Objectives: The pharmacokinetic (PK) parameters of artesunate, recently discovered to possess promising trematocidal activity, and its main metabolite dihydroartemisinin (DHA) were determined in rats infected with hepatic and biliary stages of Fasciola hepatica and compared with uninfected rats after single intragastric and intravenous (iv) doses.

Methods: Rats infected with F. hepatica for 25 and 83 days and uninfected rats were cannulated in the right jugular vein and blood samples were withdrawn at selected timepoints following 10 mg/kg of iv and a single 100 mg/kg oral dose of artesunate. Plasma was analysed for artesunate and DHA by liquid chromatography coupled to tandem mass spectrometry.

Results: Rats harbouring juvenile and adult F. hepatica infections revealed considerable changes in PK parameters of artesunate and DHA. Following oral administration, maximum plasma concentrations (Cmax) of artesunate and DHA were 1.8–2.3-fold higher in infected rats [artesunate: 1334±1404 ng/mL (no infection) versus 2454±1494 ng/mL (acute infection) and 2768±538 ng/mL (chronic infection); DHA: 3802±2149 ng/mL (no infection) versus 6507±3283 ng/mL (acute infection) and 9093±884 ng/mL (chronic infection)]. The AUCs of artesunate and DHA were 2.1–4.4-fold greater in infected rats. An opposite trend was observed after iv injection. Cmax and AUC of artesunate and DHA following iv dosing were 5784±3718 and 140938±128783 ng.min/mL and 3849±3060 and 86107±41863 ng.min/mL, respectively, in uninfected rats versus 2623±1554 and 21617±12230 ng.min/mL and 2835±980 and 64290±29057 ng.min/mL, respectively, in rats harbouring a chronic infection. The elimination half-lives (t1/2) of artesunate and DHA were considerably altered in infected rats following oral and iv administration of artesunate.

Conclusions: F. hepatica infections strongly influence the disposition kinetics of artesunate and its metabolite in rats. The clinical implications of this finding need to be carefully studied.

Keywords: food-borne trematodiasis, artemisinins, LC-MS/MS

Introduction

Fascioliasis is an infection caused by the liver flukes Fasciola hepatica and Fasciola gigantica. Fascioliasis is a zoonotic disease of global distribution, which is of considerable public health significance and veterinary importance. It is estimated that 91 million people are at risk and between 2.4 and 17 million people are infected with F. hepatica and/or F. gigantica. Approximately 300 million cattle and 250 million sheep are infected with liver flukes. Economic losses in livestock associated with fascioliasis are estimated to be $2–3 billion annually.1,2

In the absence of vaccines, triclabendazole is the main drug used to treat liver fluke infections as it is highly effective against juvenile and adult Fasciola spp.3 However, resistance to the drug in fluke populations first developed in Australian livestock in the mid-1990s and has since been reported from several European countries. No confirmed cases of triclabendazole resistance in human Fasciola infections have been documented yet, but this may happen soon, given the spread of resistance in
livestock Fasciola populations.\textsuperscript{1,3} Hence, there is a pressing need to develop novel trematocidal drugs.

The artemisinins, well known for their antimalarial\textsuperscript{4} and anti-schistosomal\textsuperscript{5} properties, are also active against Fasciola spp.\textsuperscript{6} Complete worm burden reductions were achieved in rats infected with adult F. hepatica after treatment with artesunate and artemether at single oral doses of 400 and 200 mg/kg, respectively. Administration of artesunate and artemether at a 200 mg/kg dose to rats harbouring juvenile F. hepatica resulted in worm burden reductions of 46\% and 82\%, respectively.\textsuperscript{7} Artemether has also shown promising fascicidal properties in sheep.\textsuperscript{7} In addition, a recent study in Vietnam has shown that artesunate might also play a role in the treatment of acute human fascioliasis.\textsuperscript{8}

Infections with Fasciola spp. are well known to dramatically impair the hepatic function, including the metabolism and pharmacokinetic (PK) parameters of drugs.\textsuperscript{9,10} Hence, with a possibly increasing role of the artemisinins in the treatment of fascioliasis, it is important to determine the PK parameters of these drugs during the course of Fasciola infections.

