

Pharmacological and pharmacokinetic studies on
trematocidal drugs : praziquantel and two synthetic
peroxide lead candidates

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Summary

Trematodiasis affect more than 250 million people worldwide, while the economic impact through veterinary infections is also colossal. Treatment options are however limited to two main drugs, triclabendazole for the liver fluke *Fasciola hepatica*, and praziquantel (PZQ) for the rest of the trematodes including schistosomes, the etiological agent of bilharziosis. Furthermore, the kinetic disposition of PZQ is hardly studied in diseased patients and lacking in pre-school children, where the burden of schistosomiasis is already high. The aim of the present thesis is to provide pharmacokinetic and pharmacological studies to understand the modalities of PZQ disposition in patients. Additionally, the efficacy of two novel synthetic peroxide lead candidates are evaluated against *F. hepatica* in sheep and supported by a pharmacokinetic (PK) study.

We first validated an analytical method to assess the PK profile of the synthetic peroxides OZ78 and MT04 in sheep plasma and bile. After an intramuscular administration of a 100 mg/kg dose to groups of 5 sheep naturally infected with *F. hepatica*, we observed a good fasciocidal efficacy for the peroxide candidate MT04, while the other candidate, OZ78, failed to display any activity. The PK study did not reveal a higher kinetic disposition for MT04 than for OZ78, but highlights problems in drug absorption. The presence of both peroxide candidates in sheep bile at 6 h post-treatment does not rule out a direct drug action on the worm tegument.

We recorded a high antischistosomal activity for the R enantiomer of PZQ against *S. mansoni* adults and NTS *in vitro* with IC₅₀ values below 0.04 µg/ml. The S form displayed a very limited effect *in vitro*. It produced *in vivo* an important and transient shift of the worms to the liver but at 24 h after S-PZQ administration, the worms had returned to the mesenteric veins. The R forms of the main metabolites in mice and humans, cis- and trans-4-OH, exhibited a moderate activity. *In vivo*, mice harbouring adult *S. mansoni* were cured with a 200 mg/kg dose, half of the curative dose required with racemic PZQ. Treatment with a 800 mg/kg dose of S-PZQ failed to display a significant effect.

In order to undertake PK studies, we validated an enantioselective method for PZQ and its main metabolite, R-trans-4-OH-PZQ, in blood, plasma and dried blood spots (DBS) using an analytical range from 0.01 to 2.5 µg/ml for R- and S-PZQ and from 0.1 to 25 µg/ml for the metabolite. The comparison of blood, plasma and DBS samples simultaneously withdrawn from 9 patients infected with the liver fluke *Opisthorchis viverrini* and treated with 3x 25 mg/kg PZQ allowed us to evaluate the potential of DBS as unique sampling method in future PK trials. Due to the high affinity of PZQ for plasma albumin, samples using whole blood displayed slightly lower concentrations of the parent enantiomers than plasma measurements. This difference is however small and within an acceptable range. For the metabolite, plasma concentrations did not display significant differences to blood or DBS levels. Therefore, DBS was validated as an alternative to plasma sampling. The PK profile of the 9 patients investigated in this study was discussed on the basis of plasma PK data. We observed plasma AUCs of 1.1, 9.0 and 188.7 µg/ml*h and half-lives of 1.1, 3.3 and 6.4 h for R-PZQ, S-PZQ and R-trans-4-OH, respectively. Maximal plasma concentrations of 0.2, 0.9 and 13.9 µg/ml for R-PZQ, S-PQZ and R-trans-4-OH peaked at 7 h for PZQ enantiomers and 8.7 h for the metabolite. The high levels of the R form of the main metabolite of PZQ do not rule out its participation in the opisthorchicidal effect of the drug.

In conclusion, we identified MT04 as a potential fasciocidal candidate. We also generated precise *in vivo* and *in vitro* data on the sensitivity of *S. mansoni* to PZQ enantiomers and confirmed that the eutomer of PZQ is its R-form. The LC-MS/MS method for praziquantel enantiomers allows to use DBS in future clinical trials, therefore enabling the sampling of children and the planning of larger PK trials. Finally, we presented the first PK values of PZQ enantiomers and its main metabolite in patients suffering from opisthorchiasis.

List of Abbreviations

AUC	Area under the curve
C_{\max}	Maximal concentration
CR	Cure rate
CV	Coefficient of variation
DBS	Dried blood spot
DMSO	Dimethylsulfoxide
ED _{50/90}	Effective dose (dose to reduce parasitic load by 50/90%)
EPG	Number of eggs per gram of faeces
ERR	Egg reduction rate
FDA	Food and Drug Administration
HPLC	High pressure liquid chromatography
IC _{50/90}	Inhibitory concentration (dose to kill 50/90% of the parasites)
iFCS	Inactivated foetal calf serum
IS	Internal standard
ISR	Incurred sample reanalysis
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LLOQ	Lower limit of quantification
ME	Matrix effects
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MT04	Synthetic peroxide (tetraoxane)
NTD	Neglected tropical disease
NTS	Newly transformed schistosomulae
OZ	Ozonid
PK	Pharmacokinetics
PZQ	Praziquantel
QC	Quality control
rac	Racemic/racemate
RRE	Relative recovery
SD	Standard deviation
T ₀	Time of treatment
t _{1/2}	Half-life
T _{max}	Time at maximal concentration
ULOQ	Upper limit of quantification
WB	Worm burden
WBR	Worm burden reduction

Chapter 1

General introduction

1.1 Trematode biology and life cycle

Trematodes, together with cestodes and monogenes, are part of the phylum Platyhelminthes. They are characterised by the adoption of molluscs as intermediate hosts and are further divided into two subclasses, the Apidogastrea and the Digenea. While the former is mainly composed of fish parasites, the latter constitutes the largest group of internal metazoan parasites with around 18'000 species and are present in all the classes of vertebrate hosts (Cribb *et al.*, 2003; Olson *et al.*, 2003).

The life cycle of digenetic trematodes has typical features. First, the eggs produced by adult worms embryonate, releasing miracidia that infect molluscs, in particular gasteropodes. In this first intermediate host, they undergo one or several cycles of asexual reproduction and eventually infect a second intermediary host. Access to the definitive host is mediated either by active penetration of free-swimming cercariae or by passive ingestion of encysted

cercariae (metacercariae) (Cribb *et al.*, 2003; Kostadinova and Perez-del-Olmo, 2014).

Digenetic trematodes also share distinctive morphological traits. They possess two suckers: an oral sucker and a ventral one, also called acetabulum. Excluding schistosomes, most of the trematodes are hermaphroditic. Their digenetic tegument is syncytial and contains vesicles that might be involved in the renewal of the tegument in case of damage. Digenes have a digestive caecum, either syncytial or cellular, which secretes a wide range of proteolytic enzymes. The excretory system is composed of protonephridia (flame cells) that remove the metabolic waste, such as urea, uric acid or ammonia, through an excretory pore, usually at the posterior end of the worm. Waste products can also be eliminated via diffusion through the tegument (Beaumont and Cassier, 1973; Kostadinova and Perez-del-Olmo, 2014).

1.1.1 Food-borne trematodes

Food-borne trematodes comprise parasites of great veterinary and human importance, such as the liver flukes (*Fasciola* spp., *Opisthorchis* spp., *Clonorchis sinensis*), the intestinal flukes (*Echinostoma* spp., *Fasciolopsis buski*, the family Heterophyidae) and the lung flukes (*Paragonimus* spp.). The two species of interest in the frame of this thesis are *F. hepatica* and

O. viverrini. While infection with *O. viverrini* occurs by the consumption of raw fish, *F. hepatica* metacercariae can be ingested by the consumption of water plants or contaminated water. Liver flukes dwell in the biliary ducts of the definitive host and can survive there for up to 25 years (Keiser and Utzinger, 2009).

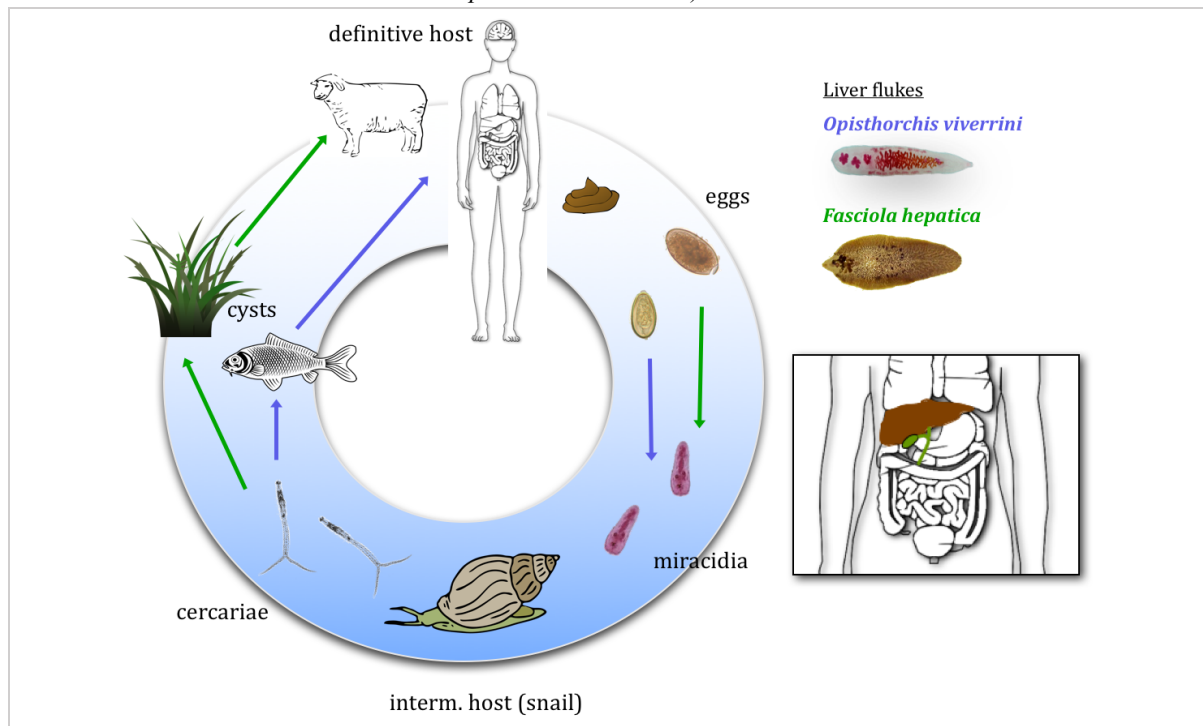


Fig. 1 The life cycle of *Opisthorchis viverrini* and *Fasciola hepatica*: the adult fluke in the bile ducts produces eggs that embryonate to miracidia. They infected a gasteropode, which will later shed cercariae that will encyst as metacercariae in fish or plants.

The genus *Fasciola* comprises two major species of human and veterinary interest, *F. hepatica* and *F. gigantica*, easily differentiated by their morphology. *F. hepatica* is shorter and stocky, with a length of 3 cm, while *F. gigantica* is even longer (5 cm) but narrower. *O. viverrini* adult worms are smaller, ca. 1 cm, and can be distinguished from *O. felineus* and *C. sinensis* by the morphology of the testes. However, microscopic discrimination of the eggs from the two co-endemic species *O. viverrini* and *C. sinensis* is problematic (Kaewkes, 2003; Keiser and Utzinger, 2009; Mas-Coma, Valero and Bargues, 2014). Adult liver flukes display a tegument covered by a glycocalyx that can reach a thickness of 20 nm in *F. hepatica* (Threadgold, 1976). The composition of the glycocalyx might be the reason to the PZQ higher resistance of *F. hepatica* and to some extent of

1.1.2 Schistosomes

Schistosomes, or blood flukes, are the only non-hermaphroditic trematodes. The elongated male measures around 1 cm and forms a groove, the gynecophoral canal, in which the female is nested. The pairing of the worms is essential, because females reach full maturity only in close contact with males. Paired schistosomes produce in Asian species up to thousand eggs per day, which are evacuated via the faeces or the urine. The first intermediate host is a

Paragonimus spp (Andrews *et al.*, 1983). Liver flukes are generally considered as blood-feeding parasites, as blood can be found in their gut content (Todd and Ross, 1966; Sukhdeo, Sangster and Mettrick, 1988). The exact composition of their diet is however not known and might be more varied than initially anticipated (Dawes, 1963). For example, the genome of *O. viverrini* revealed that it is equipped to digest not only blood but also bile components, mainly lipids, and cholangiocytes (Young *et al.*, 2014). A major difference between *F. hepatica* and *O. viverrini* is how they reach the biliary ducts. *F. hepatica* juveniles feed extensively on liver parenchyma, while *O. viverrini* arrives to the liver by the ampulla of Vater (Sripa *et al.*, 2010), hence eliciting a much lower immune reaction from the host (Bae *et al.*, 2013).

gasteropode that will release free-swimming cercariae, able to actively penetrate the skin of the definitive host, mostly humans. Immature worms travel to the lungs and then finish their development in the portal vein. Worm pairs will finally settle in the mesenteric or perivesicular veins, where they can live more than 10 years (Utzinger and Keiser, 2004; Gryseels *et al.*, 2006)

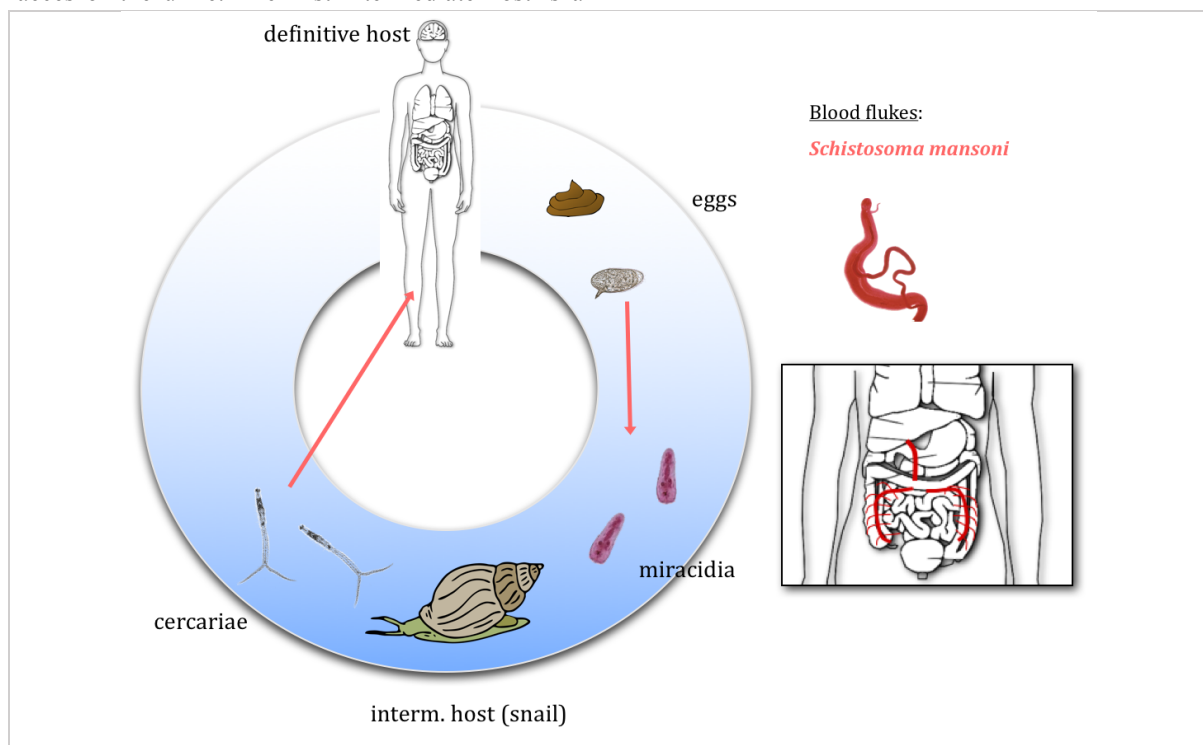


Fig. 2 The life cycle of *Schistosoma mansoni*: the adult fluke in the mesenteric veins produces eggs that will pass through the vein epithelia and liver, out to the intestinal tract. Once expelled with the faeces, the eggs will embryonate to miracidia. They infected a gasteropode, which will later shed cercariae that will actively penetrate the skin of the definitive host.

To optimise nutrient absorption and manipulation of host defences, schistosomes developed a highly specialised tegument, fundamentally different from the food-borne trematode tegument. The schistosome double lipid bilayer is also described as a simple plasma membrane covered by a membranocalyx or heptalaminated tegument and is not present in cercariae (Hockley, 1972). The absorption of glucose through the tegument is facilitated by numerous glucose transporters. Schistosomes actually consume every 5 h the equivalent of their own dry weight in glucose (Faghiri *et al.*, 2010; Da'dara *et al.*, 2012). Their high requirements in amino acids are met by the digestion of erythrocytes, mediated by a battery of cathepsin enzymes. Erythrocytes will also supply the parasite with erythrocyte-derived antigens to be displayed on the worm surface to avoid immune response (Saunders, Wilson and Coulson, 1987; Han *et al.*, 2009).

1.2 Epidemiology and clinical aspects

1.2.1 Fascioliasis

Fascioliasis is a zoonosis occurring in a vast variety of mammal hosts, such as sheep, pigs, buffaloes, horses, camels, rabbits or rodents and is linked to intensive farming activities. *F. hepatica* is present worldwide, while the repartition of *F. gigantica* seems to be limited to Asia and Africa (Toledo, Esteban and Fried, 2012; Mas-Coma, Valero and Bargues, 2014). Fascioliasis affects more than 2 million people, a number certainly underestimated (Fürst, Keiser and Utzinger, 2012; Mas-Coma, Valero and Bargues, 2014). In humans, the consumption of contaminated water or water plants on which metacercariae encyst is the cause of the infection (Mas-Coma, Valero and Bargues, 2014).

The first phase of the disease is asymptomatic and can vary between a few weeks to months. The acute phase corresponds to the migration of immature flukes through the hepatic parenchyma and is characterized by allergic reactions, abdominal pain, fever, vomiting or diarrhoea. During the chronic phase, adult worms induce biliary cholangitis, jaundice, bile duct hyperplasia, inflammation, and cholangitis accompanied with biliary obstruction. Haemorrhages into the bile duct are also common and can lead to severe anaemia and even death, especially in sheep and young cattle (Sripa *et al.*, 2010; Mas-Coma, Valero and Bargues, 2014).

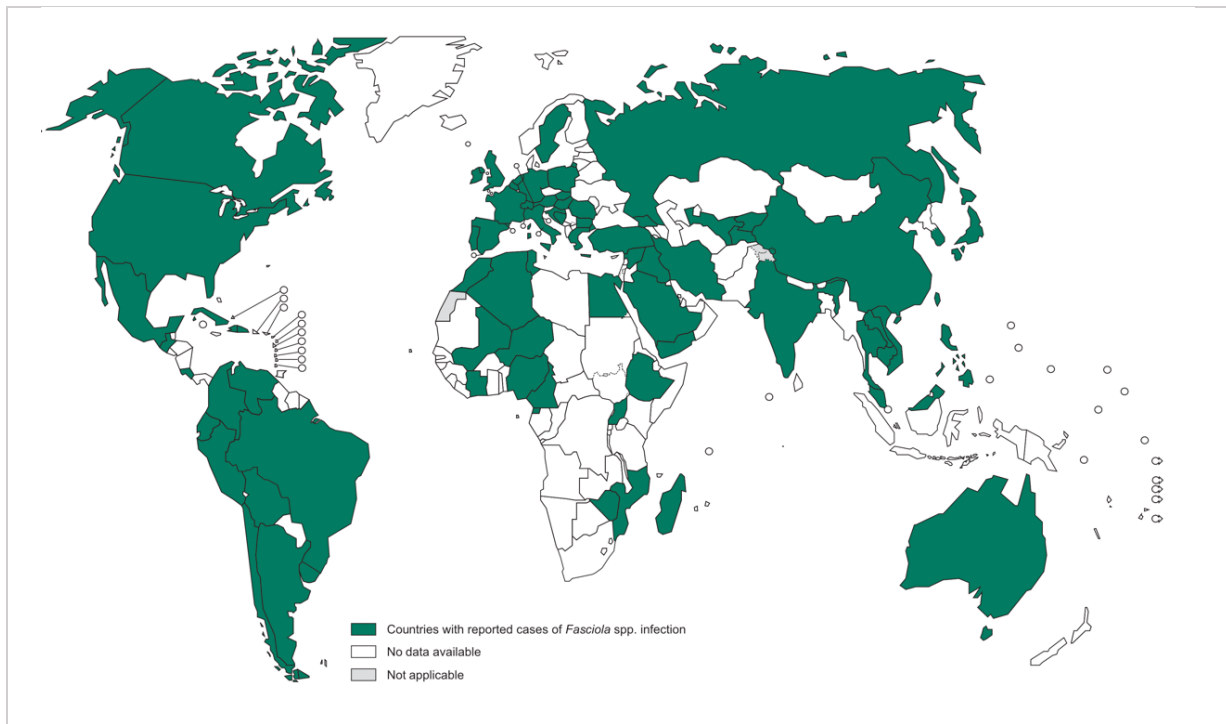


Fig. 3 The distribution of fascioliasis in 2014 (scheme from the World Health Organization, 2015)

1.2.2 Opisthorchiasis

The liver fluke *O. viverrini* infects more than 8 million people principally in Thailand, Lao PDR and Cambodia, with its focal point in the lower Mekong basin (Sithithaworn and Haswell-Elkins, 2003; Fürst, Keiser and Utzinger, 2012). The prevalence is highly variable between regions and can rise up to more than 80% in school children (Lovis *et al.*, 2010). Also, cases of reinfection are important, with prevalence rates attaining their original level only one year after treatment (Upatham *et al.*, 1988; Organization, 2015). The infection occurs by the consumption of raw or undercooked fish, a common practice in regions where

the use of fermented fish is part of many traditional dishes. For example, *koi pla*, a dish made of thin cut raw fish mixed with spices, vegetables and lemon juice, is very popular among fishers and farmers (Grundy-Warr *et al.*, 2012). This strong cultural behaviour at the basis of infection is enhanced by the high prevalence of infected fish. Also, cats and dogs have been identified as reservoirs, and are therefore maintaining the infection cycle close to human homes despite the global effort of sanitation (Sithithaworn *et al.*, 2012). The disease in its chronic state can be devastating: periductal fibrosis, bile duct hyperplasia,

jaundice, cholecystitis, and cholangiocarcinoma with consecutive renal failure (Sripa, 2003; Sripa *et al.*, 2011). *O. viverrini* and the other Asian liver fluke *C.*

sinensis are identified since 1994 as type-1 carcinogens (Andrews, Sithithaworn and Petney, 2008).

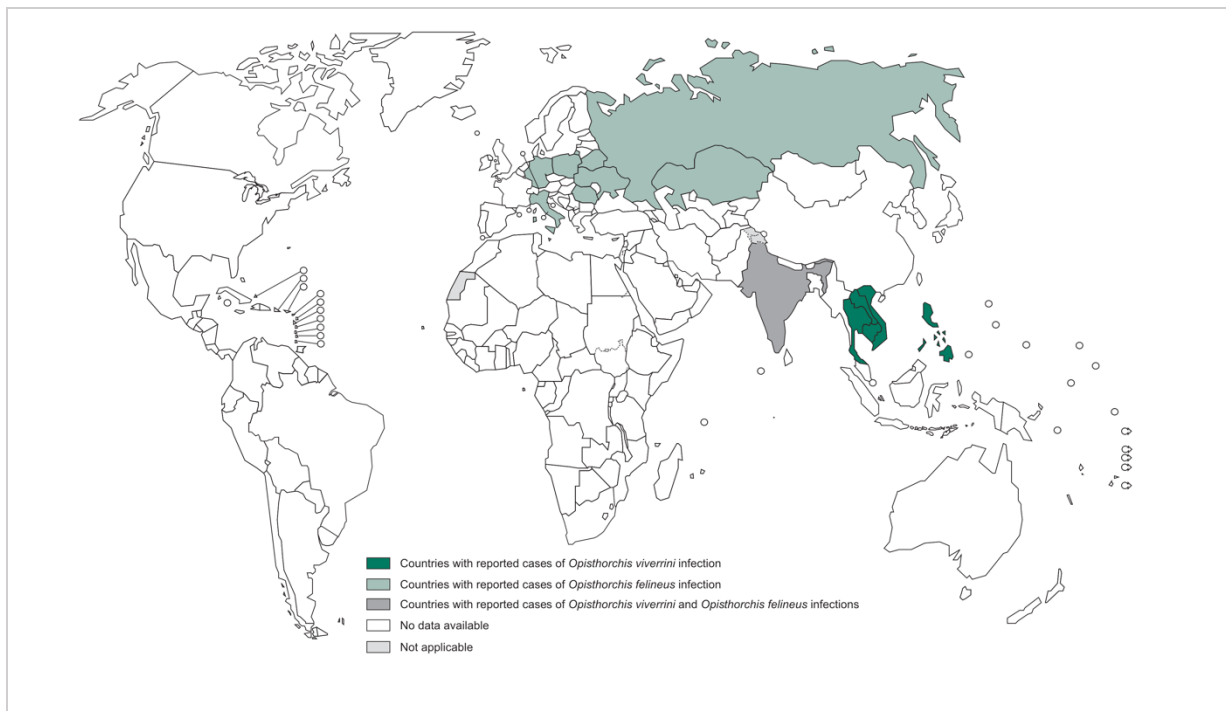


Fig. 4 The distribution of opisthorchiasis in 2014 (scheme from the World Health Organization, 2015)

1.2.3 Schistosomiasis

There are worldwide more than 200 million people suffering from schistosomiasis and more than 700 million at risk of infection (Steinmann *et al.*, 2006; Utzinger *et al.*, 2012). Three main schistosome species are of epidemiological and clinical importance: *S. mansoni*, found in Africa, the Arabian Peninsula and South America, *S. haematobium* in Africa and the Arabian Peninsula, and *S. japonicum* in China, the Philippines and Indonesia. The other two species parasitizing humans are *S. intercalatum*, restricted to west and central Africa, and *S. mekongi* in the Mekong basin (Utzinger and Keiser, 2004; Gryseels *et al.*, 2006). *S. haematobium* is responsible of urinary schistosomiasis. The adult worms live in the veins around the urinary bladder, and the eggs on their way

out produce inflammation and ulcerations of the bladder wall. Chronic patients will display fibrosis and calcification of the bladder. Infections with *S. haematobium* are also an established risk factor for bladder cancer. The other schistosome species dwell in the mesenteric veins with eggs migrating either through the liver and inducing liver fibrosis and hepatomegaly, or through the intestinal wall generating inflammations and mucosal lesions (Gryseels *et al.*, 2006). Genital schistosomiasis, caused by *S. haematobium* and *S. mansoni*, is linked to an enhanced risk of transmitting other sexual infections, such as HIV (Gryseels *et al.*, 2006; Kjetland *et al.*, 2006).

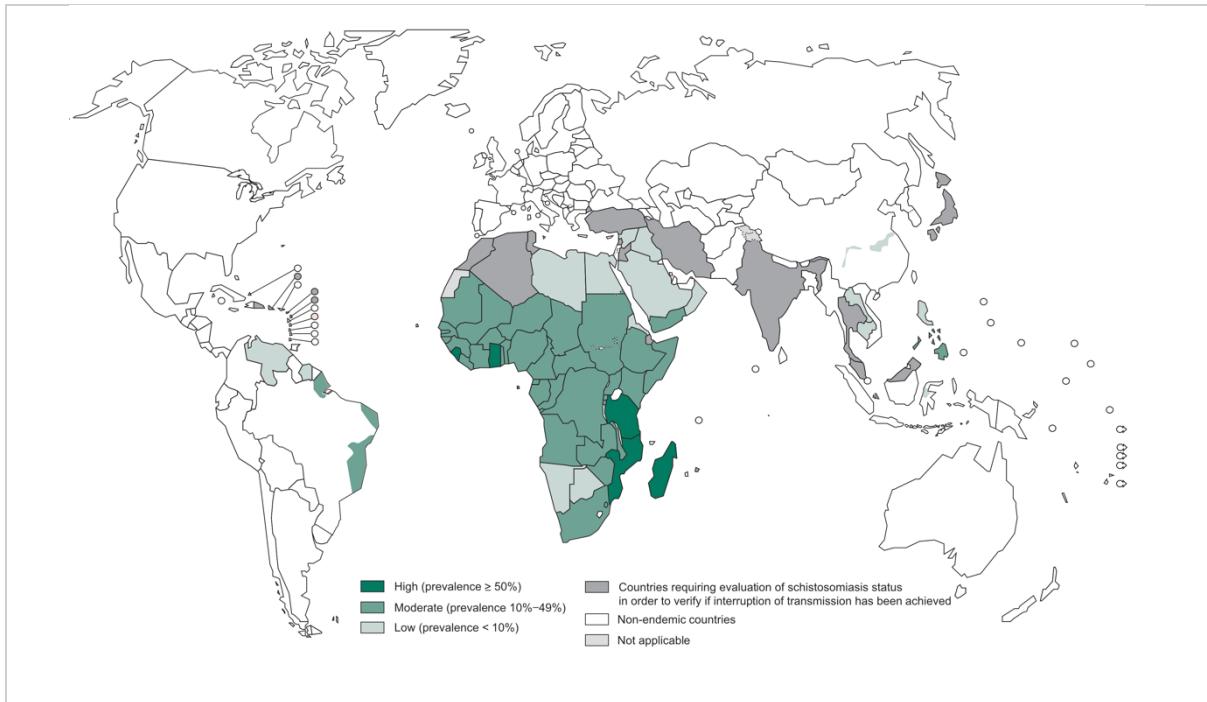


Fig. 5 The distribution of schistosomiasis in 2014 (scheme from the World Health Organization, 2015)

1.3 Chemotherapy against trematodes

1.3.1 Current drugs

Praziquantel (PZQ), first designed as a potential tranquilizer, rapidly became the major drug against trematode infections (Leopold *et al.*, 1978; Cioli and Pica-Mattocchia, 2003). It has been the pillar of schistosomiasis mass drug administration for over 30 years: for example in 2013, 181 million tablets were donated to assure schistosomiasis control (World Health Organization, 2015). The dose recommended against schistosomes is 40 mg/kg (World Health Organization, 2006). PZQ is also the drug of choice against clonorchiasis, opisthorchiasis and paragonimiasis, while *F. hepatica* is completely refractory to the treatment (Cioli and Pica-Mattocchia, 2003; Keiser, Duthaler and Utzinger, 2010). Adverse events after treatment with PZQ are mild and limited to the first 24 h post-administration, with the intensity of infection probably playing a role in their advent. Due to its excellent safety profile, PZQ can be administered to pregnant and lactating women (Cioli and Pica-Mattocchia, 2003). Its mode of action is still not completely unravelled, but seems to involve the Ca^{2+} channels of the worm (Doenhoff, Cioli and Utzinger, 2008). The main drawback of PZQ is its complete lack of activity against juvenile schistosomes. PZQ susceptibility occurs in cercariae and in schistosomula until their 2nd week of maturation in the mouse. Three- and four-week juveniles are refractory, while adults from the 6th week on are again fully susceptible to the drug (Sabah *et al.*, 1986). Therefore, to achieve a complete worm burden, a

second line of treatment a few weeks later is recommended (Utzinger *et al.*, 2000; Utzinger and Keiser, 2004). Cases of treatment failure have been observed, but it is uncertain whether they arise from worms developing resistance to PZQ or from an incomplete elimination of juvenile worms (Doenhoff, Cioli and Utzinger, 2008).

PZQ is a chiral drug extensively metabolised by the liver enzyme CYP450A4 (Li *et al.*, 2003). The R-enantiomer is mainly converted to the monohydroxylated R-trans-4-OH in humans, while S-PZQ generates a wider variety of metabolites (Lerch and Blaschke, 1998; Meier and Blaschke, 2001). R-PZQ is commonly considered to retain the antischistosomal activity of the drug (Wu *et al.*, 1991; Staudt *et al.*, 1992; Liu *et al.*, 1993). S-PZQ, on the other hand, would be responsible of most of the adverse events (Wu *et al.*, 1991) and the extremely bitter taste of the racemic formulation (Meyer *et al.*, 2009; Oliario, Delgado-Romero and Keiser, 2014). There is a strong incentive to develop a paediatric formulation for PZQ to target pre-school children, who are currently excluded from mass drug treatments against schistosomiasis (World Health Organization, 2006; Stothard *et al.*, 2013; Mutapi, 2015). PZQ treatment is to date available for a price of around 0.20 USD; hence, a final price of 0.40 USD for the enantiomeric formulation is an ideal goal that is still to be met (Woelfle *et al.*, 2011).

