# Artemisinins for the Treatment of Fascioliasis: Progress in Preclinical and Diagnostic Research

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

**Urs Philipp Duthaler**

aus Basel und Hittnau (ZH)

Basel, 2012

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. Jennifer Keiser
Prof. Dr. Jörg Huwyler
Prof. Dr. Paul Torgerson

Basel, den 21. Juni 2011

Prof. Dr. Martin Spiess
Dekan

# Table of Contents

# Acknowledgements

This PhD thesis was carried out from February 2008 to June 2011 at the Swiss Tropical and Public Health Institute in collaboration with several scientific research Institutes. At this point, I would like to express my sincere thanks to those people who have helped me with their continuous support and invaluable contributions during all these years.

First and foremost, I owe my deepest gratitude to my supervisor Jennifer Keiser for giving me the chance to carry out this PhD thesis, for introducing me to the fascinating wormy world, for guiding me through these years with enthusiasm and great scientific and personal support and for taking time whenever I needed help.

I would like to express my sincere thanks to Jörg Huwyler for serving as faculty representative, for the constant encouragement, and for taking so much time for scientific discussions and the countless LC-MS/MS troubleshooting lessons. The joint handicraft works will be unforgettable.

I am very grateful to Prof. Dr. Paul Torgerson for joining my PhD committee as a co-referee.

A special thank goes to Jürg Utzinger amongst others for the precious inputs concerning copromicroscopy, his substantial advices, catching enthusiasm, and for polishing the FLOTAC manuscript.

I would like to express my sincerest gratitude to all members of the helminth drug development unit. It was a pleasure working with all of you in the laboratory and watching how this unit was growing and developing over the past years. Therefore, huge thanks go to Mireille for supporting me so much and for keeping our laboratory in fashion. A tremendous hug and honest thanks to all my helminth PhD-fellows: Theresia, Carla, Lucienne, Katrin, and Benjamin. My deepest thanks go to Gordana for revising parts of my thesis. Special thanks to all former and current master students: Yolanda, Uzoma, Angelika, and Monika. Cheers.

I am also deeply grateful to Prof. Dr. Giuseppe Cringoli and his entire group from the University of Naples Federico II in Italy for their kind collaboration. Your hospitality was outstanding. A special thank goes particularly to Laura Rinaldi and Maria P. Maurelli for helping me with FLOTAC analysis. Furthermore, I like to thank Laura Mezzino and Vincenzo Veneziano for their excellent technical assistance with the treatments and plasma sampling for the pharmacokinetic studies in sheep.

My sincerest thanks are addressed to Massimilliano Donzelli, Peter Wegmann, Dr. Sabine Meyer, Dr. David Blaser, Dr. Manuel Haschke and Dr. Manfred Zell for their support and helpful suggestions concerning analytical and pharmacokinetic questions.

Furthermore, I owe special gratitude to Prof. Dr. Georgios Imanidis for his scientific advice, for the stimulating discussions and especially for allowing me to carry out part of my thesis in his laboratories at the University of Applied Sciences, Northwestern Switzerland. My sincerest thanks are addressed to all the Rosenthal lab mates: Michael Lanz, Martin Cavegn, Yvonne Arnold, Nalluri Venkateshwar Rao, Constantinos Markopoulos, Elizaveta Fasler, Berndt Joost, Martin Kuentz, Ursula Thormann, Cordula Stillhart, Daniel Preisig, and Martin Studer. I really enjoyed the amusing chats during lunch and coffee time.

I am deeply grateful to Thomas Smith and Maria Laura Gosoniu for their statistical support.

I would like to express my fond appreciation to all the members of Jörg Huwyler's pharmaceutical technology division of the University of Basel. A special thank goes to Pascal Detampel, Rainer Alles, Christina Erb, André Ziegler, Maxim Puchkov, Susanne Schenk, Claudia Suenderhauf, Felix Hammann, Le-Ha Dieu, Swen Seeland, Stefan Jenzer and Stefan Winzap.

My sincerest thanks are addressed to Gregori Morandi, Brendan Prideaux, and Dr. Markus Stöckli from Novartis Pharma for introducing me to the amazing world of imaging mass spectrometry.

I wish to thank the Swiss National Science Foundation for the financial support of my project (project number: PPOOA-114941).

Furthermore, I am indebted to the library team who managed to find all the publications I ordered and the ITs for their constructive support.

I am very thankful to Dafra Pharma, Mepha Pharma, Novartis Pharma and Kunming Pharmaceutical Cooperation for their kind supply of various drugs and metabolites.

My warmest appreciation goes to Karin Gysin and Pascale Steiger for taking so much care of the laboratory animals and giving me numerous helpful suggestions.

Furthermore, I would like to acknowledge Marco Tamborini for his fruitful advice and Theresa Ruf for giving me an insight into histology.

It is a pleasure to thank those numerous people, who sweetened lunch, coffee, and tea time at the SwissTPH and provided me pleasant and unforgettable moments:

Ralf, Phippu, Scheuri, Chrigu, KW, Thomas, Dania, Pax, Igor, Sebi, Esther, Till, Sonja, Fügi, Claudia, Anna, Eva-Maria, Maria, Cristian, Aurelio, Olivier, Marco, Miriam, Charlotte, Theresa, Katharina, Anita, Lukas, Dominique, Mike, Simon, Monica, Tanja, Christoph, Eva, Céline, Jolanda, Mugasa, Dominik, Sonja, Caroline, Annette, Petros, Matze, Sergio, Marcel, Yvette, Nicolas, Sophie, Sonia, Aurélie, Mirko, Mireie, David, Stefanie, Toni, Paul, Dirk and Fabien.

Finally, I am deeply grateful to my family and to all my brothers from other mothers for encouraging and supporting me during these years. Thank you all.

# Summary

The liver flukes*, Fasciola hepatica* and *gigantica* are the causative agents of fascioliasis (fasciolosis). This parasitic disease is distributed throughout the world and exhibits a broad zoonotic reservoir, with farm animals such as sheep and cattle being the most important natural end hosts from an economic point of view. Since the 1990s fascioliasis is recognized as an increasing global public health problem with an estimated number of 2.4 to 17 million individuals infected worldwide. Novel treatment options are needed, since triclabendazole is the sole drug recommended for human use and chemotherapy failure due to drug resistance is observed in livestock.

Peroxidic compounds including the semisynthetic artemisinins, artesunate and artemether, as well as the artemisinin-like synthetic 1,2,4-trioxolane OZ78 exhibit promising *in vitro* and *in vivo* fasciocidal activity against juvenile and adult flukes. Moreover, it has been shown that these peroxides were active against a triclabendazole resistant *F. hepatica* strain.

In the framework of this PhD thesis preclinical investigations were carried out to further strengthen our knowledge on the potential of the semisynthetic artemisinins, artesunate and artemether, for the treatment of *F. hepatica* infections. Moreover, the FLOTAC technique, a novel copromicroscopic technique, was evaluated for the detection and quantification of *F. hepatica* eggs in faecal samples.

A sensitive copromicroscopic technique was required to examine *F. hepatica* infections intensities in experimentally infected rats or naturally infected sheep and to estimate the egg burden reduction after treatment in chemotherapy studies. Therefore, we compared the sedimentation technique, which is the reference copromicroscopic method, with the FLOTAC techniques for the detection and quantification of *F. hepatica* eggs in faecal samples obtained from experimentally infected rats. In low infection intensities, the sedimentation technique needed a rigorous reading effort with 8 slides examined to achieve a comparable sensitivity as a single FLOTAC (85.2% vs. 92.6%). Sensitivity was not an issue for both techniques analysing high infection intensities. Overall, the sedimentation technique is more uniform and easier to handle than FLOTAC, but the high reading effort needed to obtain a good sensitivity made it less time efficient.

For this reason FLOTAC and not the sedimentation technique was chosen for future copromicroscopic measurements carried out in our laboratories.

The FLOTAC technique was successfully applied to identify sheep naturally infected with *F. hepatica*, to quantify their egg loads for allocating the animals in balanced treatment groups, and for evaluating treatment outcome on egg excretion. Sheep naturally infected with *F. hepatica* were treated with single doses of either artesunate or artemether using different routes of applications. Both drugs showed good activities against *F. hepatica* following intramuscular treatments, whereas artesunate required approximately a fourth of the artemether dose to obtain similar good activity. Oral treatments lacked activity in sheep. To strengthen the efficacy data, pharmacokinetic analyses of artemether and artesunate following different routes of application and doses were performed.

A liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated in terms of accuracy, precision and selectivity for the simultaneous quantification of artesunate, artemether and their metabolites dihydroartemisinin (DHA) and dihydroartemisinin-glucuronide (DHA-glucuronide) in sheep plasma for the later application to pharmacokinetic studies. The latter metabolite was identified in a preliminary analysis and was included in the pharmacokinetic study, since major plasma levels were observed. Plasma sample workup was performed using a simple protein precipitation method resulting in relative recoveries of 60-80%. All analytes were detected in the positive mode using selected reaction monitoring with a transition of *m/z* 267.4→163.0. The developed method demonstrated to be accurate, precise, and selective and achieved a lower limit of quantification of approximately 10 ng/ml for artesunate, DHA, and DHA-glucuronide and of 90 ng/ml for artemether.

The pharmacokinetic profiles of artesunate following intramuscular treatments were characterised by rapid drug invasion with $T_{max}$ of 15 min observed for artesunate and high $C_{max}$ and AUCs observed for all analytes. Moreover, drug elimination occurred fast with estimated $t_{1/2}$ of 58-63 min, 94-113 min, and 89-98 min for artesunate, DHA and DHA-glucuronide. On the other hand, a slow liberation of artemether from the oil-based drug formulation with more or less constant levels of artemether and its metabolites during the entire sampling period of 24 h was observed following intramuscular application. In consequence, low $C_{max}$ of all analytes were detected following artemether

im treatments compared to artesunate. However, the calculated AUCs of effective artemether treatments (160 mg/kg) were comparable with those of the effective artesunate applications (40 and 60 mg/kg). Moreover, the estimated AUCs were significantly higher for the effective im artemether dose compared to the ineffective im 40 and oral 80 mg/kg artemether doses. *In vitro* drug assays revealed that DHA reduced significantly the viability of *F. hepatica* flukes, whereas DHA-glucuronide exhibited no activity and might not contribute to the drug activity.

To further improve the efficacy and safety of peroxidic drug treatments, we investigated the effect of peroxide-triclabendazole combinations against adult and juvenile *F. hepatica* harboured in rats. Negative binomial regressions of worm and egg counts were used to analyse dose-response relationships of mono- and combination chemotherapy. $ED_{50}$ values of 113.0, 77.7, 22.9, and 2.7 mg/kg of body weight were calculated for monotherapy with artesunate, artemether, OZ78, and triclabendazole, respectively, against adult *F. hepatica*. Encouraging results were achieved using peroxides-triclabendazole combinations, since enhanced drug effects compared to monotherapy were observed. However, in the case of the artemisinins the observed treatment effect depended on the applied triclabendazole dose. Juvenile *F. hepatica* flukes were less sensitive to mono- and combination chemotherapy *in vivo* and *in vitro* compared to adult flukes. Finally, our chemotherapy studies highlighted a substantial loss of precision if egg instead of worm counts were used to estimate dose-response relationships.

To sum up, with the help of the developed LC-MS/MS method, we were able to determine the PK parameters of artesunate, artemether and their metabolites in sheep, which accurately reflected the observed activities against *F. hepatica* in sheep. The LC-MS/MS method might be further applied for the analysis of the artemisinins in different biological fluids (e.g. bile/urine) and tissues (e.g. liver/flukes). The FLOTAC techniques are time efficient and sensitive and hold promise to become a valuable diagnostic tool for laboratory work with fascioliasis and probably also for other helminthic diseases. Nevertheless, further work is necessary to achieve a better analytical robustness and ease of application. Combinations with triclabendazole improved the efficacy of the treatments with peroxidic compounds, but further chemotherapy studies against triclabendazole resistant *F. hepatica* strains, studies in ruminants and pharmacokinetic

analyses are required to thoroughly evaluate the potential of triclabendazole-peroxide combinations.

In conclusion, the artemisinins are promising lead structures for the development of novel peroxidic fasciocidal drugs, because treatment efficacy has been demonstrated against adult and juvenile *F. hepatica in vitro* and in the rat model, in sheep naturally infected with *F. hepatica* and importantly also in rats infected with a triclabendazole resistant *F. hepatica* strain.

# Zusammenfassung

Die Verursacher der Fasziolose sind die Leberegel *Fasciola hepatica* sowie *Fasciola gigantica*. Diese parasitäre Erkrankung ist weit verbreitet und besitzt außerdem ein breites zoonotisches Reservoir, aus ökonomischer Sicht stellen jedoch die Nutztiere Schaf und Rind die wichtigsten Endwirte dar. Seit den 1990er Jahren wird die Fasziolose als ein zunehmendes globales Gesundheitsproblem erkannt, schätzungsweise sind 2.4 bis 17 Millionen Menschen infiziert. Zur Behandlung der Fasziolose wird vor allem Triclabendazol empfohlen. Da im Nutztier immer häufiger Behandlungsresistenzen vorkommen, werden neue Medikamente dringend benötigt.

Die semisynthetischen Artemisinine und das synthetische Artemisinin-Analogon OZ78, ein 1,2,4-Trioxolan, sind peroxidische Verbindungen, die sowohl *in vitro* wie auch *in vivo* eine vielversprechende Aktivität gegen juvenile und adulte *F. hepatica* aufweisen. Zusätzlich konnte gezeigt werden, dass diese Peroxide auch gegen einen Triclabendazol resistenten *F. hepatica* Stamm aktiv sind.

Im Rahmen dieser Doktorarbeit wurden präklinische Untersuchungen durchgeführt, um unser Wissen über das Potenzial der semisynthetischen Artemisinine, Artesunat und Artemether zur Behandlung von *F. hepatica* Infektionen zu erweitern. Darüber hinaus wurde die FLOTAC Technik, welche eine neuartige mikroskopische Methode zur Detektion und Quantifizierung von *F. hepatica* Eiern in Stuhlproben darstellt, evaluiert.

Eine sensitive Mikroskopietechnik wurde zur Untersuchung von *F. hepatica* Infektionen in künstlich infizierten Ratten, in natürlichen *F. hepatica* Infektionen im Schaf und zur Analyse des Behandlungserfolgs hinsichtlich der Eierexkretion benötigt. Aus diesem Grund haben wir zunächst die Sedimentationstechnik (Referenzmethode) mit der FLOTAC Technik zur Detektion und Quantifizierung von *F. hepatica* Eiern in Stuhlproben von infizierten Ratten verglichen. Einen erheblichen Aufwand stellte das Auslesen von 8 Objektträgern dar, welches durchgeführt werden musste um bei tiefen Infektionsintensitäten mit der Sedimentationstechnik eine vergleichbare Sensitivität wie nach einer FLOTAC Analyse zu erreichen (85.2% vs. 92.6%). Bei hohen Infektionsintensitäten war die Sensitivität für beide Techniken keine Limitation. Prinzipiell war die Sedimentationstechnik uniformer und einfacher anzuwenden als die

FLOTAC Technik, jedoch war der benötigte Zeitaufwand um eine gute Sensitivität zu erreichen viel grösser. Vor allem aus diesem Grund wurde in unserem Labor für zukünftige Stuhluntersuchungen FLOTAC und nicht die Sedimentationstechnik verwendet.

Die FLOTAC Technik konnte erfolgreich zur Identifikation von natürlich infizierten Schafen und zur Quantifizierung der Eierausscheidung verwendet werden. Dies ermöglichte die Schafe gemäss der Infektionsintensitäten in ausgeglichene Behandlungsgruppen einzuteilen und den Behandlungserfolg anhand der Eierausscheidungsmenge abzuschätzen. Die natürlich *F. hepatica* infizierten Schafe wurden mit Einzeldosen von Artesunat oder Artemether mittels unterschiedlicher Applikationsarten behandelt. Nach intramuskulärer Verabreichung wiesen beide Wirkstoffe gute Aktivitäten auf, wobei viermal weniger Artesunat als Artemether benötigt wurde. Nach per oraler Darreichung konnte kein Effekt beobachtet werden. Daraufhin wurde die Pharmakokinetik (PK) von Artesunat und Artemether im Schaf nach verschiedenen Applikationsarten analysiert, um die beobachteten Aktivitätsdaten besser verstehen zu können.

Für die pharmakokinetische Analyse der Artemisinine musste eine Hochleistungs-flüssigkeitschromatographie Tandem-Massenspektrometrie (LC-MS/MS) Methode zur zeitgleichen Detektion und selektiven, akkuraten sowie präzisen Quantifizierung von Artesunat, Artemether und deren Metaboliten Dihydroartemisinin (DHA) und Dihydroartemisinin-glukuronid (DHA-glukuronid) entwickelt und validiert werden. Der Metabolit DHA-glukuronid wurde in diesen PK-studien ebenfalls untersucht, da in einer vorausgehenden Analyse grosse DHA-glukuronid Plasmaspiegel detektiert wurden. Nach Aufarbeitung der Plasmaproben mittels einer einfachen Proteinpräzipitations-methode wurde je nach Analyt eine Wiederfindung von 60-80% erreicht. Alle Analyten wurden mittels „selected reaction monitoring“ mit einem Übergang von *m/z* 267.4→163.0 im positiv-Modus detektiert. Es konnte gezeigt werden, dass die entwickelte Methode die Artemisinine präzise, akkurat und selektiv mit einer ungefähren Quantifizierungsgrenze von 10 ng/ml für Artesunat, DHA und DHA-glukuronid und von 90 ng/ml für Artemether bestimmen kann.

Die Pharmakokinetik von Artesunat nach intramuskulärer Gabe ist durch eine schnelle Pharmaka Invasion mit einer $T_{max}$ von 15 min sowie einer grossen Pharmaka

Disposition, $C_{max}$ sowie AUCs, beobachtet für Artesunat und seine Metaboliten, charakterisiert. Ein weiteres Charakteristikum der Kinetik ist die schnelle Elimination der Pharmaka mit geschätzten Halbwertszeiten von 58-63 min für Artesunat, 94-113 min für DHA und 89-98 min für das DHA-glukuronid. Artemether hingegen wird nach intramuskulärer Darreichung nur langsam und kontinuierlich aus der öligen Formulierung freigesetzt, dementsprechend wurden während den 24 Std der Probenentnahme mehr oder weniger konstante Artemether und Metaboliten Plasmaspiegeln gemessen. Folglich wurden, verglichen mit einer Artesunat i.m. Behandlung, nach i.m. Applikation von Artemether niedrigere $C_{max}$ aller Analyte bestimmt. Anderseits sind die berechneten AUCs der wirkungsvollen Artemether Behandlungen (160 mg/kg) vergleichbar mit denjenigen von Artesunat (40 und 60 mg/kg). Die AUCs, welche nach effektiven Artemether i.m. Behandlungen bestimmt wurden, waren signifikant grösser als diejenigen, welche entweder nach wirkungsloser intramuskulärer (40 mg/kg) oder per oraler (80 mg/kg) Artemether Gabe gemessen wurden. Mittels *in vitro* Versuchen konnte gezeigt werden, dass DHA gegen *F. hepatica* Egel aktiv ist, während das DHA-glukuronid keine Aktivität besitzt. Aus diesem Grund trägt das Glukuronid mit grosser Wahrscheinlichkeit auch nicht zur Behandlungswirkung bei.