Here, we compare the PK characteristics of artesunate and its main metabolite dihydroartemisinin (DHA) (Figure 1) in rats infected with juvenile and adult F. hepatica and in uninfected rats. Plasma drug levels were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). We furthermore evaluated two different routes of drug administration, an oral and an intravenous (iv) drug administration.

\textbf{Materials and methods}

\textit{Animal studies}

\textit{Drug.} Artesunate was obtained from Mepha AG (Aesch, Switzerland). For the oral administration (100 mg/kg), artesunate was prepared in a homogeneous suspension in 7\% (v/v) Tween-80 and 3\% (v/v) ethanol. The iv preparation (10 mg/kg) was prepared with 0.9\% (v/v) sterile NaCl. The drug formulations were prepared shortly before administration.

\textit{Ethical clearance, animals, infection, study design and PK sampling.} All animal studies presented here were approved by the local government based on Swiss national regulations (permission no. 2070).

Female Wistar rats (n=18; age, 5 weeks; weight, ~100 g) were purchased from RCC (Itingen, Switzerland). Rats were kept in groups of five in macrolon cages in environmentally controlled conditions (temperature, ~25°C; humidity, ~70\%; 12 h light and 12 h dark cycle) and acclimatized for 1 week. They had free access to water and rodent food (Rodent Blox from Eberle NAFAG; Gossau, Switzerland). Three groups were formed. Rats of groups 1 and 2 (n=12) were each infected intra-gastrically with 20 metacercarial cysts of F. hepatica. F. hepatica metacercariae (Cullompton isolate) were purchased from Mr G. Graham (Addlestone, UK). Six rats remained uninfected (group 3).

Twenty-five (group 1) and 83 (group 2) days post-infection with F. hepatica, six rats each and also the six uninfected rats (group 3) were cannulated in the right jugular vein following standard procedures. The animals were allowed to recover for 3 days. Three days after implantation of the catheter, rats in each group were further divided into two subgroups of three rats each—receiving either oral or iv artesunate. The iv doses were injected for 60 s via the jugular vein cannula, immediately followed by injection of 1 mL of saline solution.

Blood samples (0.2 mL) were withdrawn from the cannulated jugular vein into heparinized tubes at 5, 15, 30, 45, 60, 90, 120, 240 and 300 min following the iv dose. After oral treatment, blood samples were taken at 15, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min. The blood volume was replaced with an equal volume of 0.9\% saline solution. The blood samples were centrifuged and the plasma was taken and stored at ~80°C until analysed by LC-MS/MS. At the end of the experiments, all rats were sacrificed by the CO\textsubscript{2} method and the parasitic pathology was ascertained by examination of the rat livers.

\textbf{LC-MS/MS analysis of artesunate and DHA}

\textit{Solvents and chemicals.} DHA and artesunate were kind gifts from Abbott (Baar, Switzerland). The internal standard (IS) artemisinin (Figure 1) was obtained from Sigma-Aldrich (Schnelldorf, Germany). Ultrapure water was obtained from a Milli-Q\textsuperscript{®} UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate was purchased from Fluka (Buchs, Switzerland). Sodium nitrite, acetic acid (glacial) 100\% anhydrous, formic acid (98\%), methanol (MeOH) and acetonitrile (MeCN) for chromatography were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

\textit{Instrumentation.} The liquid chromatography system consisted of a Rheos 2200 quaternary pump, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and controlled by Janeiro-CNS software. Separations were done on a 2.1 mm×50 mm Atlantis\textsuperscript{®} dC18 3 µm analytical column (Waters, Milford, MA, USA) equipped with a 2.1 mm×10 mm guard column containing the same packing material and placed in a thermostated column heater at 25°C (model

![](attachment:Chemical_structures_of_artersusate_DHA_and_artemisinin(IS).pdf)
PK parameters of artesunate and DHA in rats infected with *F. hepatica*