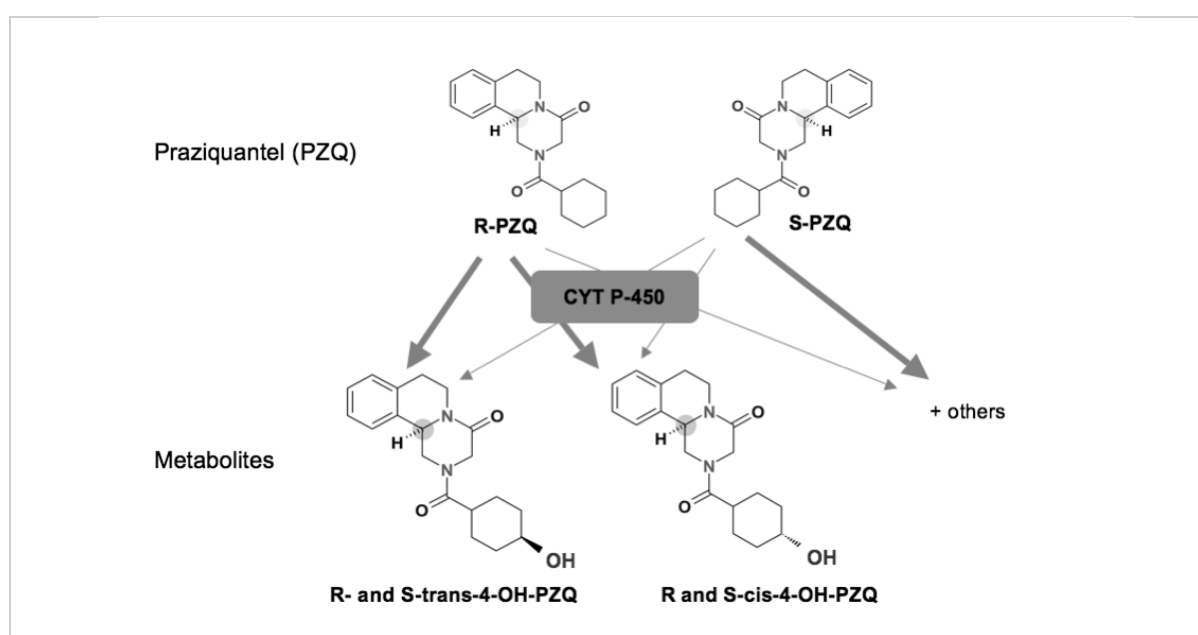


Fig. 6 The enantioselective metabolism of PZQ with the chiral centre highlighted with a circle

The fasciocidal drug triclabendazole is routinely used in veterinary medicine but is currently only registered in four countries for human use (Egypt, Ecuador, Venezuela and France). Triclabendazole displays activity against both immature and mature worms, but its spectrum is restricted to fascioliasis and eventually paragonimiasis. Successful treatment with triclabendazole is achieved in humans with an oral dose of 10 mg/kg that can be repeated in heavy infection cases (Keiser and Utzinger, 2009). Resistance has been reported since the mid-1990s in veterinary medicine in Australia and more recently in several countries of Western Europe as well

1.3.2 Alternative treatment candidates

Artemisinin, a sesquiterpene lactone containing a peroxidic group, is a molecule extracted from the leaves of the shrub *Artemisia annua*. This plant was known in the traditional Chinese pharmacopoeia for its therapeutic effect against fevers since 340 AD, and specifically for treating malarial fevers since 1596. The isolation and identification of the active compound in 1972 confirmed its broad antimalarial efficacy, even against chloroquine-resistant parasites. To optimise its antimalarial activity, the semi-synthetic analogues artemether and the water-soluble artesunate have been produced and successfully tested, although displaying a higher toxicity than artemisinin itself (Klayman, 1985; White, 2008). Due

(Fairweather, 2009). Bithionol is another marketed fasciocidal drug, but its use has been restricted to particular cases of triclabendazole failure because of its lengthy treatment (Keiser and Utzinger, 2009). Other schistocidal drugs, as oxamniquine or metrifonate, have been abandoned with the advent of PZQ. Oxamniquine presented the major disadvantage to be active only against *S. mansoni*. Hence, its was mainly used in Brazil (Utzinger and Keiser, 2004; Danso-Appiah *et al.*, 2008). Metrifonate, on the other hand, is only active against *S. haematobium*. These drugs might however be interesting in combination chemotherapy (Kramer *et al.*, 2014).

to high production prices, the treatment with semi-synthetic artemisinins remains above the financial means of many patients, who could already hardly afford chloroquine treatment. Also, long treatment schedules make patient compliance difficult (White, 2008). Most of the antimalarial activity of artemisinin is centred on the peroxidic bond, which is supposed to react with iron during haemoglobin digestion to produce reactive oxygen species (Vennerstrom *et al.*, 2004; Jefford, 2007). Therefore, fully synthetic peroxide analogues were tested (Vennerstrom *et al.*, 2004; Jefford, 2007), of which the trioxolane OZ439 was successfully brought to a first-in-man trial at a single oral dose (Moehrle *et al.*, 2013).

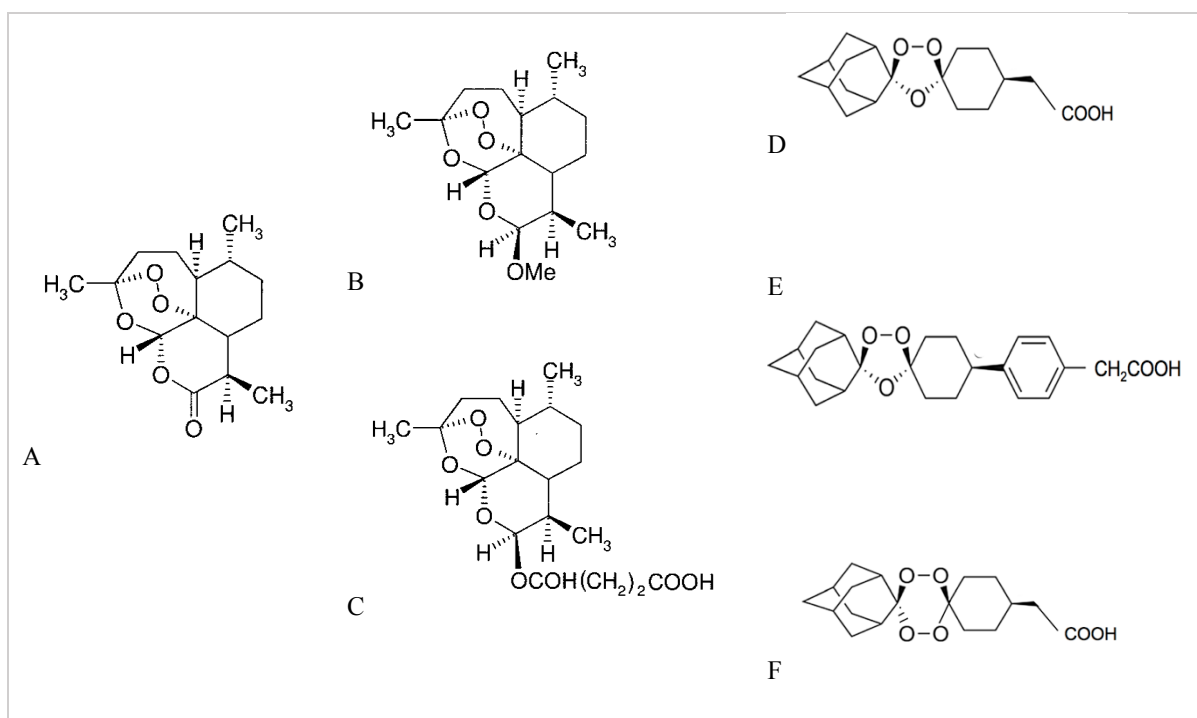


Fig. 7 Chemical structures of artemisinin (A), the semi-synthetic artemisinins artemether (B) and artesunate (C), and the synthetic peroxides OZ78 (D), OZ418 (E) and MT04 (F)

The high antimalarial potency of semi-synthetic artemisinins and synthetic peroxides lead researchers to investigate its activity against other parasites. For example, the trioxolane ozonids OZ78 and OZ418 exhibited encouraging effects in rodents against juvenile and adult *Schistosoma* spp (Xiao *et al.*, 2007; Xiao, Mei and Jiao, 2011; Keiser *et al.*, 2012; Xue *et al.*, 2014). Scaffold optimisations are currently under way to improve and stabilise schistocidal activities (Cowan *et al.*, 2015). OZ78 was also successfully tested in rats harbouring *F. hepatica*, *E. caproni* and *C. sinensis* (Keiser *et al.*, 2006; Keiser, Xiao, *et al.*,

2007; Keiser *et al.*, 2007). The fasciocidal effect of OZ78 seemed however to be limited to rats, since subcutaneous or oral treatment of infected sheep did not achieve any worm burden reduction (Keiser *et al.*, 2008). Combination chemotherapies with PZQ and tribendimidine were explored in *C. sinensis*, but the same combinations with semi-synthetic artemisinins still show comparatively better results (Keiser and Vargas, 2010). Recently, the synthetic tetraoxane MT04 displayed even higher fasciocidal effects than OZ78 *in vitro* and *in vivo* in rats (Kirchhofer *et al.*, 2011).

Tribendimidine, a derivative of amidantel, was synthesized in China in 1983, where it is approved for human use against soil-transmitted helminths (Utzing and Keiser, 2004; Xiao *et al.*, 2013). It displayed a high efficacy against *C. sinensis* and *O. viverrini* in rodents. However, it was not active against *S. mansoni* and *F. hepatica* (Keiser, Shu-Hua, *et al.*, 2007). In the frame of a phase 2a clinical trial, school children infected with *O. viverrini* were treated with the standard dose of PZQ or tribendimidine at the recommended oral dose for treating soil-transmitted helminths. The cure rates were similar, if not higher, for tribendimidine than for PZQ (Soukhathammavong

et al., 2011). Phase 2b trials for opisthorchiasis are ongoing. Infections with *C. sinensis* were also successfully cured in adults with tribendimidine, and here again, the effects of PZQ and tribendimidine was equivalent. Moreover, tribendimidine-treated patients seemed to suffer less adverse events (Qian *et al.*, 2013). In nematodes, tribendimidine acts on the acetylcholine receptor family, in a similar manner as the other nematocidal drugs levamisole and pyrantel. However, tribendimidine is still active against levamisole-resistant phenotypes, most likely since it can activate a different population of acetylcholine receptors than levamisole (Buxton *et al.*, 2014).

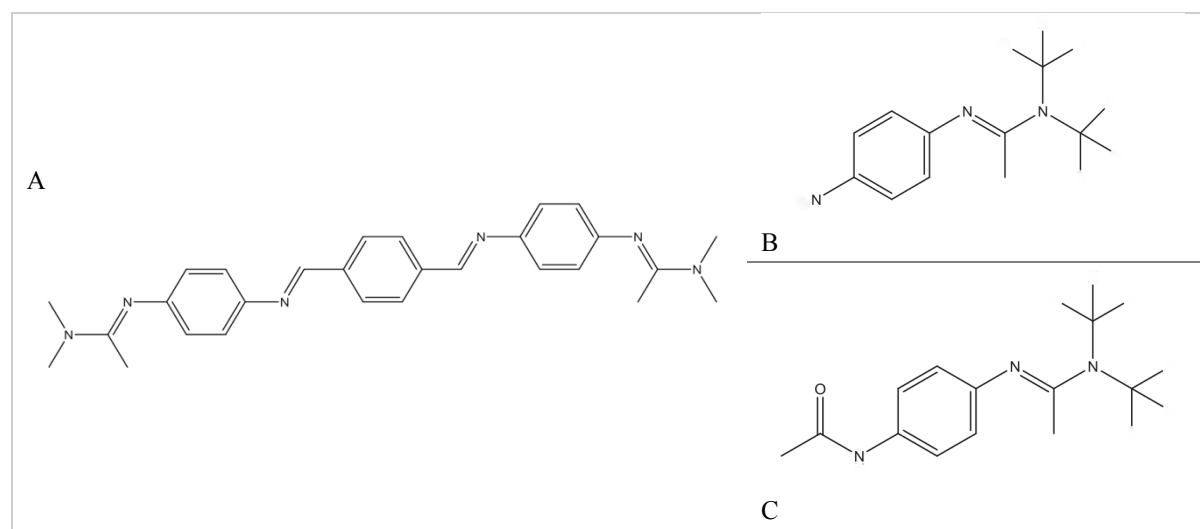


Fig. 8 Chemical structures of tribendimidine (A) and its two most significant metabolites dADT (B) and adADT (C)

Mefloquine was also first developed as a potent antimalarial until its antischistosomal activity was discovered in 2008. In contrast to PZQ, it is active against both juvenile and adult schistosomes. In an exploratory open-label trial, children co-infected with *S. mansoni* and *S. haematobium* were successfully treated with a combination of mefloquine and

artesunate (Keiser *et al.*, 2010; Xiao, 2013). Arylmethanol analogues, as enpiroline, also exhibited favourable effects *in vitro* and *in vivo* against *S. mansoni* (Ingram, Ellis and Keiser, 2012). Mefloquine, however, does not display activity against *F. hepatica* and *C. sinensis* (Keiser, Duthaler and Utzinger, 2010). Significant worm burden

reductions were observed in hamsters infected with *O. viverrini* (Keiser, Duthaler and Utzinger, 2010), but a clinical trial in humans failed to confirm an opisthorchicidal activity (Soukhathammavong *et al.*, 2011). The mode of action of mefloquine against

trematodes is not clear, but the drug seems to inhibit haeme aggregation (Ingram, Ellis and Keiser, 2012) and to interfere with the glycolysis pathway (Manneck, Keiser and Muller, 2012).

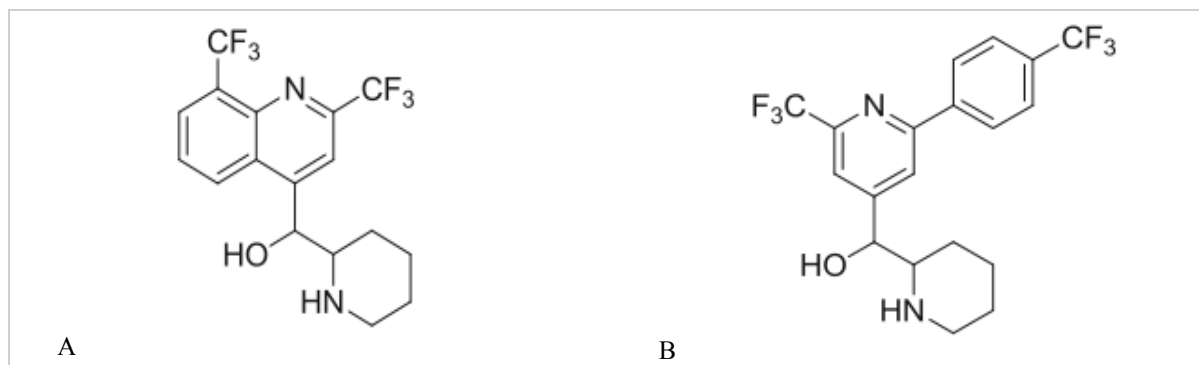


Fig. 9 Chemical structures of mefloquine (A) and one of its analogues, enpiroline (B)

Other trematocidal drug candidates might emerge from screening among drugs developed for another purpose, not only among malaria candidates but also against other protozoan infections. For example, nitazoxanide, a drug treating *Cryptosporidium* spp. and *Giardia intestinalis* infections displayed interesting fasciocidal activities. Similarly, screening among veterinary drugs might be the source of new trematocidal compounds that could be developed for

human use. Drug repurposing can also venture as far as cancer drugs. These libraries provide an enormous panel of different scaffolds that can later be optimised to specifically target parasites. For example, nilutamide, an antiandrogen used to cure prostate cancer, displayed effects against *S. mansoni* juveniles. Also, miltefosine, a drug initially treating mammalian cancer, showed activity against *S. haematobium* and is approved to treat leishmaniasis (Panic *et al.*, 2014).

1.4 Pharmacokinetic methods

The PK analyses included in the present thesis were all performed on a LC-MS/MS instrumentation and were validated according to the FDA guidelines for analytical method validation. A short introduction to these methods is therefore not superfluous. The LC-MS/MS system is an excellent tool for the quantification of drug concentrations in biofluids. Depending on their composition and the type of mobile phases carrying the sample, HPLC columns retain the molecule of interest while washing out most of the biological contaminants. By changing the concentration of the mobile phases, the analyte will elute at a specific time and will be carried on to the MS. The principle of mass spectrometry is to select

particles with a defined molecular weight using magnetic or electric fields. Since molecules in the MS are only distinguished according to their mass, the cleaner from contaminants the sample is, the more robust will be the analysis. A more precise molecular identification is possible with tandem MS (MS/MS). MS/MS spectrometers, after selecting for the mass of interest, will use a collision gas to break the molecule at its weaker chemical bounds and quantify the fragments produced. Fragmentation patterns are specific to chemical structures. Therefore, in the eventuality that a contaminant displays the same original mass as the molecule of interest, they can be segregated according to their fragmentation patterns.

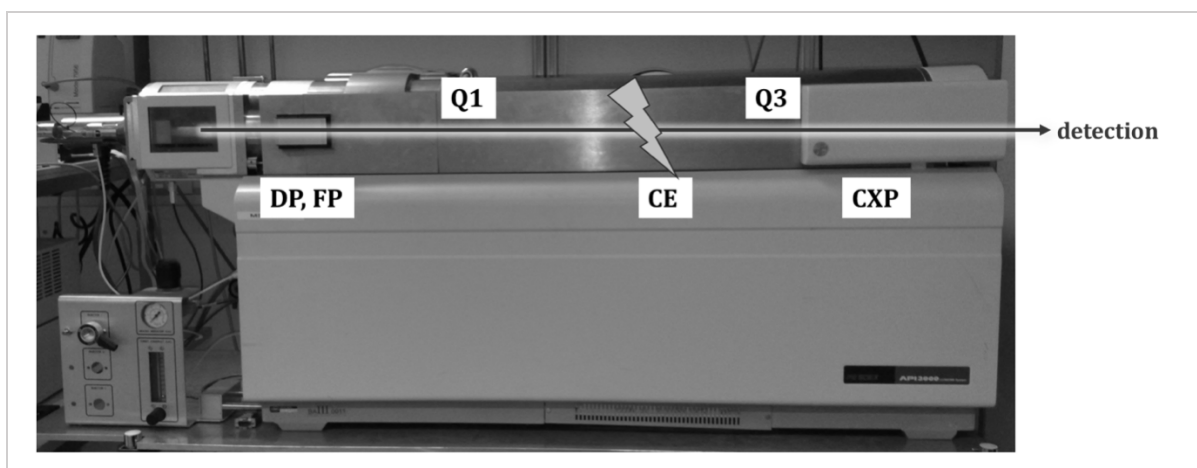


Fig. 10 Scheme of a MS/MS: declustering (DP) and focusing (FP) potentials direct the beam of molecules to the first quadrupole (Q1), where a first mass segregation occurs. The selected molecules continue to the collision chamber where an optimised collision energy (CE) fragments the parent molecule. The fragments of desired mass are then selected for in the second quadrupole (Q3) and the cell exit potential (CXP) guides them to the detector.

Further modifications of the instrumentation can be performed to maximise analyte signals, as for example column switching. This system allows to load the sample on a first, and usually shorter, chromatographical column and to wash the sample from contaminants before eluting the analytes to the main HPLC column for chromatographical separation.

The advantage of this method is to provide the main HPLC column with analytes virtually free from matrix contaminants. In the frame of this thesis, it was especially useful for analysing bile samples, and in the case of PZQ to protect as much as possible the chiral column from contaminants.

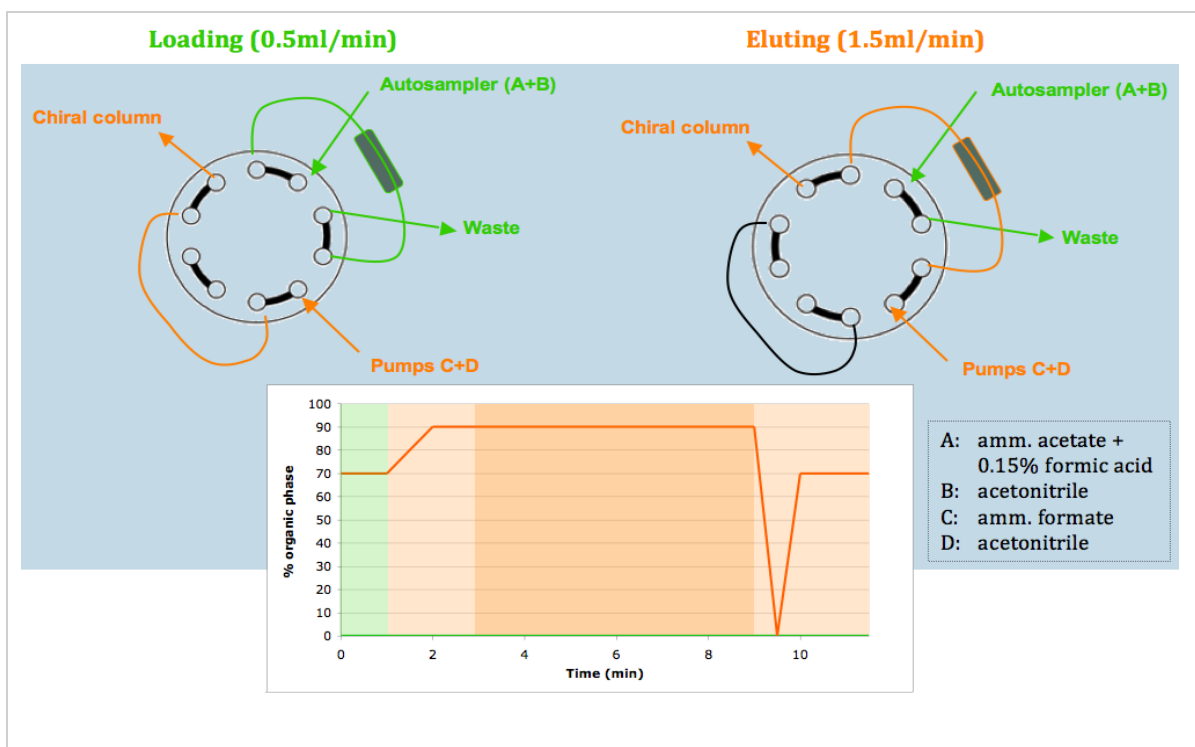


Fig. 11 Scheme of a typical column switching system on a 10-port valve: in green, the loading phase of the trapping column (sketched with a grey rectangle) with respective mobile phases; in orange, the elution phase to the chiral column. The dark orange panel on the time program shows the scanning time of the MS.

The validation of an analytical method should follow defined steps to ensure measurements as precise as possible, especially when performing extensive PK studies. The first obvious control is the stability of the analytes. The stability in the original matrix (plasma or blood) at storage temperature and time until analysis needs to be documented, as well as stability during the extraction procedure (bench-top stability), during several rounds of sample freezing and thawing and in the autosampler, while the sample batch is being analysed. The second crucial point is the selectivity of the method for the analyte. If, when analysing blank samples, a signal for the analyte is detected, the method needs to be adapted in a way that it will not interfere with analysis. The sensitivity of the method is important and needs to be tailored to the use of the method (quantify trace elements or diagnose

toxicity cases). The calibration line should be linear in the range of concentrations expected in the samples to be analysed. The accuracy of the method is evaluated by using quality control (QC) samples prepared to known concentrations and measured as normal clinical samples. The original concentration of QC samples is then compared to the one calculated from the calibration line. The precision of the method is assessed by re-measuring samples on different days and comparing how much they differ. The last step is to estimate how much of the analyte is lost during the sample extraction procedure, termed extraction recovery, and how much of the matrix contaminants are interfering with the analyte signal recorded, the relative recovery. The evaluation of recovery provides useful information on the robustness of the method and allows identifying weak points to be improved.

1.5 Goal and Objectives

Trematode infections represent an important burden in terms of global health and economic impact, and are tightly linked to poverty. Resistance is threatening the efforts to control trematodiasis, and alternative drugs lacking. Moreover, few studies on anthelmintic drug disposition in target patients are available, although

widely distributed in mass chemotherapy programs. The main goal of the present thesis is to contribute to an amelioration of the treatment options against trematodes, by investigations on lead peroxide candidates and on the standard drug praziquantel.

The following objectives were considered:

1. Validate an LC-MS/MS method to analyse the synthetic peroxide drugs OZ78 and MT04 in sheep plasma and bile, and elucidate their pharmacokinetic profile after a single intramuscular dose of 100 mg/kg (Chapter 2)
2. Investigate the effects of PZQ enantiomers and its main metabolites on *S. mansoni* adults and NTS by *in vitro* assays, microcalorimetry and *in vivo* in mice (Chapter 3)
3. Develop and validate an enantioselective method for R-PZQ, S-PZQ and the main metabolite R-trans-4-OH in plasma, blood and DBS (Chapter 4)
4. Elucidate the PK profile of *O. viverrini* infected patients in blood, plasma and DBS, and validate DBS as sampling technique for future PK trials with PZQ (Chapter 5)

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Chapter 2

Efficacy and pharmacokinetics of OZ78 and MT04 against a natural infection with *Fasciola hepatica* in sheep

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Efficacy and pharmacokinetics of OZ78 and MT04 against a natural infection with *Fasciola hepatica* in sheep

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Abstract

Fasciolosis is a parasitosis caused by the food-borne trematode *Fasciola* spp. of major veterinary significance. Triclabendazole is the first line treatment in humans and animals but cases of resistance are spreading worldwide. The synthetic peroxides OZ78 and MT04 are lead compounds for the treatment of fasciolosis. In the present study we investigated the efficacy and drug disposition following a single intramuscular dose of 100mg/kg OZ78 and MT04 in sheep harbouring a natural *Fasciola hepatica* infection. A liquid chromatography and tandem mass spectrometry (LC-MS/MS) method was developed and validated to quantify plasma and bile concentrations of both compounds. Plasma samples were analysed with an accuracy for OZ78 and MT04 from 91 to 115% and a precision lower than 8.9%. Bile samples displayed an accuracy between 92 and 101% and a precision up to 12.7%. Bile samples were collected at 0 and 6 h post-administration. Plasma mean peak concentration was 11.1 µg/ml at 1.5 h for OZ78 and 4.8 µg/ml at 4.2 h for MT04. Mean AUC of OZ78 and MT04 was 6698 and 3567 µg×min/ml, respectively. Bile concentration at 6 h post-treatment was 1.0 µg/ml for OZ78 and 1.4 µg/ml for MT04. Treatment with OZ78 showed no effect on egg burden and adult worm counts *in vivo*, whereas MT04 displayed a significant egg count reduction of 98.5% and a worm burden reduction of 92%. In conclusion, our study reveals an excellent activity of MT04 against *F. hepatica* in naturally infected sheep and a first insight into its PK behaviour.

1. Introduction

Fasciolosis is a major zoonosis of livestock in temperate regions and is responsible of an annual estimated economical loss of US\$3 billion (Fairweather, 2005). This parasitosis is caused by the liver flukes *Fasciola hepatica* and *Fasciola gigantica*, and is responsible for morbidity and mortality, including weight loss, jaundice, hepatomegaly and biliary cholic. Triclabendazole is the drug of choice in both livestock and humans and is highly active against all the parasite stages (Keiser and Utzinger, 2009). Resistance against triclabendazole has been observed in Australia since 1995 and is now recorded in several European countries (Fairweather, 2009). This situation is critical and new fasciocidal drugs are urgently needed.

Synthetic peroxides, such as ozonide (1,2,4-trioxolane) OZ277, have been synthesized in the late 1990s in the frame of antimalarial research as

alternatives to artemisinin derivatives (Vennerstrom *et al.*, 2004). The screening of libraries of structural analogues has generated other interesting antimalarial candidates and ozonide OZ439 successfully completed phase I trials in healthy human volunteers (Moehrle *et al.*, 2013). In the past few years, the ozonides have also been studied against a range of helminths and OZ78 revealed activity against diverse trematode species, such as *F. hepatica*, *Echinostoma caproni* (Keiser *et al.*, 2006), *Clonorchis sinensis* (Keiser *et al.*, 2007), *Schistosoma mansoni* and *Schistosoma japonicum* (Xiao *et al.*, 2007; Xiao, Mei and Jiao, 2011). Additionally, its toxicological profile has been proven acceptable *in vitro* and in rats and the drug has good pharmacokinetic properties (Vennerstrom *et al.*, 2004). In *F. hepatica* infections, OZ78 achieved elimination of juvenile and adult worms in rats with a single oral dose of 100 mg/kg

(Keiser *et al.*, 2006). *In vitro*, adult *F. hepatica* was affected at concentrations of 10-100 µg/ml OZ78 (Keiser *et al.*, 2006; Halferty *et al.*, 2009). Incubation for 48 h at a concentration of 100 µg/ml of OZ78 produced significant tegumental damages and displayed an impact on vitelline and testis follicles on adult worms (Halferty *et al.*, 2009).

Recently, additional dispiro 1,2,4-trioxanes and 1,2,4,5-tetraoxanes were synthesized and tested in *F. hepatica* infected rats. Among those, the tetraoxane MT04 displayed a higher activity than OZ78 against *F. hepatica*, especially against adult worms (Wang *et al.*, 2011). A single 50 mg/kg oral dose of MT04 achieved a complete burden reduction in *F. hepatica* infected rats. Furthermore, the onset of action for MT04 was observed to occur between 18 and 24 h, in contrast to 24-72 h for OZ78 (Kirchhofer *et al.*, 2011). The goal of the present study was to test the efficacy of MT04 in sheep harbouring a natural *F. hepatica* infection. For comparison, one group of sheep was treated with OZ78. In previous experiments (Keiser *et al.*, 2010), OZ78 showed no activity in sheep at a single dose of 50mg/kg given orally and subcutaneously. Since a recent investigation on the fasciocidal properties of artemether and artesunate demonstrated an increased efficacy when the drugs were administered intramuscularly to sheep (Keiser *et al.*, 2008), we opted for a single intramuscular dose of 100 mg/kg for both compounds. We also refined and validated an existing LC-MS/MS method for OZ78 and MT04 (Kirchhofer, Keiser and Huwyler, 2010) with the purpose to study their drug disposition in plasma and bile.

Table 1 LC-MS/MS parameters for the detection of OZ78, MT04 and the internal standard OZ352

Parameter	OZ 78	MT 04	OZ 352
Source temperature [°C]	400	400	400
Nebulizer gas [L/min]	12	12	12
Curtain gas [L/min]	7	7	7
Declustering potential [V]	-56	-61	-61
Focusing potential [V]	-230	-230	-220
Collision energy [V]	-24	-20	-26
Cell exit potential [V]	-15	-11	-5
Entrance potential [V]	-10	-10	-10
Ion spray voltage [V]	-4200	-4200	-4200
Polarity of analysis	negative	negative	negative
Mass and transitions [m/z]	321→277	337→153	336→291

2. Materials and methods

2.1. Chemicals and reagents

OZ78, MT04 and OZ352 (internal standard, IS) were provided by Prof. J. Vennerstrom of the College of Pharmacy, University of Nebraska Medical Center

(Omaha, USA). Their chemical structures are depicted in Fig. 1. Acetonitrile, ethanol and methanol of HPLC grade were purchased from Sigma-Aldrich (Buchs, Switzerland). Ammonium formate of HPLC grade was obtained from Fluka Analytical (Buchs, Switzerland). Ultrapure water was produced with a Millipore Milli-Q water purification system. The solution used for the intramuscular application of OZ78 and MT04 was prepared with 20 ml Lipoid S 100 (Lipoid AG, Steinhausen, Switzerland), 10 ml ethanol, 30 ml NMP (N-methylpyrrolidone Ph.Eu./CAS 70142-34-6) and Arlasolve DMI (dimethylisorbide/CAS 5306-85-4) ad 100 ml. Blank sheep plasma was obtained from the local slaughterhouse (Basel, Switzerland).

2.2. LC-MS/MS equipment and conditions

The LC-MS/MS method developed here was adapted from Kirchhofer and colleagues (Kirchhofer, Keiser and Huwyler, 2010) for the analysis of OZ78 and MT04 in both plasma and bile. The HPLC system (Shimadzu, Kyoto, Japan) included two LC-20AD pumps, a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), and an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan). Chromatographic separation for plasma samples was accomplished at 25°C and 0.3 ml/min flow using a Kinetex XB-C18 column (50 mm × 4.6 mm, 2.6 µm, Brechbühler AG, Schlieren, Switzerland) combined with a Phenomenex security guard cartridge (Brechbühler). The solvent gradient was defined as follows: 0-1 min: 30% B; 1-4 min: 30-95% B; 4-9 min: 95% B; 9-10 min: 95-30% B; and 10-11 min: 30% B, where A was 20 mM ammonium formate in ultrapure water and B pure acetonitrile. To avoid contamination of the mass spectrometer, the flow between the HPLC and the MS was interrupted during 0-4 and 8-11 min of each run using a six-port switching valve (VICI Valco Instruments, Schenkon, Switzerland).

For the analysis of bile samples, the analytical column was combined with a trapping column (Halo C18, 4.6mm×5mm, Sertec Electronics, Olten, Switzerland). The column switching system operated with the following solvent gradient: 0-1 min: 30% B, 0.3 ml/min flow through the trapping column (sample loading) and 1ml/min through the analytical column (column wash); 1-4 min: 30-95% B, 0.3 ml/min flow through the trapping and analytical column (sample elution of the trapping column and loading onto the analytical column); 4-8min: 95% B (sample elution); 8-11 min: 30% B (column regeneration); and injection loop: 20 µl. In the first minute, the trapping column was washed with a flow of 1 ml/min.

An API 3000 tandem mass spectrometer (AB Sciex, Framingham, USA) equipped with a turbo ion spray source operating in the negative ionization mode was used for the MS/MS analyses. The applied mass

transitions and the MS parameters are summarized in Table 1. Instrument control and data analyses were performed using the Analyst 1.5 software (AB Sciex, 2008).

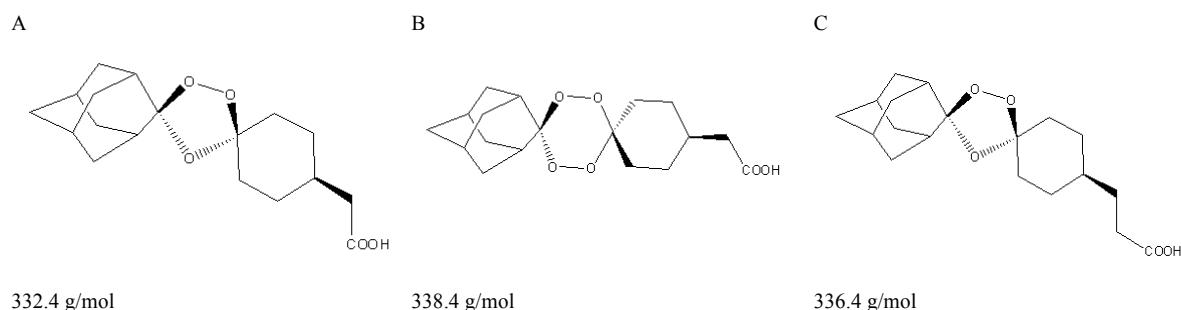


Fig. 1 Chemical structures of OZ78 (A), MT04 (B) and OZ352 (C, internal standard)

2.3. Standard, quality control and internal standard preparation

Stock solutions (OZ78 and MT04, 0.5 mg/ml) were prepared in acetonitrile. Working solutions of OZ78 and MT04 were obtained by serial dilution of stock solutions with pure water:acetonitrile 1:1 (v/v) to concentrations ranging from 300 to 6 $\mu\text{g/ml}$. The internal standard (IS) working solution was prepared at a concentration of 1 $\mu\text{g/ml}$ of OZ352 in acetonitrile (Keiser *et al.*, 2010).