Um die Effektivität und Verträglichkeit der Behandlungen mit peroxidischen Verbindungen zu verbessern, untersuchten wir Peroxid-Triclabendazol Kombinationen gegen juvenile und adulte *F. hepatica* Infektionen in Ratten. Die Analyse der Dosis-Wirkungs-Beziehung von Mono- und Kombinationstherapien wurde mittels negativ binomialer Regression der Wurm- und Eieranzahlen errechnet. Gegen adulte *F. hepatica* Egel wurden $ED_{50}$ Werte von 113.0, 77.7, 22.9 und 2.7 mg/kg Körpergewicht für Artesunat, Artemether, OZ78 und Triclabendazol ermittelt. Die Tatsache, dass verstärkte Aktivitäten bei Peroxid-Triclabendazol Kombinationsbehandlungen verglichen zu den jeweiligen Monotherapien beobachtet wurden, ist viel versprechend. Jedoch war bei Kombinationen aus Artemisininen und Triclabendazol der verstärkte Effekt abhängig von der verwendeten Triclabendazol Dosis. Desweiteren wurde beobachtet, dass juvenile Würmer im Vergleich zu adulten Würmer weniger deutlich auf Mono- und Kombinationstherapien reagierten, sowohl *in vitro* als auch *in vivo*. Unsere Chemotherapie Studien zeigten ausserdem, dass sich die Präzision von Dosis-Wirkungs-

Beziehungen basierend auf Berechnungen der Eieranzahlen bedeutend verschlechtert, wenn man sie mit Berechnungen basierend auf Wurmanzahlen vergleicht.

Zusammengefasst, ermöglichte uns die entwickelte LC-MS/MS Methode die Bestimmung der PK Parameter von Artesunat, Artemether und dessen Metaboliten im Schaf, welche die beobachteten Aktivitäten gegen *F. hepatica* zufriedenstellend reflektierten. Zusätzlich könnte diese Methode möglicherweise auch für die Analyse der Artemisinine in anderen Körperflüssigkeiten (Galle/Urin) oder Geweben (Leber/Egel) angewendet werden. Die Zeiteffizienz und Sensitivität der FLOTAC Technik ist vielversprechend und aufgrund dessen könnte diese Technik ein wertvolles diagnostisches Instrument für die Arbeit im Labor mit Fasziolose und unter Umständen auch für weitere Wurmerkrankungen werden. Jedoch sind zusätzliche Optimierungen der Methode notwendig, um dessen Robustheit und Einfachheit in der Handhabung zu verbessern. Kombinationen mit Triclabendazol verbesserten die Behandlungseffizienz der peroxidischen Verbindungen. Dennoch werden weitere Experimente benötigt, zum Beispiel Kombinationsstudien gegen Triclabendazol resistente *F. hepatica* Stämme sowie Studien im natürlichen Endwirt Schaf unterstützt von PK Analysen, um das Potenzial dieser Kombinationen voll und ganz abschätzen zu können.
Schlussfolgernd stellen die Artemisinine vielversprechende Leitstrukturen für die Entwicklung von neuartigen peroxidischen Wirkstoffen zur Behandlung der Fasziolose dar, da diese Substanzklasse gegen juvenile und adulte Würmer wirkt sowie Aktivitäten im Schaf und entscheidend auch in Ratten, infiziert mit einem Triclabendazol resistenten *F. hepatica* Stamm, gezeigt wurden.

# Table of Abbreviations

| | |
|---|---|
| **AM** | Artemether |
| **AS** | Artesunate |
| **AUC** | Area under the plasma concentration time curve |
| **CAD** | Collision gas |
| **CE** | Collision energy |
| **$C_{max}$** | Maximal plasma concentration |
| **CV** | Coefficient of variation |
| **DHA** | Dihydroartemisinin |
| **DHA-G** | Dihydroartemisinin-12-α-o-β-D-glucuronide |
| **DHA-glucuronide** | Dihydroartemisinin-12-α-o-β-D-glucuronide |
| **EBR** | Egg burden reduction |
| **$EC_{50/90}$** | Concentration required to kill 50/90% of the parasites |
| **$ED_{50/90}$** | Doses required to kill 50/90% of the parasites |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EPG** | Number of eggs per gram of stool |
| **ESA** | Excretory-secretory antigen |
| **ESI** | Electrospray ionization |
| **FEC** | Faecal egg count |
| **FECRT** | Faecal egg count reduction test |
| **FS** | Flotation solution |
| **HPLC** | High pressure liquid chromatography |
| **HPLC-ECD** | High pressure liquid chromatography with electrochemical detection |
| **IQR** | Interquartile range |
| **IS** | Internal standard |
| **KW** | Kruskal-Wallis test |
| **LC-MS/MS** | Liquid chromatography tandem mass spectrometry |
| **LLOQ** | Lower limit of quantification |
| **LRS** | Likelihood ratio statistics |
| **MW** | Molecular weight |
| **NMR** | Nuclear magnetic resonance |

| | |
|---|---|
| **NPV** | Negative predictive value |
| **NTDs** | Neglected tropical diseases |
| **OZ78** | Synthetic artemisinin-like compound, 1,2,4-trioxolane |
| **PCR** | Polymerase chain reaction |
| **PEG** | Polyethylene glycol |
| **PK** | Pharmacokinetic |
| **QC** | Quality control |
| **RRE** | Relative recovery |
| **RSD** | Relative standard deviation |
| **S.g.** | Specific gravity |
| **SAR** | Structure-activity relationships |
| **SD** | Standard deviation |
| **SE** | Standard error |
| **SEM** | Scanning electron microscopy |
| **SRM** | Selected reaction monitoring |
| $t_{1/2}$ | Elimination half-life |
| **TEM** | Transmission electron microscopy |
| $T_{max}$ | Time to achieve maximal plasma concentration |
| **ULOQ** | Upper limit of quantification |
| **USFDA** | US Food and Drug Administration |
| **WAAVP** | World Association for the Advancement of Veterinary Parasitology |
| **WBR** | Worm burden reduction |

# *Chapter 1*

# General Introduction

## Fascioliasis: Biology, Control and Epidemiology

# General Introduction

## Fascioliasis: Biology, Control and Epidemiology

## 1 Food-borne Trematodiasis

Food-borne trematodiasis is caused by trematode parasites and belongs to the group of neglected tropical diseases (NTDs), a group of diseases which affect primarily poor and marginalized people in rural settings of the developing world [1, 2]. Collectively, the NTDs cause an estimated global disease burden of more than 50 million disability-adjusted life years and therefore represent the 4$^{th}$ most important group of infectious diseases, behind respiratory infections, HIV, and diarrheal diseases [2, 3]. It is estimated that more than 10% of the world's population is at risk of infection from the major food-borne trematodes (~750 million people) with at least 40 million infections worldwide [4, 5]. Food-borne trematode infections are frequently zoonotic diseases and hence also cause a significant veterinary problem and massive economic losses to the agriculture sector (see also Section 4.2) [6-8].

The trematodes, or flukes, belong to the phylum Platyhelminthes and include approximately 70 species, which are known to infect humans via food ingestion [9]. The food-borne trematodes are often classified according to the organ the adult worm occupies in the mammalian hosts and hence comprise of intestinal (*Echinostoma* spp., *Fasciolopsis buski and* heterophyids), lung (*Paragonimus* spp.), and liver (*Clonorchis sinensis*, *Opisthorchis viverrini* and *felineus*, and *Fasciola gigantica* and *hepatica*) flukes. The latter liver fluke, *Fasciola hepatica* shall be pointed out particularly, since this thesis deals exclusively with this parasite [5]. The trematode flukes have various sizes and are characterised by a bilaterally symmetrical and dorsoventral flattened anatomy (Fig. 1) [10]. Further characteristics are the presence of an oral sucker and often a ventral sucker, which allow the worms to attach within the host organism. The flukes are always hermaphroditic and exhibit no respiratory and circulatory systems [10]. The tegument, a syncytial epithelium, surrounds the trematodes and is associated with nutrient absorption, synthesis, secretion, and osmoregulation and possesses additional sensory functions [10]. Importantly, it protects the parasite from host detergents (bile acid), digestion (enzymes), and immune system [10, 11].


**Figure 1**. **Food-borne trematodes** (Swiss Tropical and Public Health Institute archive, Joachim Pelikan)

**(A)** *Fasciola hepatica*, **(B)** *Fasciolopsis buski*, **(C)** *Clonorchis sinensis*, **(D)** *Opisthorchis viverrini*.

# 2 Biology and Life-cycle of *Fasciola* spp

The food-borne trematodes, *Fasciola hepatica* and *F. gigantica* infest a broad range of animals and accidentally also humans. Farm animals such as sheep and cattle are most commonly affected by fascioliasis and act as the main mammalian end hosts. However, *Fasciola* spp. can develop in a variety of animals such as deer, llamas, kangaroos, rabbits, beavers and rats, which demonstrates the remarkable capability of the parasite to adapt to new hosts [12, 13].

The life-cycle of *F. hepatica* is initiated with mature flukes starting to lay eggs (Fig. 2). Adult *F. hepatica* flukes are able to excrete up to 20,000 eggs per day in the biliary ducts of their hosts [14]. The eggs are oval in shape with a size of 130-145 µm in length and 70-90 µm width, and are therefore large compared to the eggs of other trematode worms. The yellow-brownish colour and the operculum are further characteristics of *F. hepatica* eggs [10, 14]. The immature *F. hepatica* eggs migrate through the ductus choledochus into the duodenum and are released in the environment with faeces **(A)**.

Approximately 5,000 eggs per gram faeces (EPG) can be excreted in heavy infections of humans as has been observed in hyperendemic areas [12, 15, 16]. Embryonation occurs outside the host and especially humidity, temperature and oxygen tension influence its the development [14]. The *F. hepatica* eggs hatch within 2 to 3 weeks and release free-swimming miracidia, which either invade molluscan intermediate hosts, typically *Galba*/*Lymnaea truncatula*, or die within 24 hours [13, 14] **(B)**. Miracidia are strongly phototropic and a positive chemotactic reaction to *L. truncatula* occurs up to a distance of 15 cm [14, 17]. The miracidia multiply asexually within the snail and develop during 4-7 weeks into sporocysts, rediae, and finally cercariae [12, 14] **(C)**. A snail infected with a single miracidium produces around 4,000 free-swimming cercariae [18]. The tadpole-like cercariae (250-300 µm) attach to freshwater vegetation and encyst to metacercariae, which stay infectious for over a month, depending on environmental conditions [14] **(D)**. Mammalians get infected by ingesting metacercariae through consumption of aquatic vegetables or contaminated water [19] **(E)**. The *F. hepatica* metacercariae excyst in the small intestine, penetrate the intestinal wall, burrow through liver parenchyma within 5 to 6 weeks and finally lodge in the bile ducts for years. The life span of *F. hepatica* flukes is an estimated 9-13 years in humans, 1-2 years in cattle, and up to 20 years in sheep [10, 12, 14]. Fully-grown *F. hepatica* (Fig. 1A) measure up to 20 to 50 mm in length whereas *F. gigantica* can be as large as 75 mm in length [20, 21]. Finally, the life cycle is completed approximately 8 weeks post infection, when mature *F. hepatica* flukes start excreting eggs in the bile [14] **(A)**.

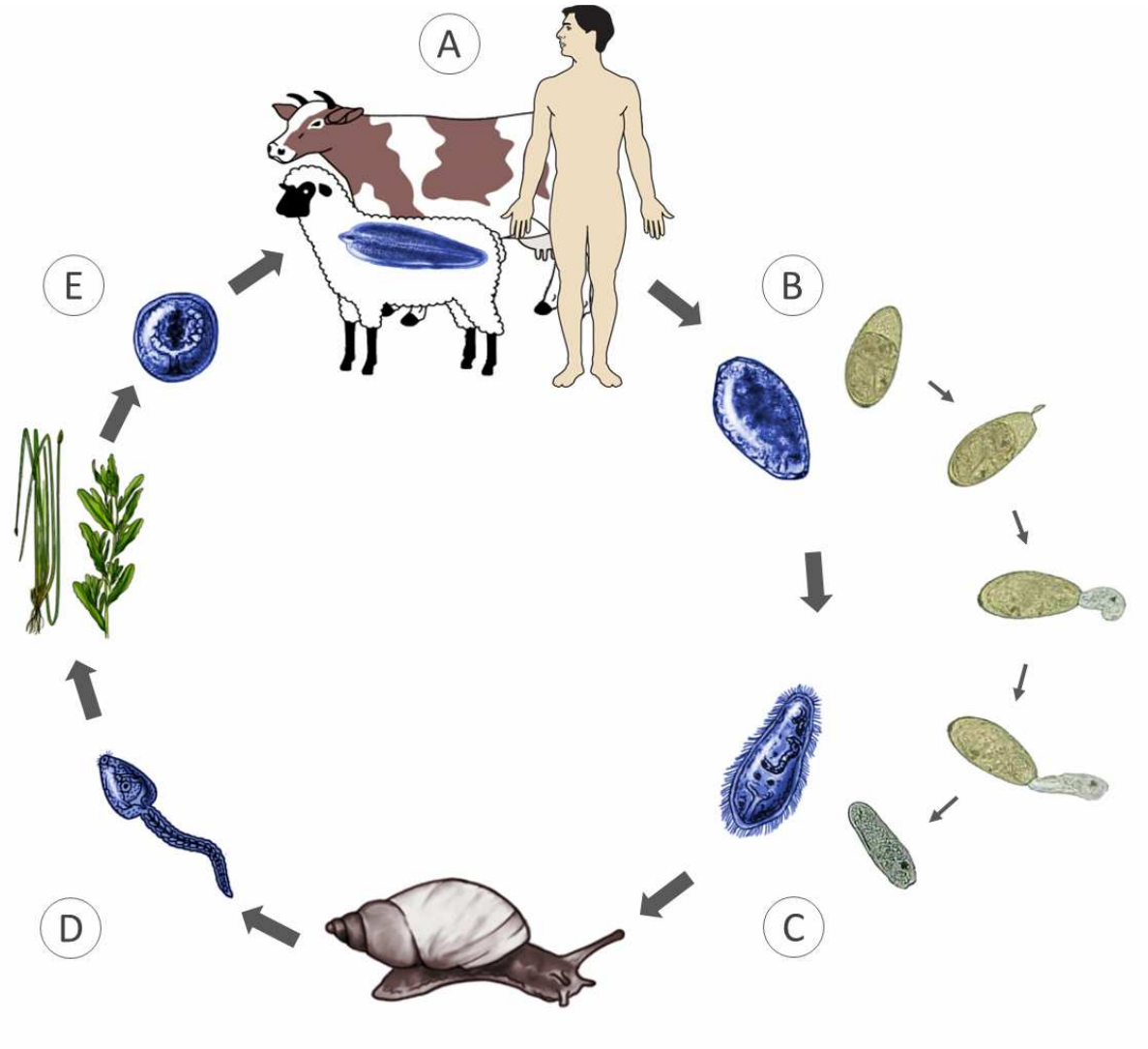



**Figure 2**. **The Life-cycle of *F. hepatica*.** Adapted from [10, 22, 23]

**(A)** End host infected with *F. hepatica*, **(B)** Excretion of eggs with faeces and miracidia hatching, **(C)** Infection of the molluscan intermediate host, **(D)** Release of cercariae and encystment to infectious metacercariae, **(E)** Infection of the end host by consumption of contaminated aquatic plants or water with metacercariae.

# 3 Pathogenesis and Clinical Aspects of Fascioliasis

Pathological studies of fascioliasis were mostly accomplished in experimental animals such as rats or sheep and not humans, since there are few opportunities for post-mortem examinations with human subjects. However, the existing data suggests that human fascioliasis parallels that in animals and the maturity rate of *F. hepatica* in sheep is similar to that in humans [13, 24, 25].

It is conceivable that the severity of the disease correlates with the number of parasites per host. Therefore, disease severity and symptoms can vary and range from asymptomatic to highly pathogenic and debilitating symptoms [24, 26]. Fascioliasis can be grossly subdivided into two pathogenic phases: the acute (parenchymal) and chronic (biliary) phase. The former occurs during the migration of the juvenile flukes through the liver parenchyma, whereas the latter arises when mature flukes reside in the bile ducts [24, 26]. Mammalians may be simultaneously infected with *F. hepatica* of different stages and present a complex pathology. In the case of human fascioliasis, commonly only a few parasites reach the bile ducts, since humans are not the natural end host [24].

## 3.1 Acute Fascioliasis

Acute fascioliasis starts once juvenile *F. hepatica* flukes have reached the host's liver (~90 h post-infection). The penetration through the intestinal wall is not associated with clinical signs and the appearance of the first symptoms can take a few days to a few months (= incubation phase) [24, 26]. Juvenile flukes digest hepatic tissue during the liver migration stage, which causes haemorrhage and inflammation. Necrosis, fibrosis, and even migration tracts of flukes can be detected in a histological liver examination (Fig. 3). Unspecific symptoms such as fever, weakness due to anaemia, abdominal pain, and gastrointestinal disturbance arise in the acute phase of infection [26, 27]. Hepatic damage potentially leads to hepatosplenomegaly, ascites, and jaundice. Sudden death can occur, in particular in sheep and goats, due to extensive blood loss caused by liver haemorrhage, liver failure, or secondary infections with *Clostridium novyi* (= black disease) [24, 26, 28]. However, black disease infections are uncommon today due to effective vaccination [24]. The mortality due to *Fasciola* spp. infections is low in humans and case reports suggest that children are more often affected [25-27].

In addition, ectopic fascioliasis can be observed if the parasites deviate during migration and enter other organs such as the diaphragm, lung, or intestine. Inflammatory respiratory lesions including pneumonia and fibrous pleuritis are regularly recorded and the pathology is more significant in heavy infections [24, 26, 29].

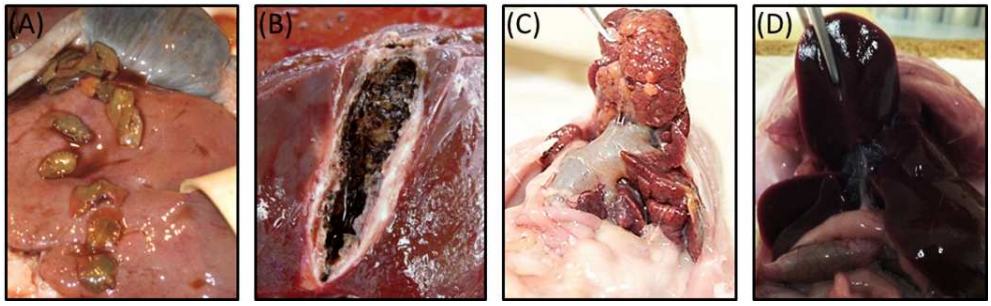



**Figure 3. Pathological picture of fascioliasis.**

**(A)** Infected sheep liver, **(B)** Calcification and thickening of a cattle's bile duct infected with *F. hepatica* **(C)** Rat experimentally infected with *F. hepatica* showing liver fibrosis, necrotic liver tissue, and dilatation of the bile duct, **(D)** Healthy rat liver and bile.

## 3.2 Chronic Fascioliasis

In the chronic phase of the disease, the parasites have entered the bile-ducts and have started to lay eggs. Fibrosis, hyperplasia, dilatation, calcification, and thickening of the bile ducts and gallbladder are characteristics of this stage (Fig. 3) [26, 27]. Periods of indistinguishable gastrointestinal disorders or even no symptoms are present in human chronic fascioliasis (= latent phase). Nevertheless, the latent phase is intermitted by obstructive periods, where parasites or debris block the bile ducts. Severe complications such as cholestatic hepatitis, cholecystitis, cholangitis, choledocho- and cholecysto-lithiasis, acute pancreatitis, and bacterial superinfections might be monitored [26]. Observed consequences may include biliary colic pain, fever, diarrhoea due to fatty food intolerance, pruritus, jaundice, and anaemia [26].

