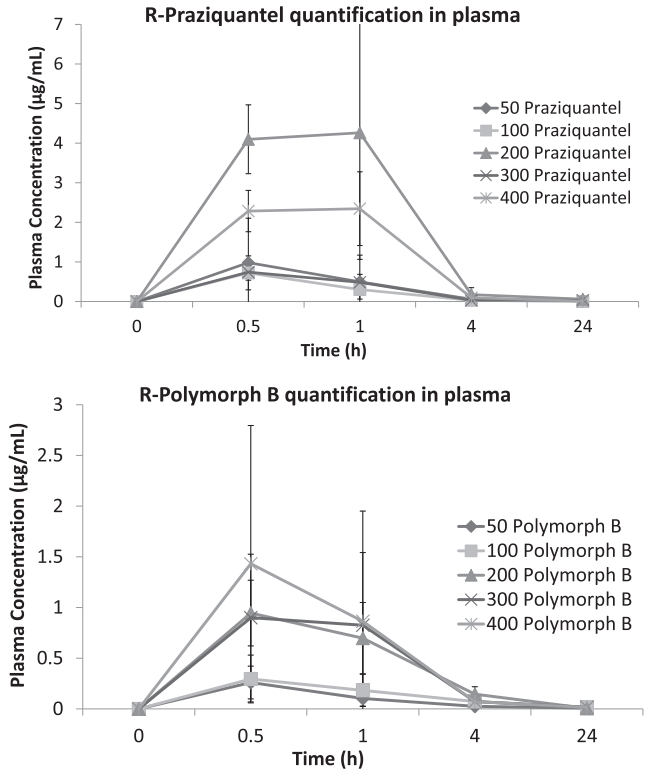

Fig. 1. Plasma concentration profiles of the standard R-praziquantel (a) and of R-polymorph B (b), Concentration time profile of the standard R-praziquantel (a) and of R-polymorph B (b) after quantification by LC-MS/MS. The error lines represent the standard deviation of the samples.

Table 1
IC_{50} values *in vitro* on *S. mansoni* newly transformed schistosomula (NTS).

IC_{50} (μg/mL)	24 h	SD	48 h	SD	72 h	SD
Polymorph B	16.45	4.88	4.45	2.96	2.40	0.50
Praziquantel	12.64	2.33	4.48	3.5	2.58	0.36

Data obtained as average of three independent experiments. SD = standard deviation.

3. Results

3.1. In vitro results with S. mansoni newly transformed schistosomula (NTS)

After 72 h, the calculated IC_{50} value for standard praziquantel on NTS was 2.58 μg/mL (8.26 μM), while for the polymorph B it was 2.40 μg/mL (7.68 μM) (Table 1). Both formulations showed decreased IC_{50} values over time, from 16.45 μg/mL (52.66 μM) (24 h) to 2.40 μg/mL (7.68 μM) (72 h) for the polymorph B and from 12.64 (24 h) to 2.58 (72 h) μg/mL (39.69 to 8.26 μM) for standard praziquantel (Table 1).

3.2. In vitro results with S. mansoni adults

The IC_{50} values determined after 24 h were 0.14 μg/mL (0.45 μM) for standard praziquantel and 0.23 μg/mL (0.74 μM) for the polymorph B. After 72 h, similar IC_{50} values of 0.07 μg/mL (0.22 μM) for the polymorph B and 0.05 μg/mL (0.16 μM) for the standard praziquantel were calculated (Table 2).

3.3. In vivo results

There was no significant difference between the number of female and male adult worms recovered from infected mice treated with praziquantel or the polymorph B for any of the treatment groups. However, both the polymorph B and standard praziquantel reduced the overall worm burden significantly (p value < 0.05) compared to the control mice (Table 3, Supplementary Fig. 1). The highest WBR was achieved at 300 mg/kg for both treatment arms (polymorph B = 89%; praziquantel = 87%). The lowest WBR was observed at 50 mg/kg dose for both standard praziquantel and the polymorph B (33.3% and 12.5% mg/kg, respectively) (Table 3). The 50% effective dose (ED_{50}) was determined as 58.75 and 122.61 mg/kg for standard praziquantel and the polymorph B, respectively.

3.4. LC-MS/MS partial validation and incurred samples reanalysis (ISR)

At least 75% of the CL were within a precision of ± 15% (LLOQ): ± 20%) and an accuracy between 85% and 115% (LLOQ: 80–120%). In every set, a minimum 75% of the CL samples showed linearity with a fitted linear regression R^2 value of above 0.996. At least four quality controls (QCs) (or 67%) were within precision of ± 15% (LLOQ: ± 20%) and an accuracy between 85% and 115% (LLOQ: 80–120%).