2.4. Plasma sample preparation

Plasma calibration samples were freshly prepared by spiking blank plasma samples with the working solutions to reach final concentrations of 20, 10, 4, 2, 1 and 0.4 $\mu\text{g/ml}$ of OZ78 and MT04, where 0.4 $\mu\text{g/ml}$ was the lower limit of quantification (LLOQ). Quality control (Qc) samples were obtained by spiking blank plasma derived from 4 different sheep to final concentrations of 15, 3, 0.75 and 0.4 $\mu\text{g/ml}$ (high, medium, low and LLOQ concentrations). To adjust for matrix effects, calibration, Qcs and PK samples to be measured were further diluted 1:1 with the plasma used for the calibration samples.

Plasma samples were extracted using Phree 96 well plate kits (Phenomenex, Brechbühler) allowing an efficient filtering and removal of both proteins and phospholipids in one step. First, 400 μl IS was dispensed in each well. Then 100 μl of each plasma sample was added. The plates were agitated and centrifuged at $500 \times g$ and 10°C for 5 min (5804 R centrifuge, Vaudaux-Eppendorf AG, Switzerland). Five microliters of the filtrate was used for the LC-MS/MS analysis.

2.5. Bile sample preparation

Calibration and Qc samples ($n = 4$) were prepared for each single bile sample with the corresponding blank sample from the same animal collected at time $T = 0$. Calibration, Qcs and samples to be measured were diluted with one volume of mobile phase A. Extraction was performed by adding 400 μl IS to 100 μl sample followed by mixing in a thermomixer (Thermomixer Comfort 1.5ml, Vaudaux-Eppendorf) for 10 min at 10°C . Samples were then centrifuged ($5424 R$ centrifuge, Vaudaux-Eppendorf) at $21,130 \times g$ and 10°C for 10 min. 20 μl of the supernatant was used for the LC-MS/MS analysis.

2.6. Method validation

The LC-MS/MS method was validated with respect to selectivity, linearity, precision, accuracy, and recovery. Selectivity was tested by running 15 blank PK plasma and 16 bile samples extracted with pure acetonitrile to test for potential interferences with endogenous substances. The calibration curves were established using the internal standard method by plotting concentrations *vs.* peak-area ratios of the analytes to the IS. Accuracy and precision of the method was assessed by analysing Qc samples covering the calibration range (LLOQ, low, middle and high concentrations). Precision was determined using the coefficient of variation, and the accuracy estimated as the percentage ratio of the measured concentration to the nominal concentration. The uniformity of the extraction recovery (partial recovery) within the calibration range was evaluated by comparing three samples spiked before and after the extraction at low, medium and high

concentrations. Similarly, suppressive or enhanceive effects of the biological matrix (matrix effects) were quantified by comparing organic solvents or the biological matrix spiked with defined amounts of analytes (Table 2). The stability of each analyte has been reported elsewhere (Kirchhofer, Keiser and Huwyler, 2010).

2.7. Study design and sample collection

The drug trial in sheep was conducted between June and July 2012 in a farm located in the Campania region of southern Italy with a known history of fasciolosis. The study had received clearance from the Centre for Veterinary Service of the University of Naples Federico II (Ref. no. 99/12), which is the central ethical committee for the control of animal care in field trials. Treatment as well as blood and bile collections was performed by accredited veterinarians. Fifteen sheep with moderate infections (based on faecal egg burden) with *F. hepatica* were selected on the study farm. At days -2, -1 and 0 before treatment, sheep were weighted and their faecal egg burden estimated with the FLOTAC technique using zinc sulphate as flotation solution (Cringoli *et al.*, 2010). Based on egg counts three equal groups (n = 5/group)

were formed: controls, OZ78 treated and MT04 treated sheep. Sheep treated with OZ78 or MT04 received a single dose of 100 mg/kg drug intramuscularly. Drug was dissolved in as little solvent as possible and total injection volume did not exceed 15 ml.

Before treatment, 15-30 ml blood was collected from the jugular vein from each sheep using 10 ml EDTA-coated vacutainers (BD, Belliver Industrial Estate, Plymouth, UK). At t = 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 24 h post-treatment, 5 ml blood samples were drawn from treated sheep. Bile samples (10 ml) were collected prior to and 6 h post-treatment by direct puncture of the gall bladder of each treated sheep and transferred to 4.5ml Nunc Cryotubes (Sigma-Aldrich).

Blood samples were cooled and centrifuged within 1 h at a nearby laboratory to obtain plasma. Plasma and bile samples were transported on dry ice and stored at -80°C until assayed. At days 19, 20 and 21 post-treatment, the faecal egg counts were again determined. At day 21, all the sheep were transported to the local slaughterhouse and euthanized. The livers were dissected to collect and count the flukes in the veterinary facility of the CREMOPAR in Eboli.

Table 2 Accuracy and precision of quality control samples of OZ78 and MT04 measured in plasma and bile

Analytes	Nominal concentration [ng/ml]	Concentration measured [ng/ml] ^a	Accuracy [%] ^b	Precision [%] ^c	
Plasma	OZ 78	15000	13710	91.4	5.0
		3000	3171	105.7	5.6
		750	863	115.1	3.8
	MT 04	400	438	109.4	0.6
		15000	14051	93.7	4.1
		3000	3121	105.1	8.9
Bile	OZ 78	750	795	106.0	3.6
		400	432	108.1	8.2
		15000	15073	100.5	7.4
		3000	2968	100.9	7.3
		750	706	92.2	9.4
		400	385	92.3	12.7
	MT 04	15000	14668	97.8	3.5
		3000	2998	99.9	3.5
		750	749	99.8	4.1
		400	407	101.7	4.4

^a Mean of measured concentrations in 4 quality control samples

^b The accuracy is calculated as the percentage ratio of the measured concentration to the nominal concentration

^c The precision is the percentage ratio of the standard deviation to the measured concentration

2.8. Statistical analysis

Geometric means were calculated for individual *F. hepatica* egg counts and arithmetic means and medians with respective ranges were used to characterize adult worm burdens. Stata (version 12.1, StataCorp, TX, USA) was used for the statistical analyses. The difference in geometric means of egg counts pre- and post-treatment was compared between control and treated groups with a Student's t-test

allowing for unequal variances. For worm burdens, medians of control group were compared to medians in treated groups using the Wilcoxon-Mann-Whitney test. To assess differences in concentrations of OZ78 and MT04 in bile 6 h after treatment, an unpaired t-test allowing for unequal variances was performed. Differences in means and medians were considered to be significant at the 0.05 level.

2.9. Pharmacokinetic analyses

On the basis of the LC–MS/MS measurements, the following PK parameters for plasma were calculated:

C_{\max}	maximal plasma concentration
T_{\max}	time to reach C_{\max}
AUC	area under the curve, from 0 to 24 h
$T_{1/2}$	elimination half-life

C_{\max} and T_{\max} of OZ78 and MT04 were observed values derived from the plasma concentration time profile. AUC and $T_{1/2}$ were calculated with the software WinNonlin (Version 5.2, Pharsight Corporation, USA) using non-compartmental analysis. The elimination half-life was defined as: $T_{1/2} = \ln 2/\lambda$, where λ was determined by performing a regression of the natural logarithm of the concentration values during the elimination period

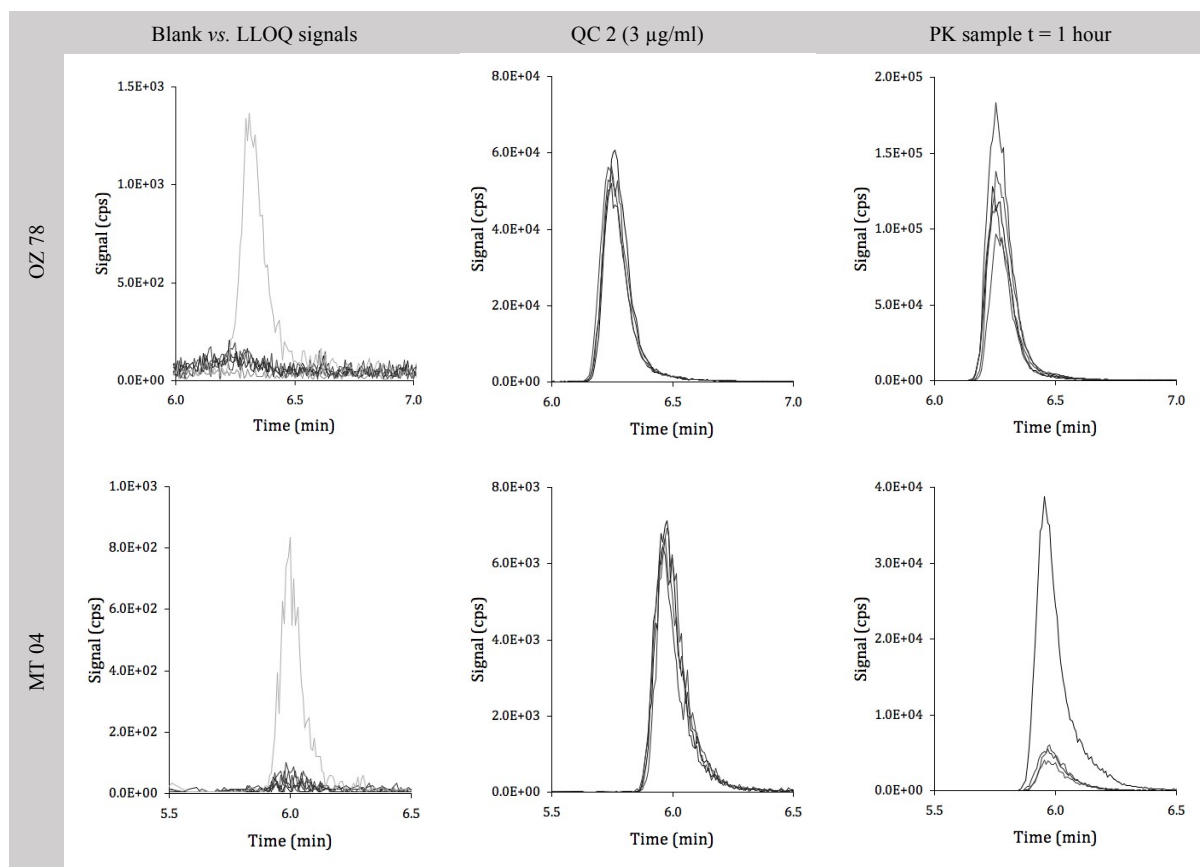


Fig. 2 Chromatograms of blank vs. LLOQ, QC 2, and PK sample $t = 1$ h in plasma

3. Results

3.1. Method validation

T_0 plasma and bile blanks were analysed to detect endogenous matrix components producing interfering peaks at the analyte retention time (Figs. 2 and 3). The bile average of noise and LLOQ signals, however, varied greatly in intensities between individuals. Mean plasma relative recovery was $82.75 \pm 3.25\%$ for OZ78 and $86.58 \pm 4.52\%$ for MT04. The total recovery observed for plasma was $83.83 \pm 4.95\%$ for OZ78 and $82.83 \pm 3.97\%$ for MT04.

The calibration lines were linear over the range of 0.4–20 g/ml with a coefficient of correlation (R^2) above 0.99 for plasma and bile. Accuracy and precision at the four Qc concentrations for plasma and bile are

summarized in Table 2. The accuracy for plasma ranged from 91 to 115%, with a precision between 0.6 and 8.9%. In bile samples, an accuracy between 92 and 101% and a precision of 3.5 to 12.7% was determined.

3.2. Efficacy of OZ78 and MT04

Table 3 summarizes the effects of OZ78 and MT04 on the faecal egg burden. Geometric mean egg counts of OZ78 and MT04 before treatment were 90.8 and 92.3 eggs per gram of faeces (EPG), respectively. The infection intensity ranged between 42 and 215.3 EPG for OZ78 and between 54 and 160.1 EPG for MT04. Between the start and the end of the study, EPG counts of the control group showed a statistically non-significant difference of 10.2% in EPG geometric means. The OZ78 treated group similarly displayed a

statistically non-significant EPG reduction of 49% with a single intramuscular dose of 100 mg/kg. However, at the same dose and administration route, we found for MT04 a highly significant EPG reduction of 98.5% after the treatment (p-value = 0.001). The effects of OZ78 and MT04 on total adult worm

burden are presented in Table 4. The mean *F. hepatica* burden in the control group was 5 worms. In the OZ78 treated group, on average 7.8 worms were recovered. In MT04 treated sheep, the mean worm burden was 0.4, which translates to a statistically significant worm burden reduction of 92%.

Table 3 *F. hepatica* faecal egg counts of control and OZ78- or MT04-treated sheep: mean group and individual egg counts pre- and post-treatment and respective percentage egg reductions

Treatment group Individual sheep	Pre-treat. means (SD) ^a Individual EPG values	Post-treat. means (SD) ^b Individual EPG values	Mean egg reduction (%) Individual egg reduction (%)	T ^c	P-value
Control	60.0 (37.1)	53.9 (30.5)	10.2		
1	46.4 (15.9)	52.4 (12.7)	0		
2	31.2 (9.2)	11.0 (3.5)	64.8		
3	55.5 (19.3)	52.9 (8.5)	4.7		
4	42.3 (15.1)	97.0 (24.0)	0		
5	124.5 (89.6)	56.2 (21.5)	54.8		
OZ 78	90.8 (72.0)	46.3 (28.8)	49.0	-1.26	0.12
6	42 (0.0)	28.7 (4.6)	31.6		
7	86.5 (19.3)	74.2 (10.3)	14.2		
8	43.4 (9.2)	23.0 (8.9)	47.0		
9	215.3 (21.6)	81.1 (25.0)	62.3		
10	66.9 (15.1)	24.3 (9.1)	63.7		
MT 04	92.3 (42.3)	1.4 (1.3)	98.5	-3.10	0.01
12	59.8 (6.0)	2.8 (1.4)	95.3		
13	99.5 (12.5)	2.5 (2.0)	97.5		
14	54.3 (13.0)	0 (0.0)	100		
15	160.1 (40.3)	1.6 (0.6)	99		
16	87.9 (18.6)	0 (0.0)	100		

^a Geometric mean (SD) of EPG data from day -2 to 0 post-treatment

^b Geometric mean (SD) of EPG data from day 19 to 21 post-treatment

^c T score obtained from the unpaired t-test

3.3. Pharmacokinetics of OZ78 and MT04 in plasma and comparative bile data

The plasma concentration-time profiles of OZ78 and MT04 are presented in Fig. 4 and corresponding PK parameters are summarized in Table 5. OZ78 and MT04 were both observed in plasma over 24h post-treatment. The OZ78 mean peak concentration of 11.1 µg/ml was reached after 1.5 h. MT04 displayed a peak concentration of 4.8 µg/ml at 4.2h. The mean AUC for

OZ78 and MT04 was 6698 and 3567 µg×min/ml, respectively. Elimination half-life was 13.1 and 13.3 h for OZ78 and MT04, respectively.

The concentration of each compound was measured in bile 6h after treatment. Mean concentrations of 1.0 ± 1.0 µg/ml OZ78 and 1.4 ± 0.6 µg/ml for MT04 were determined in bile fluids. We also detected a non-significant tendency (p-value = 0.561) of MT04 to be more concentrated than OZ78 in bile (Fig. 5).

Table 4 *F. hepatica* worm burdens (WB) of control and OZ78- or MT04-treated sheep: cure rates, group mean values and medians with ranges and worm burden reductions

Treatment group	Cure rate (%) ^a	Mean WB (SD)	Median WB (range)	Total WB reduction (%)	Z ^b	P-value
Control	0	5 (4.2)	4 (1-12)	-	-	-
OZ 78	0	7.8 (3.1)	7 (5-12)	0	-1.485	0.14
MT 04	60	0.4 (0.5)	0 (0-1)	92	2.463	0.01

^a Percentage number of animals cleared from *F. hepatica*

^b Z score obtained from Wilcoxon-Mann-Whitney test

Table 5 Pharmacokinetic parameters of OZ 78 and MT 04

Treatment group	C _{max} (µg/ml)	T _{max} (h)	AUC (µg×min/ml)	T _{1/2} (h)
OZ 78	11.1±5.1	1.5±0.9	6698.6±1634.2	13.1±6.1
MT 04	4.8±4.0	4.2±2.0	3567.5±2739.1	13.3±3.8

C_{max}: maximal concentration; T_{max}: time of peak concentration; AUC: area under the curve; T_{1/2}: time of half-life

4. Discussion

4.1. Method validation

The LC–MS/MS method used here was adapted from Kirchhofer, Keiser and Huwyler, 2010 to allow the analysis the analytes in bile. In addition, the covered concentration range was extended to 400–20,000 ng, which made the dilution of highly concentrated samples unnecessary. The overall sample handling time was shortened by simplifying the extraction procedure (acetonitrile extraction instead of evaporation and reconstitution in solvent) and the analytical runs were reduced from 14 to 11 min. The sensitivity and selectivity of our method was considered to be satisfactory, since the desired LLOQ of 400 ng/ml was achieved with a signal to noise ratio of 4:1. The recovery of analytes in plasma was high (>82%). However, different types of interfering matrix effects were observed between bile obtained from different sheep. We overcame this problem by establishing an individual calibration curve for each animal using blank bile obtained prior to the administration of drug. A precision of $\pm 15\%$ (LLOQ: $\pm 20\%$) and an accuracy between 85 and 115% (LLOQ: 80–120%) was accepted in our study.

4.2. Efficacy and PK profile of OZ78 and MT04

The treatment with 100 mg/kg OZ78 by intramuscular injection had no effect on a moderate natural *F. hepatica* infection in sheep. This result echoes the unsuccessful outcomes of our previous study in sheep which had been experimentally infected with *F. hepatica* and treated orally or subcutaneously with 50mg/kg OZ78 (Keiser *et al.*, 2010). Hence, doubling the dosage and changing the route of administration did not result in increased fasciocidal activity. On the other hand, at the same dosage and administration route, MT04 displayed a very good activity against chronic *F. hepatica* infection, achieving significant egg and worm burden reductions of 98.5 and 92.0%, respectively.

When comparing PK parameters of OZ78 and MT04, the time to reach the maximal plasma concentration (T_{max}) was 1.5 h for OZ78 and 4.2 h for MT04 with mean peak concentrations of 11.1 and 4.8 $\mu\text{g/ml}$, respectively. Elimination half-life did not differ between compounds and is approximately 13 h. However, oily and lipid formulations may lead to depot effects in muscle tissues, and since it has been shown that OZ78 and MT04 are rapidly eliminated in sheep (Keiser *et al.*, 2010), absorption is most likely

rate-limiting. Thus, the long $T_{1/2}$ observed here may be indicative of a prolonged absorption of drug.

OZ78 plasma maximal peak concentration and AUC values are higher, almost double, than those of MT04. Considering the lack of fasciocidal activity of OZ78 compared to MT04, the latter result is surprising. A high concentration of OZ78 in plasma is therefore not sufficient to guarantee a fasciocidal effect in sheep. The difference in fasciocidal activity between the two related compounds is certainly inherent to their chemical structures. MT04 is characterized by a tetraoxane moiety, while OZ78 is a trioxolane derivative. MT04 thus has an additional peroxide bond, which might explain the higher activity. A previous study by Wang and colleagues (Wang *et al.*, 2011) investigated a wide library of dispiro peroxides against *F. hepatica*. Besides the observation that spiroadamantane and carboxymethyl structures were required for fasciocidal efficacy, they noticed that trioxane and tetraoxane peroxides displayed a higher efficacy than corresponding trioxolanes. It seems thus that the peroxide bond is not the only element influencing efficacy but also that the 6-membered heterocycle (trioxane and tetraoxane) might offer advantages over the 5-ring structure (trioxolane). Moreover, it was observed that tetraoxanes had a better stability in red blood cells over corresponding trioxanes and trioxolanes (O'Neill *et al.*, 2010).

The drug disposition profiles of OZ78 and MT04 were monitored over a period of 24 h. The C_{max} and T_{max} determined here for a single OZ78 dose of 100 mg/kg were not different from the parameters observed in a previous study in sheep using a 50 mg/kg subcutaneous dose (Keiser *et al.*, 2010). The AUC found here is 50% smaller compared to the AUC observed at 50 mg/kg subcutaneous dose. It is surprising that the administration of a double dose is not reflected in a higher AUC. The elimination half-life on the other hand is much longer in our study than with the subcutaneous smaller dose. This difference might be explained by the administration route: a higher dose injected intramuscularly might diffuse more slowly due to a depot type effect, releasing the drug over time and in proportion to muscular activity. This depot effect could therefore be reflected by a smaller C_{max} and a longer elimination half-life. Thus, differences in drug bioavailability between the intramuscular and subcutaneous administration routes are likely due to differences in absorption from the injection site.

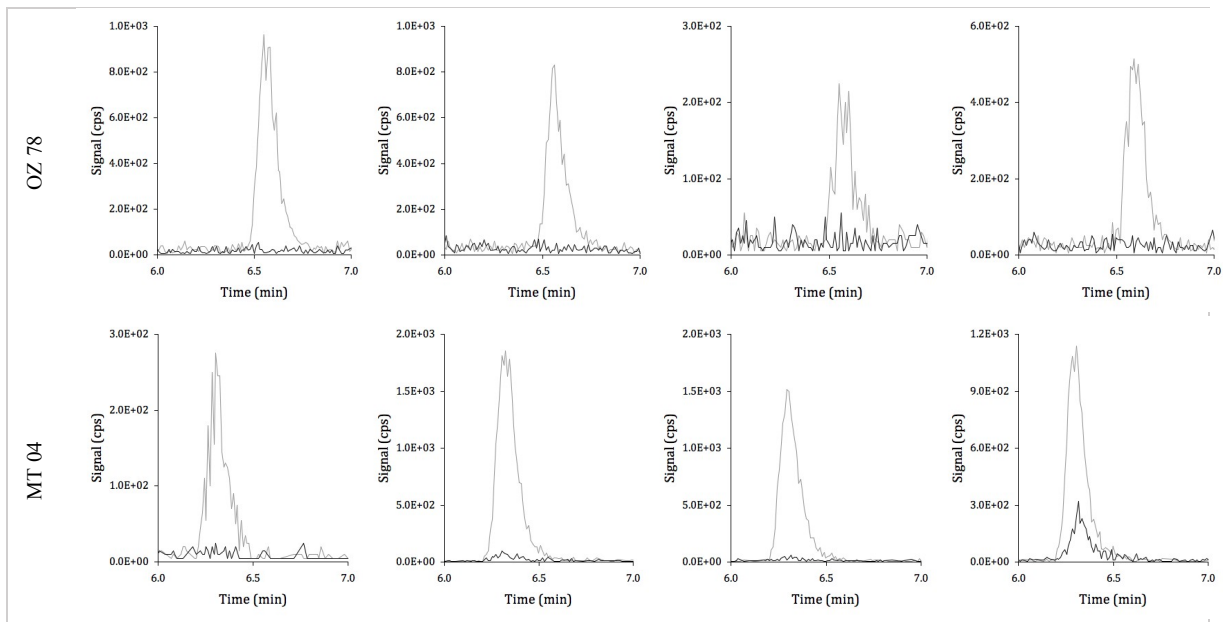


Fig. 3 Chromatograms of blank vs. LLOQ signals in bile samples from 8 sheep

Excellent fasciocidal activity was detected at a single oral dose of 100 mg/kg OZ78 in rats (Kirchhofer *et al.*, 2011). However, as mentioned above, no activity was observed in experimentally *F. hepatica* infected sheep at a dose of 50mg/kg (Keiser *et al.*, 2010) or at a higher dose, as observed here. In contrast to sheep, compounds in rats with a molecular weight >300 are well excreted into bile (Smyth and Haslewood, 1963; Abou-El-Makarem *et al.*, 1967; Toutain, Ferran and Bousquet-Melou, 2011). This difference with respect to biliary excretion of drugs could explain the discrepancy between the activity of OZ78 in rats and sheep. Following the unexpected low activity in sheep, a PK study with OZ78 and MT04 was conducted in uninfected rats. It was observed that C_{max} , T_{max} and $T_{1/2}$ did not significantly differ between sheep and rat. The AUC however was increased by a factor five in rats, which might explain the discrepancy of fasciocidal activity between both animal species. However, using uninfected rats might not reflect at best PK parameters observed in infected animals, and repeating the experiment in infected rats might allow generation of more fine-tuned hypotheses.

Besides PK measurements in plasma, we examined the concentration of OZ78 and MT04 in sheep bile 6h after treatment. The drug concentration in bile is of high interest because it mirrors the direct environment of the fluke. OZ78 and MT04 were less concentrated in bile than in plasma at the same time point. We also observed a trend towards higher concentrations of MT04 than OZ78 in bile. It is possible that MT04 undergoes greater biliary excretion, thus displaying a higher concentration than OZ78 in bile and therefore

revealing a higher efficacy against *F. hepatica*. However, to be able to draw a final conclusion on drug disposition in bile, the collection of additional sheep bile samples would have been required, which was not possible in the setup of the present study

The exact mechanism of action of peroxides against *F. hepatica* or other trematodes is not known. In malaria, peroxides are dependent upon haemoglobin digestion, leading to the formation of reactive intermediates including reactive oxygen species (Vennerstrom *et al.*, 2004; Jefford, 2007; Kořený, Oborník and Lukeš, 2013). *F. hepatica* dwells in biliary canals but feeds, as malaria, on red blood cells from the neighbouring biliary blood vessels. Peroxides might therefore also interact with the digestion process of *F. hepatica*. Keiser and Morson (Keiser and Morson, 2008) compared mortality and tegumental damage of *F. hepatica in vitro* between normal medium plus OZ78 and medium plus OZ78 supplemented with haemin. They observed that tegumental damage caused by OZ78 was more pronounced in the medium supplemented with haemin, but no change in mortality rate was recorded. It is likely that the presence of iron is not necessary for OZ78 action. Also, specimens treated *in vitro* with OZ78 displayed damages in the mid-body (no haemin) or anterior (haemin supplemented) regions, while parasites recovered from treated rats showed substantial damage to the posterior region. It is clear that *in vitro* conditions are not sufficient to mimic the *in vivo* effect of peroxides. Another study compared the uptake of albendazole, fenbendazole and triclabendazole by *F. hepatica* when adding bile in the incubation medium (Alvarez,

Mottier and Lanusse, 2004). They observed that the detergent properties of bile decreased the absorption of these drugs by the parasite. The properties of bile might also modify the lipophilicity of peroxides and possibly enhance their diffusion. It would therefore be

useful to study OZ78 and MT04 *in vitro* in the presence of sheep and rat bile salts to assess the diffusion of peroxides in the parasite, as well as to specify their mechanism of action.

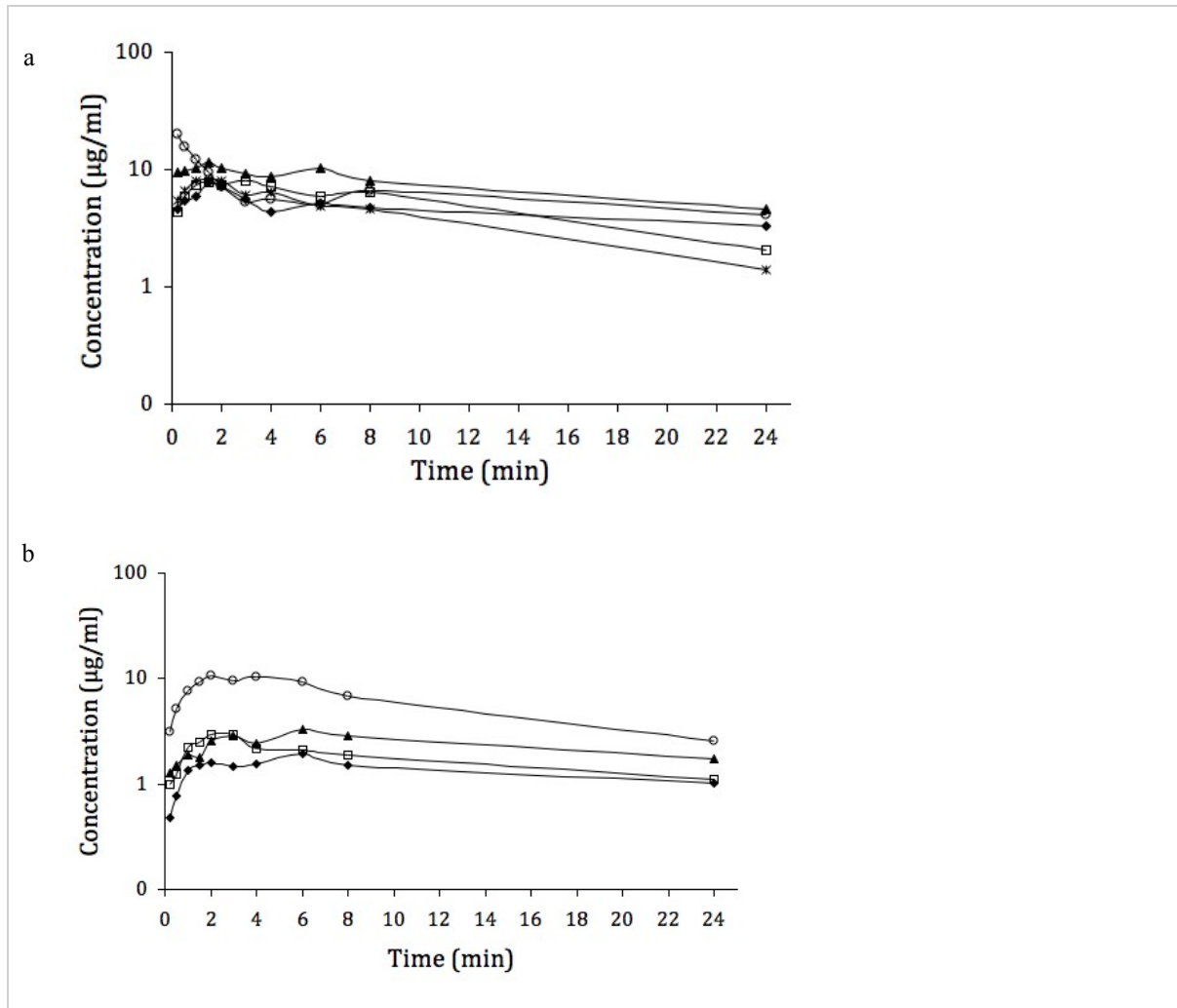


Fig. 4 Pharmacokinetic profiles in plasma of OZ 78 (a) and MT 04 (b) following a single intramuscular dose of 100mg/kg

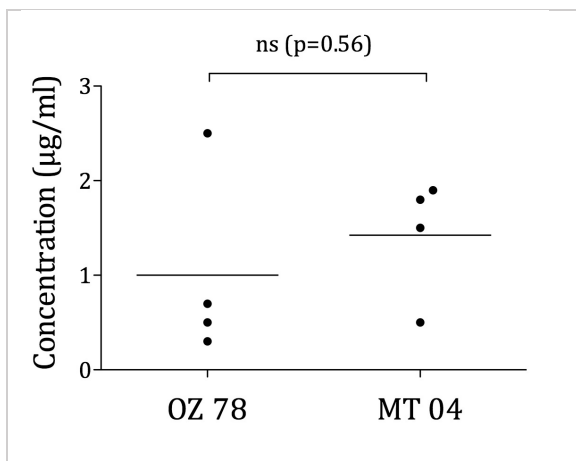


Fig. 4 Concentration of OZ 78 and MT 04 in bile 6 hours after treatment

5. Conclusion

The present study was conducted in sheep naturally infected by *F. hepatica*. Although field experiments might display different outcomes due to various factors (e.g. co-infections), the conditions of the sheep in this preliminary study are often encountered by farmers and field veterinarians. In contrast to OZ78, the tetraoxane MT04 shows better anthelmintic activity in sheep despite a lower drug concentration in plasma. Both peroxides were observed to be excreted in similar concentrations in bile, which does not explain their difference in efficacy. The result found with MT04 is encouraging and further experiments in sheep under controlled conditions and using a larger sample size should therefore be conducted. Efficacy of MT04 should also be assessed against juvenile worms.

Additionally, biliary excretion of MT04 and fluke uptake mechanism should be investigated in more detail to clarify its mechanism of action.

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Chapter 3

Activity of praziquantel enantiomers and main metabolites
against *Schistosoma mansoni*

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Activity of praziquantel enantiomers and main metabolites against *Schistosoma mansoni*

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Abstract

A racemic mixture of R and S enantiomers of praziquantel (PZQ) is currently the treatment of choice for schistosomiasis. Though the S enantiomer and the metabolites are presumed to contribute only a little to the activity of the drug, in-depth side-by-side studies are lacking. The aim of this study was to investigate the *in vitro* activities of PZQ and its main metabolites, namely, R- and S-cis- and R- and S-trans-4-hydroxypraziquantel, against adult worms and newly transformed schistosomula (NTS). Additionally, we explored the *in vivo* activity and hepatic shift (i.e., the migration of the worms to the liver) produced by each PZQ enantiomer in mice. Fifty percent inhibitory concentrations of R-PZQ, S-PZQ, and R-trans- and R-cis-4-hydroxypraziquantel of 0.02, 5.85, 4.08, and 2.42 µg/ml, respectively, for adult *S. mansoni* were determined *in vitro*. S-trans- and S-cis-4-hydroxypraziquantel were not active at 100 µg/ml. These results are consistent with microcalorimetry data and studies with NTS. *In vivo*, single 400-mg/kg oral doses of R-PZQ and S-PZQ achieved worm burden reductions of 100 and 19%, respectively. Moreover, worms treated *in vivo* with S-PZQ displayed an only transient hepatic shift and returned to the mesenteric veins within 24 h. Our data confirm that R-PZQ is the main effector molecule, while S-PZQ and the metabolites do not play a significant role in the antischistosomal properties of PZQ.