*F. hepatica* infections in farm animals might result in severe anaemia, which considerably affects the morbidity and mortality of the disease. For instance, it has been estimated that one fluke is responsible for 0.2-0.5 ml blood lost per day [24, 30, 31]. In

the chronic phase, sheep and cattle might develop oedema under the jaw ("bottle jaw") [28]. Inappetence and reduced feed conversion compromise weight gain and wool growth, hence economic loss is prominent with fascioliasis (Section 4.2) [24].

# 4 Epidemiology of Human and Veterinary Fascioliasis

As mentioned, fascioliasis is on the one hand a disease of great veterinary importance and on the other hand, a neglected human disease. Therefore, both epidemiological aspects of the disease shall be introduced here.

## 4.1 Human Fascioliasis

Human infections are usually prevalent in areas where veterinary fascioliasis is endemic, because wild and farm animals are the major reservoir of *Fasciola* spp. and contribute considerably to worldwide dissemination as well as to local transmission of the disease [12]. However, it seems that a high prevalence of fascioliasis in animals is not directly related to increased numbers of cases in human [32]. Instead, environmental factors, human habits and especially eating behaviour influence the human infection rate [20].

*F. hepatica* and *F. gigantica* display a remarkably large global distribution (Fig. 4), with human infections being reported from 51 countries in five continents [33, 34]. Distribution of *F. gigantica* is more limited than that of *F. hepatica* and is restricted to Africa, Asia, Middle East, and Eastern Europe (Fig. 4) [35]. Recent studies have estimated that 90 up to 180 million people are at risk of *Fasciola* spp. infections and between 2.4 and 17 million individuals are infected worldwide [4, 19, 36]. Nevertheless, the true number of *Fasciola* infections might be considerably higher [34], since recent outbreaks were not considered in these estimates, not all countries have reporting systems, and infections might be unrecognized due to the potential asymptomatic course of the disease. Human fascioliasis is of particular importance in the Andean countries Peru and Bolivia, Egypt, the Islamic Republic of Iran, Cuba, Vietnam, and the Western European countries Portugal, Spain, and France (Fig. 4) [4, 26, 37]. The highest prevalences and intensities of human fascioliasis were observed in the Northern Bolivia Altiplano, with

more than 40% of infected individuals in certain communities [12, 13]. For example, it has been estimated that in Bolivia as many as 360,000 humans are infected with *F. hepatica* [4]. In addition, 830,000 individuals are potentially infected with fascioliasis in the Nile Delta region of Egypt [4], with observed high mean prevalence of 12.8% (5.2-19.0%) in some villages [13, 38]. In comparison, human fluke infections are rare in Europe, however regular outbreaks of the disease occur in France, Spain, and Portugal, causing about 50-100 infections per year [12].

Most of the reported cases of human fascioliasis are due to infections of *F. hepatica* and only a few reports e.g. from Vietnam, Egypt, and Thailand indicate *F. gigantica* as an agent of human fascioliasis [8, 12, 39].

The distribution pattern of fascioliasis has a patchy character, because it depends on general physiographic and climatic conditions as well as the dissemination of the intermediate snail host populations in freshwater bodies [32]. The global distribution of human fascioliasis on a country level is depicted in Fig. 4. Note that, due to reasons highlighted above, the disease distribution is not uniform within these countries.

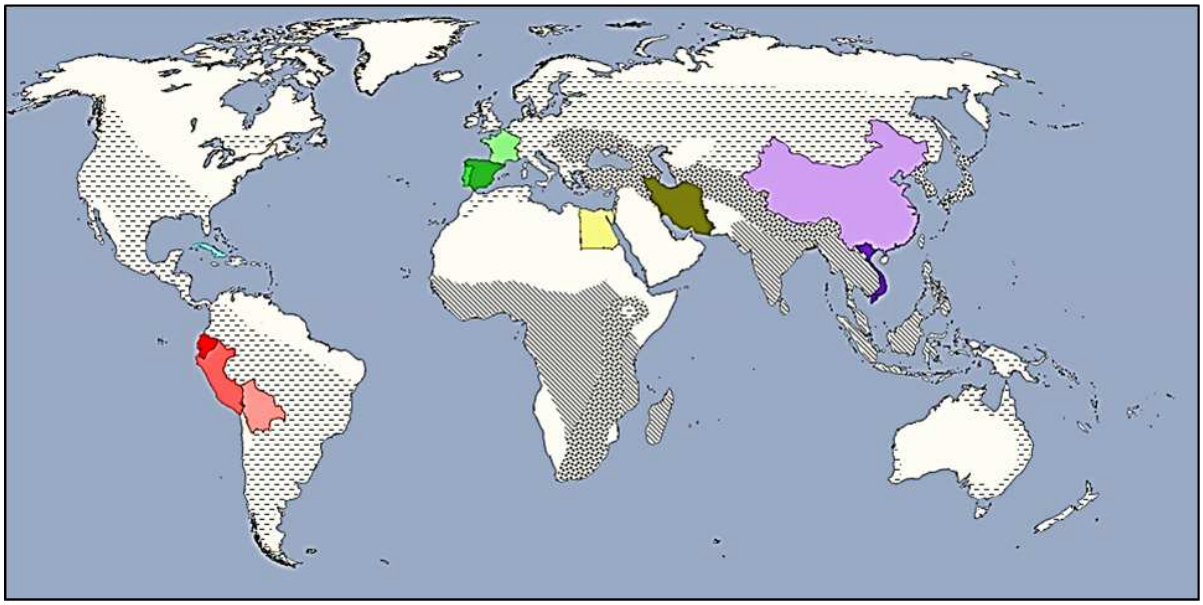


**Figure 4. Estimated distribution of human and veterinary fascioliasis** adapted from [35, 37]

| Estimates of infected individuals worldwide [4] | | Distribution of *Fasciola* spp. [35] |
|---|---|---|
| **Andean countries** | **Africa** | Distribution of *F. hepatica*, |
| Peru: 742,000 | Egypt: 830,000 | Distribution of *F. gigantica* |
| Bolivia: 360,000 | **Middle East** | Overlapping regions, where both *Fasciola* spp. may be present |
| Ecuador: 20,000 | Iran: 10,000 | |
| **Caribbean countries** | **Asia** | |
| Cuba: n.a. | China: 160,000 | |
| **Western Europe** | Vietnam: n.a. | |
| Portugal: 267,000 | | |
| Spain: 1,000 | | |
| France: n.a. | | n.a. = not available |

## 4.2 Veterinary Fascioliasis

*Fasciola* spp. are present on every continent on earth, except Antarctica and are able to live in areas from below sea level in the Caspian region up to a very high altitude of 4,200 m at the Paso del Condor in Venezuela. Hence, fascioliasis is the vector-borne disease with the greatest latitudinal, longitudinal and altitudinal distribution known (Fig. 4) [33, 34]. Livestock animals such as sheep, cattle, and buffalo are the most important end hosts from an ecological point of view [35]. It is challenging to provide a countrywide overview of fascioliasis in livestock, since numerous countries and animals are affected. Importantly, fascioliasis due to *F. hepatica* or *F. gigantica* is of veterinary significance and it appears to be independent of the country's wealth, since high prevalences were noted from all over the world. For instance, the following prevalences of *Fasciola* spp. infections in farm animals were recorded: USA; 5.9-68%, Switzerland; 18%, Spain; 29.5%, Chile; up to 94%, UK; 10%, New Zealand; 8.5%, Thailand; 47.1%, and Nigeria; 43% [35, 40]. Taken as a whole, it has been estimated that more than 250 million sheep and 300 million cattle are infected with *F. hepatica* worldwide [12, 41]. Hence, infections with *Fasciola* spp. cause an economic loss to the agriculture sector due to reductions in weight gain, milk yield and fertility and in the case of sheep, a decrease in wool production [35, 42, 43]. A recent estimate calculated a median financial loss of €299 per year per infected dairy cattle in Switzerland, where this loss arises mostly from reduced milk yield and fertility and less so from reduced meat production and treatment costs [42]. Overall, fascioliasis causes an estimated financial loss of more than US$ 3 billion per year to the agriculture sector worldwide [8, 12].

# 5 Diagnosis of Fascioliasis

Diagnostic tools build the basis for successful prevention and control of infection diseases including fascioliasis. First and foremost, an accurate diagnostic method is obviously needed to identify infected individuals in a population and thus to enable proper intervention strategies, prevalence records, and ultimately accurate estimates of the global disease burden. In addition, a sensitive and specific diagnostic method is essential for evaluating treatment outcomes and community effectiveness of interventions, as well as verifying local disease eliminations and early detection of

reappearances [44]. Direct parasitological diagnostics, immunodiagnostics, molecular diagnostics, and imaging techniques are the main ante-mortem tools to diagnose *Fasciola* spp. infections [10].

Imaging techniques such as ultrasound, computer tomography, and magnetic resonance imaging can reveal the hepatobiliary damages (biliary stones, fibrosis, and calcifications) caused by fascioliasis [10, 45]. However, these techniques alone are not sufficiently sensitive for the diagnosis (<15%) [26], since the apparent pathological picture is not specific for fascioliasis. However, these imaging devices might be important to exclude secondary damages and to survey the healing process [25].

Immunodiagnostics, in particular enzyme-linked immunosorbent assays (ELISA), are applicable as serological and coprological indirect tests. The former detects circulating IgG antibodies elicited by infected individuals against fluke antigens, such as cystein proteinase (Fas2) and cathepsin L1 (CL1), whereas the latter detects antigen in stool samples including *Fasciola* excretory-secretory antigens (FES-Ag) [26, 46, 47]. These tests are characterised by a high diagnostic sensitivity and hence are able to identify low-infection intensities. In addition, prepatent infections (~1-2 week post infection) and ectopic fascioliasis can be detected [13, 26]. However, there are difficulties to evaluate treatment outcome with ELISA tests, because circulating antibodies might not disappear immediately post treatment [48]. Furthermore, cross-reactivity with infections of other trematode species can arise [49].

In contrast to immunodiagnostics and copromicroscopy, polymerase chain reaction based methods (PCR) allow for a safe differential diagnosis of *F. hepatica* and *F. gigantica* infections [13]. This is important because differentiation between eggs from *Fasciola* spp., *Fasciolopsis*, and echinostomes is difficult by microscopy [27]. Moreover, PCR can be applied to detect *Fasciola* spp. infections within the snail population [50].

In terms of direct parasitological diagnostics, *F. hepatica* eggs are detected in stools (=copromicroscopy), duodenal fluid, or bile aspirates. Traditionally, the copromicroscopic techniques including the Kato-Katz method and the sedimentation method are used to analyse *F. hepatica* eggs in stools [51, 52]. These diagnostic approaches allow for estimating infection intensity and treatment outcome. Furthermore, they are applied to evaluate the efficacy of anthelmintics in ruminants [53]. Coprodiagnostic methods have the advantage of being cost-effective and relatively easy to perform, which render them widely applicable in resource-constrained settings

[26]. However, only chronic *F. hepatica* infections can be diagnosed, since eggs are excreted only from mature worms and hence ectopic fascioliasis and acute infections (~ first 3 months) remain undiscovered [26]. Coprodiagnostic methods are sufficiently sensitive in detecting moderate and high *Fasciola* spp. infection intensities, but the methods lack sensitivity for detecting low-intensity infections. Still, repeated sampling can improve sensitivity significantly [26, 54].

To improve the sensitivity of traditional coprodiagnostic methods, a series of novel multivalent faecal egg count (FEC) techniques, the FLOTAC techniques, have recently been developed for the qualitative and quantitative diagnosis of human and veterinary helminth infections [55]. The FLOTAC apparatus (Fig. 5) is the core of these various techniques. This device consists of two flotation chambers, which can be loaded with 5 ml faecal sample suspensions each. The principle is that parasitic elements, such as eggs, float on top of the chamber, whereas faecal debris sediment during centrifugation. Subsequent translation of the apical portion of the floating suspension separates the eggs from debris. The degree of parasitic element flotation depends on the parasite and host species as well as the applied flotation solution. Therefore, the most suitable flotation solution is selected in a calibration step, among a panel of nine flotation solutions with specific gravities (s.g.) ranging from 1.2 to 1.45 [55]. The great advantage of these techniques is that a single FLOTAC uses up to 1 gram of stool per analysis with a sensitivity of 1 egg per gram, whereas a single Kato-Katz analyses only 41.7 mg of stool [55]. For more details, the reader is referred to *chapter 2*.

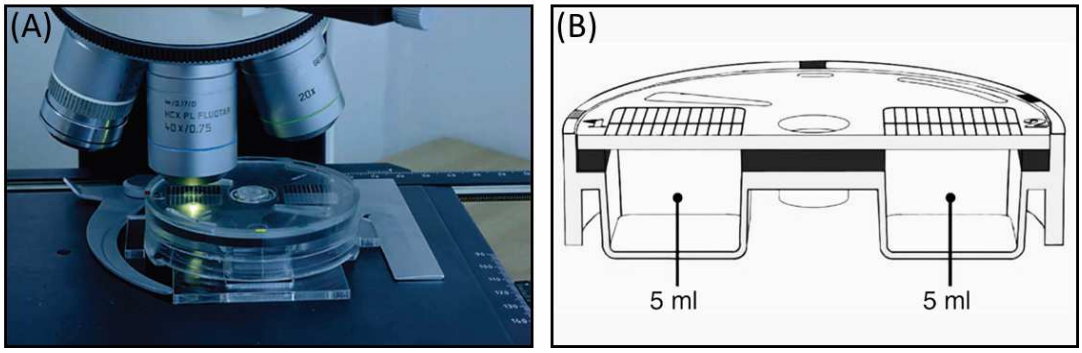



**Figure 5. An illustration of the FLOTAC techniques** adapted from [55]

**(A)** Photography of the FLOTAC apparatus

**(B)** Cross-section of the FLOTAC apparatus

# 6 Control Strategies against Fascioliasis

*Fasciola* spp. exhibits the remarkable ability to adapt to a broad variety of end hosts, ranking from humans to livestock and various wild animals, which collectively build a major reservoir for the parasite and contribute to the dissemination of the disease [12].
Therefore, an effective control strategy against fascioliasis is complex and includes both different mammal end hosts as well as molluscan intermediate hosts. A universal concept may not available, since fascioliasis affects too many various regions with diverse circumstances such as different climatic conditions (temperature, humidity), dissimilarly developed sanitation, variable human behaviour, and diverse zoonotic reservoirs.
Complete eradication of fascioliasis appears unlikely at the moment, because no highly active vaccines are available [56]. To date, there are only experimental vaccine candidates available, which provide moderate protection efficacy [12, 56]. However, it has been shown that the prevalence of fascioliasis can be diminished with adequate control strategies, including treatment with anthelmintics (section 7), molluscicides, grazing management, changing of human behaviour, community-based education, and improved access to adequate sanitation [10, 28, 35].
Human fascioliasis would be preventable if only thoroughly washed vegetables and cooked water are consumed as well as kitchen utensils are carefully sanitized. Nevertheless, the implementation appears difficult, because changing human behaviour is not easy, and the potential contamination sources are diverse and differentiate between regions. For instance, consumption of watercress, spearmint, lettuce, alfalfa juice, contaminated water (floating metacercariae), and raw liver containing juvenile worms are potential risk factors of human fascioliasis [20, 57-59]. However, improvements in sanitation such as the construction of an appropriate filtration system of piped water, have decreased human infections in some Nile delta areas [20]. Community-based education on aspects of parasite transmission such as the importance of separating animals from areas where food is grown can directly reduce cases of human fascioliasis [12].
Drug treatment programs should initially emphasize children in high-risk areas, because highest prevalence is present in children between 8 and 11 years old [12, 60]. Regular treatment of livestock animals with fasciocidals covering juvenile and adult flukes will

reduce infection intensity over time. For instance, chemotherapy reduced the prevalence of *F. hepatica* infections by up to 75% on some Irish farms and from 49% down to 1% on farms in Scotland [35, 61, 62]. The chosen treatment schedule depends on seasonal transmission dynamics and hence schedules vary from single annual treatment up to multiple (4-5) treatments per year. However, a complete elimination was not realizable using anthelmintics only, probably because of the broad wild animal reservoir of fascioliasis [35].

A further strategy includes control of the intermediate molluscan host. Molluscicides such as niclosamide were successfully used to control snail populations, but this approach might not be suitable from an ecological point of view, because of the risk of environmental impairment. Infection control might be attained with environmentally friendly approaches such as the use of natural molluscicides (*Eucalyptus* and *Euphorbiales* spp.), predator species (goose and ducks), competitor molluscs (non-host species of snails: *zonitoides nitidus*), and draining or fencing-off wet areas on pasture [35].

Taking all strategies into account, what is really important is to keep in mind both human and animal infections and choose the strategies appropriate for each region.

# 7 Treatment Options: Marketed and Experimental Drugs

## 7.1 Marketed Drugs

No new fasciocidals have been introduced on the market since the 1980s [63]. To date, the available drugs for veterinary use can be classified into the following five main chemical groupings (Fig. 6 A-E): halogenated phenols, salicylanilides, benzimidazoles, sulphonamides, and phenoxyalkanes [63]. Among all fasciocidals only the benzimidazole, triclabendazole (Egaten®) is recommended for human use [26, 64]. In addition, it is registered in only four countries worldwide [64]. In the past, bithionol was applied in humans, but it is no longer recommended, because of its prolonged treatment course (50 mg/kg/day for 20-30 days) with moderate to low cure rates and its frequent gastrointestinal adverse drug events [26, 64]. For comparison, a single oral dose of 10 mg/kg triclabendazole achieves an average worm burden reduction of more than 90% against all stages of *F. hepatica*. This broad activity of triclabendazole is unique, since no other fasciocidals show a similar broad efficacy and frequently lack activity against juvenile worms (Table 1). For instance, the activity of the related benzimidazole albendazole is also restricted to mature worms and multiple doses are needed to achieve a good response [63].

**Table 1.** Estimated stage activity of different fasciocidal drugs at recommended dose rates against *F. hepatica* in sheep. No activity Moderate activity High activity

adapted from [63] table 2

| Class | Anthelmintic | Stage specificity of anthelmintics against *F. hepatica* in sheep [age of fluke in weeks] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| **Halog. phenols** | Bithionol | | | | | | | | | | 50-70% | | 80-99% | | |
| **Salicylanilides** | Closantel | | | | | | | 50-90% | | | 91-99% | | | | |
| | Rafoxanide | | | | 50-90% | | | | | | 91-99% | | | | |
| **Benzimidazoles** | Albendazole | | | | | | | | | | 50-70% | | 80-99% | | |
| | Triclabendazole | | 90-99% | | | | | | | | 99-100% | | | | |
| **Sulophonamides** | Clorsulon | | | | | | | | | | 90-99% | | | | |
| **Phenoxyalkanes** | Diamphenethide | | 100-91% | | | | | | | | 80-50% | | | | |

Triclabendazole lacks activity against nematodes and cestodes in contrast to other benzimidazoles [63, 65]. Higher dosages of triclabendazole showed activity against different *Paragonimus* spp. (= lung fluke) [64]. A disadvantage of triclabendazole is that it accumulates in milk and therefore cannot be applied to lactating cows [28, 66]. Triclabendazole is extensively metabolized *in vivo* to triclabendazole-sulfoxide and -sulfone. Triclabendazole-sulfoxide is the main active component of triclabendazole. It has been suggested that triclabendazole-sulfone might also contribute to the fasciocidal activity [67]. The high treatment potency of triclabendazole might be of particular value in resource-constrained settings, where drug accessibility is limited and a simple treatment schedule is needed for a successful intervention. However, it is frightening that triclabendazole resistance has been documented from Australian sheep farms since the mid-1990s and thereafter also from western European countries including the United Kingdom, Netherlands, and Spain and recently also from Latin America in Argentina [67-69]. The mode of resistance is not yet fully understood; altered triclabendazole uptake and metabolism of resistant *F. hepatica* isolates are to date the most convincing mechanisms. In addition, resistant *F. hepatica* strains were observed against salicylanilides, closantel and rafoxanide [41, 63]. Fortunately, until now there is no evidence of drug resistance in human *F. hepatica* infections. Nevertheless, the risk is present, since human and veterinary fascioliasis are interlinked.