During the partial validation process, the complete sets with QCs and CLs were run on different days (inter-day validation) and analysed. The coefficient of variation between the QC replicates was below 7%.

Table 2
IC_{50} values *in vitro* on adult *S. mansoni* worms.

IC50 (μg/mL)	24 h	SD	48 h	SD	72 h	SD
Polymorph B	0.23	0.20	0.08	0.11	0.07	0.05
Praziquantel	0.14	0.16	0.06	0.09	0.05	0.06

Data obtained as average of three independent experiments. SD = standard deviation.

Table 3
Effect of standard praziquantel and polymorph B on the worm burden in mice harboring a chronic *S. mansoni* infection.

			Worm burden			Worm Burden Reduction (WBR %)		
Compound	Dose (mg/kg)	No. of mice	Female	Male	Total	Female	Male	Total (SD)
Control_mice_1	Untreated	8	12.6	12.4	25.0	–	–	–
Control_mice_2	Untreated	8	10.5	11.5	22.0	–	–	–
Control_mice_3	Untreated	8	2.1	1.9	4.0	–	–	–
Polymorph B***	50	4	10.5	10.5	21.0	13	12	13 (25)
Praziquantel***	50	3	4.8	4.8	9.5	33	33	33 (58)
Polymorph B**	100	4	3.3	4.3	7.5	69	63	66 (21)
Praziquantel**	100	4	1.5	2.3	3.8	86	80	83 (20)
Polymorph B*	200	4	2.5	8.5	11.0	80	31	56 (33)
Praziquantel*	200	4	3.3	5.5	8.8	74	56	65 (17)
Polymorph B*	300	4	0.8	2.0	2.8	94	84	89 (14)
Praziquantel*	300	4	1.3	2.0	3.3	90	84	87 (5)
Polymorph B*	400	5	3.0	3.4	6.4	76	73	74 (13)
Praziquantel*	400	4	1.5	2.5	4.0	88	80	84 (23)

* Indicates that the WBR was calculated based on mice batch 1.
** Indicates that mice batch 2 was used to calculate the WBR.
*** Indicates that mice batch 3 was used to calculate the WBR. In the group of 50 mg/kg praziquantel, one mouse was excluded from the analysis as outlier, because it was not infected. SD = Standard deviation.

The observed matrix effect values were between 93% and 114% for R-praziquantel (lowest to highest concentration) and 97% to 104% for S-praziquantel. The total recovery values were between 87% and 97% for R-praziquantel (lowest to highest concentration). A total recovery of 87% to 91% was observed for S-praziquantel (lowest to highest concentration). The 10% of ISR (17 samples) randomly chosen were within the ± 20 variation, as recommended by the FDA guidelines.

3.5. Accuracy, precision, and matrix effect

During the analysis about 2.5% of the samples exceeded the upper limit of quantification (ULOQ) (4 μg/mL). These samples were diluted three times in the extraction solution without ISTD and re-run within the same run. The samples below the LLOQ (0.01 μg/mL) were considered as 0, as recommended by the Food and Drug Administration (FDA) guidelines [31].

The inter-day accuracy of the analytical method was measured in every individual set as described above. Each set was within ± 15% and ± 20% for the LLOQ. The highest deviation was observed on the LLOQ in all three sets. The inter-assay accuracy was between 96% and 112%, with the highest variation of 12% observed at LLOQ. The inter-day precision was between 2% and 12% for the LLOQ values.

The matrix effect is summarized in Supplementary Table 1. The matrix effect of R praziquantel was 93.4% with a standard deviation of 10% for lower concentrations; at higher concentrations (2.5 μg/mL) the matrix effect was 113.6%, with a standard deviation of 1.6%. The R-praziquantel recovery was between 87% and 97% (low to high concentrations), with a maximum standard deviation of 10% at low concentrations. For S-praziquantel, the total recovery was 86.6% with a standard deviation of 16.7% for lower concentrations, while at high concentrations it was 104.3% with a standard deviation of 2.4%.