Croco-Cil, Cluzeau, France). The chromatographic system was coupled to a triple-stage quadrupole Quantum Ion Max mass spectrometer (MS) from Thermo Scientific, Inc. (San Jose, CA, USA), equipped with an electrospray ionization (ESI) interface and operated with Xcalibur 2.0 software (Thermo). The mobile phase used for chromatography was 20 mM ammonium formate in ultrapure water adjusted to pH 4.1 with formic acid (98%) (solution A) and acetonitrile (solution B). The mobile phase was delivered at a flow rate of 0.3 mL/min using the following stepwise gradient elution programme: 0 min, 75/25% of A/B; 4 min, 55/45%; 10.0 min, 40/60%; and 12 min, 100% of B. The second part of the run included 4 min of intensive washing (100% B with a flow rate of 0.5 mL/min) and re-equilibration step to the initial solvent up to 20 min (at 16.01 with 0.5 mL/min and after 19 min with a flow rate of 0.3 mL/min). The injection volume was 10 μL.

The tuning of MS/MS parameters was performed by direct infusion of each compound solution in the ESI separately: DHA, artesunate and artemisinin (1 μg/mL in 50:50 of solution A buffer/MeOH). The LC-MS/MS conditions were as follows: ESI in positive mode; capillary temperature, 350°C; in source collision-induced dissociation, 10 V; tube lens range voltage, 41–56 V; spray voltage 4 kV; and sheath and auxiliary gas (nitrogen) flow rate of 35 psi and 10 arbitrary units, respectively. The selected \\textit{m/z} transitions were 221.1→163.1 with a collision energy (CE) of 24 eV for DHA and artesunate and 283.1→247.2 with a CE of 20 eV for artemisinin (IS). Because of the specificity of product ions, no cross-talk was observed between MS transitions. The Q2 collision gas (argon) pressure was 1 mtorr (0.13 Pa).

The MS/MS acquired in selected reaction monitoring. MS acquisitions were done in centroid mode and the mass resolution was set at full-width half-maximum equal to 0.7, corresponding to ≤1 amu mass resolution.

**Calibration and quality control (QC) plasma samples.** Standard stock solutions of DHA and artesunate were prepared in methanol. The stock solutions were protected from light and stored at −20°C. Appropriate quantities of stock solutions were diluted with H₂O to obtain working solutions of 40 µg/mL DHA and artesunate in MeOH/H₂O (1:1). Plasma calibration samples of 1, 5, 10, 100, 1500 ng/mL DHA and artesunate, and three plasma QC samples of 15, 150 and 1500 ng/mL were prepared by serial dilution (with MeOH/H₂O, 1:3) of working solution and diluted 1:20 with blank plasma from rat, in accordance with the recommendations of bioanalytical method validation stating that the total added volume must be ≤10% of the biological sample volume. Blank rat plasma pool for the preparation of matrix-matched calibration and control samples was collected by cardiac puncture of rats. Rat plasma samples were stored at −80°C until analysis.

A 100 µg/mL stock solution for the IS (artemisinin) was also prepared in MeOH and kept at −20°C, and was diluted to 2000 ng/mL with MeOH on the day of analysis. The calibration curves were fitted by least-squares quadratic regression using 1/concentration (1/×) as weighting factor of the peak-area ratio of DHA and artesunate to IS versus the respective DHA and artesunate concentration in each standard samples. During the analysis, each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Control samples at three concentrations levels (low, medium and high: i.e. 15, 150 and 1500 ng/mL) were assayed throughout rat sample analysis. Samples with levels exceeding 2000 ng/mL (as found in the early samples just after artesunate administration) were appropriately diluted with blank rat plasma to yield concentration comprised within the calibration range, prior to their extraction.