To conclude, there is a strong need of back-up drugs or effective drug combinations to overcome the dilemma of drug resistance and the narrow arsenal of potent fasciocidals.

## 7.2 Drug Screening Methods against *Fasciola hepatica*

It is not possible so far to cultivate *F. hepatica in vitro* throughout the entire life cycle [23], and consequently laboratory animals are indispensable for drug screening against fascioliasis. Therefore, ethical considerations such as replace, reduce and refine life-animal (3R) have to be integrated in the drug screening process [70]. As a rule, the most effective drug candidates should be evaluated first *in vitro* before testing the *in vivo* activity in rodents.

*In vitro* screening of *F. hepatica* can be accomplished against newly excysted metacercariae obtained from infected snails or against fluke's isolates from the liver and bile of experimentally or naturally infected animals [23]. *F. hepatica* infections of sheep and cattle are frequent in endemic countries (Section 4.2) and therefore parasite

material can be obtained from animals slaughtered in abattoirs [23, 71]. The drug effect on the viability of newly excysted metacercariae or of adult and juvenile flukes is commonly evaluated using laborious phenotype-based viability assay, where the motility of the flukes is monitored [23]. Calorimetric and colorimetric viability assays hold promise to become a more efficient alternative to phenotype-based assays, which enables an easier standardisation of the experiments [23, 72].

After *in vitro* screening, the most prominent drug candidates are selected for further investigations *in vivo*. Therefore, rats are infected with 20-30 metacercariae by oral gavage and treated 3-5 weeks (juvenile flukes) or 8-12 weeks (adult flukes) post infection. The drug activity can be evaluated one week post-treatment by analysing the worm burden reduction of treated compared to untreated controls [23]. The efficacy of drug candidates, which showed promising activity in the rat model, can be further evaluated in larger animals such as naturally or experimentally *F. hepatica* infected sheep. For more detail, the reader is referred to *chapter 4*.

Promising *in vitro* activities of lead compounds can sometimes not be translated to an *in vivo* efficacy, since pharmacokinetic properties of the molecules including poor bioavailability, extensive metabolism, and fast drug clearance might avert a potential effect [73]. Therefore, the analysis of the pharmacokinetic parameters of drug candidates is essential to understand a potential *in vivo* ineffectiveness and additionally important to improve the pharmacokinetic properties of second generation analogues. The reader is referred to *chapter 5* and *6*.

## 7.3 Experimental Drugs - Artemisinins

Artemisinin (qinghaosu), a secondary plant compound of the herb *Artemisia annua* (Chinese wormwood), is highly effective against *Plasmodia* spp. [74]. In 1971, the antimalarial activity of *A. annua* extracts was discovered and the chemical structure of the active constituent artemisinin was identified in the late 1970s [75]. Artemisinin has a sesquiterpene lactone scaffold that contains a peroxide bond in a unique 1,2,4, trioxane heterocycle (Fig. 6 F & G). Semi-synthetic derivatives such as artemether and artesunate were developed, because artemisinin itself has biopharmaceutical shortcomings including poor bioavailability and low solubility in water and oil [76]. The methyl ether derivative artemether (Fig. 6 F) is characterised by a stronger antiplasmodial activity than artemisinin. Furthermore, artemether can be applied orally

or intramuscularly in an oil-based drug formulation. Artesunate (Fig. 6 G) is soluble in water because of its succinate functional grouping, and can be applied intravenously and is therefore indispensable for the treatment of severe malaria [77].

Even though the semisynthetic artemisinins possess better biopharmaceutical properties than artemisinin itself, shortcomings including low bioavailability and short half-life are still apparent. Furthermore, there is the disadvantage of growing *Artemisia annua* as a starting material [77]. Hence, totally synthetic artemisinin-like peroxides such as 1,2,4-trioxolanes (=OZ compounds, Fig. 6 H) were produced which showed excellent antimalarial activity and improved biopharmaceutical proprieties [78].

The antiparasitic activity of the semisynthetic and synthetic artemisinins are not restricted to *Plasmodia spp*, given that *Schistosoma* spp. and several other food-borne trematodes are also affected by this compound class [37, 79]. The discovery of the trematocidal effect of artemisinin occurred in the early 1980s in China, where the trematocidal properties were first recorded for *Schistosoma japonicum* and *Clonorchis sinensis* [37, 80]. Later, further promising *in vitro* and *in vivo* activities of the semisynthetic and synthetic artemisinins (OZ78) were recorded against several *Schistosoma* spp. and major food-borne trematodes including *E. caproni*, *O. viverini*, and most important for this work *F. hepatica* [37].

Artesunate and artemether kill *F. hepatica* flukes *in vitro*, and damage the tegument and the gastrointestinal system of the parasite [81, 82]. The mode of action of the artemisinins against *Fasciola* spp. has not been elucidated. However, the presence of haemin in culture medium resulted in an increased *in vitro* activity of the artemisinins, which suggests that iron plays a role in the cleavage of the peroxide bond and the generation of free radicals, a mechanism similar to one of the proposed mechanisms of action of artemisinin against *Plasmodium* [81, 83]. Besides the *in vitro* activity, artesunate and artemether were active against *F. hepatica* in experimentally infected rats [84]. For more details, the reader is referred to *chapter 3*. It is promising that artemether and the synthetic artemisinin, OZ78, were active against triclabendazole resistant *F. hepatica* isolates in the rat model [85]. In light of the veterinary importance of fascioliasis, the activity of artemether was assessed in sheep harboring natural *F. hepatica* infections. Treatment outcome relied on the route of drug administration and the applied dosage. On the one hand artemether achieved good worm and egg burden

reductions >90% at 160 mg/kg given intramuscularly, while a 40 mg/kg dose and oral treatment lacked activity [86]. More details are given in *chapter 6*.

Finally, there is evidence that artesunate may also play a role in treatment of acute human fascioliasis [87].

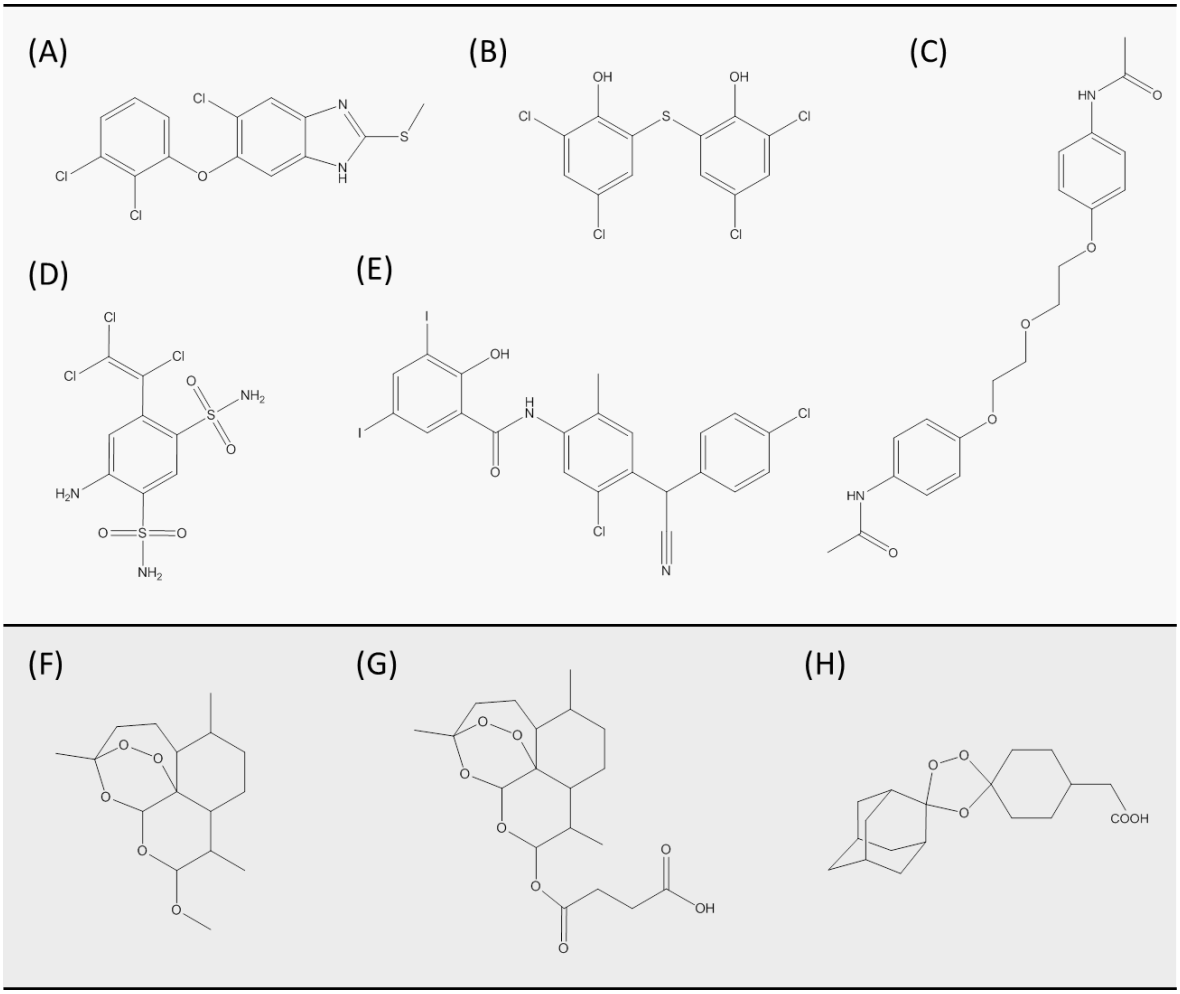



**Figure 6. Chemical structure of fasciocidal marketed drugs (A-E) and experimental fasciocidals (F-H).** adapted from [37, 88]

**(A)** Triclabendazole, **(B)** Bithionol, **(C)** Diamphenethide, **(D)** Clorsulon, **(E)** Closantel, **(F)** Artemether, **(G)** Artesunate, and **(G)** OZ 78

# 8 Aim and Objectives

There is a pressing need for discovery and development of novel drugs against fascioliasis, since to date no vaccine is available for the prevention of the disease, only a single drug, triclabendazole is recommended for treatment of human fascioliasis, and moreover triclabendazole resistance is a well-known global problem in veterinary medicine.

The following 5 objectives were accomplished to further strengthen our knowledge about treatment and diagnosis of fascioliasis.

1. To compare the sedimentation and FLOTAC techniques for the detection and quantification of *F. hepatica* eggs in rats (*chapter 2*).

2. To assess the potential of triclabendazole-peroxidic drug combination therapies in *F. hepatica* infected rats (*chapter 3*).

3. To study the anthelmintic activity of artesunate against *F. hepatica* in naturally infected sheep (*chapter 4*).

4. To develop and validate a sensitive and selective LC-MS/MS method for the simultaneous analysis of artemether, artesunate, and their major metabolites for the prospective determination of pharmacokinetic parameters (*chapter 5*).

5. To analyse pharmacokinetic parameters of artemether and artesunate in sheep naturally infected with *F. hepatica* (*chapter 6*).

# References


1. **Hotez, P.J., D.H. Molyneux, A. Fenwick, E. Ottesen, S.E. Sachs, and J.D. Sachs**, Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. Plos Med, 2007. **4**(9): p. 1553-1553.
2. **Keiser, J. and J. Utzinger**, Advances in the discovery and development of trematocidal drugs. Expert Opin Drug Dis, 2007. **2**: p. S9-S23.
3. **Hotez, P.J., D.H. Molyneux, A. Fenwick, J. Kumaresan, S.E. Sachs, J.D. Sachs, and L. Savioli**, Control of neglected tropical diseases. N Engl J Med, 2007. **357**(10): p. 1018-27.
4. **WHO**, Control of foodborne trematode infections. Report of a WHO Study Group. World Health Organ Tech Rep Ser, 1995. **849**: p. 1-157.
5. **Keiser, J. and J. Utzinger**, Chemotherapy for major food-borne trematodes: a review. Expert Opin Pharmacother, 2004. **5**(8): p. 1711-26.
6. **Anantaphruti, M.T.**, Parasitic contaminants in food. Southeast Asian J Trop Med Public Health, 2001. **32 Suppl 2**: p. 218-28.
7. **Gajadhar, A.A., W.B. Scandrett, and L.B. Forbes**, Overview of food- and water-borne zoonotic parasites at the farm level. Rev Sci Tech Oie, 2006. **25**(2): p. 595-606.
8. **Spithill, T.W., Smooker, P. M., Copeman, D.**, *Fasciola gigantica*: Epidemiology, Control, Immunology and Molecular Biology, in *Fasciolosis*, J.P. Dalton, Editor. 1999, CABI Publishing: Oxford (UK). p. 465-525.
9. **Fried, B., T.K. Graczyk, and L. Tamang**, Food-borne intestinal trematodiases in humans. Parasitol Res, 2004. **93**(2): p. 159-70.
10. **Keiser, J. and J. Utzinger**, Food-borne trematodiases. Clin Microbiol Rev, 2009. **22**(3): p. 466-83.
11. **Halton, D.W.**, Microscopy and the helminth parasite. Micron, 2004. **35**(5): p. 361-90.
12. **Robinson, M.W. and J.P. Dalton**, Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos T R Soc B, 2009. **364**(1530): p. 2763-2776.
13. **Mas-Coma, S., M.D. Bargues, and M.A. Valero**, Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol, 2005. **35**(11-12): p. 1255-78.
14. **Andrews, S.J.**, The life cycle of *Fasciola hepatica*, in *Fasciolosis* J.P. Dalton, Editor. 1999, CABI Publishing: Oxford (UK). p. 1–29.
15. **Esteban, J.G., A. Flores, R. Angles, W. Strauss, C. Aguirre, and S. Mas-Coma**, A population-based coprological study of human fascioliasis in a hyperendemic area of the Bolivian Altiplano. Trop Med Int Health, 1997. **2**(7): p. 695-9.
16. **Mas-Coma, S., R. Angles, J.G. Esteban, M.D. Bargues, P. Buchon, M. Franken, and W. Strauss**, The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. Tropical Medicine & International Health, 1999. **4**(6): p. 454-467.
17. **Neuhaus, W.**, [The chemical sense of miracidia of *Fasciola hepatica*]. Z Parasitenkd, 1953. **15**(6): p. 476-90.
18. **Krull, W.H.**, ed. The number of cercariae of *Fasciola hepatica* developing in snails infected with a single miracidium. *Proceedings of the Helminthological Society of Washington*, ed. J.R. Christie. 1941, The Helminthological Society of Washington: Washington. 55-58.
19. **Keiser, J. and J. Utzinger**, Emerging foodborne trematodiasis. Emerg Infect Dis, 2005. **11**(10): p. 1507-1514.
20. **Mas-Coma, S.**, Human fascioliasis, in *Waterborne zoonoses: identification, causes and control*, A.D. J.A. Cotruvo, G. Rees, J. Bartram, R. Carr, D.O. Cliver, G.F. Craun, R. Fayer and V.P.J. Gannon, Editor. 2004, IWA Publishing: London, UK. p. 305-322.

21. **CDC**. Parasites - Fascioliasis. 2011 [cited 2011 27.04.2011]; Available from: http://www.cdc.gov/parasites/fasciola/biology.html.
22. **Stein, M.** Animal-Health-Online "Leberegel" Survey 2003 [cited 2011 15.05.2011]; Available from: http://leberegel.de/.
23. **Keiser, J.**, *In vitro* and *in vivo* trematode models for chemotherapeutic studies. Parasitology, 2010. **137**(3): p. 589-603.
24. **Behm, C.A. and N.C. Sangster**, Pathology, Pathophysiology and Clinical Aspects, in *Fasciolosis* J.P. Dalton, Editor. 1999, CABI Publishing: Oxford (UK). p. 185-225.
25. **Chen, M.G. and K. Mott**, Progress in assessment of morbidity due to *Fasciola Hepatica* infection: A Review of Recent Literature. Tropical disease bulletin, 1990. **4**(87): p. R1-R38.
26. **WHO**, Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control. 2006: Geneva, Switzerland. p. 1-31.
27. **Haswell-Elkins, M.R., Levri, E.**, Food-borne Trematodes, in *Manson's Tropical Disease*, Z. Cook G, A, Editor. 2003, WB Saunders: London, UK. p. 1471-1486.
28. **Boray, J.C.** Liver fluke disease in sheep and cattle. Primefact 446 2007 30.04.2011]; Available from: http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0004/114691/liver-fluke-disease-in-sheep-and-cattle.pdf.
29. **Boray, J.C.**, Experimental fascioliasis in Australia. Adv Parasitol, 1969. **7**: p. 95-210.
30. **Dawes, B. and D.L. Hughes**, Fascioliasis: The Invasive Stages of *Fasciola hepatica* in Mammalian Hosts. Adv Parasitol, 1964. **2**: p. 97-168.
31. **Jennings, F.W.**, The anaemias of parasitic infections., in *Pathophysiology of Parasitic Infection*, E.J.L. Soulsby, Editor. 1976, Academic Press: New York, USA. p. 41-67.
32. **Mas-Coma, M.S., J.G. Esteban, and M.D. Bargues**, Epidemiology of human fascioliasis: a review and proposed new classification. Bull World Health Organ, 1999. **77**(4): p. 340-6.
33. **Garcia, H.H., P.L. Moro, and P.M. Schantz**, Zoonotic helminth infections of humans: echinococcosis, cysticercosis and fascioliasis. Current Opinion in Infectious Diseases, 2007. **20**(5): p. 489-494.
34. **Mas-Coma, S., M.A. Valero, and M.D. Bargues**, Chapter 2. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol, 2009. **69**: p. 41-146.
35. **Torgerson, P. and J. Claxton**, Epidemiology and Control, in *Fasciolosis*, J.P. Dalton, Editor. 1999, CABI Publishing: Oxford (UK). p. 113-149.
36. **Hopkins, D.R.**, Homing in on Helminths. Am J Trop Med Hyg, 1992. **46**(6): p. 626-634.
37. **Keiser, J. and J. Utzinger**, Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol, 2007. **23**(11): p. 555-62.
38. **Esteban, J.G., C. Gonzalez, F. Curtale, C. Munoz-Antoli, M.A. Valero, M.D. Bargues, M. el-Sayed, A.A. el-Wakeel, Y. Abdel-Wahab, A. Montresor, D. Engels, L. Savioli, and S. Mas-Coma**, Hyperendemic fascioliasis associated with schistosomiasis in villages in the Nile Delta of Egypt. Am J Trop Med Hyg, 2003. **69**(4): p. 429-37.
39. **Tran, V.H., T.K. Tran, H.C. Nguye, H.D. Pham, and T.H. Pham**, Fascioliasis in Vietnam. Southeast Asian J Trop Med Public Health, 2001. **32 Suppl 2**: p. 48-50.
40. **Rapsch, C., G. Schweizer, F. Grimm, L. Kohler, C. Bauer, P. Deplazes, U. Braun, and P.R. Torgerson**, Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. Int J Parasitol, 2006. **36**(10-11): p. 1153-8.
41. **Fairweather, I.**, Triclabendazole: new skills to unravel an old(ish) enigma. Journal of Helminthology, 2005. **79**(3): p. 227-234.