3.6. Pharmacokinetic parameters

Pharmacokinetic parameters for R and S-praziquantel and the R and S-polymorph B are summarized in Table 4. Median T_{max} values between 0.5 and 0.75 h were determined for both the R-, S-polymorph B and R-, S-praziquantel. The median values of C_{max} ranged between 0.67 and 2.73 μg/mL (2.14 to 8.74 μM) for R-praziquantel and between 0.18 and 1.56 μg/mL (0.58 to 5 μM) for R-polymorph B at 50–400 mg/kg. The C_{max} values for the S-polymorph B and S-praziquantel show a similar trend. The median values for the $t_{1/2}$ for R-praziquantel were between 2.64 and 5.93 h and between 4.02 and 10.56 h for the R-polymorph B. At the 100 mg/kg dose R-praziquantel showed a 4 times lower $t_{1/2}$ than the R-polymorph B, while the other doses showed similar $t_{1/2}$ profiles for the two formulations (Table 4). The median values for the $AUC_{0\rightarrow 24h}$ ranged between 1.25 and 6.28 h*μg/mL for R-praziquantel, with the maximum value observed at the 400 mg/kg dose and the lowest value at the 100 mg/kg dose. The $AUC_{0\rightarrow 24h}$ for the R-polymorph B reached the maximum level at 300 mg/kg (3.1 h*μg/mL) and the minimum at 50 mg/kg (0.3 h*μg/mL). The values for the S- polymorph B and S-praziquantel were lower. The apparent clearance for the R-polymorph B and R-praziquantel median values were between 22.25 and 123.94 and between 53.08 and 134.7 (mg)/(μg/mL)/h, indicating a ~30% variation in the overall profiles of the two formulations (Table 4). No relationship was observed between WBR and $AUC_{0\rightarrow 24h}$ or C_{max}. (Supplementary Fig. 2).

4. Discussion

For over 30 years, praziquantel has been the only available drug against schistosomiasis together with oxamniquine [10,34]. Oxamniquine was used extensively in South America until 10 years ago, when it was withdrawn due to resistance development [34]. In the present study, a crystalline polymorph of standard praziquantel, revealing improved physical characteristics, such as increased water solubility and dissolution, together with an appreciable physical stability, was thoroughly tested, given initial promising *in vitro* and *in vivo* findings [17]. Moreover, we conducted PK studies, for which we successfully adapted and validated an LC-MS/MS method for mouse plasma.

Indeed, different studies have demonstrated that improved praziquantel formulations are able to influence the *in vivo* drug performance. For example, a study by El-Feky *et al.* (2015) [35] showed that a clay-based nanoformulation praziquantel increased the efficacy of the drug. Moreover, El-Lakkany *et al.* (2012) showed an increased efficacy of a praziquantel–polyvinylpyrrolidone (PVP) solid dispersion, by increasing the bioavailability of praziquantel in a PK study [36].

Our results, based on *in vitro* and *in vivo* studies using the *S. mansoni* mouse model, show that the polymorph B has no benefit in terms of efficacy over the current formulation. No significant differences were observed in the *in vitro* and *in vivo* studies, contrary to preliminary *in vitro* and *in vivo* results which showed higher efficacy of the formulation derivative compared to the standard praziquantel [17].

The ED_{50} value of 58.45 mg/kg for the standard praziquantel calculated in this study is in line with previously reported values for susceptible *S. mansoni* strains ranging from 70 to 100 mg/kg [35–38] but lower than the one previously determined in our laboratory (246.5 mg/

Table 4
Pharmacokinetic parameters calculated for R- and S-praziquantel following treatment of mice infected with *S. mansoni* with praziquantel and the polymorph B.