**Sample preparation.** A rat plasma sample aliquot (100 µL) onto which 30 µL of 3 M sodium nitrite containing 1% acetic acid (pH≈4.3) was added was placed in a water bath at 37°C for 30 min. Addition of sodium nitrite was found to successfully prevent the apparent degradation of artesunate and DHA in haemolysed samples. The beneficial effect of sodium nitrite on DHA and artesunate recovery had been verified using plasma samples spiked with DHA, artesunate and artemisinin onto which 0.5%, 1% or 2% (v/v) of haemolysed red blood cells was added. Conversely, sodium nitrite was found not to alter DHA and artesunate levels in non-haemolysed plasma levels. A 100 µL volume of IS solution (2000 ng/mL) was then added and the resulting sample was subjected to protein precipitation with acetonitrile (600 µL). Sample was vortex-mixed and centrifuged for 10 min at 14000 rpm (16820 g) at 4°C (Benchtop Universal 16R centrifuge, Bäch, Switzerland). The supernatant (800 µL) was transferred into a polypropylene tube and evaporated to dryness under nitrogen at room temperature. The solid residue was reconstituted in 130 µL of MeOH, vortex-mixed and centrifuged again under the above-mentioned conditions. A 100 µL aliquot of supernatant was mixed with 100 µL of buffer (solution A) to give the reconstituted extract placed in glass HPLC micro-vials kept at 4°C in the autosampler rack during the LC-MS/MS analysis.

**PK analysis**

PK parameters for artesunate and DHA were determined by non-compartmental analysis using WinNonLin (Version 5.2, Pharsight Corporation, USA). \(C_{\text{max}}\) and \(T_{\text{max}}\) were the observed values. The AUC (from 0 to infinity) was estimated using the linear trapezoidal rule. The elimination half-life (\(t_{1/2}\)) was calculated by the equation: \(t_{1/2} = 0.693/\lambda\), where \(\lambda\) was estimated by performing a regression of the natural logarithm of the concentration values in this range on sampling time. Clearance (CL) was calculated as dose/AUC. The steady state volume of distribution (\(V_{ss}\)) was determined by the following equation: \(V_{ss} = MRT_{\text{INF}} \times CL\), where \(MRT_{\text{INF}}\) = mean residence time when the drug concentration profile is extrapolated to infinity and CL = total body clearance. Absolute bioavailability (\(F\)) for non-infected, acute infections and chronic infections of artesunate was calculated as mean \(\text{AUC}_{\text{oral}} \times \text{dose}_{\text{oral}} / (\text{AUC}_{\text{p.o}} \times \text{dose}_{\text{oral}})\), where iv stands for intravenous.

The above indices were determined for each individual animal and their arithmetic mean (±SD) was calculated.

**Statistical analysis**

PK parameters between infected and uninfected rats were compared using the Kruskal–Wallis test (KW). A \(P\) value <0.05 was considered statistically significant. Version 2.4.5 of Statsdirect statistical software (Statsdirect Ltd, Cheshire, UK) was used for the statistical analysis.

**Results**

**Performance of the LC-MS/MS assay**

The lower limit of quantification was 1 ng/mL. The inter-assay precision obtained with the rat plasma QC samples of 15, 150 and 1500 ng/mL DHA and artesunate were 12.2%, 7.4% and 11.9% for DHA and 14.1%, 7.0% and 11.8% for artesunate. Mean absolute deviation from nominal values of QC samples (15, 150 and 1500 ng/mL) during the series of analysis (\(n=15\))
PK parameters of artesunate and DHA following a single oral dose of 100 mg/kg (mean ± SD).

**Table 1.** Artesunate and DHA parameters in control rats and rats infected with *F. hepatica* (acute and chronic infection) after iv and oral administration

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Artesunate</th>
<th>Dihydroartemisinin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cmax (ng/mL)</strong></td>
<td><strong>Tmax (min)</strong></td>
<td><strong>t1/2 (min)</strong></td>
</tr>
<tr>
<td>iv, no infection</td>
<td>100 mg/kg</td>
<td>1334.0 (1404.0)</td>
</tr>
<tr>
<td>iv, acute infection</td>
<td>100 mg/kg</td>
<td>2453.6 (1493.5)</td>
</tr>
<tr>
<td>iv, chronic infection</td>
<td>100 mg/kg</td>
<td>2767.6 (537.8)</td>
</tr>
<tr>
<td>iv, no infection</td>
<td>10 mg/kg</td>
<td>5783.8 (3718.0)</td>
</tr>
<tr>
<td>iv, acute infection</td>
<td>10 mg/kg</td>
<td>1652.2 (642.7)</td>
</tr>
<tr>
<td>iv, chronic infection</td>
<td>10 mg/kg</td>
<td>2623.1 (1553.9)</td>
</tr>
</tbody>
</table>

Data are presented as arithmetic mean (±SD).