42. **Schweizer, G., U. Braun, P. Deplazes, and P.R. Torgerson**, Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet Rec, 2005. **157**(7): p. 188-93.
43. **Hawkins, C.D. and R.S. Morris**, Depression of Productivity in Sheep Infected with *Fasciola-Hepatica.* Vet Parasitol, 1978. **4**(4): p. 341-351.
44. **Bergquist, R., M.V. Johansen, and J. Utzinger**, Diagnostic dilemmas in helminthology: what tools to use and when? Trends Parasitol, 2009. **25**(4): p. 151-6.
45. **Lim, J.H., E. Mairiang, and G.H. Ahn**, Biliary parasitic diseases including clonorchiasis, opisthorchiasis and fascioliasis. Abdom Imaging, 2008. **33**(2): p. 157-65.
46. **Espinoza, J.R., O. Timoteo, and P. Herrera-Velit**, Fas2-ELISA in the detection of human infection by *Fasciola hepatica.* J Helminthol, 2005. **79**(3): p. 235-40.
47. **Rokni, M.B., J. Massoud, S.M. O'Neill, M. Parkinson, and J.P. Dalton**, Diagnosis of human fasciolosis in the Gilan province of Northern Iran: application of cathepsin L-ELISA. Diagn Micr Infec Dis, 2002. **44**(2): p. 175-179.
48. **Boulard, C., F. Carreras, and F. Van Gool**, Evaluation of nitroxynil and closantel activity using ELISA and egg counts against Fasciola hepatica in experimentally and naturally infected cattle. Vet Res, 1995. **26**(4): p. 249-55.
49. **Hillyer, G.V.**, Immunodiagnosis of Human and Animal Fasciolosis, in *Fasciolosis*, J.P. Dalton, Editor. 1999, CABI Publishing: Oxford (UK). p. 435-446.
50. **Ai, L., S.J. Dong, W.Y. Zhang, H.M. Elsheikha, Y.S. Mahmmod, R.Q. Lin, Z.G. Yuan, Y.L. Shi, W.Y. Huang, and X.Q. Zhu**, Specific PCR-based assays for the identification of *Fasciola* species: their development, evaluation and potential usefulness in prevalence surveys. Ann Trop Med Parasitol, 2010. **104**(1): p. 65-72.
51. **Cawdery, M.J. and M. Ruane**, Sedimentation method for the demonstration of the eggs of *Fasciola hepatica* in faeces. Lab Pract, 1971. **20**(12): p. 935-9.
52. **Katz, N., A. Chaves, and J. Pellegrino**, A simple device for quantitative stool thick-smear technique in Schistosomiasis mansoni. Rev Inst Med Trop Sao Paulo, 1972. **14**(6): p. 397-400.
53. **Wood, I.B., N.K. Amaral, K. Bairden, J.L. Duncan, T. Kassai, J.B. Malone, J.A. Pankavich, R.K. Reinecke, O. Slocombe, S.M. Taylor, and J. Vercruysse**, World-Association-for-the-Advancement-of-Veterinary-Parasitology (Waavp) 2nd Edition of Guidelines for Evaluating the Efficacy of Anthelmintics in Ruminants (Bovine, Ovine, Caprine). Vet Parasitol, 1995. **58**(3): p. 181-213.
54. **Rapsch, C., G. Schweizer, F. Grimm, L. Kohler, C. Bauer, P. Deplazes, U. Braun, and P.R. Torgerson**, Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. International Journal for Parasitology, 2006. **36**(10-11): p. 1153-1158.
55. **Cringoli, G., L. Rinaldi, M.P. Maurelli, and J. Utzinger**, FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat Protoc, 2010. **5**(3): p. 503-15.
56. **McManus, D.P. and J.P. Dalton**, Vaccines against the zoonotic trematodes Schistosoma japonicum, *Fasciola hepatica* and *Fasciola gigantica.* Parasitology, 2006. **133 Suppl**: p. S43-61.
57. **Marcos, L., V. Maco, A. Terashima, F. Samalvides, J.R. Espinoza, and E. Gotuzzo**, Fascioliasis in relatives of patients with *Fasciola hepatica* infection in Peru. Rev Inst Med Trop Sao Paulo, 2005. **47**(4): p. 219-22.
58. **Marcos, L., V. Maco, F. Samalvides, A. Terashima, J.R. Espinoza, and E. Gotuzzo**, Risk factors for *Fasciola hepatica* infection in children: a case-control study. Trans R Soc Trop Med Hyg, 2006. **100**(2): p. 158-66.
59. **Taira, N., H. Yoshifuji, and J.C. Boray**, Zoonotic potential of infection with *Fasciola* spp. by consumption of freshly prepared raw liver containing immature flukes. International Journal for Parasitology, 1997. **27**(7): p. 775-779.

60. **Curtale, F., Y. Abd El-Wahab Hassanein, A. El Wakeel, S. Mas-Coma, and A. Montresore**, Distribution of human fascioliasis by age and gender among rural population in the Nile Delta, Egypt. J Trop Pediatr, 2003. **49**(5): p. 264-8.
61. **Taylor, S.M., S.A. Langridge, and J. Kenny**, Anthelmintic suppression of *Fasciola hepatica* infections in sheep. Vet Rec, 1994. **135**(4): p. 86-8.
62. **Fawcett, A.R.**, A study of a restricted programme of strategic dosing against *Fasciola hepatica* with triclabendazole. Vet Rec, 1990. **127**(20): p. 492-3.
63. **Fairweather, I. and J.C. Boray**, Fasciolicides: efficacy, actions, resistance and its management. Vet J, 1999. **158**(2): p. 81-112.
64. **Keiser, J., D. Engels, G. Buscher, and J. Utzinger**, Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Investig Drugs, 2005. **14**(12): p. 1513-26.
65. **Coles, G.C.**, Anthelmintic activity of triclabendazole. J Helminthol, 1986. **60**(3): p. 210-2.
66. **Imperiale, F., P. Ortiz, M. Cabrera, C. Farias, J.M. Sallovitz, S. Iezzi, J. Perez, L. Alvarez, and C. Lanusse**, Residual concentrations of the flukicidal compound triclabendazole in dairy cows' milk and cheese. Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 2011. **28**(4): p. 438-45.
67. **Fairweather, I.**, Triclabendazole progress report, 2005-2009: an advancement of learning? J Helminthol, 2009. **83**(2): p. 139-50.
68. **Overend, D.J. and F.L. Bowen**, Resistance of *Fasciola hepatica* to triclabendazole. Aust Vet J, 1995. **72**(7): p. 275-6.
69. **Olaechea, F., V. Lovera, M. Larroza, F. Raffo, and R. Cabrera**, Resistance of Fasciola hepatica against triclabendazole in cattle in Patagonia (Argentina). Vet Parasitol, 2011.
70. **Autissier, C.**, [Ethical review in biomedical research]. Med Sci (Paris), 2008. **24**(4): p. 437-42.
71. **Shalaby, H.A., A.H. El Namaky, and R.O. Kamel**, *In vitro* effect of artemether and triclabendazole on adult *Fasciola gigantica.* Vet Parasitol, 2009. **160**(1-2): p. 76-82.
72. **Kirchhofer, C., M. Vargas, O. Braissant, Y. Dong, X. Wang, J.L. Vennerstrom, and J. Keiser**, Activity of OZ78 analogues against *Fasciola hepatica* and *Echinostoma caproni.* Acta Trop, 2011. **118**(1): p. 56-62.
73. **Ruiz-Garcia, A., M. Bermejo, A. Moss, and V.G. Casabo**, Pharmacokinetics in drug discovery. J Pharm Sci, 2008. **97**(2): p. 654-90.
74. **Klayman, D.L.**, Qinghaosu (Artemisinin) - an Antimalarial Drug from China. Science, 1985. **228**(4703): p. 1049-1055.
75. **Hsu, E.**, Reflections on the 'discovery' of the antimalarial qinghao. Br J Clin Pharmacol, 2006. **61**(6): p. 666-70.
76. **Medhi, B., S. Patyar, R.S. Rao, D.S.P. Byrav, and A. Prakash**, Pharmacokinetic and toxicological profile of artemisinin compounds: an update. Pharmacology, 2009. **84**(6): p. 323-32.
77. **White, N.J.**, Qinghaosu (artemisinin): the price of success. Science, 2008. **320**(5874): p. 330-4.
78. **Vennerstrom, J.L., S. Arbe-Barnes, R. Brun, S.A. Charman, F.C. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. Santo Tomas, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin, and W.N. Charman**, Identification of an antimalarial synthetic trioxolane drug development candidate. Nature, 2004. **430**(7002): p. 900-4.
79. **Utzinger, J., S.H. Xiao, M. Tanner, and J. Keiser**, Artemisinins for schistosomiasis and beyond. Curr Opin Investig Drugs, 2007. **8**(2): p. 105-16.

80. **Le, W.J., J.Q. You, Y.Q. Yang, J.Y. Mei, H.F. Guo, H.Z. Yang, and C.W. Zhang**, [Studies on the efficacy of artemether in experimental schistosomiasis (author's transl)]. Yao Xue Xue Bao, 1982. **17**(3): p. 187-93.
81. **Keiser, J. and G. Morson**, *Fasciola hepatica*: Tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. Experimental Parasitology, 2008. **118**(2): p. 228-237.
82. **O'Neill, J.F., R.C. Johnston, L. Halferty, G.P. Brennan, J. Keiser, and I. Fairweather**, Adult triclabendazole-resistant *Fasciola hepatica*: morphological changes in the tegument and gut following *in vivo* treatment with artemether in the rat model. J Helminthol, 2009. **83**(2): p. 151-63.
83. **Meshnick, S.R.**, Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol, 2002. **32**(13): p. 1655-60.
84. **Keiser, J., X. Shu-Hua, M. Tanner, and J. Utzinger**, Artesunate and artemether are effective fasciolicides in the rat model and *in vitro.* J Antimicrob Chemother, 2006. **57**(6): p. 1139-45.
85. **Keiser, J., J. Utzinger, J.L. Vennerstrom, Y. Dong, G. Brennan, and I. Fairweather**, Activity of artemether and OZ78 against triclabendazole-resistant Fasciola hepatica. Trans R Soc Trop Med Hyg, 2007. **101**(12): p. 1219-22.
86. **Keiser, J., L. Rinaldi, V. Veneziano, L. Mezzino, M. Tanner, J. Utzinger, and G. Cringoli**, Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol Res, 2008. **103**(3): p. 517-22.
87. **Hien, T.T., N.T. Truong, N.H. Minh, H.D. Dat, N.T. Dung, N.T. Hue, T.K. Dung, P.Q. Tuan, J.I. Campbell, J.J. Farrar, and J.N. Day**, A randomized controlled pilot study of artesunate versus triclabendazole for human fascioliasis in central Vietnam. Am J Trop Med Hyg, 2008. **78**(3): p. 388-392.
88. **Fairweather, I. and J.C. Boray**, Mechanisms of Fasciolicide Action and Drug Resistance in *Fasciola hepatica*, in *Fascioliosis*, J.P. Dalton, Editor. 1999, CABI: Oxford (UK). p. 225-276.

# *Chapter 2*

# *Fasciola hepatica*: Comparison of the Sedimentation and FLOTAC Techniques for the Detection and Quantification of Faecal Egg Counts in Rats

Urs Duthaler [a], Laura Rinaldi [b], Maria P. Maurelli [b], Mireille Vargas [a], Jürg Utzinger [c], Giuseppe Cringoli [b], Jennifer Keiser [a]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

[b] Department of Pathology and Animal Health, CREMOPAR Regione Campania, University of Naples Federico II, Naples, Italy

[c] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

**Published in Experimental Parasitology 126 (2010): p. 161-166**

Experimental Parasitology 126 (2010) 161–166

Contents lists available at ScienceDirect

Experimental Parasitology

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

# *Fasciola hepatica*: Comparison of the sedimentation and FLOTAC techniques for the detection and quantification of faecal egg counts in rats

Urs Duthaler [a,b], Laura Rinaldi [c], Maria P. Maurelli [c], Mireille Vargas [a,b], Jürg Utzinger [b,d], Giuseppe Cringoli [c], Jennifer Keiser [a,b,*]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland
[b] University of Basel, P.O. Box, CH-4003 Basel, Switzerland
[c] Department of Pathology and Animal Health, CREMOPAR Regione Campania, University of Naples 'Federico II', Via della Veterinaria 1, 80137 Naples, Italy
[d] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland

ARTICLE INFO

*Article history:*
Received 11 January 2010
Received in revised form 26 February 2010
Accepted 20 April 2010
Available online 29 April 2010

*Keywords:*
*Fasciola hepatica*
Diagnosis
Sedimentation
FLOTAC
Sensitivity
Faecal egg count
Efficiency

ABSTRACT

We compared the sedimentation and FLOTAC techniques for the detection and quantification of *Fasciola hepatica* eggs in faecal samples obtained from 120 experimentally-infected rats before intervention, and in 42 rats after drug administration. Additionally, the average time for a single test was determined. A single FLOTAC showed a higher sensitivity (92.6%) than 2, 4 and 8 sedimentation readings (63.0–85.2%) for detecting *F. hepatica* eggs in rat faeces post-treatment. On average, it took 21 min to prepare and examine a single FLOTAC, whereas 114 min were needed for the sedimentation method including the reading of 8 slides. In both treated and untreated rats, the sedimentation method resulted in higher mean faecal egg counts (FECs) than FLOTAC ($P < 0.05$). In view of the high sensitivity and efficiency, the FLOTAC technique holds promise for experimental work in the *F. hepatica*-rat model. Additional research is needed to determine the reasons for the observed differences in FECs.



## 1. Introduction

Fascioliasis (fasciolosis) is caused by the trematodes *Fasciola hepatica* and *Fasciola gigantica*. These trematodes parasitise the liver of humans and a host of domestic animals (e.g., sheep and cattle) and wild animals (e.g., rabbits, beavers, deer and rats) (Robinson and Dalton, 2009). Fascioliasis exhibits an exceptionally large latitudinal, longitudinal and altitudinal distribution. Indeed, human infections have been reported from 5 continents in 51 countries (Garcia et al., 2007; Mas-Coma et al., 2009). Recent estimates suggest that more than 90 million people are at risk of fascioliasis and between 2.4 and 17 million individuals are infected (Keiser and Utzinger, 2009). However, not all countries have established reporting systems and recent fascioliasis outbreaks were not considered in these estimates, and hence the 'true' number of *Fasciola* infections might be considerably higher (Mas-Coma et al., 2009). The worldwide incidence of fascioliasis in sheep and cattle is more than 250 and 300 million, respectively, causing an estimated economic loss of US$ 3 billion to the agricultural sector each year through losses of milk and meat yields (Mas-Coma et al., 2005; Robinson and Dalton, 2009).

There is a need for readily available, accurate, sensitive and efficient diagnostic tools, so that the true extent and global burden of fascioliasis can be estimated, and the impact of interventions quantified and monitored (Bergquist et al., 2009; Keiser and Utzinger, 2009). Today's spectrum of ante-mortem tools to diagnose infections with *Fasciola* spp. ranges from copromicroscopic techniques to immunodiagnostics (e.g., enzyme-linked immunosorbant assay (ELISA)) and molecular diagnostics (e.g., polymerase chain reaction (PCR)) (Mas-Coma et al., 2007; Keiser and Utzinger, 2009). Several immuno and molecular assays have been developed, which are able to detect low-infection intensities with a high sensitivity. However, immuno and molecular methods fail to distinguish between current or past infections, as well as infection intensities. Cross-reactivity and low specificity are further issues regarding immunodiagnostic assays.

Traditional copromicroscopic techniques, such as the Kato–Katz method (Katz et al., 1972), sedimentation (Cawdery and Ruane, 1971; Ash and Orihel, 1987; Conceicao et al., 2002) and formalin-ethyl-acetate techniques (Allen and Ridley, 1970) are reasonably sensitive to detect moderate and high *Fasciola* spp. infection intensities, but the methods lack sensitivity for detecting low-intensity infections. In contrast to immunodiagnosis, copromicroscopy is not

* Corresponding author at: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. Fax: +41 61 284 8105.
*E-mail address:* jennifer.keiser@unibas.ch (J. Keiser).

0014-4894/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2010.04.020

applicable in the prepatent stage of fascioliasis. Advantages of copromicroscopic techniques are their low costs and relative ease of performance, which render them widely applicable in resource-constraint settings.

A series of novel multivalent faecal egg count (FEC) techniques – the FLOTAC techniques – have been developed for the diagnosis of human and veterinary parasitic infections (Cringoli, 2006; Cringoli et al., 2010). The methods make use of the FLOTAC apparatus and the principle of flotation of parasitic elements and sedimentation of faecal debris during centrifugation. A single FLOTAC uses up to 1 g of stool per analysis, and hence the sensitivity of this method for low-intensity helminth infections is higher than, for example, the Kato–Katz method, which analyses only 41.7 mg of stool (Utzinger et al., 2008; Knopp et al., 2009a,b).

The aim of this study was to compare the sedimentation technique (considered as a reference coprodiagnostic method) with the recently developed FLOTAC techniques for the detection and quantification of *F. hepatica* eggs in faecal samples obtained from experimentally-infected rats before and after administration of anthelminthic drugs. In addition, operational and practical issues (e.g., time requirements to perform a single test) were investigated.

## 2. Materials and methods

### 2.1. Animals and ethical clearance

Three-week-old female Wistar rats ($n = 120$, weight ~100 g) were purchased from Harlan (Itingen, Switzerland). The rats were kept in groups of five in type-3 Makrolon cages under environmentally-controlled conditions (temperature ~25 °C, humidity ~70%, light/dark cycle 12/12 h). Rats had free access to water and rodent food (Rodent Blox from Eberle NAFAG; Gossau, Switzerland). Animals were examined daily on general wellbeing, adhering to standard procedures at the Swiss Tropical and Public Health Institute. The current study was approved by the local veterinary agency based on Swiss cantonal and national regulations (permission no. 2070).

### 2.2. Parasite, infection and faecal sampling

*F. hepatica* metacercariae (Pacific Northwest, wild strain) were purchased from Baldwin Aquatics (Monmouth, USA). After 1 week of acclimatisation, 120 rats were infected with 22 ± 2 metacercariae each by oral gavage as part of a large combination chemotherapy experiment. A single faecal sample was collected from each rat in the period 12–18 weeks post-infection.

In addition, 42 rats were subjected to experimental chemotherapy (20–32 weeks post-infection) and faecal samples collected 1 week post-treatment. The objective was to obtain faecal samples from rats with no or only low numbers of *Fasciola* eggs.

For the collection of stool, rats were housed individually for two nights to obtain a large amount of stool (~5 g). The dry faecal pellets were collected and homogenised with mortar and pistil. One part of the homogenate was subjected to the FLOTAC double technique (Cringoli et al., 2010), whereas the other part was employed for the sedimentation method, adhering to a standard operating procedure (SOP) used at the Swiss Tropical and Public Health Institute (see below).

### 2.3. Chemicals

Zinc sulphate heptahydrate ($ZnSO_4$–$7H_2O$) and sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany) and FLUKA (Buchs, Switzerland), respectively. Deionised water was produced on a milli-Q purification system (Milli-Q Advantage A10; MA; USA). After a prior calibration study, we used flotation solution 8 (FS8) from a panel of 14 different FS (Cringoli et al., 2004, 2010). FS8 contains 685 g $ZnSO_4$–$7H_2O$ and 685 ml deionised $H_2O$ and has a specific gravity (s.g.) of 1.35.