Parameter	50 mg/kg	100 mg/kg	200 mg/kg	300 mg/kg	400 mg/kg	
$t_{1/2}$ [h]	5.9 (5.3; 9.8)	2.6 (2.3; 3.5)	3.3 (2.7; 6.5)	5.9 (4.1; 8.2)	3.3 (3.0; 3.7)	**R-Praziquantel**
T_{max} [h]	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	0.8 (0.5; 1)	0.5 (0.5; 0.5)	0.8 (0.5; 1)	
C_{max} [μg/mL]	0.7 (0.2; 1.4)	0.7 (0.4; 1.0)	2.1 (1.2; 4.5)	0.8 (0.7; 0.9)	2.7 (2.1; 3.1)	
AUC $_{0\text{-}24h}$ [h*μg/mL]	1.5 (0.8; 3)	1.2 (0.5; 1.9)	5.9 (4.5; 10.4)	2.2 (1.8; 2.4)	6.3 (4.7; 8.1)	
AUC_{∞} [h*μg/mL]	2.2 (1.9; 4.3)	1.3 (0.6; 1.9)	7.1 (6.5; 10.4)	2.5 (1.8; 3.1)	6.4 (4.7; 8.2)	
Cl/F_{obs} (mg)/(μg/mL)/h	22.3 (15; 26.9)	112.8 (51.8; 180.3)	28.1 (23.4; 31.8)	123.9 (98.5; 186.5)	66.3 (49.3; 85)	
$t_{1/2}$ [h]	5.4 (3.3; 7)	10.6 (8.9; 11.1)	3.1 (2.9; 3.6)	4 (3.8; 6.3)	5.3 (4.3; 6.5)	**R-Polymorph B**
T_{max} [h]	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	0.8 (0.5; 1)	0.5 (0.5; 0.9)	
C_{max} [μg/mL]	0.2 (0.1; 0.3)	0.3 (0.2; 0.4)	0.9 (0.7; 1.1)	0.9 (0.6; 1.3)	1.6 (0.3; 2.7)	
AUC $_{0\text{-}24h}$ [h*μg/mL]	0.3 (0.3; 0.5)	1.4 (0.9; 2)	2.9 (1.9; 3.8)	3.1 (2.3; 3.6)	2.5 (1.1; 4.5)	
AUC_{∞} [h*μg/mL]	0.4 (0.3; 0.5)	1.9 (1.3; 2.4)	3 (2.2; 3.9)	3.1 (2.5; 3.7)	3.1 (2.4; 5.1)	
Cl/F_{obs} (mg)/(μg/mL)/h	134.7 (109.3; 147.3)	53.1 (41.4; 113.7)	72.6 (51.9; 91.6)	96.7 (82.8; 125.4)	131.1 (78.3; 170.1)	
$t_{1/2}$ [h]	5.5 (4.7; 6.3)	0.4 (0.4; 0.4)	6 (5.0; 7.0)	6.4 (3.1; 9.7)	4.7 (4.5; 4.8)	**S-Praziquantel**
T_{max} [h]	0.5 (0.5; 0.6)	0.5 (0.5; 0.6)	0.8 (0.5; 1.0)	0.5 (0.5; 0.5)	0.8 (0.5; 1)	
C_{max} [μg/mL]	0.2 (0.1; 0.8)	0.1 (0.1; 0.2)	2.9 (2.4; 3.2)	0.3 (0.2; 0.3)	0.5 (0.4; 0.7)	
AUC $_{0\text{-}24h}$ [h*μg/mL]	2.2 (1.0; 3.8)	0.2 (0.1; 0.8)	7.2 (5.0; 9.4)	0.8 (0.6; 0.9)	1.8 (1.3; 2.1)	
AUC_{∞} [h*μg/mL]	3.6 (2.5; 4.7)	0.3 (0.3; 0.3)	11.5 (11.1; 11.9)	1 (0.7; 1.2)	1.9 (1.3; 2.2)	
Cl/F_{obs} (mg)/(μg/mL)/h	22.6 (15.6; 29.6)	316.2 (316.2; 316.2)	17.4 (16.8; 18)	314.3 (251.8; 804.5)	222.4 (182.8; 335.1)	
$t_{1/2}$ [h]	5.4 (1.1; 47.4)	20.4 (17.2; 21.2)	1.5 (1.0; 2.7)	5.4 (4.7; 7)	7.1 (5.1; 13.2)	**S-Polymorph B**
T_{max} [h]	0.5 (0.5; 0.6)	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	0.8 (0.5; 1)	0.5 (0.5; 0.9)	
C_{max} [μg/mL]	0.1 (0; 0.2)	0.1 (0.1; 0.2)	0.4 (0.2; 0.6)	0.2 (0.2; 0.3)	0.7 (0.2; 0.8)	
AUC $_{0\text{-}24h}$ [h*μg/mL]	0.2 (0.1; 0.8)	0.6 (0.6; 1.4)	0.9 (0.4; 1.5)	0.8 (0.5; 1.1)	1.3 (0.5; 1.5)	
AUC_{∞} [h*μg/mL]	0.3 (0.2; 8.2)	1.6 (1.3; 3.2)	1 (0.6; 1.6)	0.9 (0.6; 1.1)	1.5 (1.5; 1.6)	
Cl/F_{obs} (mg)/(μg/mL)/h	196.5 (127; 385.5)	61.7 (41.1; 82.6)	236.1 (129.4; 366.4)	371.5 (271.1; 507.9)	260.8 (245.4; 269.7)	

Median values are reported, and an interquartile range is provided: 1st quartile and 3rd quartile.

kg) [39]. While a slightly higher ED_{50} of 122.61 mg/kg was calculated for the polymorph B (mainly due to a slightly lower activity at 50 mg/kg) no significant difference was observed between the two treatments (as aqueous suspensions) at the individual dosages.