**a** Plasma concentrations at *t = 15* could not be measured for one rat; hence, this value might be overestimated.

**b** Significantly different from control.

The t1/2 of artesunate was significantly shorter (38.4 versus 10 000 min) following iv administration in rats infected with *F. hepatica* compared to uninfected rats.

Effective concentrations of artesunate and DHA were significantly increased in rats infected with *F. hepatica* compared to those infected with *F. hepatica* (acute infection).

In rats infected with *F. hepatica* following iv administration, the effective concentrations of artesunate and DHA were significantly increased compared to those infected with *F. hepatica* (acute infection).

**Figure 2.** Arithmetic mean plasma concentration–time profiles of artesunate following a single oral dose of 100 mg/kg artesunate. Error bars represent SD.

**Figure 3.** Arithmetic mean plasma concentration–time profiles of DHA following a single oral dose of 100 mg/kg artesunate. Error bars represent SD.

The 95% confidence interval (9.8% to 11.2%) for the effective concentrations of artesunate and DHA at the 100 mg/kg dose was 8.7% to 9.9%.
PK parameters of artesunate and DHA in rats infected with *F. hepatica*

68.3 min) (KW = 3.857, P = 0.049), while \( t_{1/2} \) of DHA was slightly increased in rats with an acute infection (61.7 versus 53.7 min).

Rats that had been infected with *F. hepatica* for 86 days (chronic, biliary infection) had a more than 2-fold increased \( C_{\text{max}} \) of artesunate and DHA, which was statistically significant for DHA [3802 ± 2149 ng/mL (control) versus 9093 ± 884 ng/mL (infected); KW = 3.857, P = 0.049]. The AUCs of the parent drug [81065 ± 5629 ng-min/mL (infected rats) versus 32439 ± 29808 ng-min/mL (control rats)] and the metabolite [691102 ± 336175 ng-min/mL (infected rats) versus 155592 ± 66921 ng-min/mL (control rats)] were significantly elevated (KW = 3.857, P = 0.049). Similar to the rats with an acute infection, \( t_{1/2} \) of artesunate was significantly shorter (42.6 versus 68.3 min) (KW = 3.857, P = 0.049), while \( t_{1/2} \) of DHA was increased in these rats (74.6 versus 53.7 min).

The ratio of AUC\(_{\text{DHA}}\) to AUC\(_{\text{artesunate}}\) had a range of 4.7–8.5, the highest value determined in chronic *F. hepatica* infections. In infected rats, the oral bioavailability (\( F \)) of artesunate was considerably greater than the controls (49.7% and 37.5% versus 2.3%).

**Intravenous administration.** Following iv administration of 10 mg/kg artesunate to control and infected rats, the mean plasma concentration–time profiles of artesunate and DHA are shown in Figures 4 and 5. The PK parameters are summarized in Table 1.

Rats harbouring an acute *F. hepatica* infection showed the following not significant changes in mean PK parameters of artesunate and DHA when compared with control rats. \( C_{\text{max}} \) values were lower [artesunate: 1652 ± 643 ng/mL (infected) versus 5784 ± 3718 ng/mL (control); and DHA: 2860 ± 1269 ng/mL (infected) versus 3849 ± 3060 ng/mL (control)]. The AUCs of artesunate [13665 ± 5573 ng-min/mL (infected) versus 140938 ± 128783 ng-min/mL (control)] and DHA [52984 ± 14888 ng-min/mL (infected) versus 86107 ± 41863 ng-min/mL (control)] were smaller, translating into higher CL. The \( t_{1/2} \) of artesunate and DHA were similar in acute infection and control rats [38.6 (infected) versus 34.5 min (control) and 32.3 versus 31.6 min for artesunate and DHA, respectively]. In addition, there was no significant difference in the \( V_{\text{ss}} \) of DHA and artesunate in rats with an acute infection and uninfected rats.