1. Introduction

Schistosomiasis or bilharzia is caused by blood flukes of the genus *Schistosoma* and is part of the group of neglected tropical diseases affecting more than 207 million people in tropical areas (Steinmann *et al.*, 2006; Utzinger *et al.*, 2011; Colley *et al.*, 2014).

The exclusive treatment to date for schistosomiasis is praziquantel (PZQ), which was discovered in the 1970s by Merck and Bayer. PZQ is administered as a racemic mixture of R and S enantiomers in tablets of 600 mg. The recommended dosage to treat schistosomiasis is 20 mg/kg three times in 1 day, and since PZQ does not act on juvenile worms, follow-up treatment 4 to 6 weeks later is strongly advised (Gryseels *et al.*, 2006). In preventive chemotherapy programs, PZQ is administered as a single 40-mg/kg dose to at-risk populations (Organization, 2006). PZQ undergoes significant first-pass metabolism through the liver enzyme cytochrome P450 (CYP) 3A4 and to

a lesser extent through 1A2 and 2C19 (Li *et al.*, 2003). R-PZQ is metabolized at a much higher rate than S-PZQ. R-PZQ is transformed mainly into cis- and trans-hydroxypraziquantel (4-OH-PZQ), while S-PZQ is converted to other monohydroxylated metabolites. In rat liver microsomes, the main metabolite is cis-4-OH-PZQ (Lerch and Blaschke, 1998; Meier and Blaschke, 2001), while in humans it is trans-4-OH-PZQ (Melo *et al.*, 2005).

The difference in the antischistosomal activity of each PZQ enantiomer has been known since 1983 (Andrews *et al.*, 1983), and several studies have observed greater activity of R-PZQ than of S-PZQ *in vitro* and *in vivo* (Tanaka *et al.*, 1989; Xiao and Catto, 1989a; Staudt *et al.*, 1992). A clinical trial with *Schistosoma japonicum*-infected patients also recorded a higher efficacy of R-PZQ than of racemic PZQ at the same dosage (Wu *et al.*, 1991; Xu *et al.*,

1994). Additionally, treatment with R-PZQ resulted in fewer adverse events than the standard treatment (Wu *et al.*, 1991). However, since higher drug concentrations in plasma and slightly longer half-lives are achieved with the metabolites than with PZQ (Lima *et al.*, 2011), it is possible that the metabolites contribute to the antischistosomal activity of PZQ. The efficacy of racemic trans-4-OH-PZQ was evaluated *in vitro* by Staudt and colleagues (Staudt *et al.*, 1992), who observed similar antischistosomal properties of the trans metabolite and R-PZQ against adult worms. In this study, we comparatively assessed the *in vitro* activities of R-PZQ, S-PZQ, and the metabolites cis- and trans-4-OH-PZQ against adults and newly transformed schistosomula (NTS). Drug effects were evaluated by using both microscopic readouts and isothermal microcalorimetry. Since the metabolites are also chiral molecules, we evaluated for the first time the *in vitro* efficacy of the respective R and S enantiomers. We also studied the *in vivo* activity of each parent enantiomer in mice and estimated the hepatic shift of the worms after each treatment.

2. Materials and methods

2.1 Mice and infection

All *in vivo* experiments were performed at the Swiss Tropical and Public Health Institute (Basel, Switzerland) and followed Swiss and cantonal animal welfare regulations (license no. 2070). Female NMRI mice (age, 3 weeks; weight, ca. 14 g) were purchased from Charles River (Sulzfeld, Germany) or Harlan Laboratories (Blackthorn, United Kingdom). The animals were allowed to adapt for 1 week under controlled conditions (22°C, 50% humidity, 12 h of light, and free access to water and a rodent diet) before experimental handling.

NMRI mice were infected subcutaneously with 80 to 100 cercariae, as previously described (Keiser, 2009).

2.2 Drugs and media

RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml; Life Technologies), and streptomycin (100 µg/ml; Life Technologies) was used for adult schistosome *in vitro* and microcalorimetry experiments. For NTS *in vitro* culture, medium 199 (Life Technologies) supplemented with iFCS and antibiotics was used.

Racemic PZQ was purchased from Sigma-Aldrich (Buchs, Switzerland). Enantiomers of PZQ and cis- and trans-4-OH-PZQ were acquired from Merck Serono (Darmstadt, Germany) and synthesized by Matthew Todd (University of Sydney, Sydney,

Australia) (Woelfle *et al.*, 2011). Racemic cis- and trans-4-OH-PZQ were obtained from Gilles Gasser (University of Zurich, Zurich, Switzerland) (Patra *et al.*, 2013). For *in vitro* studies, each compound was dissolved in dimethyl sulfoxide (DMSO; Fluka, Buchs, Switzerland) at a concentration of 10 mg/ml. For *in vivo* studies, the drugs were dissolved in 7% (vol/vol) Tween 80 and 3% (vol/vol) ethanol before oral treatment.

2.3 In vitro studies

NTS were obtained from cercariae by mechanical transformation (Keiser, 2009). Six to 12 h later, the schistosomula (100 NTS/well) were incubated in flat-bottom 96-well plates (BD Falcon) containing the drug solution in medium at 1.2, 3.7, 11.1, 33.3, and 100 µg/ml. Control NTS were incubated with the highest concentration of drug solvent used in the assays (2% DMSO). The plates were incubated at 37°C in 5% CO₂ for 72 h, and compound activity was microscopically assessed by using a motility scale ranging from 3 (normal activity) to 0 (no activity and granularity present) (Manneck, Haggemüller and Keiser, 2009).

To test the effect of each compound on adult worms, the drugs were diluted in medium in flat-bottom 24-well plates (BD Falcon) at concentrations ranging from 0.01 to 10 µg/ml for racemic PZQ and R-PZQ and from 0.4 to 100 µg/ml for S-PZQ and the metabolites. Control wells consisted of drug-free medium with 2% DMSO. At 7 to 8 weeks post-infection, *S. mansoni*-infected mice were euthanized with CO₂ and dissected and adult worms were collected from the hepatic portal and mesenteric veins. Four to 6 worms of both sexes were deposited in each well and incubated at 37°C. After 4 and 72 h, the worm condition was microscopically evaluated on a scale of 3 (normal activity and no tegumental alteration) to 0 (dead, highly granulated) (Keiser, 2009). To test the recovery of adult worms following a short exposure to S-PZQ, we incubated adult worms in medium with 100, 200, 300, or 400 µg/ml S-PZQ for 1 or 2 h and next transferred them to drug-free medium for up to 72 h. These motility values at 72 h were compared to the values of worms incubated in S-PZQ for 72 h and control worms incubated in drug-free medium. Fifty percent inhibitory concentrations (IC_{50s}) and IC_{90s} were determined with CompuSyn software by using the motility values obtained at different dosages. The eudysmic ratio (Testa and Trager, 1990) was calculated as follows: eudysmic ratio IC₅₀distomer/IC₅₀eutomer, where the eutomer, the active enantiomer, is R-PZQ and the distomer is S-PZQ.

2.4 Isothermal microcalorimetry

The microcalorimetry experiments were performed in triplicate on a 48-channel isothermal microcalorimeter (TAM48; TA Instruments, New Castle, DE). First, glass ampoules were filled with 2,900 μ l of medium and four worms of both sexes were added to each vial. Ampoules were then placed in the channels for the equilibration phase. Twelve hours later, 100 μ l of a prewarmed drug solution prepared in medium was injected with a 1-ml syringe (BD Plastipak, Becton, Dickinson S.A., Madrid, Spain). End concentrations reached 0.04, 0.2, and 1 μ g/ml for racemic and R-PZQ and 1, 5, and 50 μ g/ml for S-PZQ and the metabolites. Ampoules containing schistosomes in the presence of DMSO alone (final concentration of 2%) served as negative controls, while ampoules containing dead worms, obtained by dipping them in 70% ethanol for 5 min and rinsing them in a medium solution, served as positive controls. Schistosome motility data derived from noise amplitudes were recorded for 5 days and analyzed with R software and Excel (Manneck *et al.*, 2011). The noise amplitudes produced by worm movements and metabolism decay exponentially as the worms die, until they reach the background noise level recorded in the dead-worm positive controls. The intersection of both curves determines the endpoint of worm motility (Manneck *et al.*, 2011).

2.5 In vivo studies

At 49 days post-infection (chronic *S. mansoni* infection), groups of three to six mice were treated orally with 400 mg/kg racemic PZQ, 400 or 800 mg/kg S-PZQ, or 100, 200, or 400 mg/kg R-PZQ. At 14 days post-treatment, the mice were euthanized and dissected. The worms in the veins and liver were sexed and counted (Xiao *et al.*, 2007). Mean worm burdens of treated mice were compared to those of untreated mice, and worm burden reductions (WBRs) were determined. IC_{50s} and eudysmic ratios were calculated as described above. The hepatic shift was investigated as follows. Groups of five mice infected with adult schistosomes were treated with 400 mg/kg S-PZQ, 400 mg/kg racemic PZQ, or 200 mg/kg R-PZQ. After 30 min, 1 h, 4 h, 24 h, and 7 days, one mouse in each group was euthanized and dissected and the worm burdens in its veins and liver were evaluated. Statistical tests were performed with Stata (version 12.1; StataCorp LP, College Station, TX). Differences

in worm burdens were assessed by using an unpaired t-test and allowing for unequal variances by comparing the control groups with the treated groups. The significance threshold was set at a P-value of 0.05.

Table 1 Calculated *in vitro* IC₅₀ and IC₉₀s of racemic and enantiomeric PZQ and 4-OH metabolites against adult *S. mansoni* at 4 and 72 h postincubation

Compound	IC ₅₀ (μ g/ml)		IC ₉₀ at 72 h (μ g/ml)	Eudysmic ratio
	4 h	72 h		
Rac-PZQ	0.1	0.05	0.4	293
R-PZQ	0.04	0.02	0.04	
S-PZQ	5.7	5.9	17.9	
Rac-trans-4-OH-PZQ	16.7	7.9	3694 ^a	
R-trans-4-OH-PZQ	13.4	4.1	58.4	
S-trans-4-OH-PZQ	NA ^b	NA	NA	
Rac-cis-4-OH-PZQ	15.8	4.8	81.4	
R-cis-4-OH-PZQ	4.5	2.4	48.7	
S-cis-4-OH-PZQ	NA	NA	NA	

^a Extrapolated value determined by CompuSyn.

^b NA, not active at 100 μ g/ml.

3. Results

3.1 In vitro studies

Table 1 summarizes the *in vitro* IC₅₀ and IC₉₀ of racemic and optically pure PZQ and 4-OH-PZQ metabolites against adult *S. mansoni* after 4 and 72 h of incubation. R-PZQ displayed the highest activity, with an IC₅₀ of 0.04 μ g/ml after 4 h of incubation. The IC₅₀ of R-PZQ after 72 h was half of the value for the racemic mixture, while the IC₅₀ of S-PZQ was higher by a factor 100. The IC_{50s} of the metabolites at 72 h showed the same pattern; the R conformation was twice as active as the racemic form, while no activity of the S metabolites at 100 μ g/ml was detected. When the activities of the cis and the trans configurations were compared, the cis metabolites displayed slightly better activity than trans metabolites but the IC_{50s} of the metabolites were nevertheless much higher than that of racemic PZQ. The eudysmic ratio of PZQ *in vitro* at 72 h post-exposure was estimated at 293.

The antischistosomal activity of S-PZQ following short-term incubation is depicted in Fig. 1. Worms incubated for 1 or 2 h in high concentrations of S-PZQ recovered almost completely and displayed high motility values (1.25 to 2.5) after 3 days, in contrast to worms incubated for a full 72 h in S-PZQ, which did not score above 0.5.

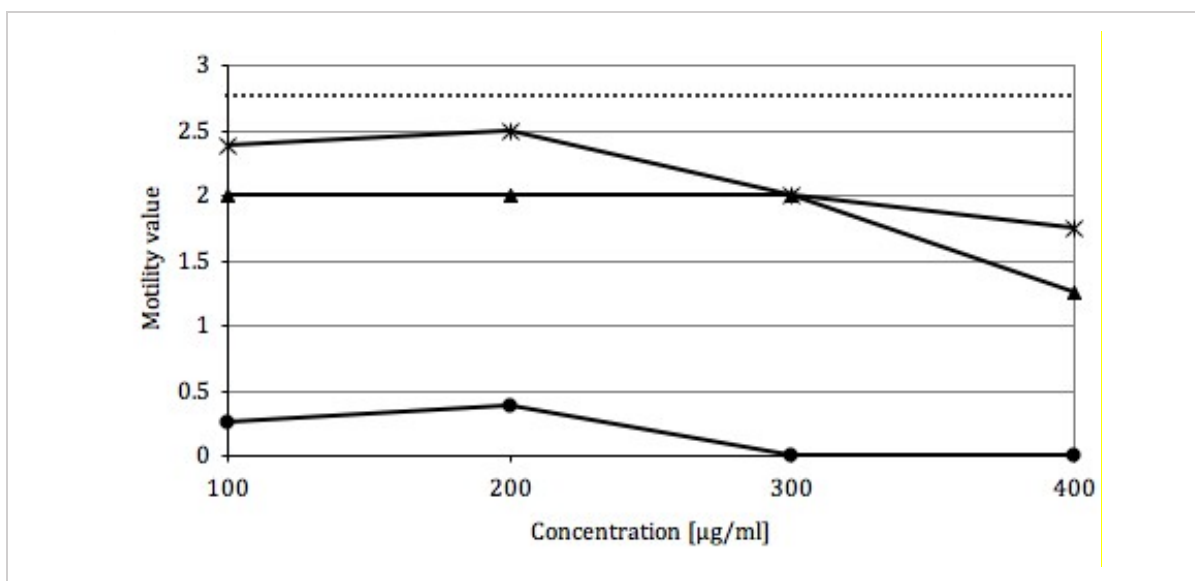


Fig. 1: Motility values of adult worms (n=4-6, in triplicate) after 1 h (*) or 2 h (▲) incubation in *S*-PZQ followed by incubation in drug-free medium until 72 h, compared with adults incubated 72 h in *S*-PZQ (●) and controls 72 h in drug-free medium (dashed line).

The results of the *in vitro* assays against NTS are displayed in Table 2. The IC₅₀ of R-PZQ was estimated at 0.03 µg/ml. *S*-PZQ showed markedly lower activity, with an IC₅₀ of 40.0 µg/ml. The eudysmic ratio calculated against NTS was 1,196. The IC₅₀s of the trans metabolites were determined as 133 and 28.5 µg/ml for the racemic and R derivatives, respectively, while the cis form of the R enantiomer showed moderate activity (IC₅₀ of 34.3 µg/ml).

Table 2 IC₅₀ and IC₉₀ values of racemic and enantiomeric PZQ and 4-OH metabolites against NTS 72 h post-incubation *in vitro*

	IC ₅₀ at 72 h (µg/ml)	IC ₉₀ at 72 h (µg/ml)	Eudysmic ratio
Rac-PZQ	1.5	34.5	1196
<i>R</i> -PZQ	0.03	18.8	
<i>S</i> -PZQ	40.0	522.5 ^a	
Rac- <i>trans</i> -4-OH-PZQ	133.1 ^a	5852.2 ^a	
<i>R-trans</i> -4-OH-PZQ	28.5	747.4 ^a	
<i>S-trans</i> -4-OH-PZQ	NA ^b	NA	
Rac- <i>cis</i> -4-OH-PZQ	911.1 ^a	2448837 ^a	
<i>R-cis</i> -4-OH-PZQ	34.3	1161.8 ^a	
<i>S-cis</i> -4-OH-PZQ	NA	NA	

^a Extrapolated value determined by CompuSyn.

^b NA, not active at 100 µg/ml.

3.2 Isothermal microcalorimetry

The worm motility endpoints after PZQ enantiomer and metabolite treatments are summarized in Table 3. With R-PZQ, worm motility ceased in the first 3 h post-injection at concentrations as low as 0.04 µg/ml,

while the same effect was observed for racemic PZQ only at 0.2 µg/ml. Worms exposed to the racemic and R metabolites at a concentration of 1 µg/ml did not display a decrease in motility. For racemic and *R-cis*-4-OH-PZQ, the motility endpoints at 5 µg/ml were estimated, as depicted in Fig. 2, as 96.7 and 3 h post-injection, respectively. Racemic *trans*-4-OH-PZQ was not active at 5 µg/ml, but *R-trans*-4-OH-PZQ produced a motility endpoint of 75 h post-injection.

Table 3 Endpoints of worm motility in hours post-injection (SD) determined by noise amplitudes for different concentrations of racemic and enantiomeric PZQ and 4-OH metabolites

	0.04 µg/ml	0.2 µg/ml	1 µg/ml
Rac-PZQ	> 120	< 3	< 3
<i>R</i> -PZQ	< 3	< 3	< 3
<i>S</i> -PZQ	> 120	> 120	> 120
	1 µg/ml	5 µg/ml	50 µg/ml
Rac- <i>trans</i> -4-OH-PZQ	> 120	> 120	< 3
<i>R-trans</i> -4-OH-PZQ	> 120	75 (5)	< 3
<i>S-trans</i> -4-OH-PZQ	> 120	> 120	> 120
Rac- <i>cis</i> -4-OH-PZQ	> 120	96.7 (16.1)	< 3
<i>R-cis</i> -4-OH-PZQ	> 120	< 3	< 3
<i>S-cis</i> -4-OH-PZQ	Not tested	Not tested	> 120

At a very high concentration of the racemic and R metabolites of 50 µg/ml, the motility of worms stopped within 3 h. None of the *S* derivatives interfered with worm motility after incubation for 5 days at 50 µg/ml.

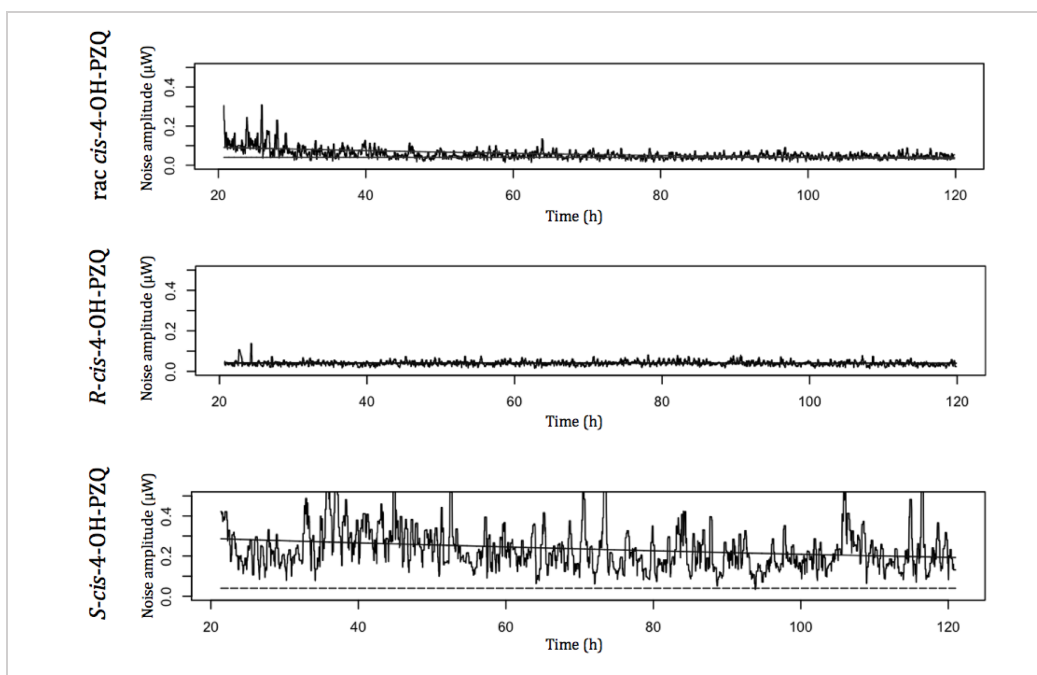


Fig. 2 Example of heat production recorded by microcalorimetry: rac cis-4-OH and R-cis-4-OH-PZQ at 5 µg/ml and S-cis-4-OH-PZQ at 50 µg/ml

3.3 *In vivo* studies

In Table 4, the WBRs after different single oral doses of R- and S-PZQ are presented. Racemic PZQ produced a WBR of 94.1% at 400 mg/kg, while no significant effect was observed at 100 mg/kg. R-PZQ showed a WBR of 52% at 100 mg/kg and WBRs of 98% at 200 and 400 mg/kg. S-PZQ displayed a low WBR of 19.6% at 800 mg/kg. When the worm burdens at 400 mg/kg were compared, there were significant differences between racemic PZQ and R-PZQ and the control group (P -values < 0.001). There was no statistically significant difference between the worm burdens of controls and those of S-PZQ-treated mice ($P=0.68$). The 50% effective doses (ED_{50} s) of R- and S-PZQ were 95.4 and >1,000 mg/kg, respectively, and the corresponding eudysmic ratio was >10.

The observed hepatic shift obtained with PZQ enantiomers for a single mouse per time point is illustrated in Fig. 3. Racemic PZQ acted rapidly; at 30 min post-treatment, only a few living worms were still observed in the mesenteric veins, while from 1 h onward, all of the worms were found dead in the liver. Treatment with R-PZQ at half the dose of racemic PZQ produced fairly similar effects. Living worms in veins, however, were observed until 4 h post-treatment. In contrast, treatment with S-PZQ resulted

in a high number of dead worms in the liver at 30 min post-treatment, after which the number of worms killed decreased over time, and after 4 h post-treatment only a small number of worms were found dead. At the 4-h examination point, all of the worms had migrated to the liver following treatment with S-PZQ. By 24 h post-treatment, the majority of the worms had returned to the mesenteric veins.

Table 4 Total and female worm burden reduction (WBR) obtained with racemic PZQ, R-PZQ and S-PZQ at different dosages in mice harboring adult *S. mansoni*

Dose	No. of mice	WBR [%] (SD)	ED_{50} (mg/kg)	Eudysmic ratio
Rac PZQ				
400 mg/kg	4	94.1 (8.6)	246.5	
100 mg/kg ^b	6	15 (9.5)		
R-PZQ				
400 mg/kg	3	100.0 (0)	95.4	
200 mg/kg	6	98.1 (2.3)		
100 mg/kg	6	52.0 (30.8)		
S-PZQ				
800 mg/kg	6	19.6 (22.2)	3066777 ^a	32136
400 mg/kg	4	18.0 (21.4)		

^a Extrapolated value determined by CompuSyn

^b Data from Keiser, Manneck and Vargas, 2011

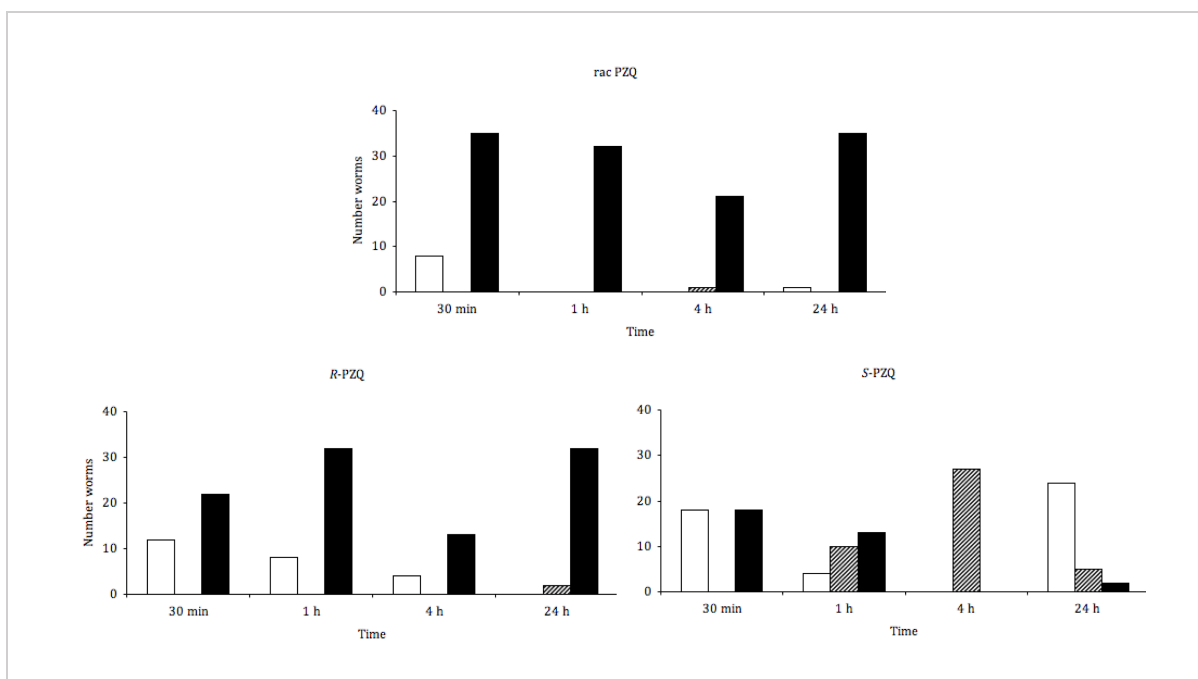


Fig. 3 *In vivo* hepatic shift after treatment with rac PZQ 400 mg/kg, R-PZQ 200 mg/kg or S-PZQ 400 mg/kg: number of worms alive in the mesenteric veins (white), alive in the liver (dashed) and dead in the liver (black)

4. Discussion

In the framework of a public-private partnership including Merck Serono, Astellas Pharma, the Swiss Tropical and Public Health Institute, and TI Pharma, efforts are ongoing to develop a pediatric formulation of PZQ. The project is currently in the preclinical phase, and in this work, we have for the first time conducted thorough side-by-side *in vitro* and *in vivo* studies with PZQ enantiomers and metabolites that will aid the development process.

Our data show that the antischistosomal activity is driven mainly by the R configuration. We observed that R-PZQ and the R-hydroxylated metabolites reveal 100- and 1,000-fold higher activities than their S counterparts *in vitro*. The racemic compounds display IC_{50} s twice as high as those of their respective R configurations. Note that the IC_{50} s observed against NTS were much higher than those against adults, which is in line with previous findings (Xiao, Catto and Webster, 1985; Ingram *et al.*, 2012). Nevertheless, R enantiomers are again more active than S conformations against NTS.

Microcalorimetry findings are consistent with our IC_{50} s determined microscopically against adults *in vitro*. The loss of motility produced by R-PZQ at 0.04 and 0.2 μ g/ml and by racemic PZQ between 0.04 and 0.2 μ g/ml correlates nicely with the IC_{50} s (0.02 and 0.05 μ g/ml, respectively). As observed in the *in vitro* microscopic assays, S-PZQ is not active at 1 μ g/ml. Microcalorimetric measurements confirmed that the

R-cis and R-trans forms are the active metabolites (e.g., with the R-cis and R-trans forms at 5 μ g/ml, a loss of motility was observed at 96.7 and 75 h post-injection, respectively). These observations are in agreement with our IC_{50} data (2.4 and 4.1 μ g/ml for the R-cis and R-trans forms, respectively) based on microscopic viability scores.

A similar pattern was observed *in vivo*. A single oral dose of 400 mg/kg of racemic PZQ shows activity similar to that of R-PZQ at 200 mg/kg. In contrast, treatment with S-PZQ at 800 mg/kg did not result in a significant WBR and none of the treated mice were cured.

Dissection of mice at different time points after treatment allowed us to investigate the hepatic shift caused by PZQ and its enantiomers. The hepatic shift of worms into the liver had been characterized earlier for PZQ, as well as for several other drugs, including mefloquine (Keiser *et al.*, 2009), artemether (Xiao and Catto, 1989b), oxamniquine (Foster and Cheetham, 1973), or older antischistosomal drugs (Buttle and Khayyal, 1962). Treatment with the racemate and R-PZQ efficiently immobilized or killed the majority of the worms, which were carried by blood flow to the liver, where they disintegrated over time. In contrast, treatment with S-PZQ killed only a few worms. Worms migrated to the liver and returned to the mesenteric veins 24 h post-treatment. The typical translocation of the worms into the liver might be explained by a loss of grip on the mesenteric vein wall

due to the chemical action of the compound, and when the therapeutic effect ceases, they migrate back to the mesenteric veins (Buttle and Khayyal, 1962). The return of worms to the mesenteric veins has been described for subtherapeutic doses or inefficient compounds (Bueding and Fischer, 1970). The transient hepatic shift observed in S-PZQ-treated mice is therefore strong additional evidence of its inefficacy.

In order to place our *in vitro* findings in context, we have summarized the pharmacokinetic (PK) parameters of R-PZQ, S-PZQ, and the R-trans and S-trans enantiomers obtained in humans (Lima *et al.*, 2011) in Table 5. The maximal concentration (C_{max}) of R-PZQ (0.16 $\mu\text{g/ml}$) is 8 and 4 times as high as its IC_{50} (0.02 $\mu\text{g/ml}$) and IC_{50} (0.04 $\mu\text{g/ml}$) at 72 h and still 4 times as high as its IC_{50} (0.04 $\mu\text{g/ml}$) at 4 h. Besides, the high ratio of the area under the curve (AUC) to the IC_{50} of 43.5 of R-PZQ might also describe its excellent antischistosomal activity. On the other hand, the concentrations of S-PZQ and the R-trans enantiomer in plasma do not exceed the calculated IC_{50} calculated in our work at any time (IC_{50} s approximately 11 and 3 times as high as the C_{max} s, respectively). Though the AUC of the R-trans metabolite is much higher than those of R-PZQ and S-PZQ, the AUC/ IC_{50} ratio is only 2.1. Furthermore, our *in vitro* recovery experiments with S-PZQ, even at concentrations up to 700 times its C_{max} (Lima *et al.*, 2011), demonstrated that the worms were still alive and recovered from 2 h of exposure. As

mentioned before, the S-trans metabolite is not active at 100 $\mu\text{g/ml}$.

Published PK data for the cis metabolite are not yet available, but in light of its high IC_{50} compared to that of R-PZQ, it is also unlikely that it significantly contributes to the antischistosomal activity of PZQ.

Changes in the activity of CYP enzymes can dramatically change the PK parameters of PZQ and thereby its therapeutic activity. For example, co-administration of CYP 3A4 inducers such as dexamethasone dramatically reduces plasma PZQ levels in patients with neurocysticercosis (Vazquez, Jung and Sotelo, 1987; Na-Bangchang, Vanijanonta and Karbwang, 1995; Li *et al.*, 2003). Albendazole is an inhibitor of CYP enzymes, and when it is administered concomitantly with PZQ, plasma R-PZQ levels are increased (Lima *et al.*, 2011). The expression of CYP is also modulated during chronic schistosomiasis, with markedly lower activity in infected mice, probably resulting from the immune response to the infection (Gotardo *et al.*, 2011). Interestingly, resistant isolates of *S. mansoni* do not inhibit host CYP as much as susceptible isolates do. This mechanism of resistance produces faster first-pass metabolism, hence a shorter time of exposure to the parent drug (Botros *et al.*, 2006). These results support the evidence that R-PZQ is the active molecule and metabolites do not have a major role in the activity of PZQ.

Table 5 PK parameters after an oral dose of 23.3 mg/kg in human volunteers^a: maximal concentration (C_{max}), time to maximal concentration (t_{max}), half-life ($t_{1/2}$), area under the curve (AUC), and ratio C_{max}/IC_{50} ^b and AUC/ IC_{50} ^b

	C_{max} ($\mu\text{g/ml}$)	t_{max} (h)	$t_{1/2}$ (h)	AUC ($\mu\text{g}\times\text{h/ml}$)	C_{max}/IC_{50} (ratio)	AUC/ IC_{50} (ratio)
R-PZQ	0.16	2.67	1.55	0.87	8	43.5
S-PZQ	0.52	2.55	1.46	2.99	0.09	0.5
R-trans-4-OH-PZQ	1.31	2.72	1.70	8.80	0.31	2.1
S-trans-4-OH-PZQ	0.78	3.05	1.91	5.60	NA ^c	NA ^c

^a adapted from Lima *et al.*, 2011 (oral dose of 23.3 mg/kg)

^b IC_{50} values from adults after 72 h

^c NA, not applicable (no IC_{50})

We conclude that the activity of PZQ is based almost exclusively on R-PZQ and that neither S-PZQ nor the metabolites significantly contribute to the therapeutic effect. Our results favor the development of a child-friendly formulation of R-PZQ, since an enantiopure formulation displays two major advantages; first, it would allow clinicians to reduce the dosage by half, and second, it would ease administration to children, who are bothered by the bitter taste of S-PZQ (Meyer *et al.*, 2009).

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Chapter 4

Development and validation of an enantioselective LC-MS/MS method for the analysis of the anthelmintic drug praziquantel and its main metabolite in human plasma, blood and dried blood spots

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Development and validation of an enantioselective LC–MS/MS method for the analysis of the anthelmintic drug praziquantel and its main metabolite in human plasma, blood and dried blood spots

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Keywords: praziquantel, R-trans-4-OH-PZQ, enantiomer, dried blood spot, LC–MS/MS, pharmacokinetics

Abstract

Praziquantel (PZQ) is the treatment of choice against various trematode and cestode infections. To study the pharmacokinetics of PZQ in patients infected with the liver fluke *Opisthorchis viverrini*, we developed and validated an enantioselective liquid chromatography coupled to tandem mass spectrometry method for the analysis of R- and S-PZQ and its R-trans-4-OH-PZQ metabolite in human plasma, blood and dried blood spots (DBS). The analytes were detected in the positive mode using selected reaction monitoring (R- and S-PZQ: m/z 312.2 → 202.2; R-trans-4-OH-PZQ: m/z 328.0 → 202.0). Prior to the chiral separation with a cellulose tris(3-chloro-4-methylphenylcarbamate) column, the analytes were purified from matrix contaminants and concentrated on a C-18 trapping column. The analytical range for each PZQ enantiomer was 0.01–2.5 µg/ml, and 0.1–25 µg/ml for the metabolite. The method met the requirements regarding precision (±15%, ±20% at the lower limit of quantification-LLOQ), intra- and inter-assay accuracy (85–115%, 80–120% at LLOQ), and linearity ($R^2 \geq 0.998$). The analytes were stable in stock solutions as well as in plasma, blood and DBS. For DBS, the influences of hematocrit and blood spot size were considered as minor. Our validation results show that the method presented here is precise, accurate and selective, and can be used for pharmacokinetic studies. Moreover, the enantioselective separation was achieved with a run time of 11.5 min and a simple sample processing method.