### 2.4. Sedimentation method

The sedimentation method was carried out according to a SOP. In brief, 1 g of dry faecal homogenate was suspended in 20 ml 0.9% (m/V) NaCl and incubated for 1 h. The faecal suspension was filtered into a cone glass through a screen (aperture: 250 μm) coated with triple layered medical gauze. One-hundred and eighty millilitre of 0.9% (m/V) NaCl was used to rinse the residue. The faecal suspension was allowed to stand for 1 h for sedimentation of *F. hepatica* eggs. Subsequently, the supernatant was decanted and the sediment transferred and weighted in a 15 ml conic tube. Eight samples (30 μl each) of the sediment were placed on microscope slides under a cover slip (21 × 26 mm) and examined under a light microscope at a 100× magnification (Laborlux K Leitz; Wetzlar, Germany). The number of eggs per gram of stool (EPG) was estimated according to the following formula:

$$\mathrm{EPG} = \left(\frac{M_{(tot)}}{M_{(a)}}\right) \cdot N_{(a)},$$

where $M_{(tot)}$ is the total weight of faeces suspension (expressed in g), $M_{(a)}$ is the weight of the analysed faecal suspension (expressed in g) and $N_{(a)}$ is the number of eggs counted under the microscope. *F. hepatica* FECs were estimated following the reading of 2, 4 and 8 sedimentation slides.

### 2.5. FLOTAC double technique

The FLOTAC double technique was used with details of the protocol presented elsewhere (Cringoli et al., 2010). In brief, two different faecal samples were examined simultaneously using a single FLOTAC apparatus. Hence, two faecal samples were assigned to the two flotation chambers of the FLOTAC apparatus, using the same FS. Two gram of dry faecal homogenate was suspended in tap water with a dilution ratio ranging between 1:50 and 1:200 (depending on the *F. hepatica* infection intensity) and incubated for 1 h. The suspension was homogenised, filtered through a wire mesh (aperture: 250 μm) and the debris was discarded. Six millilitre of the filtered suspension was placed into a conic tube and centrifuged (Eppendorf 5810 R; Hamburg, Germany) at 170*g* for 3 min at room temperature. The supernatant was discarded and the faecal pellet was re-suspended in 6 ml of FS8. The s.g. of FS8 was checked with a hydrometer at room temperature.

The resulting agitated suspension was then taken up by a pipette to load one of the two chambers of the FLOTAC (volume of each chamber: 5 ml). The apparatus was then centrifuged at 120*g* for 5 min (Jouan B4i Multifunction; St. Nazaire, France) and translated. The multiplication factor (Ø = 20, dilution/volume) ranged from 10 (dilution/volume: 50/5) to 40 (dilution/volume: 200/5). *F. hepatica* eggs were counted under a light microscope (Laborlux K Leitz; Wetzlar, Germany) along grids in the reading disc at a 100× magnification.

### 2.6. Sample processing time

The sample processing time of the two techniques was measured, using multiple replicates. Our base-case scenario was how long it takes to have a single stool sample examined either with 8 sedimentations or a single FLOTAC. The weighting, incubation and filtration of the stool preparation are equal for both methods. The time of filtration and weighting was measured 5 times. The FLOTAC preparation consists of the assembly, loading and cleaning

of the apparatus and was determined 5 times. The reading time of 1 FLOTAC chamber and 1 sedimentation slide was estimated based on 30 replicates for each method.

### 2.7. Statistical analysis

Statistical analysis was done with version 2.7.2 of StatsDirect statistical software (StatsDirect Ltd.; Cheshire, UK) and version 9.0 of STATA (Stata Corp.; College Station, TX, USA). The arithmetic mean EPG of both techniques were compared for paired data using the non-parametric Wilcoxon's signed ranks test. For unpaired data, the Kruskal–Wallis test was used. The coefficient of variation (CV), which is the standard deviation (SD) in percentage of the mean, was calculated for both techniques based on all analysed untreated rat samples. The agreement between the two diagnostic techniques was assessed using the kappa ($\kappa$) statistics (Landis and Koch, 1977). The following cut-offs were used: $\kappa < 0$, no agreement; $\kappa = 0.01–0.2$, poor agreement; $\kappa = 0.21–0.4$, fair agreement; $\kappa = 0.41–0.6$, moderate agreement; $\kappa = 0.61–0.8$, substantial agreement; and $\kappa = 0.81–1.0$, almost perfect agreement. The combined results from the two diagnostic methods were considered as diagnostic 'gold' standard. This assumption maximises specificity (i.e., 100%) and allows quantification of the sensitivity (proportion of 'true' positives in the population) and negative predictive value (NPV; proportion of rats with a negative test result that are correctly diagnosed) of each method.

## 3. Results

### 3.1. Sample population

Of the 120 rats experimentally infected with *F. hepatica* metacercariae, 7 rats died before the establishment of a chronic infection within 8–12 weeks post-infection. A single stool sample was obtained from the remaining 113 rats (in the period 12–18 weeks post-infection), and was subjected to a single FLOTAC and 8 sedimentation readings. Faecal samples were again obtained from 42 rats, 1 week after they had undergone treatment with experimental trematocidal drugs. Stool samples were subjected to a single FLOTAC and multiple sedimentation readings (2, 4 and 8 readings).

### 3.2. Comparison of sedimentation and FLOTAC

#### 3.2.1. Untreated rats

No *F. hepatica* eggs were observed in 6 faecal samples neither by FLOTAC nor multiple sedimentations, and hence the experimental infection in these 6 rats was considered unsuccessful. Fig. 1 shows FECs of the remaining 107 rats before anthelminthic drug administration, determined with either 8 sedimentation readings or a single FLOTAC. Both methods detected all 107 faecal samples as positive. A mean FEC of 10,051 EPG (range: 106–24,850 EPG; 95% CI: 8969–11,133 EPG) was estimated after subjecting each of the 107 faecal rat samples to 8 sedimentation readings. Had only 2 sedimentations been performed on each faecal sample, the mean FEC would have been slightly higher (10,196 EPG; range: 0–27,538 EPG; 95% CI: 9040–11,351 EPG). A single FLOTAC revealed a mean of 6756 EPG (range: 6–20,000 EPG, 95% CI: 5904–7608 EPG). Hence the mean FEC of 8 sedimentations was significantly higher than that of a single FLOTAC ($P < 0.001$). Both methods resulted in similar CVs; 65% for FLOTAC and 56% for the sedimentation method.

The 113 rats were classified into the following infection intensity groups: (i) no infection (no eggs found); (i) low (FEC: 1–999 EPG); (iii) moderate (FEC: 1000–9999 EPG); and (iv) heavy (FEC: $\geqslant$10,000 EPG). Table 1 shows the results obtained with both methods after stratification into these infection intensity classes. Whilst three-quarter (74.3%) of the animals were classified as lightly or moderately infected according to a single FLOTAC analysis, 8 sedimentation readings classified half (49.5%) of the rats in the low and moderate infection intensity groups. In case only 2 or 4 sedimentation slides per stool sample were examined, the respective percentage was 51.3% and 47.8%. In addition, the *F. hepatica* infection of 1 rat was not detected, if only 2 or 4 sedimentation slides were examined. According to a single FLOTAC or 8 sedimentation readings, 20.3% and 45.1%, of the rats, respectively, were determined as heavily infected.

#### 3.2.2. Treated rats

Taken together, a single FLOTAC and 8 sedimentation readings detected 27 out of 42 rats (64%) with *F. hepatica* eggs in their stool after experimental chemotherapy (Fig. 2). The results for both diagnostic methods combined were considered as diagnostic 'gold' standard. A single FLOTAC revealed *F. hepatica* eggs in 25 faecal samples, resulting in a sensitivity of 92.6%. Eight sedimentation slides revealed 23 *F. hepatica*-positive rats, resulting in a sensitivity of 85.2%. Examination of only 2 or 4 sedimentations resulted in considerably lower sensitivities; 63.0% and 77.8%, respectively.

A mean FEC of 108 EPG (range: 19–407 EPG, 95% CI: 70–146 EPG) and 176 EPG (range: 27–435 EPG, 95% CI: 123–229 EPG) were recorded for a single FLOTAC and 8 sedimentation slides, respectively. The difference in these mean EPG values showed statistical significance ($P = 0.019$). Examination of 2 rather than all 8

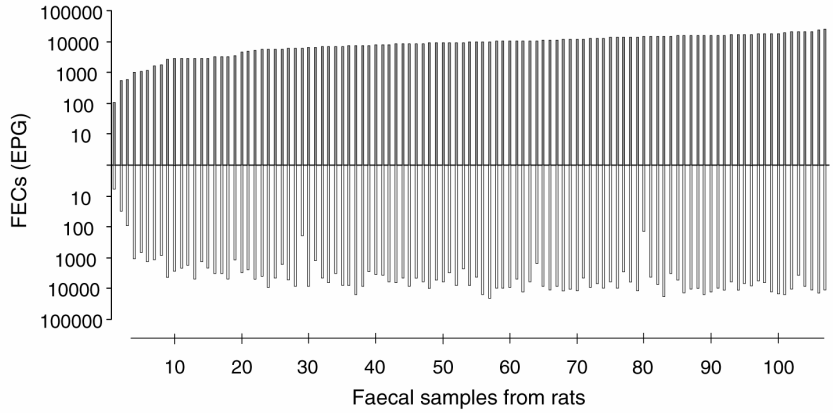



**Fig. 1.** *Fasciola hepatica* faecal egg counts (FECs) of 107 untreated rats determined with a single FLOTAC and 8 sedimentation readings. FLOTAC: white bars; sedimentation: grey bars.

**Table 1**
*Fasciola hepatica* infection intensities of 113 rats determined by the FLOTAC and sedimentation method.

| Infection intensity (EPG) | FLOTAC | | | Sedimentation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 chamber | | | 8 slides | | | 4 slides | | | 2 slides | | |
| | No. | % | Mean EPG | No. | % | Mean EPG | No. | % | Mean EPG | No. | % | Mean EPG |
| No infection (0) | 6 | 5.3 | – | 6 | 5.3 | – | 7 | 6.2 | – | 7 | 6.2 | – |
| Low infection (1–999) | 7 | 6.2 | 277 | 4 | 3.5 | 559 | 3 | 2.7 | 490 | 4 | 3.5 | 591 |
| Moderate infection (1000–9999) | 77 | 68.1 | 5402 | 52 | 46.0 | 6054 | 51 | 45.1 | 6026 | 54 | 47.8 | 6310 |
| Heavy infection (⩾10,000) | 23 | 20.3 | 13,263 | 51 | 45.1 | 14,872 | 52 | 46.0 | 14,992 | 48 | 42.5 | 15,581 |

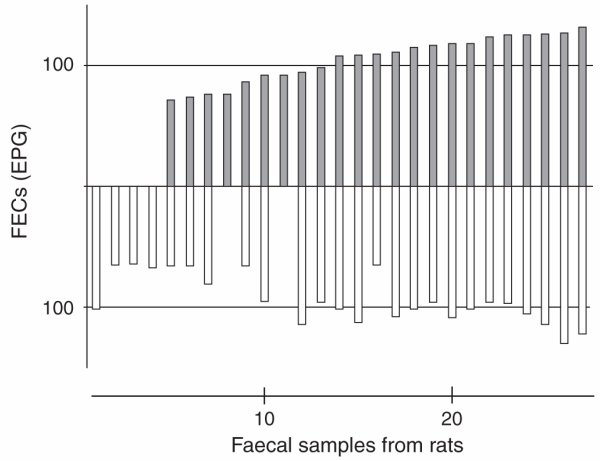



**Fig. 2.** *Fasciola hepatica* faecal egg counts (FECs) of 27 treated rats determined with a single FLOTAC and 8 sedimentation readings. FLOTAC: white bars; sedimentation: grey bars.

sedimentation slides revealed a mean FEC of 263 E (range: 109–531 EPG, 95% CI: 199–327 EPG).

Whilst a single FLOTAC had a NPV of 88.2% (95% CI: 63.6–98.5%), 8 sedimentation slides resulted in a NPV of 79.0% (95% CI: 54.4–94.0%). The respective NPVs using only 2 or 4 sedimentations were 60.0% (95% CI: 38.7–78.9%) and 71.4% (95% CI: 47.8–88.7%), respectively.

The kappa agreement between a single FLOTAC and 8 sedimentation slides was 0.71 ($P < 0.001$), hence showed a substantial agreement. Reading only 2 or 4 sedimentation slides also resulted in substantial agreement with a single FLOTAC ($\kappa = 0.63$ for 2 and $\kappa = 0.71$ for 4 sedimentation readings) (Table 2).

### 3.3. Sample processing time

The faecal sample processing steps and the required time for the sequential steps are illustrated in Fig. 3. Briefly, the time needed to prepare a faecal sample, to read it under a microscope and clean the devices thereafter was calculated for 8 sedimentation slides (needed for high sensitivity; see Table 2) and a single FLOTAC analysis. Overall an estimated 21 and 114 min were required to determine FECs with a single FLOTAC and 8 sedimentation slides, respectively. The weighting, incubation and filtration of the stool are equal for both methods (incubation: 60 min, filtration and weighting: 4:16 min; $n = 5$). After the stool had been filtered, it took ~17 min to prepare the FLOTAC. In more detail, the FLOTAC preparation steps included two centrifugation steps of 3 and 5 min, the assembly, loading and cleaning of 1 FLOTAC chamber took, on average, 3:16 min ($n = 5$), and an estimated 5:27 min were necessary to read 1 FLOTAC chamber under a microscope ($n = 30$). On the other hand, following the filtration of rat stool, the sedimentation method required an estimated 110 min to analyse one faecal sample (60 min for the sedimentation process and 50 min for reading all 8 slides).

## 4. Discussion

Coprological methods continue to be the most widely used approach for the detection and quantification of *Fasciola* and other helminth eggs in laboratory animals, domestic and sylvatic animals and humans (Cringoli et al., 2004, 2010; Bergquist et al., 2009). In the present study, we compared the current method of choice – i.e., sedimentation technique (Cawdery and Ruane, 1971; Ash and Orihel, 1987; Conceicao et al., 2002) – with a more recently developed method that is currently undergoing broad-scale validation – i.e., the FLOTAC techniques (Cringoli et al., 2010) – for the detection and quantification of *F. hepatica* eggs in faeces obtained from experimentally-infected rats before and after drug administration. FLOTAC has been used successfully in the diagnosis of *F. hepatica* infections in naturally-infected sheep, which underwent treatment with standard (Cringoli et al., 2006) and experimental drugs (Keiser et al., 2008, 2009). Considering the post-treatment faecal samples, we found that a single FLOTAC is more sensitive than multiple sedimentation readings. Additionally, a single FLOTAC test is 5–6 times faster than preparing and reading 8 sedimentation slides, which is necessary to obtain a reasonable sensitivity for *F. hepatica* diagnosis, particularly when FECs are low. However, multiple sedimentation readings resulted in significantly higher *F. hepatica* FECs in rats prior to and after experimental treatment.

The following issues are offered for discussion. First, we observed a lower sensitivity of the sedimentation technique than FLOTAC in detecting *F. hepatica* eggs in faecal samples obtained

**Table 2**
Diagnostic performance of FLOTAC and 2, 4 or 8 sedimentations in 42 *F. hepatica*-infected rats, which had undergone experimental anthelminthic treatment.

| Method | No. of tests performed | Multiplication factor | Sensitivity (95% CI) | NPV (95% CI) | EPG (95% CI) | $\kappa$ agreement |
|---|---|---|---|---|---|---|
| FLOTAC | 1 chamber | 20 | 92.6 (75.7–99.1) | 88.2 (63.6–98.5) | 108 (70–146) | – |
| Sedimentation | 8 slides | 35 | 85.2 (66.3–95.8) | 79.0 (54.4–94.0) | 176 (123–229) | 0.71* |
| Sedimentation | 4 slides | 70 | 77.8 (57.7–91.4) | 71.4 (47.8–88.7) | 207 (150–264) | 0.71* |
| Sedimentation | 2 slides | 140 | 63.0 (42.4–80.6) | 60.0 (38.7–78.9) | 263 (199–327) | 0.63* |

CI, confidence interval; EPG, eggs per gram of stool; NPV, negative predictive value.
* $P < 0.001$.

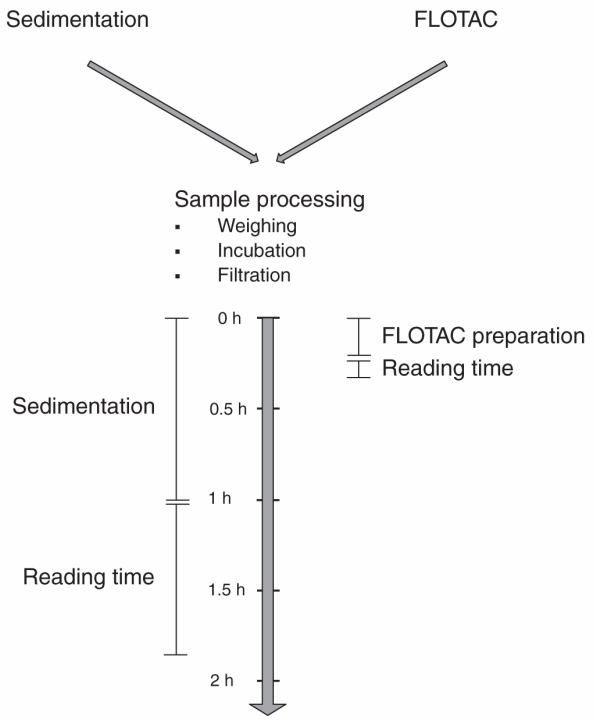



**Fig. 3.** Sample processing steps calculated for sedimentation and FLOTAC. Left-hand side: sedimentation; right-hand side: FLOTAC.

from experimentally-infected rats following experimental chemotherapy. Whilst a single FLOTAC failed to detect 2 infections amongst 42 treated-rats (sensitivity of 92.6%) an intensive sedimentation approach with 8 slides examined microscopically failed to detect 4 *F. hepatica* infections. A less rigorous sedimentation effort with only 2 or 4 slides examined further compromised the overall sensitivity to 63.0% and 77.8%, respectively. Prior to treatment when FECs were generally high, examination of 2 or 4 sedimentation slides resulted in one false negative.

Our findings are in agreement with previous studies for the diagnosis of human helminth infections; a single FLOTAC was more sensitive than duplicate or triplicate Kato–Katz thick smears for detecting low-intensity soil-transmitted helminth infections (Utzinger et al., 2008; Knopp et al., 2009a,b). FLOTAC also showed a superior diagnostic sensitivity to the ether-concentration technique in diagnosing human hookworm infections (Utzinger et al., 2008). Regarding animal infections, FLOTAC revealed a higher sensitivity than the McMaster, the Parasep Solvent Free and an ether-based concentration technique in detecting *Trichuris* infections in non-human primates (Levecke et al., 2009). FLOTAC was more sensitive than the Baermann and McMaster techniques in the diagnosis of lungworm larvae in dogs (Rinaldi et al., 2007a), cats (Gaglio et al., 2008) and sheep (Rinaldi et al., 2010). Finally, FLOTAC resulted in a higher sensitivity than the cellophane tape test in diagnosing oxyurids in rabbits (Rinaldi et al., 2007b).