**In chronically *F. hepatica*-infected rats, the terminal half-life of artesunate was increased (75.6 min compared with 34.5 min in control rats), while \( t_{1/2} \) of DHA was only slightly longer (43.5 min versus 31.6 min). Maximum plasma concentrations of artesunate and DHA were decreased [artesunate: 2623 ± 1554 ng/mL (infected) versus 5784 ± 3718 ng/mL (control); and DHA: 2835 ± 980 ng/mL (infected) versus 3849 ± 3060 ng/mL (control)]. Finally, the AUCs of the parent compound [21617 ± 12230 ng-min/mL (infected) versus 140938 ± 128783 ng-min/mL (control)] and the metabolite [64290 ± 29057 ng-min/mL (infected) versus 86107 ± 41863 ng-min/mL (control)] were considerably, but not significantly, smaller translating into higher CL. No significant differences were observed in the \( V_{\text{ss}} \) comparing chronically infected rats and rats without infection.

There was evidence of a secondary artesunate peak in uninfected rats 3 h after artesunate administration, as shown in Figure 4, which points to an enterohepatic circulation.

The ratio of AUC\(_{\text{DHA}}\) to AUC\(_{\text{artesunate}}\) was 0.6 for the control rats and 3.8 and 2.9 for acute and chronic *F. hepatica* infections of rats, respectively.

**Discussion**

Though several studies have analysed hepatic functions during the course of *F. hepatica* infections in rats,\(^{12}\) to our knowledge, this is the first PK study in *F. hepatica*-infected rats. Evaluation of pharmacological and PK properties at early stages of drug discovery is crucial as it can accelerate the conversion of hits and leads into candidates for further development. Promising fasciocidal properties of the artemisinins and the synthetic peroxide (trioxolane) OZ78 have been recently reported in rodent models,\(^{6,13}\) sheep\(^{7}\) and even humans;\(^{6}\) hence, further preclinical and clinical investigations are warranted.

We have chosen to evaluate in a first step the PK properties of artesunate, as we were surprised that in our recent study this drug was not tolerated by rats harbouring adult *F. hepatica*, at single oral doses of 200 mg/kg and above.\(^{6}\) For comparison, in uninfected rats, the LD\(50\) value of artesunate was reported to be 351 mg/kg following a single iv administration of the drug.\(^{14}\)
PK data for artesunate have been reported in adults and children with uncomplicated and severe malaria, and in healthy volunteers. It was found that malaria, infection had a significant effect on the PK of the artemisinins. For example, peak plasma concentrations of DHA and the relative oral bioavailability of DHA were higher in malaria patients. The PK properties of artesunate have also been well studied in healthy rats.

In our artesunate PK study in *F. hepatica*-infected rats, two study timepoints were selected during the course of the *Fasciola hepatica* infection, one during the acute phase of infection (day 28), when flukes migrate through and hence cause great damage to the liver parenchyma (hepatic stage), and one during the chronic stage (day 86), when adult flukes have moved to the central bile duct and the liver tissue is regenerating (biliary stage). It was demonstrated that cytochrome P450 activities decrease in the acute phase of *F. hepatica* infection in rats and return to normal values by week 9 of infection. In our study, the parasitic pathology was ascertained by autopsy, but it might be useful in future studies to also integrate basic liver function tests and an assessment of cytochrome P450 activities with PK studies to be able to correlate the degree of parasite-induced metabolism impairment with the changes observed in PK parameters.

We found both in rats suffering from acute and chronic *F. hepatica* infections considerable changes in all PK parameters of artesunate and DHA. Following oral administration, artesunate is rapidly hydrolysed to DHA in the stomach, as well as by blood esterases and the hepatic cytochrome CYP3A4. DHA is cleared predominantly by hepatic biotransformation to glucuronides and metabolites, lacking the peroxide bridge. The considerably higher *C*<sub>max</sub> and AUC levels of artesunate and DHA we observed in infected rats following oral artesunate might be due to an impaired metabolism and elimination of DHA due to hepatic damages related to *F. hepatica* pathology. High and toxic levels of DHA might explain the high mortality rate in infected rats observed in our previous experiments.