1. Introduction

Praziquantel (PZQ), a pyrazino-isoquinoline available as a racemic mixture of R- and S-enantiomers, is the drug of choice for the treatment of most trematode and cestode infections, including the liver fluke *Opisthorchis viverrini*, a parasite affecting more than 8 million people in Southeast Asia. PZQ undergoes an important enantioselective first-pass metabolism in the liver, mainly through CYP450 3A4 (Li *et al.*, 2003). R-PZQ is mostly metabolised to R-trans-4-OH-PZQ in humans or R-cis-4-OH-PZQ in mice, while S-PZQ breaks down into various mono- and di-hydroxy metabolites additionally to S-trans- and S-cis-4-OH-PZQ (Lerch and Blaschke, 1998; Meier and Blaschke, 2001; Melo *et al.*, 2005). PZQ disposition is heavily dependent on the fasting state or the co-administered food type, as well as on the liver function level (Castro *et al.*, 2000; Gotardo *et al.*, 2011). Therefore, it is of primordial importance to evaluate the

pharmacokinetics of PZQ in patients suffering from worm infections affecting the liver, such as opisthorchiasis and schistosomiasis, and relate treatment efficacy with dosage regimens and plasma concentrations.

The dried blood spotting (DBS) technique is a useful alternative for the collection of pharmacokinetic (PK) blood or plasma samples. In short, blood drops are collected by pricking a patient's finger, deposited on a filter paper and kept dried until analysis. This method provides many advantages over alternative sampling methods: no cold chain is required, handling and storage of samples are simple, and minimal blood withdrawal volumes are needed (Spooner, Lad and Barfield, 2009). The ease of sample collection allows handling a much larger group of patients and performing population PK studies in rural areas without clinical settings. However, the concentrations

measured in DBS are not necessarily equal to blood or plasma concentrations. For example, the drug concentrations in capillary blood might be different to that in venous blood, or the analyte might display a strong plasma protein binding (Emmons and Rowland, 2010; Xu *et al.*, 2013). To date different high-pressure liquid chromatography (HPLC) and liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) methods were published for chiral separation of PZQ enantiomers (Westhoff and Blaschke, 1992; Kelly, He and Stewart, 1993; Liu and Stewart, 1997; Polisel Jabor, Rocha and Bonato, 1997; Lima *et al.*, 2009), but they focused on the analysis of plasma samples only. The objective of this study was to develop a rapid, precise and accurate LC–MS/MS method to analyze R-PZQ, S-PZQ and R-trans-4-OH-PZQ metabolite in human plasma, blood and DBS. This method can be applied to future studies comparing PZQ concentrations in different bio-fluids and to support PK studies in patients in disease-endemic countries.

2. Materials and methods

2.1. Chemicals and reagents

Racemic (rac) PZQ was purchased from Sigma-Aldrich (Buchs, Switzerland). Enantiomers of PZQ and cis- and trans-4-OH-PZQ (trans-4-OH) were obtained from Merck Serono (Darmstadt, Germany). As internal standard (IS), eleven-fold deuterized PZQ (PZQ-d11) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Chemical structures of PZQ, PZQ-d11 and trans-4-OH are displayed in Fig. 1. Acetonitrile, ethanol and methanol of MS grade were obtained from Carl Roth GmbH (Allschwil, Switzerland). Ammonium formate, ammonium acetate and formic acid of MS grade were purchased from Sigma-Aldrich (Buchs, Switzerland). Ultrapure water was supplied with a Millipore Milli-Q water purification system (Merck Millipore, Darmstadt, Germany). Blank human plasma and blood

were obtained in lithium heparin-coated vacutainer tubes (BD, Allschwil) from the local blood donation centre (Basel).

2.2. LC–MS/MS equipment and conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of four LC-20AD pumps, a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), and an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan), coupled to an API 3000 triple quadrupole mass spectrometer (MS) (AB Sciex, Framingham, MA, USA) with a Turbo Ionspray interface. A first chromatographic separation was achieved at 25°C through a column trapping system (HALO C-18, 4.6 × 5 mm, Optimize Technologies, Oregon City, OR, USA) using 10 mM ammonium acetate and 0.15% formic acid in ultra-pure water as mobile phase at a flow rate of 0.3ml/min. The sample injection volume was set at 20 µl. After 1 min, the analytes were eluted via a ten-port switching valve (VICI Valco Instruments, Schenkon, Switzerland) from the trapping to the main column, a Lux Cellulose-2 column [cellulose tris(3-chloro-4-methylphenyl-carbamate) phase, 150 × 4.6 mm, 3 µm, Phenomenex, Torrance, CA, USA], for enantioselective separation. The elution gradient was defined as follows: 1-2 min, B 70-90%; 2-9.5 min, B 90%; 9.5–10 min, B 90–0%; 10–10.5 min, B 0–70%; 11–11.5 min, B 70% with mobile phase A consisting of 20 mM ammonium formate in ultrapure water and mobile phase B of pure acetonitrile. The flow rate was 1.5ml/min. Carry-over problems were controlled by rinsing the syringe and the loop twice with pure water and acetonitrile - isopropanol (1:1, v/v) after each injection. To avoid contamination of the MS, a second ten-port switching valve (VICI Valco Instruments) was used to divert the effluent of the analytical column from the MS during 0–3 and 9.5–11.5 min of each run. Due to the high flow rate of 1.5 ml/min, 50% of the flow was diverted through a splitter

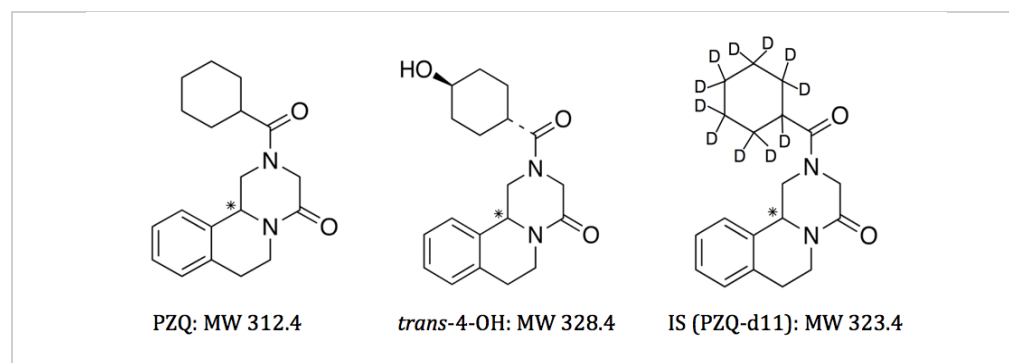


Fig. 1 Chemical structures of PZQ (A), the main metabolite trans-4-OH (B) and the internal standard PZQ-d11 (C), with the chiral centre designated with *

The analytes were detected by selected reaction monitoring (SRM) in the positive mode. Parameters were optimized by direct infusion of 1 µg/ml of PZQ or the trans-4-OH metabolite in acetonitrile at a rate of 10 µl/min (Harvard apparatus infusion pump 11, Holliston, MA, USA). Spectrometer parameters were as follows: nebulizer gas (N₂) flow was 12 µl/min, curtain gas (N₂) flow was 12 µl/min, source temperature was 400°C, ion spray voltage was 5500 V, collision gas (N₂) flow was 4 µl/min, entrance potential 10 V and dwell time was 300 ms. Analyst 1.5 software package (AB Sciex) was used for instrument control and data collection.

2.3. Standard, quality control and internal standard preparation

Stock solutions (racemic PZQ, R-PZQ and S-PZQ: 1 mg/ml; cis- and trans-4-OH in racemic or enantiomeric forms: 10 mg/ml; and IS: 1.25 mg/ml) were prepared in methanol and kept at -20°C. For the preparation of calibration and quality control (QC) samples, PZQ and R-trans-4-OH stock solutions were mixed to obtain a stock mix solution of 60 µg/ml of R- and S-PZQ and 600 µg/ml of R-trans-4-OH. Working solutions of R-PZQ, S-PZQ and R-trans-4-OH were obtained by serial dilution of the stock mix solution with pure water – acetonitrile (1:2, v/v) to concentrations ranging from 0.6 to 60 and 6 to 600 µg/ml, respectively. The extraction solution for blood and plasma consisted of 0.5 µg/ml IS diluted in acetonitrile. For DBS, the extraction solution containing IS was further diluted with ultrapure water [IS solution-water (4:1, v/v)].

2.4. Plasma, blood and DBS sample preparation

Calibration samples were freshly prepared and included in each analytical run by spiking blank samples with the working solutions to reach final concentrations of 2.5 down to 0.01 (lower limit of quantification-LLOQ) µg/ml for R- and S-PZQ, and of 25 to 0.1 (LLOQ) µg/ml for R-trans-4-OH. QC samples were similarly obtained by spiking blank samples to final concentrations of 1.75, 0.175, 0.0175 and 0.01 µg/ml (high, medium, low and LLOQ concentrations) for R- and S-PZQ, and to 17.5, 1.75, 0.175 and 0.1 µg/ml for R-trans-4-OH. For plasma and blood QC samples, each QC concentration was prepared in 6 replicates from different human donors. DBS QC sample replicates were prepared with 6 different hematocrit values ranging from 25 to 50% and the DBS calibration line with a hematocrit of 35%, following the procedure recommended by Koster *et al.*, 2015. DBS blood samples (20 µl) were deposited

on DMPK-C DBS cards (Whatman, GE Healthcare Life Sciences, Cardiff, UK) dried overnight and stored at room temperature in plastic bags containing silica gel desiccants.

2.5. Extraction procedure

Hundred µl of plasma or blood samples underwent protein precipitation with 700 µl of acetonitrile containing IS. The samples were vortex-mixed and agitated in a thermomixer for 20 min at 25°C and 18,400 × g. DBS samples were cut out of the filter card with a puncher of 5mm diameter and extracted with 300 µl of DBS extraction solution containing IS (see above). The filter-disks were vortex-mixed, agitated in a thermomixer for 20 min at 25°C, and sonicated for 40 min. Extracts were transferred to 96-well filter plates (2 µm PVDF membrane, Corning Life Sciences, Tewksbury, MA, USA) and centrifuged at 25°C and 1,500 × g for 10 min. The collecting plates were then sealed with pre-slit caps (Septra Seals®, Thermo Fisher Scientific, Hudson, NH, USA) and directly placed in the LC-MS/MS autosampler at 10°C.

2.6. Method validation

The LC-MS/MS method was validated for selectivity, linearity, precision, accuracy, recovery and stability according to the Food and Drug Administration (FDA) guidelines for bioanalytical method validation (Food and Drug Administration, 1999). The selectivity of the method was evaluated by running blank plasma, blood and DBS samples to estimate eventual interferences with endogenous matrix components. The LLOQ was set in order to achieve a sufficient sensitivity with a minimum signal to noise peak area ratio of 5, based on DBS samples, which supply a limiting amount of blood. Carry-over effects were tested by injecting a blank sample after the highest sample of the calibration line. Calibration curves normalized with IS peak areas were fitted by linear regression. The weighting factor for the linear regression ($1/x^2$) was selected to yield the lowest total error. Accuracy and precision of the method were assessed by analysing QC samples at LLOQ, low, medium and high concentrations. Precision was estimated using the coefficient of variation (CV) between 6 replicates, and accuracy was calculated as the percentage ratio of the measured concentration to the nominal concentration. A precision of ±15% (LLOQ: ±20%) and an accuracy between 85 and 115% (LLOQ: 80-120) was accepted in our method.

To estimate the uniformity of the extraction procedure (relative recovery) within the calibration range, sample extracts were compared to blank extracts

spiked with analytes at corresponding expected concentrations (low, medium and high QC concentrations). Similarly, suppressive or enhanceive effects of the biological matrix (matrix effects) were quantified by comparing the signal spiked in blank extract with the signal spiked in buffer [IS solution-water (7:1, v/v) for plasma and blood; DBS extraction solution for DBS]. The total recovery of the method was calculated as the signal in sample extracts compared to the signal in spiked buffer. Each concentration was prepared in 6 replicates from different human donors. The percentage recovery data should be constant across the calibration range.

The short-term stability of each analyte stock solution was estimated by comparing signals from stock solutions left 6 h at room temperature with freshly prepared solutions. The long-term stability of stock solutions was evaluated between 3 month-old solutions stored at 4°C and freshly prepared solutions. The bench-top stability of plasma and blood samples was tested between samples left 4 h at room temperature and fresh samples. The freeze-thaw stability was similarly determined using 3 cycles of freezing at -80°C and thawing at room temperature. The stability of extracts in the autosampler was evaluated between extracts stored at 10°C in the autosampler for 72 h and freshly extracted samples. The hematocrit bias in DBS was estimated by comparing QC samples prepared with blood from different hematocrit values (25 to 50%), as described above. The effect of the blood spot size was tested by comparing the signals from spots of blood of 15 vs. 30 µl.

2.7. Application of method

Blood, plasma and DBS sample collection was performed in the frame of a dose-finding study with tribendimidine against *O. viverrini* in humans in Lao PDR. Ethical clearance was obtained from the ethics committee of Basel, Switzerland (EKBB reference no. 375/11), and from the National Ethics Committee in Laos. The trial is registered at Current Controlled Trials (ISRCTN96948551). In short, a patient was treated with 3 oral doses of 25 mg/kg PZQ, with the second and third dose administered at 4 and 8 h after the first treatment. Eleven blood samples were collected at 0, 2, 4, 6, 8, 8.5, 9, 10, 11, 12 and 24 h after the first treatment. About 4 ml of venous blood was withdrawn from the antecubital arm vein through an i.v. catheter and collected in heparin-coated vacutainer tubes (BD). Within 30 min after sampling,

1 ml of blood was stored in a cryotube and the remaining blood centrifuged to obtain plasma. Plasma and blood samples were transported on dry ice to Basel (Switzerland) where they were kept at -80°C until assayed. Six DBS samples were collected as well at 0, 4, 8, 9, 11 and 24 h post-treatment. The samples were taken by puncturing the middle or ring finger with a sterile one-way finger pricker (Accu-Check Safe-T-Pro Plus, Roche, Switzerland). Lithium heparin coated capillaries (Alere™ Cholestech LDX®, V=40 µl) were used to collect and deposit the blood on DBS cards. The cards were allowed to dry overnight and stored in plastic bags with desiccant at room temperature.

3. Results and discussion

3.1. Method development

Different LC and LC-MS/MS methods were published for chiral separation of PZQ enantiomers in plasma (Westhoff and Blaschke, 1992; Kelly, He and Stewart, 1993; Liu and Stewart, 1997; Polisel Jabor, Rocha and Bonato, 1997; Lima *et al.*, 2009). However, these methods present long run times of at least 20 min and the use of normal-phase eluents requires long sample preparation procedures. The aim of our study was therefore to develop a less time-consuming analytical method suitable for running a large number of clinical samples. Sample processing duration and analytical run time were therefore critical points to be optimized in our method. In addition, while the earlier methods focussed on plasma, we aimed to validate a method that could be used for 3 different bio-fluids, i.e. blood, plasma and DBS, for a future bridging study. The intention behind using DBS is to be able to perform larger scale PK studies in remote settings in Africa and Southeast Asia, including young children. DBS methods clearly have limitations of sensitivity, given such the small volumes of blood assayed. For comparison, earlier developed methods using plasma reached an LLOQ of 1.25 ng/ml for the parent enantiomers and 12.5 ng/ml for the metabolite, which was not possible for DBS samples. However, such sensitivity is not absolutely needed because of the high PK disposition after a standard treatment. Finally, we increased the upper analytical range compared to the previous methods to fit better to PK profiles obtained in patients treated with the standard dose of PZQ, and therefore avoid sample dilution for S-PZQ and R-trans-4-OH.

Table 1 Recovery of R-PZQ, S-PZQ and R-trans-4-OH at low, medium and high QC concentrations in blood, plasma and DBS (n=6)

Matrix	Nominal conc. [$\mu\text{g/ml}$]	RRE [%] ^a \pm CV	ME [%] ^b \pm CV	Total recovery [%] ^c \pm CV	
R-PZQ	Blood	1.75	80.8 \pm 4.9	106.2 \pm 7.5	85.9 \pm 6.5
		0.175	79.4 \pm 9.0	105.0 \pm 6.2	83.4 \pm 2.3
		0.0175	86.2 \pm 5.1	99.6 \pm 8.3	85.9 \pm 6.9
	Plasma	0.01	99.4 \pm 4.0	101.3 \pm 4.9	100.7 \pm 2.1
		1.75	96.5 \pm 3.8	102.3 \pm 3.6	98.7 \pm 1.3
		0.175	93.9 \pm 4.4	106.0 \pm 3.8	99.5 \pm 1.3
	DBS	0.0175	81.2 \pm 7.1	97.3 \pm 3.8	79.0 \pm 3.5
		0.01	80.2 \pm 5.8	100.8 \pm 2.0	80.8 \pm 5.2
		1.75	84.9 \pm 7.5	97.4 \pm 1.4	82.7 \pm 1.7
S-PZQ	Blood	1.75	81.2 \pm 4.4	107.9 \pm 7.0	87.7 \pm 5.1
		0.175	79.3 \pm 8.0	106.2 \pm 6.7	84.2 \pm 2.8
		0.0175	86.5 \pm 3.8	100.4 \pm 8.6	86.8 \pm 7.4
	Plasma	0.01	98.4 \pm 4.6	99.0 \pm 3.8	97.3 \pm 2.7
		1.75	96.4 \pm 3.8	102.1 \pm 3.9	98.4 \pm 1.3
		0.175	94.1 \pm 4.3	104.8 \pm 2.7	98.6 \pm 1.0
	DBS	0.0175	80.9 \pm 7.2	95.8 \pm 5.3	77.5 \pm 3.0
		0.01	79.1 \pm 5.9	98.8 \pm 3.3	78.2 \pm 5.0
		1.75	84.2 \pm 8.5	96.9 \pm 1.5	81.6 \pm 1.9
R-trans-4-OH	Blood	17.5	79.8 \pm 8.2	127.0 \pm 6.4	104.0 \pm 6.6
		1.75	89.2 \pm 9.5	123.5 \pm 6.8	110.2 \pm 4.5
		0.175	87.5 \pm 9.8	111.8 \pm 5.0	94.6 \pm 6.0
	Plasma	0.16	86.9 \pm 9.6	83.3 \pm 5.0	71.0 \pm 13.0
		17.5	86.0 \pm 8.4	85.3 \pm 5.5	76.9 \pm 7.8
		1.75	81.2 \pm 6.3	74.4 \pm 6.8	70.4 \pm 4.5
	DBS	0.175	79.2 \pm 8.6	105.7 \pm 5.3	83.7 \pm 4.0
		0.1	79.8 \pm 2.6	105.2 \pm 8.0	83.9 \pm 6.0
		17.5	79.6 \pm 5.4	96.9 \pm 5.8	77.1 \pm 1.6

^a The relative recovery (RRE) is the signal in normally-extracted samples compared to the signal spiked after extraction

^b The matrix effects (ME) are the signal spiked after extraction compared to the signal spiked in buffer

^c The total recovery is the signal in normally-extracted samples compared to the signal spiked in buffer

First, the MS parameters were optimized in the positive mode for PZQ, trans-4-OH and IS, and for each analyte the mass transition with the highest signal was selected. The major analyte parameters are summarized in the Supplementary Table T1.

An efficient sample extraction method without evaporation and reconstitution was developed. For plasma and blood samples, a simple protein precipitation with acetonitrile was found to be

satisfactory. For DBS samples, the composition of the extraction solution was a critical factor. The use of pure acetonitrile led to incomplete extraction of analytes, while high water content increased sample background, due to extraction of water-soluble elements of blood. The dried blood was thus partially dissolved using a mixture of water and acetonitrile containing IS (1:4, v/v), followed by sonication for 40 min.

Table 2 Accuracy and precision obtained in DBS QC samples with hematocrit values from 25 to 50% (n=3) calculated with a calibration line of hematocrit 35%

QC concentration	Hematocrit [%]	R-PZQ [%] \pm CV	S-PZQ [%] \pm CV	R-trans-4-OH [%] \pm CV
Low	25	90.9 \pm 5.3	89.5 \pm 8.7	86.3 \pm 11.3
	30	96.2 \pm 8.9	91.5 \pm 9.9	90.3 \pm 5.8
	35	99.4 \pm 2.2	96.8 \pm 3.9	100.1 \pm 4.4
	40	103.6 \pm 0.6	103.6 \pm 6.2	111.0 \pm 6.3
	45	109.2 \pm 2.2	108.9 \pm 5.3	113.6 \pm 3.3
	50	113.5 \pm 2.2	111.1 \pm 1.5	103.4 \pm 8.8
High	25	97.9 \pm 2.4	101.4 \pm 8.5	97.9 \pm 6.7
	30	100.7 \pm 8.4	104.3 \pm 9.8	94.8 \pm 4.8
	35	110.1 \pm 9.0	109.4 \pm 10.3	92.6 \pm 0.8
	40	109.1 \pm 5.8	109.7 \pm 4.6	97.8 \pm 8.4
	45	111.5 \pm 2.0	114.5 \pm 2.2	109.8 \pm 5.8
	50	114.1 \pm 1.4	114.0 \pm 2.1	106.4 \pm 1.3

To purify and concentrate the analytes prior to chiral analysis, an in-line chromatographic separation using a C-18 trapping column was implemented. The adsorption of analytes on the trapping column was accomplished with an aqueous buffer. Ammonium acetate at 10 mM plus 0.15% formic acid displayed a 3-fold higher signal than non-acidic buffer or pure water.

The choice of the cellulose-2 column was based on the phase similarity to the Chiralcel OD columns used in previous enantioselective methods for praziquantel (Westhoff and Blaschke, 1992; Kelly, He and Stewart, 1993; Polisel Jabor, Rocha and Bonato, 1997). To optimize run times, acetonitrile was preferred over methanol for the organic mobile phase to separate PZQ enantiomers. For the aqueous phase, 20mM ammonium formate was chosen. Addition of acid modifiers, as formic or acetic acid, had a negative effect on the peak shape and was therefore not advised. By using a flow rate of 1.5 over 0.7 ml/min, a loss of half of the signal was observed. However, this was considered as acceptable in view of a 50% run time gain. A gradient from 70 to 90% mobile B in 1 min allowed us to achieve a baseline separation of the R-PZQ peak from the metabolite peaks.

Since PZQ enantiomers, as well as the mono-hydroxylated isomers cis- and trans-4-OH, have the same mass, their chromatographic baseline separation is necessary. Separation of R- and S-PZQ was achieved with a retention time of 5.5 and 7.5 min, respectively (Fig. 2). R-trans-4-OH, the main metabolite had the shortest retention time of 3.5 min. S-trans, and R- and S-cis-4-OH, which were not of interest, did not interfere with the analytics.

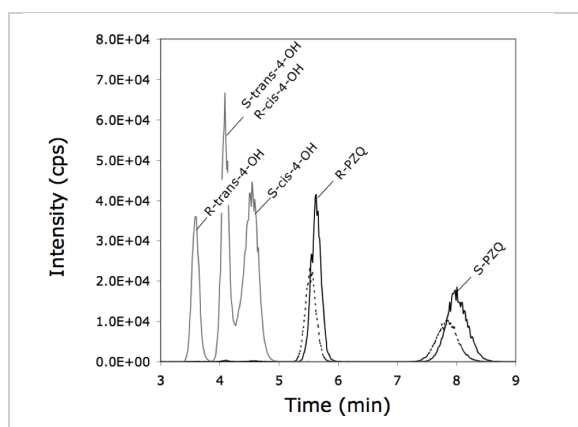


Fig. 2 Chromatogram of the chiral separation of PZQ (black line) and cis- and trans-4-OH enantiomers (grey line) at a concentration of 0.6 µg/ml, and IS (dotted line) at 0.5 µg/ml

The performance of other DBS card types, such as the 903 Protein Saver (Whatman) and the Bond Elut DMS (Agilent Technologies, Santa Clara, CA, USA), was also evaluated compared to the Whatman DMPK-C

cards used in the present study. Both card types from Whatman are made from a cellulose-based collection paper. The Agilent cards, on the contrary, are a non-cellulose technology and are designed to achieve a better signal and reduce the hematocrit bias (Agilent, no date). For Agilent cards, an extraction with acetonitrile-water (1:1, v/v) in addition to the standard 4:1 extraction was tested, without optimizing further the extraction method for this type of substrate. The choice of DBS paper turned out to be an important factor. Due to the physico-chemical properties of the Agilent cards, the blood drops were not spreading as much as on the cellulose-based cards and only a puncher of 3 mm diameter could be used. Both Whatman cards show similar signal intensities over the calibration line with low coefficients of variation (CV) (Supplementary Table T2). The Agilent cards, on the contrary, display much lower signals than the DMPK-C, and the recoveries of analytes vary with molecular structure and concentration tested, as observed by Cobb and colleagues (Cobb *et al.*, 2013) for different compound families. The linearity of DBS with the different methods reflected the results observed with the signal difference, with Whatman 903 being a good surrogate for DMPK-C, while Agilent cards exhibited non-linear calibration lines.

3.2. Method validation

The method was validated according to the guidelines for bio-analytical method validation of the FDA. The LLOQ was set at 0.01 µg/mL for R- and S-PZQ and at 0.1 g/ml for R-trans-4-OH. The method was sensitive at LLOQ concentrations, with peak area ratios of signal-to-noise at LLOQ higher than 5:1 for all the analytes. The carry-over peaks were as well lower than 5x the LLOQ signal. No co-eluting peaks from endogenous matrix components were observed in blank samples. Representative chromatograms of LLOQ vs. noise signals, and of a patient sample are depicted in the Supplementary Fig. F1, and in Fig. 3, respectively.

The calibration lines consisted of 8 points (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1 and 2.5 µg/ml for R- and S-PZQ; 0.1 0.25, 0.5, 1, 2.5, 5, 10 and 25 µg/ml for R-trans-4-OH) and were characterized by correlation coefficients for all the analytes higher than $R^2 = 0.998$. The accuracy and precision of the method was assessed intra- and inter-assay for each analyte, as summarized in the Supplementary Table T3. The accuracy observed in all the analytes was within the limits of 85 and 115% (80 and 120% at LLOQ) and the precision level observed was not above 15% (20% at LLOQ).

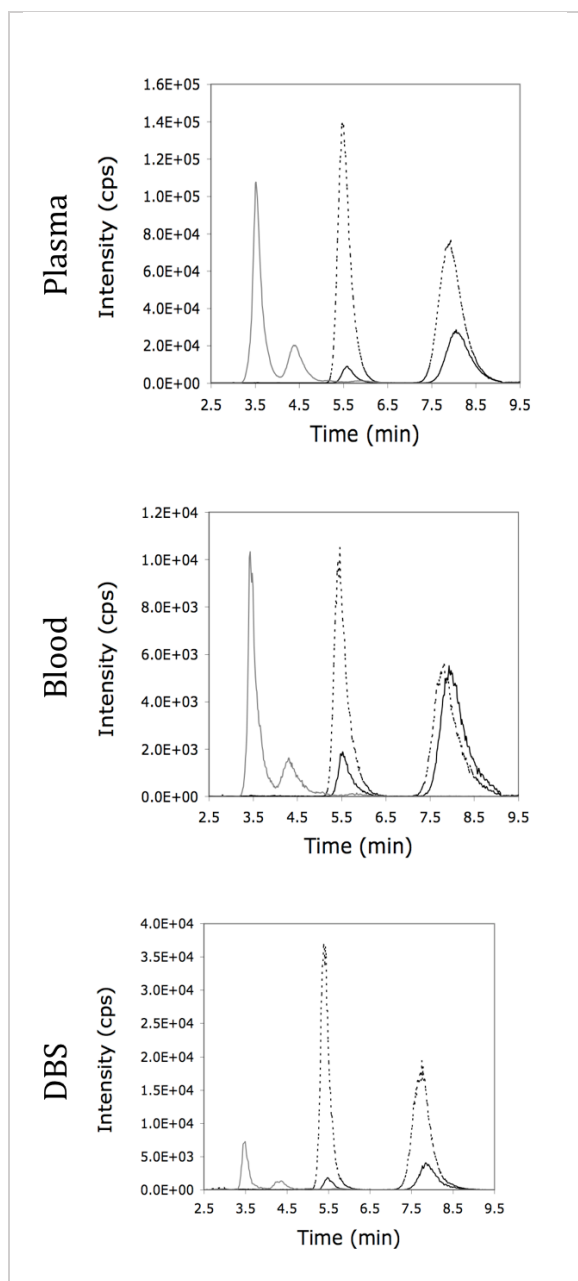


Fig. 3 Representative LC-MS/MS chromatograms of trans-4-OH (grey line), PZQ (black line) and IS (dotted line) in plasma (A), blood (B) and DBS (C) of a patient treated with 3x25 mg/kg PZQ at 2 h post-treatment

The recovery of analytes after the extraction procedure, as well as eventual enhancing or suppressing matrix effects, and overall total recovery were estimated for all the analytes across the calibration range (Table 1). Extraction recovery and matrix effects were similar for R- and S-PZQ for the different matrices. Matrix effects for the parent molecule were negligible. For the metabolite, the extraction recovery was similar to the parent analytes in blood and plasma, while in DBS the recovery efficiency was lower. Interestingly, for R-trans-4-OH, the matrix effects were minimal in DBS, while enhancing effects could be observed in blood matrix

and suppressive effects in plasma. The overall recoveries of all analytes were above 70% and constant across the 3 concentrations tested.

The stability of analytes was tested in the stock solutions as well as in the different matrices (Supplementary Table T4). Our stability results of PZQ and the main metabolite in plasma were consistent with previously published studies (Riditid *et al.*, 2002; Hanpitakpong *et al.*, 2004; Bonato *et al.*, 2007; Lima *et al.*, 2009, 2011). The analyte signals after 3 cycles of freezing and thawing was unchanged compared to fresh samples. Bench-top stability, i.e. the stability of analytes after 4 h at room temperature, was acceptable. Stability in the autosampler was not an issue, since extracted samples could be kept without variation in signal intensity for 72 h in the autosampler prior to analysis. Plasma and blood samples were stable for at least 6 months at -80°C . DBS samples could be kept at room temperature at least for 6 months without alteration of the analyte signals.

The validation of a method using DBS calls for additional parameters to be evaluated (Spooner, Lad and Barfield, 2009; Xu *et al.*, 2013). First, the blood hematocrit, by its influence on blood viscosity, can change the spread of the blood drop on the filter paper and consequently the recovery of analytes from DBS. In our study, a small hematocrit bias was observed: accuracy of all the analytes increased with increasing hematocrit. However, when using calibration standards with a middle-range hematocrit (i.e. 35%), accuracy values were within the recommended limits (Table 2). The DBS sampling technique might be as well prone to bias by repartition of the analytes over the spot area. Due to the physical behaviour of the drop depositing on the paper, the analyte might concentrate on the outer ring of the blood spot, leaving the punching area with a lower analyte concentration. The spread of the analytes in the blood spot was tested in different spot sizes. The percentage differences of signals obtained from spots of 30 vs. 15 μl spots ranged between 101.1 and 114.4% at low, medium and high concentrations for all the analytes. The spot size effect was therefore minor and we assume that all the analytes were uniformly distributed over the whole DBS spotting area.

3.3. Method application

The method reported here was applied to study the kinetic disposition of an *O. viverrini*-infected patient orally treated with 3×25 mg/kg PZQ. The PK profiles of R- and S-PZQ and R-trans-4-OH are illustrated in Fig. 4. The concentrations observed for R-PZQ are

overall lower by a factor of 2 compared to S-PZQ, with a T_{max} at 6 h after treatment. R-trans-4-OH displays a slower kinetic disposition with a peak concentration between 8 and 8.5 h. The C_{max} of the metabolite is almost 9 times higher than S-PZQ and almost 18-fold higher than R-PZQ. The C_{max} of S-PZQ in our study is, as expected, 3-fold higher than the one reported in a previously published study (Lima *et al.*, 2011) with healthy volunteers treated with a third of the dose used here (25 mg/kg vs. 3×25 mg/kg). R-PZQ, however, displays a lower C_{max} than expected from the aforementioned article (i.e. lower than 3x the C_{max}), probably because we did not sample at 2.5 h, the T_{max} recorded by Lima and colleagues. Also, the administration in 3 doses over 8 h instead of a single

dose might account for the differences of PZQ disposition between the present study and the article by Lima and colleagues. For the metabolite, on the contrary, the C_{max} observed in our study is much higher than expected, which might be explained with the different dosing regimen and the metabolite accumulating over time, probably due to a poor elimination. Blood and DBS display very similar PK profiles, while plasma shows a slightly higher disposition than blood or DBS for the parent molecule and slightly lower disposition for the metabolite, likely arising from a lower plasma affinity of the metabolite compared to the parent analyte. Overall, blood, plasma and DBS PK profiles are consistent for each analyte, demonstrating the applicability of our method.