One limitation of our study, which might explain this finding, was the comparison of the two crystalline forms as water suspension: Once the drugs were suspended in water for the oral administration, the conformation of the polymorph B might have changed, thereby possibly altering its properties. To fully exclude this possibility it would have been advantageous to use the crystalline polymorph and the standard praziquantel as powders embedded in mini gelatin capsules or loaded in microparticles suitable for oral administration. However, the high praziquantel doses required to achieve antischistosomal activity (up to 400 mg/kg) are not compatible with mini capsule usage in the mouse model (the main animal model for research on schistosomiasis [40]), since the maximum capacity of each commercially available mini-capsule is 2–4 mg. Capsules can only be administered to rodents larger than 150 g. Therefore, comparison of liquid formulations, as done in this study, is a widely used approach in drug formulations and delivery studies in rodents [41].

The praziquantel quantification method in mouse plasma was successfully adapted from a human-based validated LC-MS/MS quantification method [32,42]. The PK analysis was based on a non-compartmental analysis using a trapezoidal linear model, given the small number of time points used in the study (30, 60, 240 min and 24 h). The trapezoidal linear model was preferred to the log-linear model due to the general opinion of this model being more accurate in the calculation for drugs with a fast metabolism [43]. A limitation of the PK study was the rather small sample size for each treatment arm (four mice) which could influence the error rate in the study. To limit this experimental error, the test and the control mice were chosen randomly within the same infection batch.

The data presented here show that, surprisingly, the polymorph B has an overall lower exposure profile for both R- and S- enantiomers, indicating a lower bioavailability of the drug, in comparison with standard praziquantel. Moreover, the polymorph B shows lower plasma peak concentrations of the R- and S-enantiomers compared to standard praziquantel formulation (Table 4). The polymorph B has previously been shown to have a faster dissolution rate compared to standard praziquantel [17,44] and therefore a better absorption would have been expected [45]. However, the fast first pass metabolism typical of praziquantel, might result in lower disposition profiles of the drug over time [16,37,46]. Earlier PK sampling time points (e.g. 5 min) should have been considered to obtain a better picture of the absorption phase.

Though the AUC was 40% lower for the polymorph B compared to standard praziquantel, the WBR observed was similar (Table 3). No relationship was observed between AUC and C_{max} and WBR (Supplementary Fig. 2), confirming previous findings from Abla *et al.* (2017), hypothesizing that the portal vein drug concentration is primarily responsible for the activity of praziquantel [37].

Similar to other studies a great variability was observed for both compounds in the PK profiles and parameters among individual mice [37]. Overall, the short praziquantel T_{max} value obtained in this study is in agreement with other *in vivo* studies, while in humans the T_{max} is generally ranging between 3 and 4 h [46,47]. The C_{max} values we found (0.21–4.55 μg/mL (0.67 – 14.56 μM) for R-praziquantel) are considerably lower than the ones found in other studies, as for example El-Feky et al. (2015) and Botros et al. (2006) [35,48], which report values of 24.36 and 33.33 μg/mL (77.98 and 106.59 μM), respectively, based on PK analysis of Swiss albino mice's plasma. However, both groups are reporting those results following a 500 mg/kg dose only, which makes a direct comparison difficult. The overall lower C_{max} observed in our study could be explained by the different strains and sex of the mice used in the different studies and, as mentioned, the slightly lower doses used, but also by other parameters, such as infection rate and mouse age [16,46,48–50]. However, we obtained a similar C_{max} as the reported values from Abla et al. (2017) for the dose of 200 mg/kg of standard praziquantel (about 1.3 μg/mL) and a similar value for the WBR (about 68%) [37]. However, interestingly the AUC value of R praziquantel reported by the same group is 2.6 times lower than the ones reported in this study at the dose of 200 mg/kg (median value of 5.93 h*μg/mL in our study compared to 2.2 h*μg/mL reported by Abla and colleagues) even though experimental conditions were identical (Table 3 and Table 4).

5. Conclusion

In this study we generated activity and PK data on a new alternative

praziquantel formulation based on a crystalline polymorph of the racemic drug, the polymorph B, obtained via a solvent-free process of neat grinding. The polymorph B showed similar *in vivo* efficacy to the standard praziquantel formulation, but in general lower plasma levels. However, due to the excellent physical stability and doubled water solubility of the polymorph, this product remains a valid option to enhance the pharmaceutical performance of the antischistosomal drug praziquantel. Indeed, the possibility to generate additional praziquantel solid forms or other suitable formulations may represent new interesting approaches for overcoming the numerous praziquantel pharmaceutical and biopharmaceutical drawbacks.

Acknowledgements

This work was supported by a grant from the European Research Council (ERC 614739-A_HERO).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.06.029.

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