A second issue is that the sedimentation technique is relatively easy to perform and only few laboratory equipments are necessary, such as a cone glass, saline solution, mortar and pistil. The FLOTAC technique requires well-equipped laboratories, including a centrifuge, different FS and a densitometer. The preparation before reading a FLOTAC needs more time (~17 min) than the sedimentation technique. Since the reading of the slides is the most time consuming step in the sedimentation technique, and up to 8 slide readings were required to achieve a high sensitivity in the post-treatment faecal samples, on average, 49:52 min were necessary for the reading of all 8 slides, compared to 5:27 min for the examination of 1 FLOTAC chamber. A recent study measured a similar reading time for the examination of a single FLOTAC chamber (Levecke et al., 2009). Considering that 4 FLOTAC apparatus (using the double technique; 8 faecal samples can be analysed simultaneously) fit into the centrifuge and 8 sedimentations could be carried out in parallel, the overall examination time would be reduced to 14 min (FLOTAC) and 62 min (sedimentation) per sample, respectively.

Third, our observation of significantly higher *F. hepatica* FECs both amongst the 107 untreated ($P < 0.001$) and a random sample of 42 treated rats ($P = 0.019$) based on multiple sedimentations rather than a single FLOTAC is in line with previous studies comparing FLOTAC with multiple Kato–Katz thick smear readings for the diagnosis of human hookworm and other soil-transmitted helminth infections (Utzinger et al., 2008; Knopp et al., 2009a,b). However, studies performed in sheep and cattle naturally infected with *F. hepatica* showed higher mean FECs with FLOTAC when compared to sedimentation and other copromicroscopic techniques (unpublished data). Similarly, in other animal studies, the mean FECs of different helminths when using FLOTAC were greater than those obtained with the copromicrocopic techniques of choice (Rinaldi et al., 2007a,b, 2010; Gaglio et al., 2008). New research is needed to elucidate possible reasons that might explain the differences in *F. hepatica* FECs observed between FLOTAC and sedimentation both before and after experimental chemotherapy. Given the high FECs in the experimentally-infected rats prior to treatment, there might be some density-related issues, since eggs need to compete with faecal debris for space in the upper layer of the FLOTAC chamber during the centrifugation step. Moreover, depending on the stool composition from the rats, different quantities of faecal debris floated to the top, influencing the subsequent examination of the reading disc under a microscope. Slides examined under a microscope that derived from the sedimentation technique showed similar appearance throughout the study. There might be interactions between the individual components of a floating faecal suspension (e.g., FS components, fixative, residues of the host alimentation and parasite eggs), and hence detailed investigations are needed to further our understanding of such potential interactions that might bias outcomes (Cringoli et al., 2010). The larger multiplication factor used for estimating EPGs obtained with the sedimentation method compared to FLOTAC (35 versus 20) might have caused an overestimation of the EPG value when using the sedimentation method (Cringoli et al., 2004; Mes et al., 2007). Considering only the 14 FLOTAC samples for which a multiplication factor of 40 was used (these samples were characterised by very high EPGs and had to be further diluted), no significant differences were observed between the FECs of FLOTAC and the sedimentation method ($P = 0.583$), but the sample size arguably was small. Table 2 confirms that the use of a higher multiplication factor (140 versus 35), applied if only 2 instead of 8 sedimentation slides were read, resulted in a significantly higher EPG (263 versus 176; $P = 0.034$). The absence of a grid slide system in the examination of samples processed with sedimentation might have resulted in less accurate FECs as there is a risk of double counting eggs or missing them altogether. Considering that eggs might have been counted twice and in view of the high multiplication factor, FECs might have been in considerably overestimated.

It will be interesting to compare the sedimentation method and the FLOTAC technique for the diagnosis of *F. hepatica* and/or *F. gigantica* in humans, placing emphasis on the diagnostic performance of each method (e.g., sensitivity and FEC estimates), as well as practical and operational issues (e.g., laboratory equipment, human resources and time considerations). Results from such a

comparative investigation might shed new light on whether findings from the *F. hepatica*-rat model can be extrapolated to humans, taking into consideration variables such as differences in egg density, specific gravity, stool consistency and debris. In order to further enhance the diagnostic 'gold' standard, the concurrent use of a PCR approach should also be considered (Marcilla et al., 2002; Rokni et al., 2010).

In conclusion, our data suggests that the FLOTAC technique is more sensitive than multiple sedimentations and requires less time when a single stool sample is to be examined. On the other hand, the sedimentation technique is more uniform, revealed higher FECs and is easier to apply under field conditions compared to FLOTAC. Hence, FLOTAC might be the method of choice to diagnose infections with *Fasciola* spp. in the laboratory environment (e.g., in drug efficacy studies), where the sensitivity and speed of a method are essential.

## Acknowledgments

U.D., M.V., J.U. and J.K. are financially supported by the Swiss National Science Foundation (project no. PPOOA-114941 to U.D., M.V. and J.K. and PPOOB-102883 and PPOOB-119129 to J.U.).

## References

Allen, A.V.H., Ridley, D.S., 1970. Further observations on the formol–ether concentration technique for faecal parasites. Journal of Clinical Pathology 23, 545–546.

Ash, L.R., Orihel, T.C., 1987. Parasites: a guide to laboratory procedures and identification. American Society of Clinical Pathologists, Chicago, 260–261.

Bergquist, R., Johansen, M.V., Utzinger, J., 2009. Diagnostic dilemmas in helminthology: what tools to use and when? Trends in Parasitology 25, 151–156.

Cawdery, M.J., Ruane, M., 1971. Sedimentation method for the demonstration of the eggs of *Fasciola hepatica* in faeces. Laboratory Practice 20, 935–939.

Conceicao, M.A., Durao, R.M., Costa, I.H., da Costa, J.M., 2002. Evaluation of a simple sedimentation method (modified McMaster) for diagnosis of bovine fascioliosis. Veterinary Parasitology 105, 337–343.

Cringoli, G., 2006. FLOTAC, a novel apparatus for a multivalent faecal egg count technique. Parassitologia 48, 381–384.

Cringoli, G., Rinaldi, L., Veneziano, V., Capelli, G., Scala, A., 2004. The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and *Dicrocoelium dendriticum* in sheep. Veterinary Parasitology 123, 121–131.

Cringoli, G., Rinaldi, L., Veneziano, V., Genchi, C., 2006. A dual anthelmintic treatment strategic scheme for the control of fasciolosis in dairy sheep farms. Parasitology Research 99, 700–705.

Cringoli, G., Rinaldi, L., Maurelli, M.P., Utzinger, J., 2010. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nature Protocols 5, 503–515.

Gaglio, G., Cringoli, G., Rinaldi, L., Brianti, E., Giannetto, S., 2008. Use of the FLOTAC technique for the diagnosis of *Aelurostrongylus abstrusus* in the cat. Parasitology Research 103, 1055–1057.

Garcia, H.H., Moro, P.L., Schantz, P.M., 2007. Zoonotic helminth infections of humans: echinococcosis, cysticercosis and fascioliasis. Current Opinion in Infectious Diseases 20, 489–494.

Katz, N., Chaves, A., Pellegrino, J., 1972. A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. Revista do Instituto de Medicina Tropical de São Paulo 14, 397–400.

Keiser, J., Utzinger, J., 2009. Food–borne trematodiases. Clinical Microbiology Reviews 22, 466–483.

Keiser, J., Rinaldi, L., Veneziano, V., Mezzino, L., Tanner, M., Utzinger, J., Cringoli, G., 2008. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitology Research 103, 517–522.

Keiser, J., Veneziano, V., Rinaldi, L., Mezzino, L., Duthaler, U., Cringoli, G., 2009. Anthelmintic activity of artesunate against *Fasciola hepatica* in naturally infected sheep. Research in Veterinary Science 88, 107–110.

Knopp, S., Glinz, D., Rinaldi, L., Mohammed, K.A., N'Goran, E.K., Stothard, J.R., Marti, H., Cringoli, G., Rollinson, D., Utzinger, J., 2009a. FLOTAC: a promising technique for detecting helminth eggs in human faeces. Transactions of the Royal Society of Tropical Medicine and Hygiene 103, 1190–1194.

Knopp, S., Rinaldi, L., Khamis, I.S., Stothard, J.R., Rollinson, D., Maurelli, M.P., Steinmann, P., Marti, H., Cringoli, G., Utzinger, J., 2009b. A single FLOTAC is more sensitive than triplicate Kato–Katz for the diagnosis of low-intensity soil-transmitted helminth infections. Transactions of the Royal Society of Tropical Medicine and Hygiene 103, 347–354.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174.

Levecke, B., De Wilde, N., Vandenhoute, E., Vercruysse, J., 2009. Field validity and feasibility of four techniques for the detection of *Trichuris* in simians: a model for monitoring drug efficacy in public health? PLoS Neglected Tropical Diseases 3, e366.

Marcilla, A., Bargues, M.D., Mas-Coma, S., 2002. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. Molecular and Cellular Probes 16, 327–333.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant–borne trematode zoonoses. International Journal for Parasitology 35, 1255–1278.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2007. Plant–borne trematode zoonoses: fascioliasis and fasciolopsiasis. In: Murell, K.D., Fried, B. (Eds.), Food–Borne Parasites, Fish and Plant–Borne Parasites, vol. 11. Springer Verlag, New York, pp. 293–334.

Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Advances in Parasitology 69, 41–146.

Mes, T.H., Eysker, M., Ploeger, H.W., 2007. A simple, robust and semi-automated parasite egg isolation protocol. Nature Protocols 2, 486–489.

Rinaldi, L., Calabria, G., Carbone, S., Carrella, A., Cringoli, G., 2007a. *Crenosoma vulpis* in dog: first case report in Italy and use of the FLOTAC technique for copromicroscopic diagnosis. Parasitology Research 101, 1681–1684.

Rinaldi, L., Russo, T., Schioppi, M., Pennacchio, S., Cringoli, G., 2007b. *Passalurus ambiguus*: new insights into copromicroscopic diagnosis and circadian rhythm of egg excretion. Parasitology Research 101, 557–561.

Rinaldi, L., Maurelli, M.P., Musella, V., Santaniello, A., Coles, G.C., Cringoli, G., 2010. FLOTAC: an improved method for diagnosis of lungworm infections in sheep. Veterinary Parasitology 169, 395–398.

Robinson, M.W., Dalton, J.P., 2009. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 364, 2763–2776.

Rokni, M.B., Mirhendi, H., Mizani, A., Mohebali, M., Sharbatkhori, M., Kia, E.B., Abdoli, H., Izadi, S., 2010. Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method. Experimental Parasitology 124, 209–213.

Utzinger, J., Rinaldi, L., Lohourignon, L.K., Rohner, F., Zimmermann, M.B., Tschannen, A.B., N'Goran, E.K., Cringoli, G., 2008. FLOTAC: a new sensitive technique for the diagnosis of hookworm infections in humans. Transactions of the Royal Society of Tropical Medicine and Hygiene 102, 84–90.

# *Chapter 3*

## *In Vivo* and *In Vitro* Sensitivity of *Fasciola hepatica* to Triclabendazole Combined with Artesunate, Artemether, or OZ78

Urs Duthaler [a], Thomas A. Smith [b], Jennifer Keiser [a]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

[b] Department of Public Health and Epidemiology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

**Published in Antimicrobial Agents and Chemotherapy 54, 11 (2010): p. 4596-4604**

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2010, p. 4596–4604 Vol. 54, No. 11
0066-4804/10/$12.00 doi:10.1128/AAC.00828-10


# *In Vivo* and *In Vitro* Sensitivity of *Fasciola hepatica* to Triclabendazole Combined with Artesunate, Artemether, or OZ78▽

Urs Duthaler,[1,2] Thomas A. Smith,[2,3] and Jennifer Keiser[1,2]*

*Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, Switzerland[1]; University of Basel, Basel, Switzerland[2]; and Department of Public Health and Epidemiology, Swiss Tropical and Public Health Institute, Basel, Switzerland[3]*

Received 17 June 2010/Returned for modification 4 August 2010/Accepted 13 August 2010

**Triclabendazole resistance is continually documented from livestock, and hence new treatment strategies for *Fasciola hepatica* infections are needed. We investigated the effect of triclabendazole combined with artesunate, artemether, or OZ78 compared to that of monotherapy against adult and juvenile *F. hepatica* in rats. *In vitro* experiments with triclabendazole and its sulfoxide and sulfone metabolites, each in combination with the peroxides, complemented our study. *F. hepatica*-infected rats were subjected to single drugs or drug combinations 3 to 4 weeks (juvenile flukes) and >8 weeks (adult flukes) postinfection. Negative binomial regressions of worm and egg counts were used to analyze dose-response relationships and whether the effects of drug combinations were synergistic or antagonistic. The *in vitro* assays were evaluated by means of viability scales based on fluke motility. Fifty percent effective dose values of 113.0, 77.7, 22.9, and 2.7 mg/kg of body weight were calculated for monotherapy with artesunate, artemether, OZ78, and triclabendazole, respectively, against adult *F. hepatica*. Likelihood ratio tests revealed synergistic interactions ($P < 0.05$) of combinations of triclabendazole (2.5 mg/kg) plus artesunate or artemether on adult worm burden. Antagonistic effects on the adult burden and egg output were observed when a lower triclabendazole dose (1.25 mg/kg) was combined with the artemisinins. No significant interactions ($P = 0.07$) were observed for OZ78 and triclabendazole combinations and between the triclabendazole effect and the effects of the other partner drugs on juvenile worms. Our *in vitro* studies of adult worms agreed with the *in vivo* results, while the *in vitro* analysis of juvenile worms revealed greater interactions than observed *in vivo*. In conclusion, single-agent triclabendazole demonstrated a more potent *in vivo* and *in vitro* fasciocidal activity than the experimental drugs artesunate, artemether, and OZ78. When combined, synergistic but also antagonistic effects depending on the doses administered were observed, which should be elucidated in more detail in future studies.**

The food-borne trematodes *Fasciola hepatica* and *F. gigantica* are the causative agents of fascioliasis (fasciolosis). *Fasciola hepatica* parasitizes a wide spectrum of domestic and wild animals (e.g., sheep, cattle, rats, and deer), and it causes a huge economic loss of $3 billion annually to the agriculture sector worldwide through losses of milk and meat yields (26, 31). In addition, an estimated 90 million people are at risk of fascioliasis and up to 17 million individuals are infected (20).

Due to its excellent safety profile and the high activity against both juvenile and adult liver flukes, triclabendazole (Fasinex, Egaten) is the drug of choice for the treatment of human and veterinary fascioliasis. It is worrying, however, that triclabendazole resistance has been documented from Australian sheep farms since the mid-1990s and recently also from several western European countries (10). Furthermore, *F. hepatica* strains resistant to three alternative fasciocidal drugs (rafoxanide, closantel, and luxabendazole) have been isolated (11). Due to the rapid spread of resistance, the small arsenal of fasciocidal drugs, and the absence of effective vaccines in field conditions, there is a pressing need for the discovery and development of novel drugs or drug combinations (27). The use of drug combinations is an excellent strategy to avoid or delay drug resistance, since different drug targets are attacked simultaneously. Furthermore, drug combinations often are characterized by an increased activity and tolerability compared to that of monotherapy. Therefore, drug combinations are widely used in the treatment of infectious diseases (e.g., malaria, HIV, and tuberculosis), cancer, and chronic disorders (e.g., cardiovascular disease, diabetes, and pain management) (5, 8, 12, 30, 39). Combination chemotherapy has been tested in experimental studies using triclabendazole-susceptible and -resistant strains of *F. hepatica.* The combined treatment of triclabendazole plus clorsulon or luxabendazole was found to act synergistically. For example, the combination of triclabendazole and clorsulon, administered at one-fifth their recommended dosages, was highly effective against triclabendazole-resistant *F. hepatica* in sheep. This combination reduced 95% of the worms, while the single drugs achieved a worm burden reduction (WBR) of only approximately 30% (11, 28).

Recently, the semisynthetic artemisinins artemether and artesunate and the synthetic peroxide OZ78 were described to have excellent fasciocidal properties in rats (19, 21). Importantly, artemether and OZ78 cured a triclabendazole-resistant *F. hepatica* infection in the rat model (22). Contradictory results were obtained in sheep. While OZ78 failed to cure a chronic *F. hepatica* infection in sheep (16), artemether and artesunate achieved high worm burden reductions in naturally *F. hepatica*-infected sheep (18, 23).

* Corresponding author. Mailing address: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. Phone: 41 61 284-8218. Fax: 41 61 284-8105. E-mail: jennifer.keiser@unibas.ch.

▽ Published ahead of print on 23 August 2010.

The aim of the present investigation was to study the potential of the combined treatment of triclabendazole with artesunate, artemether, or OZ78 in *F. hepatica*-infected rats harboring juvenile or adult infections. Comparisons were made to monotherapies, and negative binomial regression was used for the analysis of egg burden and worm counts. *In vitro* studies complemented our work. We investigated the *in vitro* effect of triclabendazole and its sulfoxide and sulfone metabolites, each combined with the three peroxidic drugs (artesunate, artemether, and OZ78) against juvenile and adult *F. hepatica* flukes.

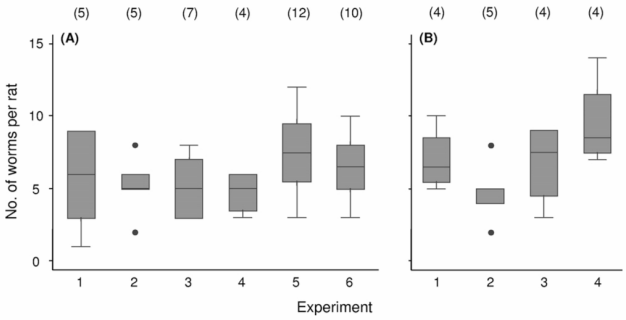



FIG. 1. Distribution of adult (A) and juvenile (B) *F. hepatica* worm counts in untreated control rats. The upper and lower limits of the boxes correspond to the interquartile range, the value in the middle to the median, and the limits of the whiskers correspond to the adjacent values, i.e., the largest value below the 25th percentile and the smallest value above the 75th percentile. Numbers in parentheses are the numbers of control animals in each experiment.

## MATERIALS AND METHODS

***F. hepatica* rat model.** Female Wistar rats ($n$ = 222; age = 3 weeks; weight ~100 g) were purchased from Harlan (Itingen, Switzerland). Rats were kept in groups of five in type-4 Makrolon cages under environmentally controlled conditions (temperature, ~22°C; humidity, ~70%; light/dark cycle, 12/12 h) with free access to water and rodent food (Rodent Blox from Eberle NAFAG; Gossau, Switzerland). *F. hepatica* metacercariae (Pacific Northwest wild strain) were purchased from Baldwin Aquatics (Monmouth, OR). After 1 week of adaptation, each rat was infected by oral gavage with 22 ± 2 metacercariae. The present study was authorized by the local veterinary agency based on national regulations (permission no. 2070).

**Drugs.** Triclabendazole, triclabendazole-sulfoxide, and triclabendazole-sulfone were the products of Novartis Animal Health (Basel, Switzerland). Artesunate was obtained from Mepha AG (Aesch, Switzerland). Artemether was a gift of Dafra Pharma (Turnhout, Belgium). OZ78 was synthesized at the College of Pharmacy, University of Nebraska Medical Center (Omaha, NE). The drugs were freshly prepared as suspensions containing 7% (vol/vol) Tween 80 (Sigma-Aldrich, Buchs, Switzerland), 3% (vol/vol) ethanol 96% (Merck, Darmstadt, Germany), and tap water for the *in vivo* studies. For the *in vitro* experiments, stock solutions (3 and 10 mg/ml) were prepared in 100% (vol/vol) dimethylsulfoxide (DMSO).