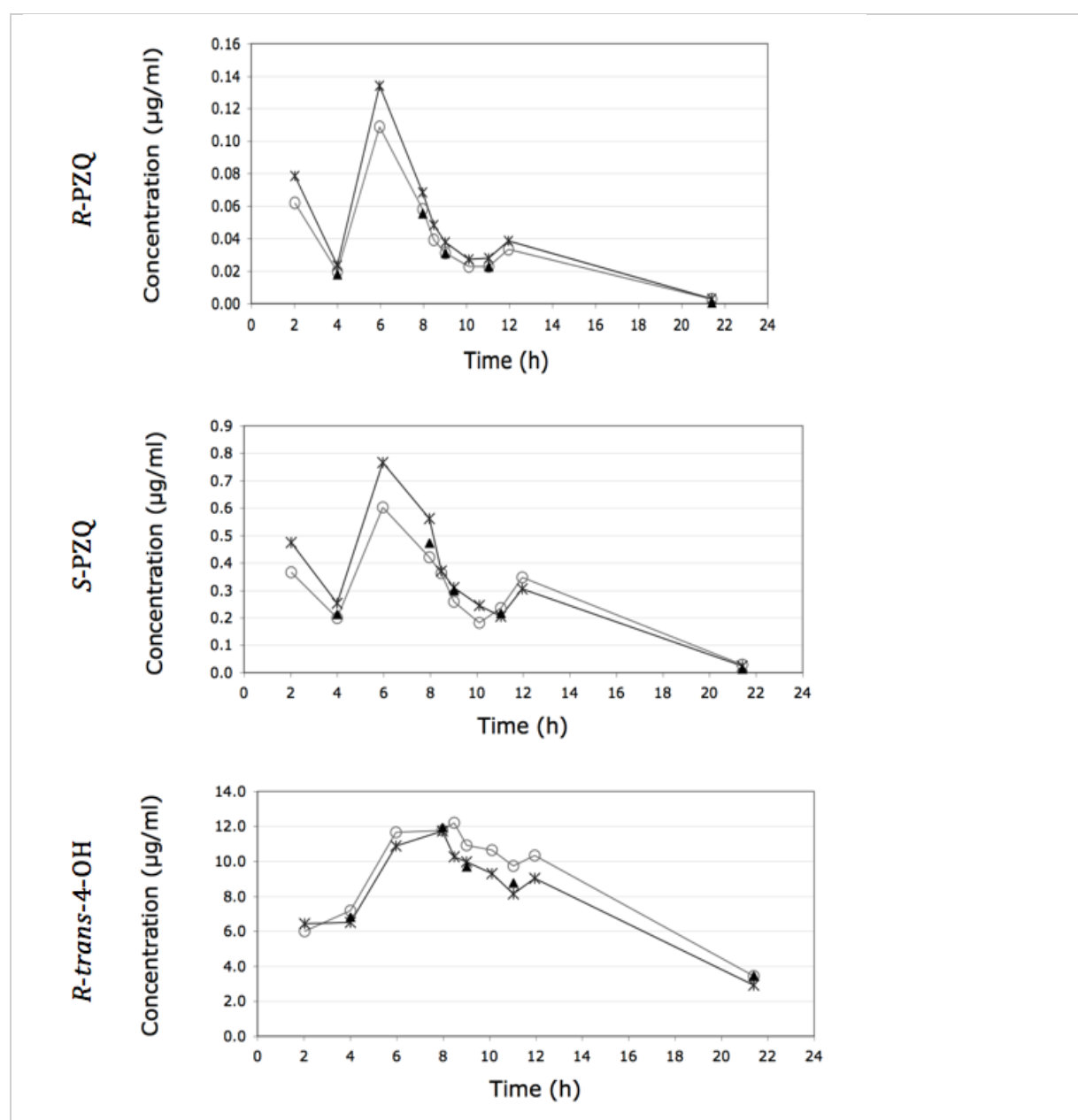


Fig. 4 Pharmacokinetic profiles of R-PZQ (A), S-PZQ (B) and R-trans-4-OH (C) in blood (O), plasma (*) and DBS (▲) in a patient treated with 3x25 mg/kg PZQ

4. Conclusion

The LC–MS/MS method reported here for the enantioselective analysis of PZQ and R-trans-4-OH metabolite is selective, precise and accurate. The short run time of 11.5 min and the simple and reproducible extraction methods for plasma, blood and DBS are valuable advantages to the analysis of a large number of samples. A sensitivity of 0.01 µg/ml for R- and S-PZQ and of 0.1 µg/ml for R-trans-4-OH was achieved in DBS, plasma and blood. Applicability of the method was demonstrated by the analysis of the PK profile of a patient treated with 3 × 25 mg/kg PZQ over 24 h. This method in combination with the DBS technique, will allow us to collect and analyze PK samples from clinical trials in rural areas of Africa and Southeast Asia.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2015.10.011>.

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Supplementary tables

Table T1 MS/MS conditions optimized for each analyte

Parameter	R- and S-PZQ	R-trans-4-OH	PZQ-d11
Mass and mass transitions [<i>m/z</i>]	312→202	328→202	323→203
Declustering potential [V]	66	56	66
Focusing potential [V]	240	190	240
Collision energy [V]	25	27	25
Cell exit potential [V]	16	16	16

Table T2 DBS calibration line linearity and differences in signal intensities between the standard Whatman DMPK-C cards and other card types

		Agilent (4:1) ^b	Agilent (1:1)	Whatman 903 (4:1)
R-PZQ	Mean difference ^a [%] ± CV	31.4 ± 19.6	50.0 ± 16.4	101.6 ± 10.1
	Linearity (R ²)	0.9837	0.9959	0.9994
S-PZQ	Mean difference [%] ± CV	33.3 ± 63.4	18.4 ± 14.2	104.5 ± 6.6
	Linearity (R ²)	0.9699	0.9720	0.9994
R-trans-4-OH	Mean difference [%] ± CV	41.2 ± 52.1	180.3 ± 42.5	105.3 ± 7.1
	Linearity (R ²)	0.9622	0.9693	0.9995

^a Mean of the percentage ratio of the tested cards to the Whatman DMPK-C card signals across all the calibration points (n=8)

^b In brackets, the extraction solution ratio acetonitrile - water (v/v)

Table T3 Intra- (n=6) and inter-assay (n=12) accuracy^a and precision^b in QC samples at LLOQ, low, medium and high concentrations

	Matrix	Nominal concentration [$\mu\text{g/ml}$]	Intra-assay [%] \pm CV	Inter-assay [%] \pm CV
R-PZQ	Blood	1.75	103.8 \pm 2.8	103.2 \pm 3.6
		0.175	99.6 \pm 5.8	97.3 \pm 5.4
		0.0175	91.8 \pm 8.4	91.3 \pm 7.3
		0.01	102.2 \pm 3.4	94.7 \pm 10.9
	Plasma	1.75	108.8 \pm 4.6	103.0 \pm 8.5
		0.175	102.8 \pm 5.9	99.3 \pm 5.8
		0.0175	106.0 \pm 4.4	103.7 \pm 6.0
		0.01	107.0 \pm 0.6	105.2 \pm 8.3
	DBS	1.75	98.9 \pm 8.6	100.0 \pm 9.9
		0.175	96.0 \pm 9.1	99.6 \pm 7.7
		0.0175	101.6 \pm 9.5	104.4 \pm 8.2
		0.01	103.6 \pm 14.3	102.4 \pm 12.7
S-PZQ	Blood	1.75	104.4 \pm 2.8	103.6 \pm 3.6
		0.175	100.5 \pm 6.2	97.6 \pm 5.7
		0.0175	96.6 \pm 6.8	94.4 \pm 6.7
		0.01	103.5 \pm 8.1	99.2 \pm 9.0
	Plasma	1.75	108.8 \pm 3.8	102.6 \pm 9.2
		0.175	97.9 \pm 9.2	96.3 \pm 5.2
		0.0175	106.1 \pm 3.8	102.9 \pm 5.9
		0.01	106.4 \pm 8.9	103.3 \pm 9.3
	DBS	1.75	100.9 \pm 9.9	100.9 \pm 10.6
		0.175	101.5 \pm 6.0	99.9 \pm 7.9
		0.0175	103.2 \pm 7.7	101.8 \pm 9.4
		0.01	100.4 \pm 10.1	91.8 \pm 12.7
R-trans-4-OH	Blood	17.5	104.5 \pm 6.3	104.5 \pm 5.7
		1.75	100.2 \pm 11.4	100.0 \pm 8.5
		0.175	105.0 \pm 8.7	99.7 \pm 10.9
		0.1	88.2 \pm 3.9	96.3 \pm 14.8
	Plasma	17.5	100.0 \pm 4.9	100.8 \pm 4.7
		1.75	104.2 \pm 5.5	105.2 \pm 4.4
		0.175	97.2 \pm 7.5	100.5 \pm 8.6
		0.1	98.9 \pm 5.6	96.1 \pm 10.5
	DBS	17.5	91.5 \pm 2.0	102.0 \pm 9.5
		1.75	94.5 \pm 9.4	101.0 \pm 9.3
		0.175	101.8 \pm 8.4	108.6 \pm 10.7
		0.1	100.7 \pm 10.1	101.2 \pm 11.6

^a The accuracy is calculated as the percentage ratio of the measured concentration to the nominal concentration^b The precision is the percentage ratio of the standard deviation to the measured concentration**Table T4** Sample stability assays (n=3)

	Analytes	QC	R-PZQ [%] \pm CV	S-PZQ [%] \pm CV	R-trans [%] \pm CV
Freeze-thaw	Blood	High	95.4 \pm 9.4	99.7 \pm 0.1	106.0 \pm 10.6
		Low	94.6 \pm 1.0	101.2 \pm 1.1	93.2 \pm 0.5
	Plasma	High	102.6 \pm 8.7	100.6 \pm 3.7	87.7 \pm 12.1
		Low	94.6 \pm 1.1	91.6 \pm 0.9	107.0 \pm 0.8
Bench-top	Blood	High	102.4 \pm 9.3	101.4 \pm 0.9	110.0 \pm 8.8
		Low	111.2 \pm 1.3	109.5 \pm 0.2	100.2 \pm 0.8
	Plasma	High	102.9 \pm 6.5	100.2 \pm 1.8	105.7 \pm 7.5
		Low	94.7 \pm 0.9	95.8 \pm 0.3	104.4 \pm 0.7
Long-term (6 months)	Blood (-80°C)	High	102.4 \pm 2.4	109.9 \pm 2.3	90.1 \pm 5.4
		Low	86.2 \pm 0.6	88.2 \pm 1.2	89.6 \pm 5.1
	Plasma (-80°C)	High	101.6 \pm 5.0	99.7 \pm 2.1	88.5 \pm 2.3
		Low	100.6 \pm 0.9	104.8 \pm 0.6	98.6 \pm 0.4
	DBS (25°C)	High	96.8 \pm 13.4	100.0 \pm 9.6	110.6 \pm 11.2
		Low	89.2 \pm 5.4	107.3 \pm 9.8	109.0 \pm 7.3
Autosampler (10°C)	Blood	High	100.0 \pm 2.3	98.2 \pm 1.7	87.0 \pm 2.8
		Low	97.2 \pm 6.9	97.8 \pm 4.9	113.5 \pm 8.7
	Plasma	High	94.0 \pm 3.9	89.5 \pm 5.6	85.8 \pm 5.8
		Low	88.5 \pm 6.1	85.3 \pm 8.0	94.2 \pm 3.1
	DBS	High	107.5 \pm 5.3	108.9 \pm 5.2	113.6 \pm 5.0
		Low	108.6 \pm 3.7	112.1 \pm 7.0	101.9 \pm 2.4
Stock solutions	Bench-top		101.8 \pm 2.8	100.0 \pm 2.0	104.7 \pm 0.6
	Sonication		107.5 \pm 1.7	101.9 \pm 1.3	101.9 \pm 3.2
	3 months		93.5 \pm 8.3	93.7 \pm 8.1	91.5 \pm 13.5

Supplementary figures

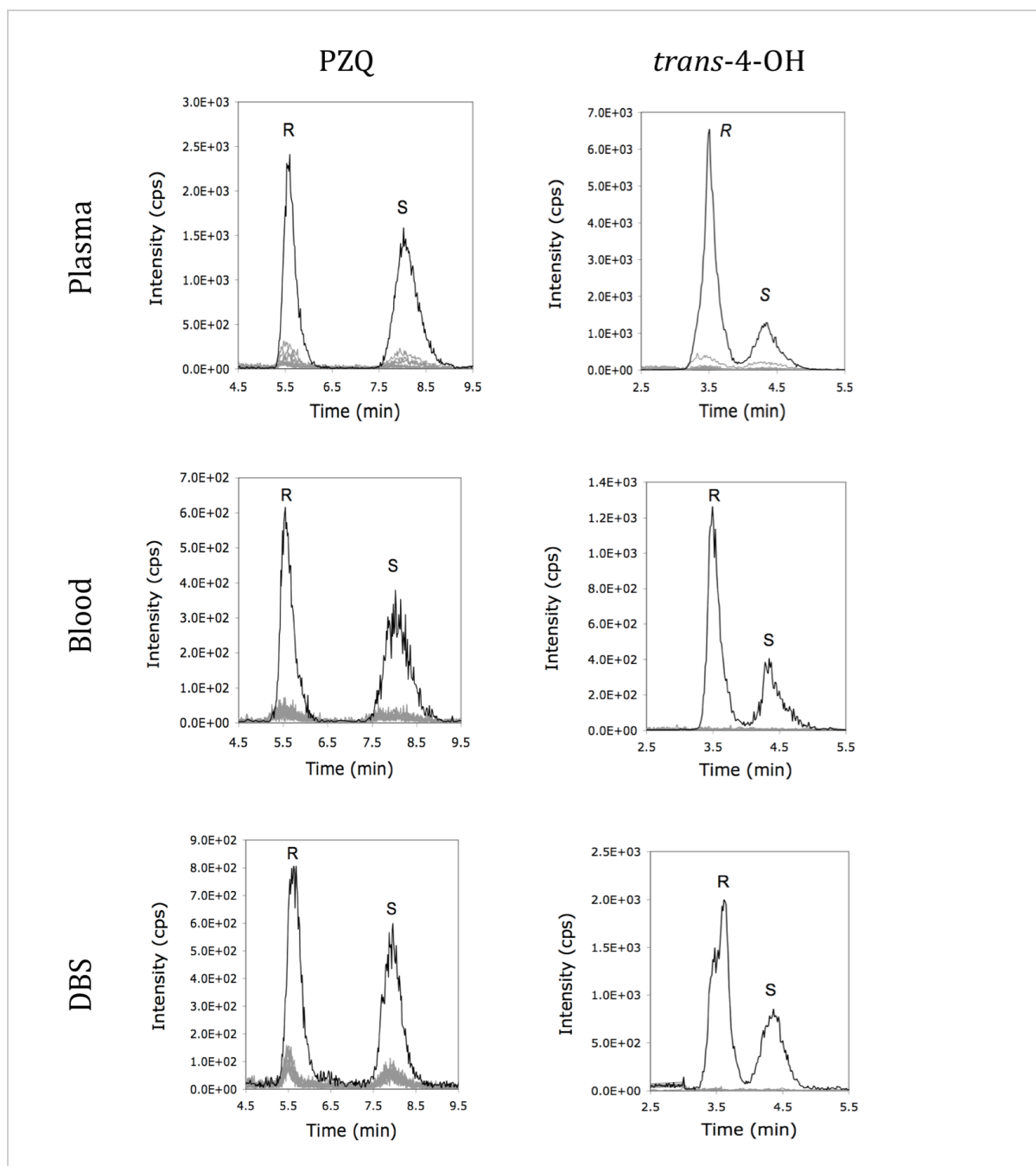


Fig. F1 Chromatograms of blank (grey line) vs. LLOQ (black line) signals of PZQ and *trans*-4-OH enantiomers in plasma (A, B), blood (C, D) and DBS (E, F)

Chapter 5

Pharmacokinetic study of praziquantel enantiomers and its main metabolite measured in blood, plasma and dried blood spots in *Opisthorchis*-infected patients

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Pharmacokinetic study of praziquantel enantiomers and its main metabolite R-trans-4-OH-PZQ in plasma, blood and dried blood spots in *Opisthorchis viverrini*-infected patients

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Abstract

Background Praziquantel (PZQ) is the treatment of choice for infections with the liver fluke *Opisthorchis viverrini*, a major health problem in Southeast Asia. However, pharmacokinetic (PK) studies investigating the disposition of PZQ enantiomers (R- and S-PZQ) and its main metabolite, R-trans-4-OH-PZQ, in diseased patients are lacking. The implementation of a dried blood spot (DBS) sampling technique would ease the performance of PK studies in remote areas without clinical facilities. The aim of the present study is to provide data on the disposition of PZQ enantiomers and R-trans-4-OH-PZQ in opisthorchiasis patients and to validate the use of DBS compared to plasma and blood sampling. **Methodology/Principal Findings** PZQ was administered to nine *O. viverrini*-infected patients at 3 oral doses of 25 mg/kg in 4 h intervals. Plasma, blood and DBS were simultaneously collected at selected time points from 0 to 24 h post-treatment. PK parameters were determined using non-compartmental analysis. Drug concentrations and areas under the curve (AUC_{0-24h}) measured in the 3 matrices were compared using Bland-Altman analysis. We observed plasma AUC_{0-24h}s of 1.1, 9.0 and 188.7 µg×h/ml and half-lives of 1.1, 3.3 and 6.4 h for R-PZQ, S-PZQ and R-trans-4-OH, respectively. Maximal plasma concentrations (C_{max}) of 0.2, 0.9 and 13.9 µg/ml for R-PZQ, S-PZQ and R-trans-4-OH peaked at 7 h for PZQ enantiomers and at 8.7 h for the metabolite. Individual drug concentration measurements and patient AUC_{0-24h}s displayed ratios of blood or DBS versus plasma between 79–94% for R- and S-PZQ, and between 108–122% for R-trans-4-OH. **Conclusions/Significance** Pharmacodynamic (PD) *in vitro* studies on PZQ enantiomers and R-trans-4-OH-PZQ are necessary to be able to correlate PK parameters with efficacy. DBS appears to be a valid alternative to conventional venous sampling for PK studies in PZQ-treated patients.

Author summary Opisthorchiasis, caused by the food-borne trematode *Opisthorchis viverrini*, affects more than 8 million people in Southeast Asia, and in its chronic phase it might lead to cholangiocarcinoma. Praziquantel (PZQ) is the sole drug available to treat the disease and is administered as a racemic mixture of R and S enantiomers, of which R-PZQ is considered active. As PZQ is rapidly metabolized, its disposition and efficacy in patients might considerably vary according to disease state, sex or age. However, pharmacokinetic (PK) studies on the disposition of PZQ enantiomers and its main metabolite, R-trans-4-OH, in diseased patients are lacking. To allow the collection of PK samples in a large number of patients, we implemented a dried blood spot (DBS) technique, which is less invasive than venipuncture. The aim of our study is to provide first data on the disposition of PZQ enantiomers and the main metabolite of PZQ in opisthorchiasis patients and to validate the use of DBS over venous sampling. Standard PZQ treatment was administered to nine *O. viverrini* infected patients, and plasma, blood and DBS were simultaneously collected within 24 h post-treatment. We observed a 100-fold higher disposition of the metabolite compared to R-PZQ, which questions its role in the opisthorchidal activity of PZQ. DBS sampling appears to be a valid alternative to venous sampling and will be a valuable tool for future PK studies in PZQ-treated patients.

1. Introduction

Opisthorchiasis is caused by the trematode *Opisthorchis viverrini*, a liver fluke affecting about 8 million people in Southeast Asia, particularly in the Mekong basin (Sithithaworn and Haswell-Elkins, 2003; Fürst, Keiser and Utzinger, 2012). Infection occurs following consumption of raw or undercooked fish harboring *O. viverrini* metacercariae (Grundy-Warr *et al.*, 2012). In the early phase, the disease is mostly asymptomatic but in the acute stage periductal fibrosis and liver enlargement are common, mostly a result of inflammation due to worm feeding. The chronic stage triggers severe clinical symptoms including jaundice, biliary obstructions, and cholangiocarcinoma as a serious complication (Mairiang and Mairiang, 2003; Sripa, 2003; Sripa *et al.*, 2010; Sayasone *et al.*, 2012).

Praziquantel (PZQ) is the drug of choice for opisthorchiasis and is manufactured as a racemic mixture of R and S enantiomers. The recommended treatment regimen is 3 oral doses of 25 mg/kg, usually administered between 4 and 6 h apart (World Health Organization, 1995). The disposition of PZQ is highly influenced by the fasting state, the co-administered food type, as well as the liver function (El Guiniady *et al.*, 1994; Castro *et al.*, 2000). Though no studies have been conducted against *O. viverrini* yet, R-PZQ is considered to be the active molecule in the treatment of schistosomiasis, while the inactive S-PZQ is suspected to be responsible for the bitter taste of the drug and for the mild to moderate adverse events caused by the treatment (Wu *et al.*, 1991; Staudt *et al.*, 1992; Meyer *et al.*, 2009; Oliaro, Delgado-Romero and Keiser, 2014). In humans, PZQ undergoes an enantioselective first-pass metabolism through the cytochrome CYP450 3A4 isoform (Li *et al.*, 2003) and is mainly transformed into the monohydroxylated metabolite R-trans-4-OH-PZQ (R-trans-4-OH), while S-PZQ is metabolized to several different monohydroxylated molecules (Lerch and Blaschke, 1998; Meier and Blaschke, 2001; Melo *et al.*, 2005). R-trans-4-OH displays minor anthelmintic activity, with an IC₅₀ hundred times higher than R-PZQ against *Schistosoma mansoni* (Meister *et al.*, 2014).

The disposition of PZQ enantiomers and metabolites has not yet been studied in opisthorchiasis patients. In fact, the only pharmacokinetic (PK) study of PZQ involving patients with opisthorchiasis focuses on the racemic drug (Na Bangchang *et al.*, 1993). Enantioselective disposition was performed exclusively in healthy volunteers with a low dose (Lima *et al.*, 2011). Therefore, studies on the

enantioselective disposition of PZQ in the diseased population are warranted for a better understanding of the modalities of drug action and disposition.

Dried blood spot (DBS) sampling is a microsampling technique, involving the collection of capillary blood through a finger prick. The method is therefore less invasive compared to venipuncture. The blood drops are dried on a filter paper and stored at ambient temperature until assayed. Compared to blood or plasma sampling, this method does not require sample freezing and offers easy handling and storage, hence allowing the performance of PK studies in remote areas without clinical set-ups. Blood quantities withdrawn with DBS are minimal (20 µl vs. 3–4 ml for plasma or blood), which provides an advantage for research with children. Finally, the ease of sample collection enables including a larger number of patients and is therefore ideal for population PK studies (Spooner, Lad and Barfield, 2009; Deglon *et al.*, 2013; Demirev, 2013; Xu *et al.*, 2013). The major caveat when replacing plasma with DBS sampling is the use of a different matrix where the drug partition might not be equivalent (Emmons and Rowland, 2010; Xu *et al.*, 2013). Validating this alternative sampling technique for future trials hence calls for a formal comparison of drug concentrations measured in plasma and DBS.

The aim of our study was to elucidate for the first time the kinetic disposition of both PZQ enantiomers and its main metabolite in *O. viverrini*-infected patients. Additionally, we assessed the difference between concentrations determined in plasma, blood and DBS sampling for the analysis of PZQ PK profiles using Bland-Altman analysis.

2. Materials and methods

2.1 Chemicals and reagents

Racemic (rac) PZQ was obtained from Sigma-Aldrich (Buchs, Switzerland). PZQ enantiomers as well as the metabolite trans-4-OH were donated by Merck Serono (Darmstadt, Germany). Eleven-fold deuterized PZQ (PZQd11, internal standard-IS) was acquired from Toronto Research Chemicals (Ontario, Canada). The chemical structures of PZQ, PZQd11 and trans-4-OH are depicted in Fig 1. Acetonitrile, ethanol and methanol of MS grade were purchased from Carl Roth GmbH (Allschwil, Switzerland), and ammonium formate, ammonium acetate and formic acid of MS grade from Sigma-Aldrich (Buchs, Switzerland). Ultrapure water was provided using a Millipore Milli-Q water purification system (Merck Millipore,

Darmstadt, Germany). Blank human plasma and blood were supplied in lithium heparin-coated vacutainer

tubes (BD, Allschwil, Switzerland) from the local blood donation centre (Basel, Switzerland).

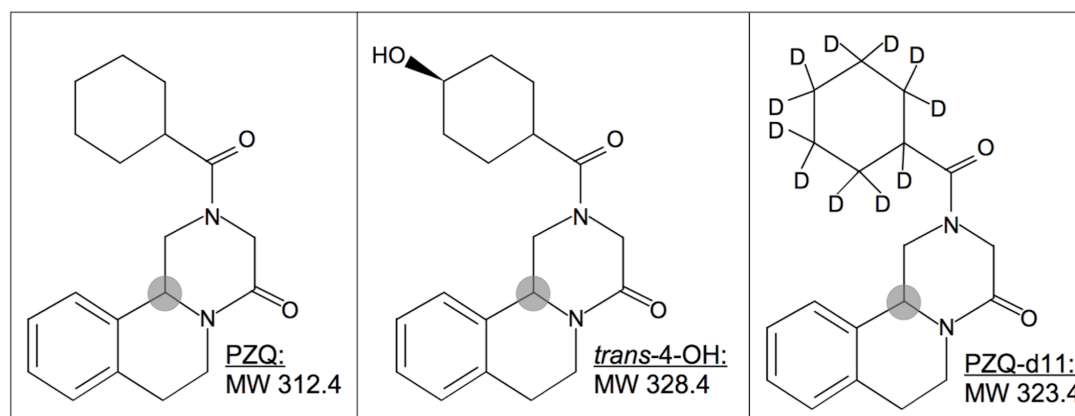


Fig. 1 Chemical structures of PZQ, the main metabolite *trans*-4-OH and PZQ-d11 (internal standard), with the chiral centre represented with a shaded circle.

2.2 PK sample collection

The plasma, blood and DBS sample collection was performed in the framework of a PK and dose-finding study of tribendimidine against *O. viverrini* in humans. Ethical clearance was obtained from the ethics committee of Northern and Central Switzerland (EKNZ reference no. 375/11), and from the National Ethic Committee for Health Research, Ministry of Health (MoH) of Lao PDR (reference no. 009/NECHR). The trial is registered at Current Controlled Trials (ISRCTN96948551). In short, 9 *O. viverrini*-infected patients were treated with 3 oral doses of 25 mg/kg PZQ, with the second and third dose administered 4 and 8 h after the first dose, respectively. The trial was performed at the Champasak Provincial Hospital in Pakse, Lao PDR, and prior to treatment, a standardized food dish (rice) was provided to all patients. Adverse events were monitored at 3, 24 and 48 hours post-treatment using a standardized questionnaire. Prior to treatment, patients underwent physical examinations and laboratory tests, such as liver and kidney parameters and complete blood counts. About 4 ml of venous blood was collected at 0, 2, 4, 6, 8, 8.5, 9, 10, 11, 12 and 24 h after the first dose from the antecubital arm vein through an intravenous catheter into EDTA-coated vacutainer tubes (BD). Within 30 min after sampling, 1 ml of blood was pipetted into a cryotube and the remaining blood centrifuged to obtain plasma. Plasma and blood samples were transported on dry ice to Basel where they were kept at -80°C until analysis. DBS samples were collected at 0, 4, 8, 9, 11 and 24 h post-first-dose from patients 1 to 5, and at 0, 2, 6, 8.5, 10 and 12 h post-first-dose from patients 6 to 9. The samples were obtained by puncturing the middle or

ring finger with sterile one-way finger prickers (Accu-Check Safe-T-Pro Plus, Roche, Switzerland). Lithium heparin coated capillaries (Alere Cholestech LDX, V = 40 µl) were used to collect and deposit blood on DMPK-C cards (Whatman, GE Healthcare Life Sciences, Cardiff, UK). The cards were dried overnight and stored in plastic bags with desiccant at room temperature.

2.3 Analytical method

The LC-MS/MS method for the analysis of R- and S-PZQ and R-*trans*-4-OH and its validation for plasma, blood and DBS is described elsewhere (Meister *et al.*, 2016). Briefly, plasma and blood calibration samples were freshly prepared and included in each analytical run by spiking blank samples to reach final concentrations from 2.5 to 0.01 (lower limit of quantification-LLOQ) µg/ml for R- and S-PZQ, and of 25 to 0.1 (LLOQ) µg/ml for R-*trans*-4-OH. QC samples were similarly prepared by spiking 6 different blanks to obtain high, medium, low and LLOQ concentrations. For the extraction of analytes, 100 µl of plasma or blood samples underwent protein precipitation with 700 µl of IS solution (500 ng/ml IS in pure acetonitrile), and were shaken in a thermomixer for 20 min at 25°C and 1400 rpm. DBS samples of 5 mm diameter were extracted with 300 µl of DBS extraction solution (IS solution: ultrapure water, 4:1, v/v), shaken in a thermomixer for 20 min at 25°C, and sonicated for 40 min prior analysis.

A first chromatographic separation was achieved through a column trapping system (HALO C-18, 4.6 x 5 mm, Optimize Technologies, OR, USA) using 10 mM ammonium acetate and 0.15% formic acid in ultrapure water at a flow rate of 0.3 ml/min. After 1

min, the analytes were eluted from the trapping to the chiral column (Lux Cellulose-2 (150x4.6mm, 3µm, Phenomenex, CA, USA)) with an elution gradient of 70 to 90% B, with mobile phase A consisting of 20 mM ammonium formate in ultrapure water and mobile phase B of pure acetonitrile.

2.4 Treatment efficacy and pharmacokinetic analysis

Statistical analyses were performed with Prism software (GraphPad, CA, USA). Parasite egg counts were determined with duplicate Kato-Katz smears from two stool samples prior to the treatment and between 19 and 25 days after treatment for the estimation of treatment efficacy. Cure rates were defined as the percentage patients who were egg-negative after treatment. The number of eggs per gram of stool (EPG) was evaluated by adding up the egg counts from the quadruplicate Kato-Katz thick smears and multiplying this number by a factor of six. Geometric mean egg counts were calculated before and after treatment to determine the corresponding percentage egg-reduction rate (ERR).

To evaluate the reproducibility of the measurements, incurred sample reanalysis (ISR) was performed with a total of 170 samples originating from 5 patients in the 3 matrices (56% of total sample size). The percentage difference between the original and the reanalyzed measurement was calculated as follows:

$$\text{percentage difference} = (\text{repeat} - \text{original}) \times 100 / \text{mean}(\text{repeat}, \text{original})$$

As acceptance criterion for ISR, at least 66.7% of the samples (2 out of 3) should not deviate by more than 20%, as recommended in the European guidelines on bioanalytical method validation and the draft of the FDA guidelines (European Medicines Agency, 2011; Food and Drug Administration, 2013).

PK parameters, including the area under the concentration-time curve (AUC_{0-24}), the maximal concentration (C_{max}), the time to maximal concentration (T_{max}) and the half-life ($t_{1/2}$) were calculated for each patient with the Excel add-in

PKsolver (Zhang *et al.*, 2010) using non-compartmental analysis with the linear trapezoidal rule.

2.5 Agreement between matrices

Concordance of drug concentrations observed in blood or DBS compared to plasma was evaluated using Pearson's correlation coefficient and Bland-Altman plots, with percentage ratios between the two matrices (blood/plasma or DBS/plasma) plotted against mean concentrations (Altman and Bland, 1983; Bland and Altman, 1986, 2012). For matrix differences in AUC values, Bland-Altman analysis also applied.

The limits of agreement at 95% for the ratios were calculated as follows:

$$\text{limits of agreement} = \text{mean percentage ratio} \pm 1.96 \times \text{standard deviation}$$

For the drug concentration data, the calculation of the limits of agreement were adapted to take into account multiple measurements per individuals, following the method described by Bland and Altman (Bland and Altman, 2007) using Stata software (version 12.1, College State, TX, USA).

2.6 In vitro assessment of blood/plasma partition of PZQ

The partitioning of PZQ between plasma and erythrocytes was assessed *in vitro* using human blood from the local blood donation center. Whole blood samples (hematocrit adjusted to 35%) were spiked in triplicate with the analytes of interest to reach end concentrations of 0.05 and 0.5 µg/ml for R- and S-PZQ and 0.5 and 5 µg/ml for R-trans-4-OH. The samples were incubated 1 h at room temperature. Hundred microliters of each sample were aliquoted and the remainder was centrifuged at 1500 g for 20 minutes to obtain plasma. Whole blood and plasma samples were extracted using acetonitrile containing IS and analyzed as described above. Analyte peaks were normalized with IS peaks and ratios of blood to plasma were calculated for each concentration and analyte.

Table 1 Characteristics of participants and *O. viverrini* cure rates

Median age (range) [years]	Sex [% females]	Median weight (range) [kg]	Pre-treatment [EPG] geometric mean	Post-treatment [EPG] geometric mean	Cure rate [%]
40 (25-46)	67	56 (40-77)	3653	0	100

3. Results

3.1 Sample collection and analysis

A total of 91 plasma and 91 blood samples were collected. For DBS, 45 samples were analysed. Due to technical problems, five DBS samples for patient 9

were collected at 6, 7, 8.5, 10 and 12 h post-treatment, and an extra venous blood sample was withdrawn at 7 h post-treatment. To estimate the repeatability of the analytical measurements, an incurred sample reanalysis was performed. Between 88.2 and 100% of the samples in plasma, blood and DBS were within the

ISR acceptance criterion (within 20% difference). All samples from the 9 patients presenting obvious measurement errors or displaying a high discrepancy between matrices were reanalyzed (n = 21).

For R-PZQ, concentrations ranged from 0.01 to 0.85 µg/ml, to 0.90 µg/ml and to 1.08 µg/ml for DBS, blood and plasma, respectively. For S-PZQ, the following concentration ranges were observed: 0.01–1.59 µg/ml in DBS, 0.01–1.83 µg/ml in blood, and 0.02–2.34 µg/ml in plasma. The metabolite R-trans-4-OH displayed concentrations ranging from 3.01 to 19.01 µg/ml in DBS, 1.62 to 22.05 µg/ml in blood, and 1.41 to 17.85 µg/ml in plasma.