An opposite trend was observed after iv injection (considerably lower *C*<sub>max</sub> and AUC of artesunate in infected rats, when compared with non-infected rats). The differences in *C*<sub>max</sub> levels might be explained with an outlier datapoint in one of the rats sampled; hence, follow-up studies are necessary to confirm this finding. Lower AUC levels of artesunate following iv administration in infected rats might be due to an impaired enterohepatic circulation in infected rats. Extensive enterohepatic circulation was demonstrated for artesunate following iv administration in healthy rats. In our study, a second plasma peak of artesunate was observed in uninfected rats also pointing to enterohepatic circulation of this drug. To confirm whether enterohepatic circulation is impaired in infected rats, plasma exposure levels should be studied in infected and uninfected bile duct cannulated rats. The lower *C*<sub>max</sub> and AUC of DHA might be attributed to the damage of CYP3A4, which has been described to metabolize artesunate into DHA.

Finally, interestingly we observed a very low oral bioavailability of artesunate in uninfected rats (2.5%), which is lower than that in two previous studies, which reported mean oral bioavailability of 27% to 29.5% for artesunate. The oral bioavailability of artesunate was higher in infected rats, namely 37.5 and 49.7%. However, these results need to be interpreted with care, as we used a Tween/alcohol suspension for the oral drug administration, which might have influenced the solubility and hence biodisposition of artesunate.

While most PK studies on the artemisinins have used HPLC using post-column alkali decomposition or electrochemical detection, we developed a sensitive and selective LC-tandem MS assay procedure for the specific and quantitative analysis of artesunate and DHA in plasma. Similar to a recently established LC/MS method for artesunate and DHA in plasma, our method is reproducible and accurate and has a detection limit of 1 ng/mL for artesunate and DHA.

Since several of our samples were haemolysed and first attempts of the analysis of these samples failed (only very low, if any, DHA or artesunate levels could be detected), a possible alteration of artesunate and DHA levels by haemoglobin (Hb) during the extraction procedure was hypothesized. Since DHA is reported to react with ferrous (Fe<sup>2+</sup>)—but not ferric (Fe<sup>3+</sup>)—haeme from Hb, sodium nitrite, a known methaemoglobin-forming agent, was added to all haemolysed plasma samples. Addition of sodium nitrite to haemolysed samples was able to successfully prevent most of DHA or artesunate degradation and, importantly, proportionally to artemisinin added as IS. Thus, the DHA/IS and artesunate/IS ratios in haemolysed samples in the presence of sodium nitrite were similar to those determined in non-haemolysed samples.

In conclusion, our study has shown that *F. hepatica* infections strongly influence the disposition kinetics of artesunate and its metabolite in rats. The clinical significance of this finding is unclear and should be studied in fascioliasis, schistosomiasis and other patients suffering from liver diseases that are treated with artemisinins, since alterations in plasma concentrations may lead to toxic adverse events or sub-curative drug levels causing treatment failures. Drug levels of the artemisinins should also be monitored in patients suffering from malaria and concurrent liver diseases or elderly patients. Neutropenia, anaemia, haemolysis, elevated levels of liver enzymes, neurotoxicity and embryotoxic effects have been described following artesunate treatment in healthy volunteers and malaria patients, and there is concern that the severity of these adverse events might increase in patients suffering from liver diseases. In addition, PK parameters of novel fascicidal agents should be studied in the presence of hepatic impairment at early phases of drug development to allow identifying changes in drug disposition, which might lead to a decrease in therapeutic efficacy or drug-induced toxicity. Further studies on the PK properties of artemether and the synthetic trioxolane OZ78 in *F. hepatica*-infected rodents and sheep are ongoing in our laboratories. These studies will assist in the selection of an ideal peroxidic trematocidal drug development candidate.

**Acknowledgements**

We thank Dr Franz Schuler, Hoffmann-La Roche, for his support with the rat catheterization.

**Funding**

J. K. (Project No. PPOOA-114941) and L. D. (REQUIP Grant No. 326000-121314/1) are grateful to the Swiss National Science Foundation for financial support.
PK parameters of artesunate and DHA in rats infected with *F. hepatica*

Transparency declarations

None to declare.

References