3.2 Treatment efficacy and kinetic disposition of PZQ in patients

All participants were adults, 3 males and 6 females aged 25 to 46 years with a median weight of 56 kg (Table 1). Prior to treatment, 8 patients displayed

moderate *O. viverrini* infections (between 1,000 and 10,000 EPG) and 1 patient a heavy infection (13,920 EPG). All patients were asymptomatic. Hookworm co-infections were present in 5 participants (participants 1, 4, 6, 7 and 8), while patient 2 presented a co-infection with the whipworm *Trichuris trichiura*. Liver and kidney parameters were in the normal range for all the patients. Blood counts were also normal, except for patient 2 who displayed slightly elevated white blood cell counts (11.7×10^3 cells/l) and a moderate anaemia (hemoglobin concentration = 9.2 g/dl). All patients were treated as planned and tolerated the treatment well, with the exception of patient 2, for whom the treatment was interrupted due to adverse events (vomited within 30 min after the second dose). Between 19 and 25 days post-treatment, the participants were screened again for the presence of *O. viverrini* eggs in stool: all patients were cleared from infection, hence cured (Table 1).

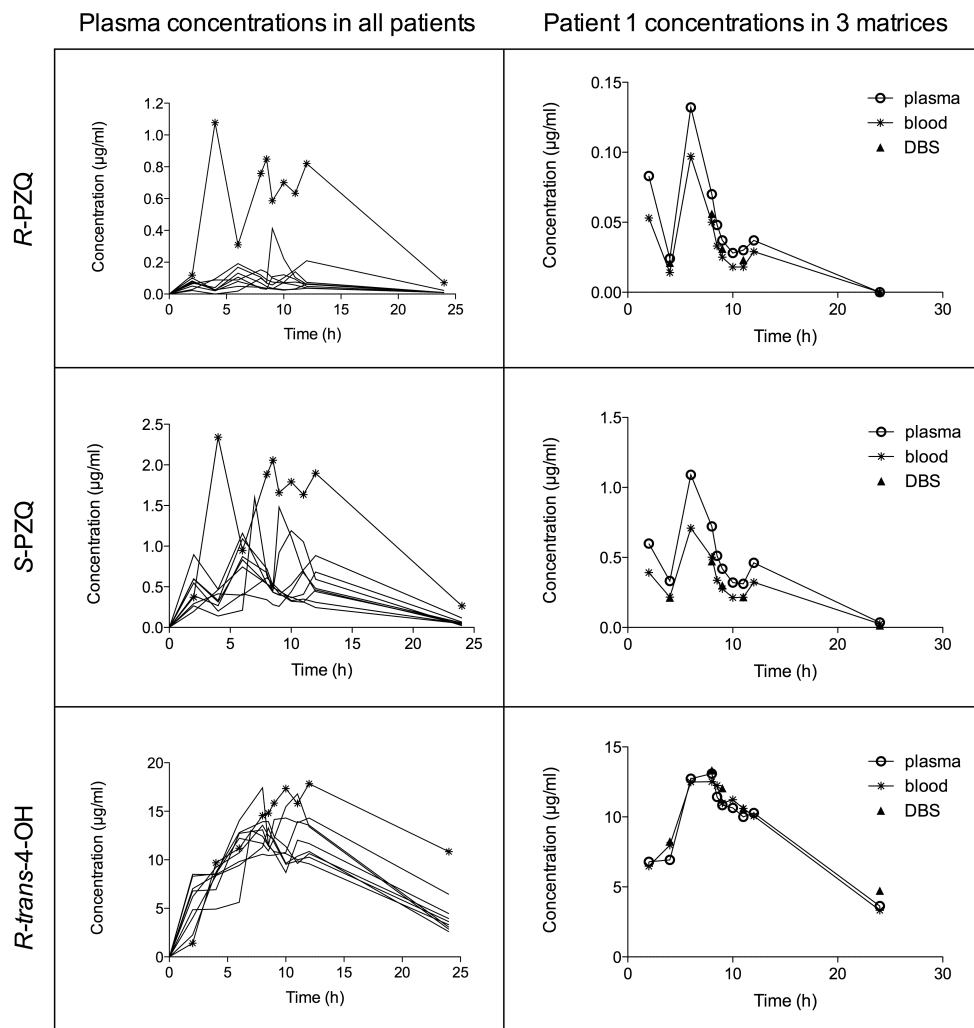


Fig. 2 Plasma concentrations for R-PZQ (1), S-PZQ (2) and R-trans-4-OH (3) over time and across patients (patient 2 sketched with a star) and analyte concentrations across the 3 matrices in patient 1

Table 2 Median (range) of AUC_{0-24h}, t_{1/2}, C_{max} and T_{max} in plasma across 9 patients

Analyte	AUC _{0-24h} [$\mu\text{g}\times\text{h}/\text{ml}$]	t _{1/2} [h]	C _{max} [$\mu\text{g}/\text{ml}$]	T _{max} [h]
R-PZQ	1.1 (0.8-10.7)	1.1 (1.0-3.0)	0.2 (0.1-1.1)	7.00 (4.0-11.8)
S-PZQ	9.0 (6.1-26.3)	3.3 (1.9-3.7)	0.9 (0.6-2.3)	7.00 (4.0-11.8)
R-trans-4-OH	188.7 (157.2-257.4)	6.4 (4.1-7.1)	13.9 (13.1-17.9)	8.7 (8.0-12.0)

Patient variability in plasma concentrations was high, with patient 2 displaying clearly higher concentrations than the other subjects, despite not taking the last dose (Fig 2). Median PK parameters calculated from plasma concentrations are summarized in Table 2. R-PZQ displays the smallest AUC_{0-24h} (1.1 $\mu\text{g}/\text{ml}/\text{h}$) and a short estimated t_{1/2} (1.1 h) compared to the other analytes. S-PZQ exhibits a nearly 5 x higher C_{max} (0.9 $\mu\text{g}/\text{ml}$) and an AUC_{0-24h} more than 8 x higher than R-PZQ (9.0 $\mu\text{g}/\text{ml}/\text{h}$). Both enantiomers peak at the same time (7 h). The main metabolite R-trans-4-OH has an increased exposure compared to the parent molecule. For example, its AUC_{0-24h} (188.7 $\mu\text{g}\times\text{h}/\text{ml}$) is 20x greater than S-PZQ and 170x greater than R-PZQ. The metabolite's estimated t_{1/2} and T_{max} are 6.4 h and 8.7 h, respectively. Patient 2 displays 2–10 fold higher R-PZQ, S-PZQ and R-trans-4-OH AUC_{0-24h} values compared to the other patients.

3.3 Sample measurement agreement between matrices

When comparing the analyte concentrations obtained in the different matrices by Pearson's correlation coefficient, blood versus plasma and DBS versus plasma data displayed correlation coefficients above 0.92 (all p < 0.01, Table 3). The mean concentration curves are consistent for plasma, blood and DBS, as exemplified with patient 1 (Fig 2).

The modified Bland-Altman approach for multiple measurements per individual was used on drug concentrations, although the values obtained with this method did not differ from the conventional approach. The Bland-Altman plots (Fig 3) show that percentage ratios were generally consistent across concentrations. The mean percentage ratios of R-PZQ in blood or DBS compared to plasma, display ratios of 79.0 and 89.6%,

respectively (Table 3). There is therefore a tendency for plasma samples to have slightly higher concentrations of R-PZQ than blood or DBS. For S-PZQ, the same pattern is observed, with slightly higher ratios than R-PZQ: 93.9 and 92.1% percentage ratios for blood and DBS, respectively. The metabolite R-trans-4-OH displays on the contrary higher ratios of blood or DBS to plasma of 122.0 and 110.6%, respectively. However, the 95% limits of agreement (LoA) all include 100%, except for R-PZQ in the blood versus plasma comparison (LoA = 59–99%). The LoA intervals are large and range up to 55–133% for the parent enantiomers and 94–145% for the metabolite in the blood-plasma ratios. LoA are slightly larger for DBS-plasma ratios.

Bland-Altman plots of AUC percentage ratios between plasma and blood or DBS are consistent across AUC values (Fig 4). The percentage ratios of blood or DBS to plasma for each PK parameter exhibit values between 80 and 120%, except in t_{1/2} DBS to plasma ratios for R-PZQ (122%) and R-trans-4-OH (75%, Tables 4 and 5). As for drug concentrations, the mean ratios of DBS or blood versus plasma tend to be lower than 100% for the parent enantiomers but higher than 100% for the metabolite. Only the t_{1/2} DBS to plasma ratios do not precisely follow this pattern, probably because they are calculated with 5 samples per patient instead of 10, driving therefore a higher estimation error. The LoA of the AUC ratios lie between 64–107% for the parent enantiomers and 87–136% for the metabolite in the blood-plasma ratios with a slightly wider range for DBS-plasma ratios. As for drug concentration results, all the LoA include 100%, except for R-PZQ in the blood to plasma ratio.

Table 3 Concordance of blood and DBS compared to plasma measurements, evaluated with Pearson's correlation and percentage ratio with their 95% limits of agreement (LoA)

Analyte	Matrix	Correlation coefficient	Percentage ratio ^a (LoA ^b) [%]
R-PZQ	blood (n=91)	0.995	79.0 (59.1; 99.0)
	DBS (n=45)	0.994	89.6 (55.6; 123.6)
S-PZQ	blood (n=91)	0.963	93.9 (54.5; 133.3)
	DBS (n=45)	0.970	92.1 (44.4; 139.8)
R-trans-4-OH	blood (n=91)	0.948	122.0 (94.0; 145.0)
	DBS (n=45)	0.921	110.6 (77.0; 144.3)

^a The percentage ratio is computed as blood or DBS values divided by plasma measurements averaged across patients and time points and presented as percentage to the plasma values

3.4 In vitro assessment of blood/plasma partition of PZQ

Blood to plasma ratios were consistent across both concentrations measured. R-PZQ displayed ratios of 83.1 ± 3.6 and $77.7 \pm 3.9\%$ for 0.05 and 0.5 $\mu\text{g/ml}$, respectively. The partition of S-PZQ in plasma was

similar to R-PZQ, with ratios of 81.3 ± 5.4 and $74.5 \pm 1.5\%$ for low and high concentrations, respectively. The metabolite R-trans-4-OH showed a partition in plasma higher than the parent enantiomers, with values of 92.0 ± 6.0 and $87.5 \pm 6.2\%$ for 0.5 and 5 $\mu\text{g/ml}$, respectively.

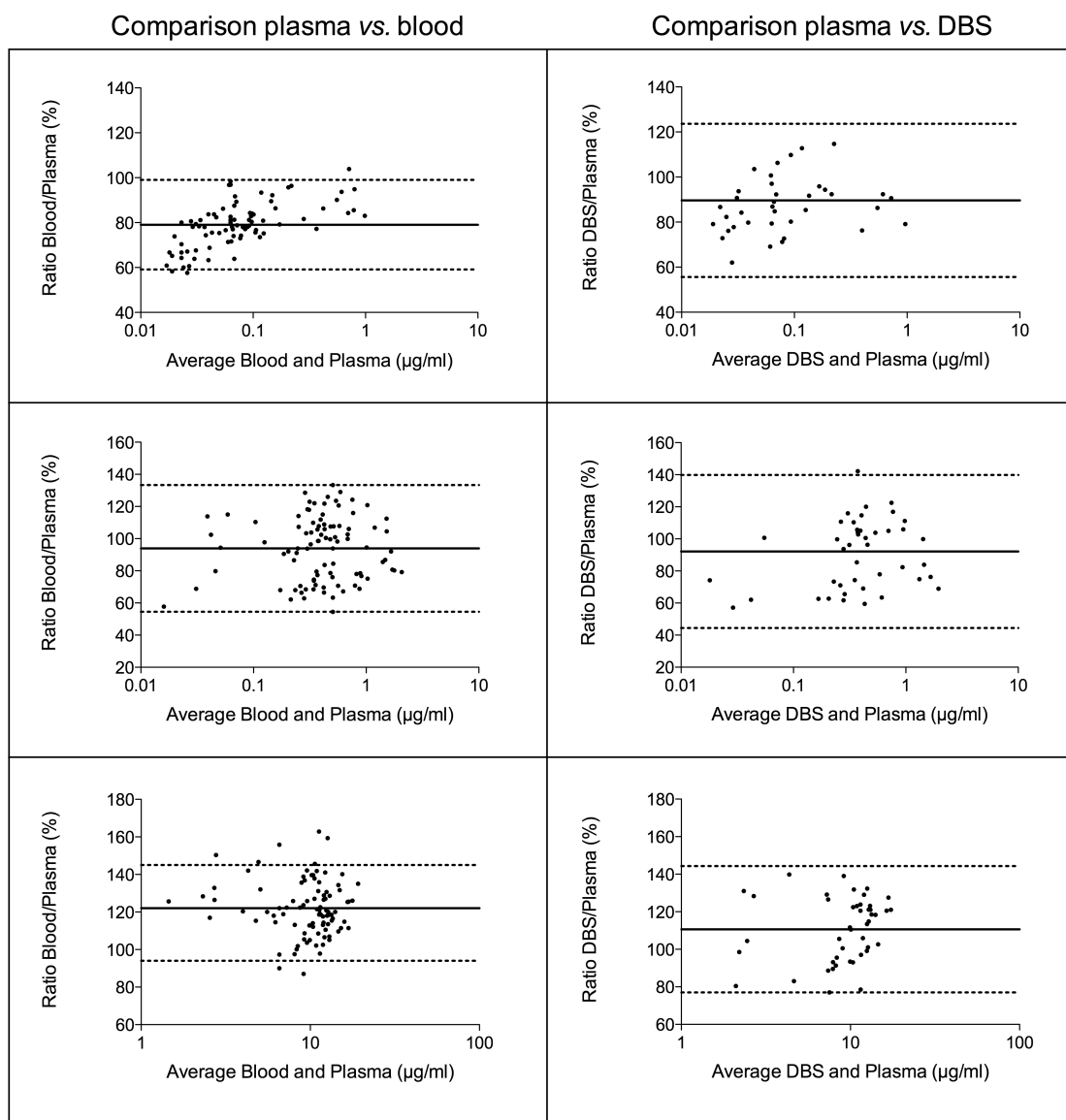


Fig. 3 Bland-Altman plots of measurement performed with blood or DBS compared to plasma values for R-PZQ, S-PZQ and R-trans-4-OH with mean ratio sketched with a solid line and 95% limits of agreement with dashed lines

4. Discussion

PZQ is the only drug available for the treatment of opisthorchiasis, yet surprisingly preclinical and clinical work including PK studies are sparse. We conducted for the first time a PK study in patients infected with *O. viverrini* treated with three doses of PZQ and studied the enantioselective drug disposition in blood, plasma and DBS.

The only other PK study conducted with *O. viverrini*-infected patients so far examined the kinetic disposition of the racemic drug after a single oral dose of 40 mg/kg (Na Bangchang *et al.*, 1993). Patients were of similar age and weight as in our study, but with a higher proportion of males. The authors observed a C_{max} for racemic PZQ of 0.9 and 1.1 $\mu\text{g/ml}$ in early (asymptomatic) and acute (moderately advanced) infection, respectively which does not

differ from the value observed in our study, 1.1 $\mu\text{g/ml}$ for R- and S-PZQ combined. A half-life value of 2.3 and 3.8 h previously observed for the racemic parent compound in early and acute infection (Na Bangchang *et al.*, 1993) is as well consistent with a half live of S-PZQ of 3.3 h (R-PZQ is eliminated much faster, due to different enzymes kinetics than S-PZQ) in our study. Given that a dose of 40 mg/kg corresponds to 53% of the dose administered in our trial, the AUC_0 .

$_{24\text{h}}$ range observed in our study (6.1–26.3 $\mu\text{g/ml}$) is closer to that previously reported for patients with acute opisthorchiasis (2.5–15.6 $\mu\text{g/ml}$) than that in patients with asymptomatic opisthorchiasis (1.6–5.0 $\mu\text{g/ml}$) (Na Bangchang *et al.*, 1993). This result is not surprising, given the disease prevalence in the region and the age of the patients, for which acute cases are expected to be frequent (Lovis *et al.*, 2010; Sayasone *et al.*, 2011, 2012).

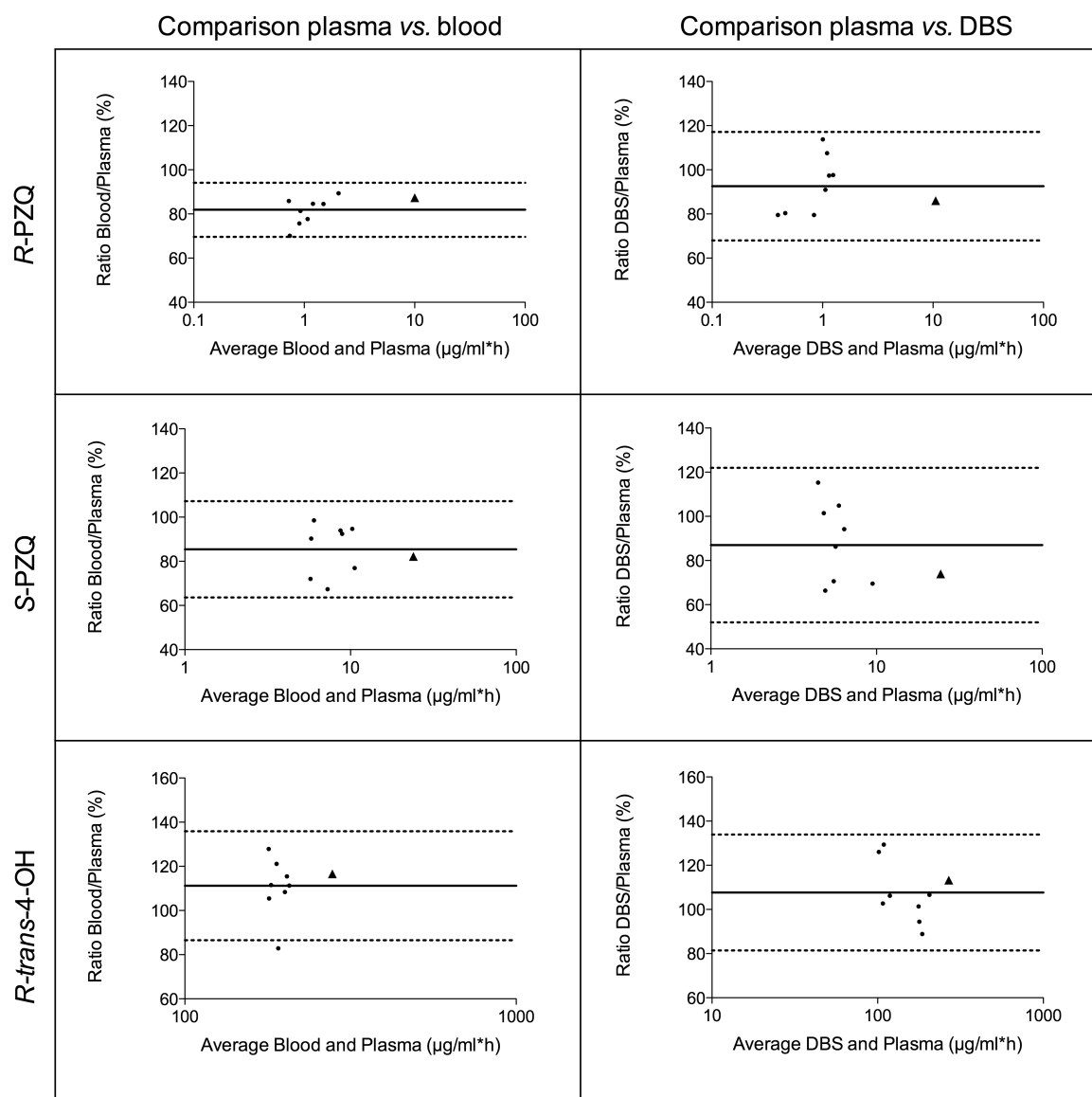


Fig. 4 Bland-Altman plots of AUC values obtained from blood or DBS compared to plasma AUCs for R-PZQ, S-PZQ and R-trans-4-OH with mean ratio sketched with a solid line and 95% limits of agreement with dashed lines. Patient 2 is depicted with a triangle (▲)

We observed a high variability in analyte concentrations between patients. This is often observed in PK studies with PZQ and is likely due to the high first-pass metabolism of PZQ in the liver or gut, with the activity of CYP 450 being highly dependent on the health, genetic and nutritional status of the patient (Olliaro, Delgado-Romero and Keiser,

2014). Multiple dosing can also add to variability, since differences among patients in absorption and elimination as well as competition/saturation effects are common and exacerbate each other.

The high $\text{AUC}_{0-24\text{h}}$ values of the PZQ enantiomers and metabolite determined for patient 2 (taking only two doses instead of three) might be explained by several

factors. Firstly, this is the only patient suffering from a co-infection with whipworms and hookworms at follow up. Changes in drug metabolism due to immune reactions due to three co-existing parasites might be possible, as some immunomodulators were found to decrease hepatic activity (Tekwanl, Shukla and Ghatak, 1988; Renton, 2001). Secondly, patient 2 displays the highest weight to height ratio (body mass index of 35.2 kg/m²), which could lead to an overestimation of the effective drug dose, as it is often the case in overweighted patients (Pai, 2012). Finally, this patient might have developed liver and intra-hepatic bile duct pathologies, thereby altering drug metabolism, as observed in patients infected with another liver fluke, *Fasciola hepatica* (Tekwanl, Shukla and Ghatak, 1988). Although measurement of liver enzyme parameters and the physical examination did not identify this patient as a symptomatic opisthorchiasis case, ongoing liver pathology can not be ruled out (Na Bangchang *et al.*, 1993). In fact, the detection of hepatic abnormalities due to opisthorchiasis, such as fibrosis or moderate hepatomegaly, is recommended to be performed via ultrasonography (not done in the present study), as liver enzymes do not seem to be a reliable indicator for the pathology of this disease (Mairiang and Mairiang, 2003; Sripa *et al.*, 2011).

Not surprisingly, patient 2 suffered from adverse events during the treatment course, as high C_{max} levels are often correlated with adverse events (Olliario *et al.*, 2011). It might be worth highlighting that this patient as well all other study participants were cured following PZQ treatment. The high efficacy noticed with a triple dose of PZQ is in accordance with

previous studies (Keiser and Utzinger, 2004). The patient with the highest infection intensity (patient 7: 13,920 EPG at baseline) displayed parent and metabolite AUC_{0-24h} values similar to the other patients with moderate EPG values (between 1,000 and 10,000 EPG at baseline), hence infection intensity does not seem to correlate with PZQ disposition.

The most striking result observed in the disposition of PZQ is the high concentration of R-trans-4-OH, culminating at 13.9 µg/ml. For comparison, a study from Lima and colleagues (Lima *et al.*, 2011) conducted in healthy volunteers treated with a single oral dose of 25 mg/kg PZQ displayed a 10x lower C_{max} of R-trans-4-OH. This finding, which might be explained with changes in metabolism due to the liver disease, raises the question of the role of the metabolite in the opisthorchicidal activity of PZQ. *In vitro* and *in vivo* studies should be conducted to assess the activities of R- and S-PZQ and R-trans-4-OH against *O. viverrini*.

The incurred sample reanalysis revealed a proportion higher than 2/3 of the samples falling into the acceptance criterion of deviating no more than 20%. These results demonstrate that the measurements are reliable and that there are no major problems in sample handling, processing or analysis. The hematocrit of 35% used for the calibration line and the 25–50% range of hematocrits used for the quality controls reflects values in our patients (mean hematocrit of 35.5 ± 4.1%), and more generally values encountered in Southeast Asia. For example, in Thailand, the mean hematocrit in men is between 42-47% and in women it is between 37-39% (Tanphaichitr *et al.*, 1980).

Table 4 Mean percentage ratios of AUC_{0-24h} values in blood or DBS compared to plasma and lower and upper 95% limits of agreement (LoA) with their respective 95% confidence intervals (CI)

Analyte	Mean ratio ^a [%] (CI)		Lower LoA ^b [%] (CI)		Upper LoA [%] (CI)	
	blood	DBS	blood	DBS	blood	DBS
R-PZQ	81.9 (76.8;86.6)	92.5 (82.9;102.2)	69.6 (61.4;77.9)	68.0 (51.3;84.7)	94.1 (85.8;108.9)	117.1 (100.4;133.8)
S-PZQ	85.4 (76.8;93.9)	87.0 (73.2;100.7)	63.6 (48.8;78.4)	52.0 (28.2;75.7)	107.2 (92.4;122.0)	122.0 (98.2;145.7)
R-trans-4-OH	111.2 (101.5;120.9)	107.6 (97.9;117.4)	86.5 (69.8;103.3)	81.4 (63.6;99.2)	135.9 (119.1;152.6)	133.9 (116.1;151.7)

^a The percentage ratio is computed as blood or DBS AUC_{0-24h} values divided by plasma AUC_{0-24h} values averaged across patients and presented as percentage to the plasma values.

^b The 95% limits of agreement for AUC_{0-24h} values were calculated with the conventional Bland-Altman method.

Table 5 Percentage ratios of t_{1/2}, C_{max} and T_{max} in blood or DBS compared to plasma

Analyte	t _{1/2}		C _{max}		T _{max}	
	blood [%]	DBS [%]	blood [%]	DBS [%]	blood [%]	DBS [%]
R-PZQ	89.4	121.6	89.4	92.0	100.0	101.0
S-PZQ	81.8	100.0	81.8	96.5	100.0	99.4
R-trans-4-OH	105.2	75.3	105.2	103.5	115.4	111.5

All the LoA intervals included 100%, indicating no difference between DBS or blood compared to plasma concentrations, except for R-PZQ in blood. The LoAs observed were wide, which can be explained by the additive measurement errors in each matrix. When validating a bioanalytical method, the accepted measurement variability is of $\pm 15\%$. This translates to indicative maximal LoA of 71–129% (calculated using the conventional Bland-Altman formula with $SD = 15\%$), which is broadly similar to the results observed in this study. The wider LoA and confidence intervals for DBS-plasma compared to blood-plasma AUC_{0-24h} ratios reflect the half as small sample size for the estimation of DBS AUC_{0-24h} compared to blood AUC_{0-24h} samples. In light of these observations, we estimate that there is a general agreement between matrices and that DBS is a valid surrogate to venous sampling.

In the Bland-Altman comparisons of blood versus plasma or DBS versus plasma, R- and S-PZQ displayed drug concentrations and AUC_{0-24h} percentage ratios of around 80% and R-trans-4-OH ratios higher than 100%. The higher concentrations observed for R- and S-PZQ when quantified in plasma compared to blood or DBS might arise from a very high affinity of the drug for plasma proteins. PZQ is highly protein-bound (~80%) (Olliaro, Delgado-Romero and Keiser, 2014). Hence, red blood cells might have a slight diluting effect on PZQ concentrations, depending on the blood hematocrit (Emmons and Rowland, 2010). For example, tasquinimod, an anticancer drug characterized by a very high plasma binding (>98%), revealed a blood:plasma ratio of 66% (Isaacs *et al.*, 2014; Svensson *et al.*, 2015). This phenomenon was also observed in a study comparing DBS and plasma sampling with piperacillin and tazobactam in infants with DBS:plasma ratios between 50 and 60% (Cohen-Wolkowicz *et al.*, 2014). Therefore, our results for the parent enantiomers are in line with previous observations in drugs with high plasma affinity and displayed an agreement between plasma and blood or DBS of around 80–90%. In contrast, R-trans-4-OH did not follow such pattern and displayed higher concentrations in blood or DBS than in plasma, which likely indicates a lower affinity for plasma proteins than its parent molecule and a higher repartition in erythrocytes.

The *in vitro* evaluation of PZQ partition between plasma and erythrocytes highlighted a higher affinity of the enantiomers to plasma, which echoes the observations in patients described above. Considering

that PZQ is bound to 80% to plasma proteins (Olliaro, Delgado-Romero and Keiser, 2014) and that acetonitrile precipitation extracts both the unbound and bound fractions, the blood to plasma ratios between 75 and 83% indicate that penetration of the free fraction into erythrocytes is very limited to almost absent. On the other hand, the metabolite R-trans-4-OH displays *in vitro* an almost even distribution in all blood compartments, while in patient samples this ratio is slightly more biased towards erythrocytes.

In conclusion, we have shown that DBS is a valid alternative to plasma sampling for PK studies with PZQ. Additional studies are warranted to estimate the kinetic disposition of patients after different PZQ dosing schemes and to investigate the PK/PD relationship, in particular the role of R-trans-4-OH in the opisthorchicidal activity of PZQ.

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Author contributions

Conceived and designed the experiments: JKe UD PO SS JH. Performed the experiments: IM JKe. Analyzed the data: IM FV. Contributed to the reagents, materials and analysis tools: JH. Wrote the paper: IM JKe.

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Competing interests

The authors have declared that no competing interests exist.

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Chapter 6

General discussion

6.1 Synthetic Peroxides

6.1.1 Key findings

The first part of my project aimed to elucidate the pharmacokinetic profile of OZ78 and MT04 in sheep naturally infected with *F. hepatica* (Chapter 2). This study was the second testing of OZ78 in sheep after a first unsuccessful experience (Keiser *et al.*, 2010). It should therefore be regarded as an additional attempt to overcome the loss of activity of OZ78 when translating the excellent rat experiments (Kirchhofer *et al.*, 2011) to sheep. Briefly summarizing the results of this investigation, after a single intramuscular dose of 100 mg/kg OZ78 or MT04, we observed C_{\max} and AUC values for OZ78 twice as high as those for

6.1.2 Rat and sheep, what is the difference for OZ78?

Fasciocidal activity of OZ78 is very good in rats, with a single oral dose of 100 mg/kg resulting in a complete juvenile and adult WBR, and a dose of 50 mg/kg displaying a WBR slightly above 50% (Kirchhofer *et al.*, 2011). When translating the optimal dose from the rat to the sheep, it was estimated that treating sheep with 50 mg/kg OZ78 was sufficient if not higher than necessary (Keiser *et al.*, 2010). The outcome of this first experiment in sheep was however disappointing; no noticeable differences in WB could be observed between treated and control sheep. Also, PK values observed in sheep were much lower than expected from the rat. For example, in uninfected rats, the C_{\max} observed in plasma was 70.1 $\mu\text{g/ml}$ after an oral dose of 50 mg/kg OZ78. In infected sheep treated orally with 50 mg/kg OZ78 (corresponding to slightly more than a 100 mg/kg dose in rats, as previously mentioned), the C_{\max} observed in plasma was only 45.8 $\mu\text{g/ml}$. As emphasized by Keiser *et al.*, 2010, the digestive system of rats and sheep displays considerable differences and can account for the treatment failure in sheep. The keratinised rumen is actually not designed for a good absorption of drugs, which can be substantially delayed by up to 15h due to the slow turn-over rate of the rumen (Toutain, Ferran and Bousquet-Melou, 2011). In the case of OZ78, absorption in another part of the gut can be considered minimal; the T_{\max} and $t_{1/2}$ of 1h obtained after oral treatment are extremely rapid and point to a ruminal absorption of OZ78. Drug binding to cellulose from an herbivorous diet can also interfere with absorption. Since cellulose breakdown in the sheep gut occurs only after 50 h (Toutain, Ferran and Bousquet-Melou, 2011), it might explain in part the lower AUC in sheep compared to rat.

Oral and subcutaneous administration routes of OZ78 in sheep have shown drastically different PK profiles

MT04, as well as a much more rapid T_{\max} . Despite its superior kinetic disposition, OZ78 failed again to cure infections with *F. hepatica*, while MT04 displayed a good fasciocidal activity. Here, I would like to bring additional elements to contribute answering to the questions left open in Chapter 2: why do we observe so different efficacy outcomes for OZ78 between rat and sheep? Is the mode of action of these peroxides linked to haem detoxification or does bile play a role as well? And finally, is MT04 still a valid fasciocidal candidate?

at 50 mg/kg dose (Keiser *et al.*, 2010). Oral application was characterised by a higher C_{\max} and a short $t_{1/2}$, while subcutaneous administration had a slower absorption rate but a longer $t_{1/2}$ and a twofold AUC value than oral application. The slow release observed with subcutaneous application is certainly due to the oily vehicle forming a depot under the skin, as suggested by Keiser *et al.*, 2010. In the second study in sheep reported in Chapter 2, we obtained C_{\max} and T_{\max} values close to and an AUC smaller for intramuscular administration than for subcutaneous application, although the dose was doubled. There are obviously clear problems with the absorption of the peroxides with intra-muscular application. The fact that none of the PK parameters could be enhanced by doubling the dose and changing the administration route in the second sheep experiment points to a rate-limiting absorption. Also a significant difference to keep in mind regarding the two studies in sheep is the use of both experimental and natural infections. Naturally infected sheep can display a much more severe liver pathology consecutive to chronic infections, while experimentally infected sheep would not present such extensive bile-duct calcifications and abscesses. Moreover, other concurring infections in naturally infected sheep (observed though not monitored) could have affected the kinetic disposition of the drug even more drastically than a simple experimental infection with *F. hepatica*. The role of the disease severity in causing a lower drug disposition is not easily explained. Investigating the PK profile of OZ78 in rats using intramuscular application instead of an oral dose would certainly help assessing whether a rate-limiting phenomenon or another factor is responsible for the low PK profile observed after a treatment with 100 mg/kg.

6.1.3 Haem or bile? Do we have enough hints to guess how peroxides act on *F. hepatica*?

The synthetic peroxide library was first generated to provide potential antimalarial drug candidates on the basis of the chemical structure of artemisinin, and more precisely on the pharmacophoric peroxide bond. This peroxide bond is supposed to interfere with the detoxification process of haem, generating free radicals attacking the parasite (Vennerstrom *et al.*, 2004; Jefford, 2007). Yet it is not only *Plasmodium* spp., but also blood-feeding parasites such as *F. hepatica* that need to deal with haem toxicity, as a by-product of erythrocyte digestion. Indeed, synthetic peroxides displayed very promising results against the liver flukes *F. hepatica* (Keiser *et al.*, 2006; Jennifer Keiser *et al.*, 2007) and *C. sinensis* (J Keiser *et al.*, 2007; Keiser *et al.*, 2009; Keiser and Vargas, 2010), the blood flukes *Schistosoma* spp. (Xiao *et al.*, 2007) and the intestinal fluke *E. caproni* (Keiser *et al.*, 2006; Kirchhofer *et al.*, 2011). In *in vitro* studies with *F. hepatica*, the effects of OZ78 on the worm tegument were already observed in medium without haemin. The addition of haemin to the culture medium containing OZ78, although producing more tegumental damage, did not accelerate worm death. It was hence postulated that an iron-independent pathway might contribute to the mechanism of action in worms (Keiser and Morson, 2008; Halferty *et al.*, 2009). An investigation of the effects of artemisinins on a yeast model reveals at least two mechanisms of action; the first involves iron, as suggested earlier, while the second directly targets mitochondria under non-fermentative conditions (Moore *et al.*, 2011).

The direct environment of adult *F. hepatica*, the bile, might well act in synergy with synthetic peroxides to damage and ultimately kill the worms. Since OZ78 had already displayed inefficacy against *F. hepatica* in sheep, we hypothesised that its concentration in bile might be not sufficient to exert a fasciocidal effect. Therefore, in parallel to investigating the pharmacokinetics of OZ78 and MT04 in sheep plasma, we were able to assess their presence in sheep bile at 6 h post-treatment. We observed that their concentration in bile was lower than in plasma by a factor of 5 and 2, respectively. However, since peroxides are secreted in bile in measurable amounts, a synergistic influence of

bile on peroxide activity could not be ruled out. In a pilot project aiming to gain more insight into the mode of action of peroxides, we compared the *in vitro* fasciocidal effects of OZ78 and MT04 in presence of bile (5%) or haem-containing elements, as haemin and haemoglobin, at 80 µg/ml (unpublished observations). Peroxide concentration was set at 10 µg/ml, a value close to the C_{max} observed in sheep plasma for OZ78, and two-fold higher than the C_{max} of MT04. Adult worms recovered from cattle livers were incubated in triplicate in medium containing bile, haemoglobin or haemin and the peroxides. Their viability was assessed after 72 h incubation using a motility scale ranging from 3 (perfectly healthy) to 0 (dead).

Surprisingly, the worms were already greatly affected when incubated with bile or haemin without the addition of peroxides. It is interesting to observe such deleterious effects of incubating worms with only 5% bile, considering that bile is their usual environment. Haemoglobin, on the other hand, is not as reactive as haemin, however a moderate effect on the worms was also observed. Worm viability in a haem-containing medium was not more compromised when peroxides were added. As observed by Keiser and Morson, 2008, haemin did not accelerate worm death when in contact with synthetic peroxides. In bile-containing medium, however, 10 µg/ml MT04 contributed to a slightly faster death of the worms. As previously mentioned, the presence of bile is clearly deleterious to the worm and adding peroxides to this incubation medium results in an earlier fasciocidal action. To summarize, MT04 at a concentration of 10 µg/ml, a two-fold higher concentration than the observed C_{max} in sheep plasma, was not able to significantly act on the worms without the contribution of additional factors, such as haemin or bile. Repeating the experiment using much higher concentrations of peroxides, i.e. 100 µg/ml, might help to estimate whether the effect of bile is simply additive or synergistic. The help of scanning electron microscopy would also contribute to characterise the specific effects of bile with and without peroxides on the worm tegument, as it was done for haem.

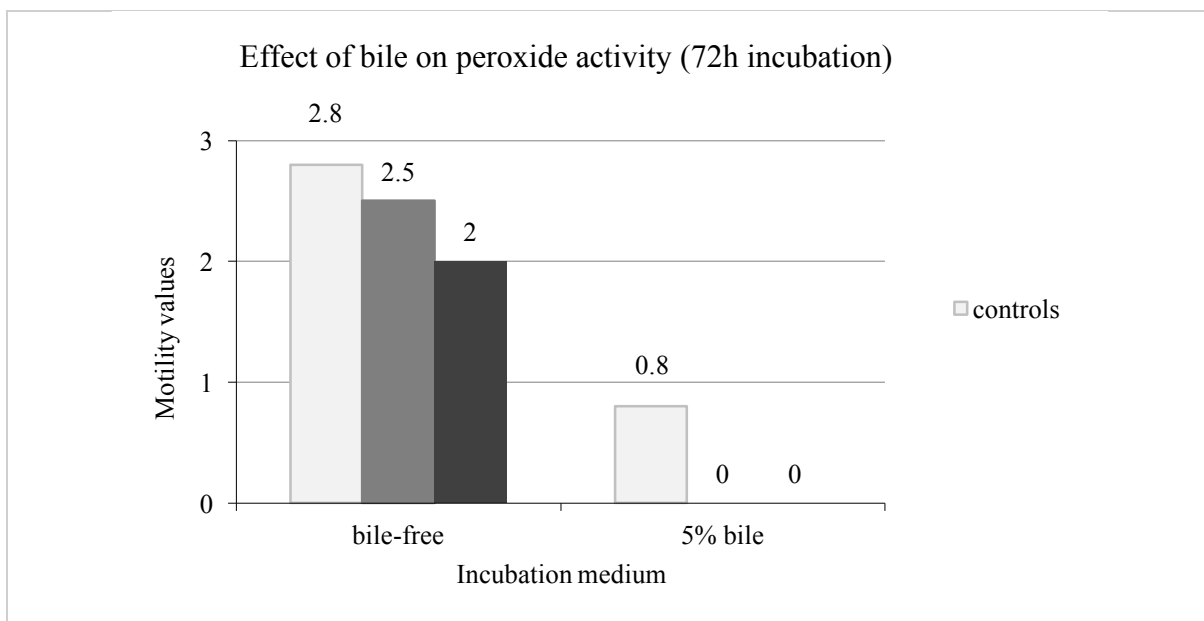


Fig. 1 Comparison of motility values of worms incubated in bile-free medium vs. medium with 5% bile and the effect on worm motility of adding peroxides to the medium.

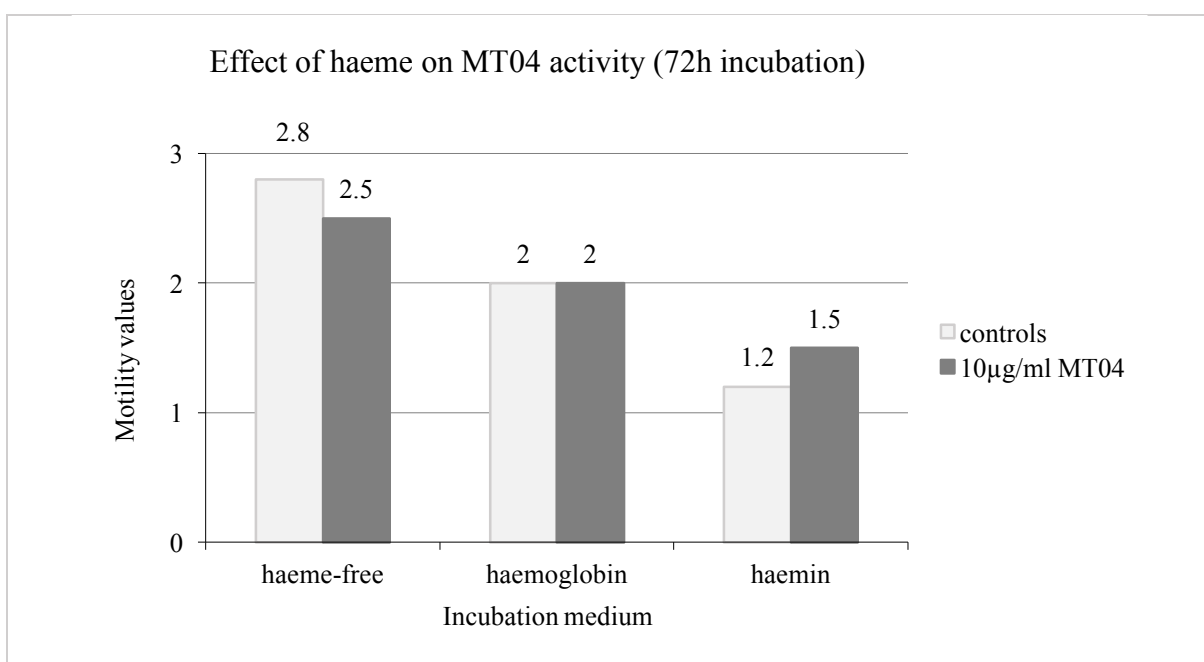


Fig. 2 Comparison of worm motility between normal medium vs. medium containing different haem forms and effect of the addition of peroxides on worm motility

Infection with liver flukes produces a tremendous reaction in the liver of the host, with a diminution of antioxidant enzymes and an increase in free radical production (Karsen *et al.*, 2011; Bahrami, Esmailzadeh and Oryan, 2014). Hepatic fibrosis and biliary cholestasis can develop and have been shown to exacerbate the oxidative stress of the host (Bahrami, Esmailzadeh and Oryan, 2014). The liver and bile ducts become therefore a hostile environment for flukes, which have developed a collection of antioxidant enzymes as protection. They release these

enzymes in great quantities in their excretory-secretory (ES) products. Since they burrow deep in the bile duct tissues to reach blood vessels, the surrounding epithelium provides them with a protective pouch where they create a micro-environment with high concentrations of antioxidant enzymes. When in contact with synthetic peroxides or artemisinin derivatives, the gut function of worms is greatly disturbed, impeding the absorption of essential nutrients to keep pace with their high metabolic rate (Halferty *et al.*, 2009; O'Neill *et al.*, 2015). Hence

starvation conditions might decrease the release of ES products, which might primarily affect the most posterior end of the parasite, the furthest to the mouth and most exposed to fresh bile. The starvation of the worm might also interfere with the natural renewal of the tegument, and here again the posterior end of the

worm is less exposed to the protective effect of ES products. Considering all of the above, synthetic peroxides are therefore highly dependent on external factors, as haemin and bile, to achieve their fasciocidal effect. The exact mechanism of action however still needs further confirmation.

6.1.4 What is the validity of MT04 as a drug candidate against trematodes?

Due to their broad spectrum of activity, ranging from malaria and trematodes to herpes and cancer (Ho *et al.*, 2014), synthetic peroxides can present problems with toxicity. It was observed on several occasions that tetraoxanes are more problematic than trioxolanes (Kirchhofer, 2012; Cowan *et al.*, 2015). Studies with a hepatic cell line demonstrated a limited toxicity of OZ78 but a higher toxicity of MT04, however still lower than observed for artesunate (Kirchhofer, 2012). In an experiment assessing the effect of artesunate on naturally infected sheep, two deaths were recorded. In

the two studies using OZ78 in sheep, no lethal cases were observed with this compound (Keiser *et al.*, 2010; Meister *et al.*, 2013). However, when treating naturally infected sheep with 100 mg/kg MOT04 intramuscularly, one sheep (sheep number 4, one out of five) died 1 h after administration (not reported in Chapter 2). We were able to retrieve 3 blood samples from this individual between 25 min and 1 h post-injection. The PK analyses revealed an extreme concentration of MT04 in plasma from the first sample on.

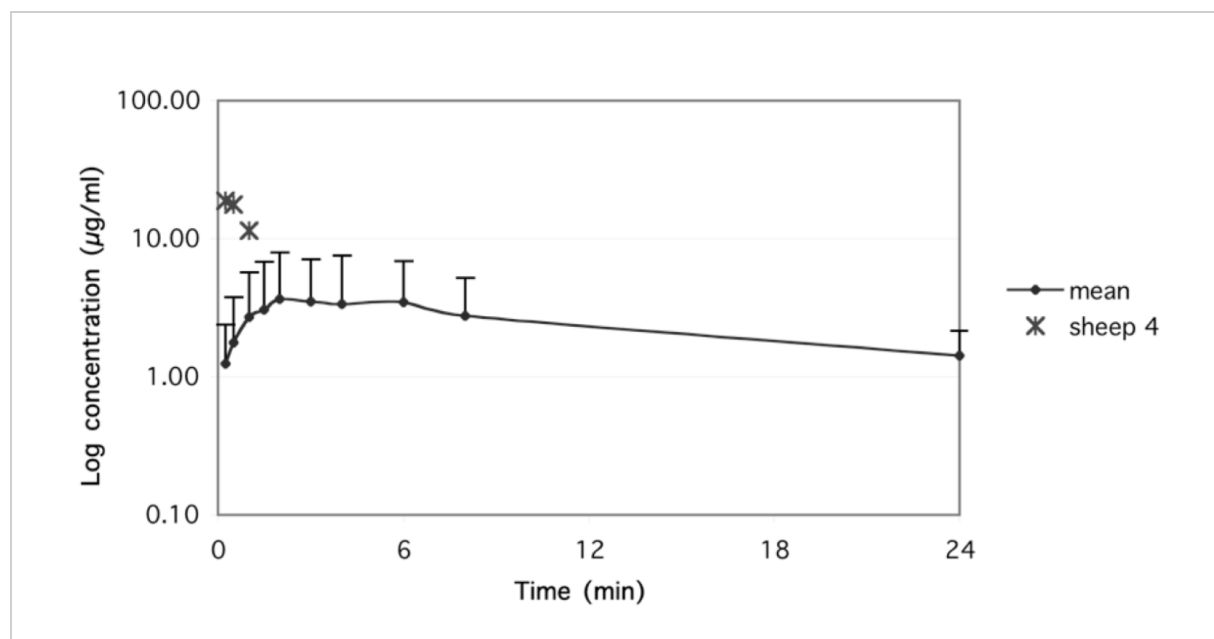


Fig. 3 Log mean pharmacokinetic profile of MT04 in sheep after an intramuscular dose of 100 mg/kg with PK values of the sheep that displayed fatal toxicity (sheep 4)

The most plausible explanation is a complete shutdown of the liver, which was not able to display a baseline detoxification/elimination of MT04. This event stresses the fragile condition of a chronically infected liver, probably not compatible with MT04 treatment, at least at the concentration of 100 mg/kg. A way to decrease the risk of developing deleterious effects due to MT04 treatment would be to supplement the animal diet with antioxidants. For example, a diet consisting of flaxseed oil displayed a clear

amelioration of the liver pathology and an appreciable WBR (Martinez-Perez *et al.*, 2014). It still needs to be demonstrated that antioxidants would not interfere with MT04 activity. However, a protection of the liver with antioxidants would be greatly beneficial to fasciocidal therapy and would promote a faster recovery. Another solution would be to combine MT04 with other drugs of different mechanisms of action to lower the quantity of MT04 administered, as already tested with *C. sinensis* (Keiser *et al.*, 2009). In

the case of *F. hepatica*, triclabendazole, acting on microtubules, could be a good candidate in regions with a low prevalence of phenotypes resistant to triclabendazole. A third alternative would be to combine MT04 with closantel, a flukicide marketed for veterinarian use that targets the parasite gut. It would be interesting to see whether these drugs would act synergistically since they target differently the same organ, the worm gut. Additionally, closantel is active only on biliary stages of the parasite (Hanna *et al.*, 2015), while MT04 is also as well effective against migrating larvae (Kirchhofer *et al.*, 2011). In

conclusion, the fatal case of toxicity encountered with MT04 treatment in sheep should not cause its withdrawal as a fasciocidal drug candidate. Due to its double peroxidic bond, MT04 is already more potent than any trioxolane (Kirchhofer *et al.*, 2011). Scaling down the treatment to 50 or 70 mg/kg would probably reduce its toxicity without compromising its activity, if indeed a rate-limiting phenomenon was present with the 100 mg/kg dose. An oral treatment combined with a sustained-release applicator placed in the rumen could also be an alternative to explore.

6.2 Praziquantel

6.2.1 Key findings

Investigations on PZQ occupy a consequent part of my thesis (Chapters 3 to 5). The focus was to elucidate the role of each PZQ enantiomer and its main metabolite on trematocidal activity. The *in vitro* and *in vivo* activity assays were conducted on *S. mansoni* and the PK studies on *Opisthorchis*-infected patients. The use of *S. mansoni* over liver flukes for the activity assays is, besides from practical reasons (it is very difficult to obtain *O. viverrini*, since there are no labs maintaining its life cycle), that the scientific interest towards schistosomiasis is much higher. These activity results will be of great importance when performing PK studies on schistosomiasis, patients, currently undergoing in our team.

The outcomes of the *in vitro* and *in vivo* assay designate R-PZQ as the eutomer, the enantiomer displaying the therapeutic effects, while S-PZQ does not seem to play any schistocidal role. The R configurations of the *cis* and *trans* main monohydroxylated metabolites of PZQ displayed

some activity but at much higher concentrations than for R-PZQ. Their significance in the schistocidal activity of PZQ should be evaluated using PK studies in patients. The development of an enantioselective analytical method for PZQ is the first step in this direction. When validating the use of DBS technology as a PK tool, we analysed the PK profile of 9 patients infected with *O. viverrini* and treated orally with 3x 25 mg/kg PZQ. We were surprised to observe that concentrations of R-*trans*-4-OH-PZQ were much higher than expected. Therefore, it is paramount to investigate the respective part of the metabolite and R-PZQ in clearing trematode infections. Since the most detailed investigations on PZQ activity were done in *S. mansoni*, the first question that comes to mind is whether other PZQ-sensitive parasites would react to PZQ enantiomers and metabolites in the same way as *S. mansoni*. Then, in the second part of the discussion about PZQ, I would like to consider the main current challenges and limitations of PZQ.

6.2.2 Are investigations on PZQ activity transposable to other PZQ-sensitive parasites?

PZQ is the standard treatment against most of flatworms. Aside from schistosomiasis and opisthorchiasis, PZQ cures other trematodes infections, such as the liver fluke *C. sinensis*, the lung flukes *Paragonimus* spp., and the intestinal flukes *Fasciolopsis buksi*, *Echinostoma* spp., *Heterophyes heterophyes* and *Metagonimus* spp. Interestingly, *Fasciola* spp. is the only known trematode genus refractory to PZQ therapy. PZQ is also highly efficient against most of the cestode species (Andrews, 1985; Cioli and Pica-Mattoccia, 2003).

The investigation performed on the *in vitro* and *in vivo* activity of PZQ enantiomers and its main metabolites against *S. mansoni* (please see Chapter 3) were in line with the general opinion that R-PZQ was the main effector molecule. However, Irie *et al.*, 1989, as well as Tanaka *et al.*, 1989 found a clear effect of R-PZQ against *S. japonicum*, while for *S. mansoni* S-PZQ was driving most of the effects. The results obtained with *S. mansoni* are in complete disagreement with simultaneous and later publications (Xiao and Catto, 1989; Staudt *et al.*, 1992), but should not be given much importance as the experimental design and some inconsistencies in the result tables might be the cause of the confusion. Results with *S. japonicum*, on the other hand, are consistent with other publications, where a treatment with R-PZQ could cure *S. japonica*-infected patients at half the dose of PZQ racemate (Wu

et al., 1991; Liu *et al.*, 1993). With the exception of studies with *S. mansoni* and *S. japonicum*, no further investigations of the activity of each PZQ enantiomer were conducted in other parasites. Since *S. mansoni* and *S. japonicum* display a clear agreement regarding enantiomer activity, we can postulate that a similar pattern occurs for all *Schistosoma* spp., and by extension to all PZQ-sensitive parasites. It actually seems very likely that the essential and specific receptor that PZQ targets conserved its chiral direction over evolution. *In vitro* investigations on R-PZQ activity with *O. viverrini* and *E. caproni* as representatives of trematode diversity would help in assessing the validity of this hypothesis. Of note, studies with *S. haematobium* and different *S. mansoni* isolates have been launched in our laboratories.

The relative trematocidal activities between parent and metabolite enantiomers, and their comparison to PK values are also essential to understanding the modalities of PZQ action. Here again, investigations on the activity of PZQ metabolites are lacking in parasites other than *S. mansoni*. Although the polarity of the worm receptor to PZQ is not expected to change, different sensitivities to parent and metabolite molecules might be observed in PZQ-sensitive parasites. It would be very valuable to assess metabolite activity in *O. viverrini*, since we are performing an extensive population PK study in *O.*

viverrini-infected patients. The comparison of efficacy outcomes with PK data from the dose-finding study

6.2.3 Praziquantel, new challenges, same limitations?

Since its advent on the market in the 1980s, PZQ treatment is based on kinetic and efficacy data collected in adults, while small children do not benefit from carefully tailored dosages. After decades of mass drug treatments in endemic countries, there is a strong incentive to include children, and especially pre-school children, in targeted treatment programs. This population, although neglected by treatment programs, displays extremely high infection rates. Moreover, infections in the early age can compromise a child's development and lead to an increased risk of irreversible chronic complications already at an early age (Stothard *et al.*, 2013; Mutapi, 2015). The main drawbacks of PZQ when treating children are the tablet size and its very bitter taste. Tablets are manufactured as 600 mg tablets, which are difficult to swallow. To circumvent this difficulty, tablets are crushed to powder. However, the repulsive bitter taste of PZQ represents a challenge to swallow for many children, even mixed in very sweet and flavoured syrup. In 2012, a public-private partnership including Merck, Astellas and the Swiss TPH was created to develop a paediatric formulation of PZQ for pre-school children. This collaboration focuses on 3 main points: adapting the treatment dose to acceptable efficacy and safety levels, finding a way to mitigate the bitter taste and decreasing tablet size. These two last points rely on an enantiomeric formulation of PZQ. A tablet containing only the eutomer R-PZQ would be half as big as the racemic tablet for a most likely equivalent therapeutic potential. In a clinical trial in adults infected with *S. japonicum*, a treatment with R-PZQ achieved similar efficacy and fewer side effects than a conventional PZQ treatment (Wu *et al.*, 1991). Moreover, removing S-PZQ from the tablets would ameliorate the overall taste (Meyer *et al.*, 2009). The efforts invested for a paediatric formulation could ultimately be beneficial for other patients suffering from trematodiasis if an enantiomeric formulation of PZQ would reduce the adverse events to nearly zero. Obviously, evidence of the relationship between S-PZQ and the type of adverse events (allergy, nausea, drowsiness) still needs to be collected. The challenge for the enantiomeric preparation compared to the original formulation is its cost. The additional steps to obtain pure R-PZQ are not so straightforward and efforts to optimise this process are underway (Woelfle *et al.*, 2011). Chiral switch, i.e. switching from a racemic drug to an enantiomeric

currently under analysis could be strengthened by the support of *in vitro* observations.

formulation, is of high interest for many modern drugs, because of the possibility to extend or present a new drug patent (Agranat, Caner and Caldwell, 2002). Although patenting is not in the scope of NTD drugs, the enormous knowledge currently generated for cost-effective strategies to produce enantiomeric drugs would also be valuable for PZQ. A competitive price for the enantiomeric formulation is the cornerstone to a sustained effort against schistosomiasis.

Resistance against PZQ is the subject of worries since the first report of PZQ treatment failure in Senegal in 1994. For example, the refractory juvenile worms that survived a PZQ treatment later produce offspring with a decreased susceptibility to PZQ. However, since decades of intensive use and drug pressure in Africa and Asia, PZQ resistance does not appear to be occurring as fast as for other drugs (Caffrey, 2007; Wang, Wang and Liang, 2012). Actually, it seems that PZQ-resistant phenotypes are disadvantaged in terms of fitness compared to susceptible strains. As a consequence, traits conferring PZQ resistance are not able to fix permanently in the genome (Greenberg, 2013). Some authors have proposed that schistosomiasis control instead of elimination programs hinders the emergence of resistance by maintaining foci of highly competitive PZQ susceptible strains. They anticipate that when implementing steps towards elimination and later perhaps even eradication of schistosomiasis, resistant phenotypes would then be able to take and hold the niche left by susceptible isolates. Monitoring resistance development becomes a difficult task when discussing PZQ. The mechanism of action of this drug remains a mystery, at least in part (Greenberg, 2005, 2013). After exposure to PZQ, worms suffer a rapid influx of Ca^{2+} ions followed by muscular paralysis and tegumental damage, exposing parasite antigens to immune response. The identification of Ca^{2+} channels as PZQ receptors is however disputed. For example, cytocholasin D antagonises the effect of PZQ but does not block the influx of Ca^{2+} in the worm. Moreover, the expression of Ca^{2+} channel genes is not different between the refractory juveniles and the susceptible adults (Doenhoff, Cioli and Utzinger, 2008; Aragon *et al.*, 2009; Greenberg, 2013). It seems that ABC transporters are involved in the uptake of PZQ and that a P-glycoprotein could be used as an efflux mechanism to evacuate PZQ (Messerli *et al.*, 2009). Elucidating

the mode of action of PZQ would allow for predicting parasite's strategies to develop resistance to PZQ. The identification of the target of PZQ, the means by which it accesses its receptor and the clarification of the downstream cascade of reactions would be highly valuable to generate new lead structures and new drug combinations minimising the resistance risks. It is not enough to stress out the importance of gathering more PK data in the target populations and link them to treatment efficacy. A thoroughly determined dosage is key against the emergence of resistance.

For liver flukes, the situation is slightly more encouraging than for schistosomiasis, as clinical trials using the novel drug tribendimidine display equivalent results to PZQ trials (Soukhathammavong *et al.*, 2011). For chlonorchiasis and opisthorchiasis, a combination of social interventions and chemotherapy would in theory prevent new infections. Changes in eating habits are however extremely difficult to induce

in regions where raw or fermented fish consumption is a strong cultural component (Sithithaworn and Haswell-Elkins, 2003; Sripa *et al.*, 2010; Grundy-Warr *et al.*, 2012). Interventions as health education demonstrated their efficacy but a sustained and more generalised effort is warranted. Until a strong societal change can occur, mass chemotherapy with PZQ or eventually later tribendimidine is vital to avoiding chronic complications, especially the cholangiocarcinoma. There are signs that PZQ treatment induces a transient increase in oxidative stress in hamsters (Pinlaor *et al.*, 2008), but evidence needs to be collected for patients and its significance on the onset of cholangiocarcinoma should be evaluated. A comparative study of the adverse events of PZQ and tribendimidine with an emphasis on liver oxidative stress level should contribute to ameliorating the mass drug administration programs.

6.3 Technical Considerations

As part of the general discussion of my thesis, it seems to me important to address some of the techniques I had the privilege to work with and to collaborate in developing in our research group, as for example the dried blood spot (DBS) technology. I would also like to give an outlook on the enantioselective analysis of

6.3.1 Chiral analysis

The enantioselective LC-MS/MS analysis of drugs faces significant challenges, such as the choice of the right chiral selector, sensitivity issues at the desired LLOQ, price or available column dimensions. For the chiral method development of PZQ, the major pitfall was to run plasma and blood samples. In contrast to traditional C18 columns that are relatively robust to sample impurities, the chiral columns used in our method display enlarged and split peaks after a few weeks of running blood or plasma samples extracted with acetonitrile. The implementation of a column switching system and regular column washes were not sufficient to give chiral columns a lifespan comparable to traditional ones. With respect to these observations, the choice of more sophisticated extraction methods, such as liquid-liquid extraction or solid phase extraction, should be weighed regarding handling time and respective costs of column and extraction material. Although some experience will still be gained over the next months, it seems that DBS extracts are much more compatible with chiral analysis than other acetonitrile-extracted biofluids. This weakness of chiral columns, combined with a price twice as high as for traditional

6.3.2 DBS technology

Dried blood spots are a remarkable technology for PK sampling. In the last decade, their use spread from metabolic disease screening to pharmacokinetic studies, therapeutic drug monitoring, immunoassays and epidemiological disease surveillance (Demirev, 2013). The first challenge for DBS analysis is the evidently smaller amount of sample that can be extracted. For plasma, usually 100 μl are extracted with 300 μl organic solvent. In our DBS method, the typical volume of a blood spot is 20 μl . From this spot, only a portion of maximum 15 μl is punched out and further extracted with 300 μl organic solvent. There are many strategies to optimise analyte concentrations in the extract, such as evaporation and reconstitution or the pooling of samples, but the fact is that the starting amount of sample is nearly 10 times lower than in conventional plasma extraction. Even with the improvement of instrumentation and column quality, it is difficult to reach the same sensitivity as for plasma-

chiral drugs and its relevance to drug development for neglected tropical diseases. Finally, the evaluation of the population PK analysis as a tool for drug development seems essential, as it is the next logical step of my investigations.

columns, encourages us to carefully consider the choice of chiral analysis.

Enantioselective analysis can however bring valuable data, as it should be the case for our PZQ method. The possibility to study the kinetic disposition of each enantiomer of a drug gives a more detailed insight for adapting dosing strategies and for understanding factors ruling plasma concentrations. For example, albendazole (ABZ), the main drug against round worms, might be a good candidate. ABZ is metabolised by the CYP450 to ABZ-sulfoxide (ABZ-SO), possessing a chiral center on the sulfure atom. ABZ-SO is further metabolised to ABZ-sulfone (ABZ-SO₂) (Virkel *et al.*, 2004). It seems that activity is shared between the parent ABZ and the ABZ-SO. For example, in neurocysticercosis, the R-form of ABZ-SO is the effective enantiomer (Paredes *et al.*, 2013). The conditions in which drug metabolism generates a particular enantiomeric ratio would be an additional point to investigate. Moreover, an enantioselective LC-MS/MS method is already available (del Nozal *et al.*, 2002).

based methods; hence, when translating a plasma method to DBS, a 10 times higher LLOQ would be a reasonable starting point.

The second major issue with DBS is the hematocrit. Blood viscosity alters the spread of the blood drop on the DBS card and therefore influencing the analyte concentration over the spot area (Xu *et al.*, 2013). There have been several attempts to overcome this difficulty, by monitoring the blood spot potassium content (correlating with hematocrit) (De Kesel *et al.*, 2014), or by using pre-cut DBS cards (Meesters and Hooff, 2013) or the newcomer microsampling device (Spooner *et al.*, 2015). Aside from blood viscosity problems caused by hematocrit, there are inherent caveats in switching from plasma to DBS. First, the drug partition might not be equivalent between plasma and blood or DBS. For example, some drugs, such as caffeine, do not bind to plasma proteins but have a high affinity for erythrocytes. Therefore, switching from

traditional plasma measurements to DBS would make the direct comparison between DBS-generated PK profiles and previously published studies more difficult. The kinetic profile measured in plasma will then be very different from the one observed in whole blood or DBS. In other cases, some drugs can have an equal affinity for plasma proteins and for erythrocytes. If the blood-cell partition remains constant across concentrations, the implications of switching to DBS are minimal. However, if there is a saturation of the binding sites of erythrocytes at high drug concentrations, PK measurements in plasma could be altered with plasma haemolysis. In this case, the use of

6.3.3 Population PK

When dealing with patients with impaired liver function, as is the case for trematodiasis, it is essential to understand the driving factors behind drug disposition. To address this issue, we worked in close collaboration with PK/PD modelists from the Swiss TPH to implement population PK clinical trials with tribendimidine and praziquantel in *O. viverrini* infected patients in Laos (currently being analysed). With population PK, in contrast to traditional PK analysis, it is possible to handle PK data originating from sparse sampling schemes, and at the same time to integrate and identify important factors influencing kinetic disposition in patients. The focus of this approach is to estimate PK parameters in the target population under field conditions, and to identify and quantify sources of kinetic variability, i.e. genetic, demographic, environmental or pathophysiological. This method generates a model describing PK parameters and integrating significant covariates. This model can later be applied and refined in further PK

blood or DBS over plasma is actually warranted. For PZQ, the drug is highly bound to plasma (80% (Gonzalez-Esquivel *et al.*, 2005)) and does not display an affinity for erythrocytes. In this situation, the haematocrit value will influence the concentrations observed: the higher the number of erythrocytes, the higher the “dilution” of the drug will occur (Emmons and Rowland, 2010; Xu *et al.*, 2013). When setting up a new DBS method for drugs in current use, most of the aforementioned parameters are already known. For new drugs or drug candidates, a drug partition assay could be easily implemented if needed.

investigations. The advantage of this method is its great flexibility for handling sparse PK data, as well as pooling different trials together, as long as the raw data are available (Food and Drug Administration, 1999; Ette and Williams, 2004). Also, a tangible advantage of population PK is to optimise the sampling time points to meet field limitations, as the number of samples taken per patient or the specific time windows where sampling is difficult or impossible. This was the case for our tribendimidine study, where modelists provided us with optimised sampling time points according to previously generated PK data. Hence, we could only sample 5 time points per patient, instead of the conventional 8-10 time points used in traditional PK studies, without a significant loss of resolution. With the experience that is currently gained with these two trials, I am convinced that population PK will soon become a fundamental tool to assess PK data in diseased patients, especially in combination with the DBS technology.

6.4 References

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Chapter 7

General conclusion

The aim of this thesis was to contribute to drug development against trematodiasis, with an emphasis on pharmacokinetic studies. Analytical methods were developed to study kinetic disposition of praziquantel in *Opisthorchis*-infected patients and of lead peroxide candidates in sheep suffering from fascioliasis. In the former case, PK studies aimed to elucidate the disposition of each enantiomer of a drug, while in the latter, PK investigations intended to understand problems with drug efficacy. Also, *in vitro* and *in vivo* studies of PZQ enantiomers on different parasitic stages will be an appreciable support for a future paediatric formulation of PZQ. Finally, the implementation of DBS is a critical step for performing large-scale PK trials in rural settings, as population PK studies, and allowing future PK investigations in small children, where the amount of blood to be sampled is strictly limited.

Investigating on one hand the gold standard PZQ and on the other hand two lead candidates, offers an excellent occasion to stress out the importance of gaining knowledge on existing drugs while promoting the advent of new drugs. In the same direction, as much as human and veterinary medicine are independent disciplines, drug development actively profits from investigation in either field to find new drug candidates. Even with the recent progress in genomics and proteomics, the biology of helminths is still poorly understood, which makes the endeavour of finding new targets extremely difficult and time-consuming. This is urgent matter, as resistance risks are increasing with time and only very few drug candidates are being developed to provide alternatives to existing drugs. The burden of the neglected tropical diseases is extremely high, especially among children, hence jeopardizing efforts towards future social and economic progresses. The growing awareness of the public and private sector towards neglected tropical diseases however gives some hope to see trematode infections step backwards.