***In vivo* drug treatment.** At least 4 rats per drug and dosage harboring adult *F. hepatica* (>8 weeks postinfection) were treated orally with triclabendazole, artesunate, artemether, or OZ78 alone. The drugs were given in the following dosages: artesunate and artemether, 200 and 100 mg/kg of body weight; OZ78, 100, 50, and 25 mg/kg; triclabendazole, 10, 5, 2.5, and 1.25 mg/kg ($n$ = 44 rats in total). For the combination chemotherapy experiments, 4 rats were each given 1.25 mg/kg triclabendazole plus either artesunate (25, 50, 100 mg/kg), artemether (25, 50, 100 mg/kg), or OZ78 (25 and 50 mg/kg) ($n$ = 32 rats in total). Finally, 4 rats each were treated with 2.5 mg/kg triclabendazole in combination with artesunate (6.25, 12.5, 25, 50, 100, and 200 mg/kg), artemether (6.25, 12.5, 25, 50, and 100 mg/kg), or OZ78 (12.5, 25, and 50 mg/kg) ($n$ = 56 rats in total).

Four rats harboring juvenile *F. hepatica* (3 to 4 weeks postinfection) were treated each with single oral doses of either triclabendazole (2.5 and 5 mg/kg), artesunate (25 and 50 mg/kg), artemether (25 and 50 mg/kg), or OZ78 (25 and 50 mg/kg) ($n$ = 32 rats in total). The following drug combinations were studied (4 rats per dosage): 2.5 mg/kg triclabendazole plus 25 mg/kg artesunate, artemether, or OZ78; 5 mg/kg triclabendazole plus 50 mg/kg artesunate, artemether, or OZ78 ($n$ = 24 rats in total).

Untreated rats ($n$ = 34) served as controls, as illustrated in Fig. 1. One week posttreatment, the number of *F. hepatica* worms per rat was determined by the examination of the bile duct and liver following the necropsy of rats (13).

**Egg excretion analysis.** A fecal sample was collected from each chronically *F. hepatica* infected rat ($n$ = 132) 1 day before treatment and 7 days after treatment to calculate the egg burden reduction. Additionally, one fecal sample was collected from 85 of the 132 rats 2 months prior to treatment. The reason for including this additional analysis was to obtain information on the variation in egg output independently of drug effect. The rats were housed individually for 1 night to obtain > 2 g of stool. The FLOTAC double technique was used to analyze egg counts as described in previous publications (4, 7). A flotation solution prepared with 685 g $ZnSO_4 \cdot 7\ H_2O$ (Merck, Darmstadt, Germany) and 685 ml deionized $H_2O$ with a specific gravity of 1.35 was used. Each fecal sample was analyzed using a dilution ratio of 1:100 (multiplication factor of 20), resulting in a diagnostic sensitivity of 20 eggs per gram fecal sample (EPG).

**In vitro drug assays.** Adult (>8 weeks postinfection) *F. hepatica* flukes were recovered from the central bile duct of rats and incubated for 2 h in RPMI 1640 culture medium (Gibco, NY) at 37°C in an atmosphere of 5% $CO_2$. Adult worms were placed in single wells of 12-well plates (Costar 3512) containing 3 ml RPMI 1640 and supplemented with 1% (vol/vol) antibiotics (50 μg/ml streptomycin and 50 U/ml penicillin; Gibco, NY) and 80 μg/ml hemin (17). Adult *F. hepatica* were incubated in the presence of either triclabendazole, triclabendazole-sulfoxide and triclabendazole-sulfone (15 μg/ml each), artesunate, artemether, or OZ78 (50 μg/ml each). For the drug combination studies of adult *F. hepatica*, triclabendazole or one of its two metabolites (15 μg/ml each) was combined with a peroxidic drug (50 μg/ml each).

Juvenile *F. hepatica* (3 to 4 weeks postinfection) were recovered from the liver of infected rats. The rat livers were pressed between transparent plastic films, and the flukes were collected using a binocular. The juvenile flukes were incubated in 48-well plates (Costar 3548, Corning, NY) in 1 ml supplemented RPMI 1640, with one *F. hepatica* in each well. The effects of single treatments of triclabendazole, triclabendazole-sulfoxide and triclabendazole-sulfone (30 μg/ml each), artesunate, artemether, and OZ78 (100 μg/ml each) were studied. For the drug combination experiments using juvenile *F. hepatica*, each triclabendazole derivative (30 μg/ml each) was combined with one of the semisynthetic artemisinins or OZ78 (100 μg/ml each).

Five to six flukes were examined per drug and drug combination in two (adult *F. hepatica*) and two or three (juvenile *F. hepatica*) independent experiments ($n$ = 10 to 18 flukes per concentration), and the arithmetic mean was calculated. Six control flukes, without drug exposure, were included in each experimental set. The control well contained the highest concentration of DMSO used.

The viability of adult flukes was scored after 24, 48, and 72 h using the following scale: 3, normal movements; 2, reduced activities; 1, very weak activities, detected only by means of microscopic magnification (20×); and 0, death of worm (absence of movements for 2 min using a microscope [20×]). The viability of juvenile *F. hepatica* was graded as follows: 2, normal activity (microscopic magnification, ×20); 1, weak activity (microscopic magnification, ×80); and 0, death (absence of movements for 2 min under a microscope [×80]).

**Statistical analysis.** For the statistical analyses we included three datasets with a total of 83 worm counts from previous experiments carried out with *F. hepatica*-infected rats in our laboratories (19, 21). This data set contained single-drug treatments of 20 rats harboring juvenile *F. hepatica* infections (3 to 4 week postinfection), 37 rats harboring adult *F. hepatica* infections (>8 weeks postinfection), and 26 control animals. Worm burdens were analyzed using negative binomial (NB) regression in STATA version 10 (Stata Corp., College Station, TX). For any rat in experiment $i$, $\sim NB(\hat{y}, 1 + \alpha\hat{y})$, where $y$ is the worm count, $\alpha$ is the overdispersion parameter (so that $1 + \alpha\hat{y}$ is the overall dispersion), and the expected worm count is $\hat{y} = \exp(x\beta + y_i)$, where $\beta$ measures the drug effect, $x$ is the drug concentration, and $\gamma_i$ is a fixed effect for the experiment. Additional fixed-effect terms were included into this model to estimate the effects of multiple drugs and drug interactions. Likelihood ratio tests were applied to assess statistical significance of these terms and the term synergism or antagonism used if significant deviations from the null hypothesis of independence were obtained (24).

Egg counts 7 days after treatment were analyzed using a similar model, but with the logarithm of the pretreatment egg count included in the model as an offset, so that the estimated drug effects refer to the comparison to pretreatment counts rather than to the comparison to the no-drug dose. The analysis of the log

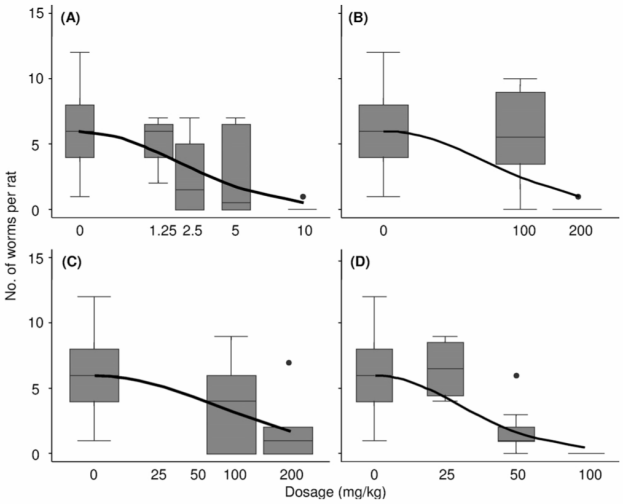



FIG. 2. Effects of monotherapy on adult *F. hepatica* in rats. Shown are triclabendazole (A), artemether (B), artesunate (C), and OZ78 (D). Box plots are as described in the legend to Fig. 1. The dosages are arranged on a square-root scale. Black lines indicate the fitted dose-response curve assuming a linear effect of dose on the logarithm of the *F. hepatica* worm burden.

linearity of the relationship between drug dose and egg counts involved comparing negative binomial models to linear effects of drug dose with a reference model in which the effects of each drug were represented by separate terms for each dose level. Posttreatment egg counts and surviving worms were plotted on a square root scale, because the distribution is right skewed and egg counts included many zeros, which cannot be displayed if a logarithmic transformation is used.

All negative binomial models were fitted using the *nbreg* command in STATA v10.0.

## RESULTS

A total of 305 *F. hepatica*-infected rats were studied, of which 212 rats harbored a chronic (>8 weeks postinfection) and 93 an acute (3 to 4 weeks postinfection) *F. hepatica* infection. The number of adult flukes in control rats ($n = 43$) varied from 1 to 12, with a median of 6 (Fig. 1A). In the absence of drug treatment, numbers of juvenile worms were similar to those of adult worms (Fig. 1). A range of 2 to 14 juvenile flukes with a median of 7 was observed in 17 control rats (Fig. 1B).

**Effect of *in vivo* monotherapies against adult *F. hepatica*.** Worm burdens were recorded from 212 rats, of which 81 underwent monotherapy (Fig. 2). All drugs tested were effective in killing the flukes, with doses required to reduce worm burden by 50 and 95% given in Table 1. Triclabendazole has a 50% effective dose ($ED_{50}$) and $ED_{95}$ of 2.7 mg/kg (95% confidence limits [95% CL], 2.0, 4.4 mg/kg) and 11.7 mg/kg (95% CL, 8.4, 19.1 mg/kg), respectively. For OZ78, an $ED_{50}$ of 22.9 mg/kg (95% CL, 16.9, 35.4 mg/kg) and an $ED_{95}$ of 99.0 mg/kg (95% CL, 73.1, 153.2 mg/kg) were calculated. Artesunate and artemether achieve worm burden reductions of 50% administered at 113.0 mg/kg (95% CL, 69.9, 295.3) and 77.7 mg/kg (95% CL, 52.8, 146.8), respectively. Dosages of 488.6 mg/kg (artesunate) (95% CL, 302.1, 1276.1 mg/kg) and 335.7 mg/kg (artemether) (95% CL, 228.2, 634.5 mg/kg) result in 95% worm burden reductions.

The estimate of the overdispersion parameter for this model was $\alpha = 0.33$ (standard error, 0.11), indicating substantial overdispersion in the worm counts, justifying the use of negative binomial rather than Poisson models to analyze these data.

Tests of the log linearity of the dose effect confirmed that a linear relationship between the logarithm of the number of surviving worms and the drug dose gives a good fit to the data for triclabendazole and artesunate, but the dose responses estimated for artemether and OZ78 showed significant deviation from log linearity (Table 1).

**Effect of *in vivo* monotherapies against juvenile *F. hepatica*.** The chemotherapy of juvenile *F. hepatica* was studied in four experiments, including 17 controls and 52 animals receiving monotherapy (Fig. 3). The effects of monotherapy with artesunate, artemether, and OZ78 were similar to those for adult worms, with highly statistically significant effects (likelihood ratio statistics [LRS], 1 degree of freedom of 9.6 [$P = 0.002$], 27.4 [$P < 0.001$], and 27.8 [$P < 0.001$], respectively). One hundred mg/kg of OZ78 cured all rats and 200 mg/kg artemether and artesunate resulted in 85.5% (95% CL, 64.1, 94.1%) and 56.4% (95% CL, 22.3, 75.5%) worm burden reductions, respectively. The effect of triclabendazole was less pronounced, with even the highest dose (5 mg/kg) having only a small effect against juvenile worms (WBR, 12.8; 95% CL, −36.8, 44.5%), even though this dose was effective in most animals against adult worms. Nevertheless, the negative binomial regression indicated a significant dose-response relationship (LRS, 8.0; $P = 0.005$).

**Effect of *in vivo* combination chemotherapy against adult *F. hepatica*.** Eighty-eight rats were given combinations of triclabendazole with one of the three peroxidic drugs (artesunate, artemether, or OZ78) as presented in Fig. 4. Triclabendazole (1.25 mg/kg) on its own had rather little effect on the worm burden (worm burden reduction was estimated as 13.5% [95% CL, −35.5, 44.8%], allowing for experimental variation). In contrast, 2.5 mg/kg triclabendazole on its own had a moderate effect on worm burden (estimated as 58.8%; 95% CL, 20.9, 78.5%). The administration of 2.5 mg/kg triclabendazole to rats showed huge variations in the treatment outcome: while approximately half of the rats were cured, no effect was seen in the remaining rats.

Combinations with a dose of 1.25 mg/kg triclabendazole appeared to slightly inhibit the effects of both artemether and artesunate. The formal analysis of interactions in the negative binomial regressions to test the effects of the drug combinations, including the data for all doses of these drugs, indicated significant deviations from the null hypothesis of the independence of drug effects for the combination of artemether ($P = 0.012$) and artesunate ($P = 0.019$) with triclabendazole (Table 1). This suggests that combinations of artemether or artesunate with the higher dose of triclabendazole (2.5 mg/kg) increases worm burden reduction over those expected for a hypothesis of independence. However, the estimates of effect sizes in these regressions are imprecise.

For OZ78 there was, overall, no significant interaction with triclabendazole. The dose-response curve of OZ78 combined with 1.25 mg/kg triclabendazole was similar to that for its effect as monotherapy. However, there was a shift toward a significant interaction ($P = 0.07$) at lower dosages, with 25 mg/kg OZ78 showing no killing on its own (WBR estimate, adjusting

TABLE 1. Estimates of effects of drug therapy against adult *F. hepatica*[a]

| Drug effect | Triclabendazole | Artemether | Artesunate | OZ78 |
|---|---|---|---|---|
| Estimates of effect on worm burdens | | | | |
| *N* | 24 | 17 | 16 | 22 |
| Dose effect (β, kg/mg) (95% CL) | −0.26 (−0.35, −0.16) | −0.0089 (−0.0131, −0.0047) | −0.0061 (−0.0099, −0.0023) | −0.030 (−0.041, −0.020) |
| $ED_{50}$ (mg/kg) (95% CL) | 2.7 (2.0, 4.4) | 77.7 (52.8, 146.8) | 113.0 (69.9, 295.3) | 22.9 (16.9, 35.4) |
| $ED_{95}$ (mg/kg) (95% CL) | 11.7 (8.4, 19.1) | 335.7 (228.2, 634.5) | 488.6 (302.1, 1276.1) | 99.0 (73.1, 153.2) |
| Estimates of effect on egg counts | | | | |
| Dose effect (β, kg/mg) (95% CL) | −0.54 (−0.85, −0.23) | −0.007 (−0.019, 0.005) | −0.011 (−0.023, 0.002) | −0.042 (−0.081, 0.002) |
| $ED_{50}$ (mg/kg) (95% CL) | 1.3 (0.8, 3.0) | 102.7 (36.9, inf*) | 65.8 (30.1, inf*) | 16.6 (8.5, 356.2) |
| Significance tests | | | | |
| Log linearity of dose effect on worm burdens | | | | |
| Degrees of freedom | 4 | 2 | 2 | 3 |
| LRS (*P* value) | 5.2 (0.3) | 23.7 (0.00001) | 0.1 (1.0) | 9.7 (0.02) |
| Interaction of dose effect with log-linear effect of triclabendazole on worm burdens | | | | |
| Degrees of freedom | | 3 | 1 | 2 |
| LRS (*P* value) | | 10.9 (0.012) | 5.5 (0.019) | 5.5 (0.07) |
| Dose effect on egg counts | | | | |
| Degrees of freedom | 1 | 1 | 1 | 1 |
| LRS (*P* value) | 11.2 (0.0008) | 1.28 (0.3) | 3.0 (0.08) | 5.3 (0.021) |
| Log linearity of dose effect on egg counts | | | | |
| Degrees of freedom | 4 | 6 | 5 | 3 |
| LRS (*P* value) | 23.5 (0.0001) | 6.5 (0.4) | 5.9 (0.3) | 8.1 (0.045) |

[a] *N* is the number of rats treated with monotherapy of the drug (across all doses); CL, confidence limits; $ED_{50}$ is the dose required to kill 50% of the parasites; $ED_{95}$ is the dose required to kill 95% of the parasites; inf*, confidence limits includes no dose dependence. All estimates were derived from negative binomial regression models. Tests of log linearity were carried out by comparing a model to separate terms for each distinct drug dose, with a model that assumed the logarithm of the worm burden to decrease linearly with drug dose. LRS, likelihood ratio ($\chi^2$).

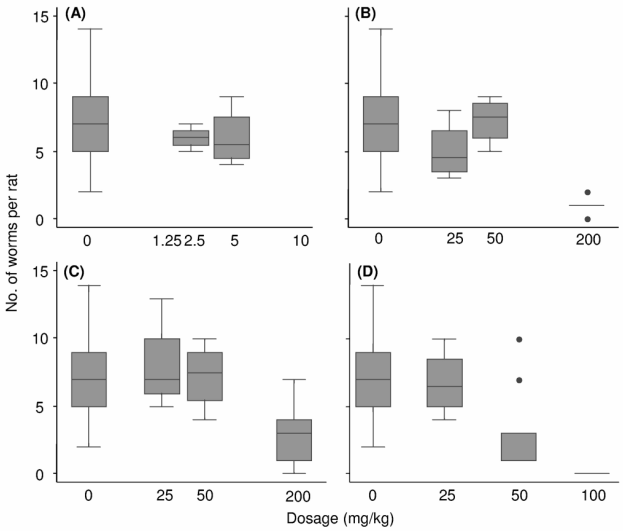



FIG. 3. Effects of monotherapy on juvenile *F. hepatica* in rats. Shown are triclabendazole (A), artemether (B), artesunate (C), and OZ78 (D). Box plots are as described in the legend to Fig. 1. The dosages are arranged on a square-root scale.

for experimental variation of −7.1% [95% CL, −60.6, 28.6%]) but substantial killing in combination with both dosages of triclabendazole (1.25 mg/kg plus 25 mg/kg; WBR, 87.3%; 95% CL, 45.8, 97.0%). Using 2.5 mg/kg triclabendazole plus 25 mg/kg OZ78, all worms were killed.

**Effect of *in vivo* combination chemotherapy against juvenile *F. hepatica*.** Twenty-four rats received combinations of triclabendazole and the peroxidic drugs. In each case 4 rats were treated with 2.5 or 5 mg/kg triclabendazole plus either 25 or 50 mg/kg artemether, artesunate, or OZ78. Twenty-five or 50 mg/kg of artesunate and artemether on their own resulted in low worm burden reductions (0 to 30%) against juvenile *F.*

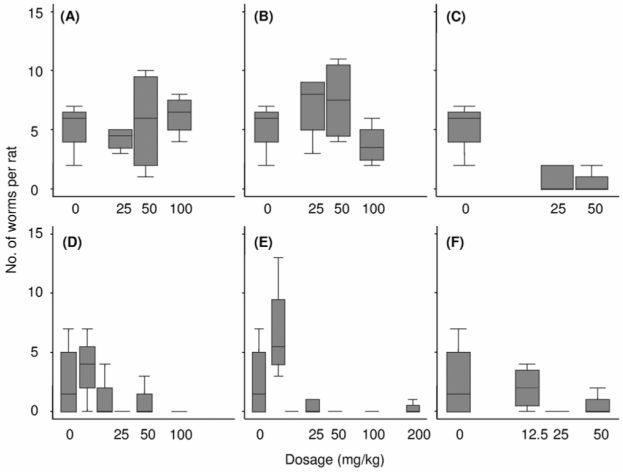



FIG. 4. Effects of combination chemotherapy with 1.25 mg/kg (A to C) and 2.5 mg/kg (D to F) triclabendazole on adult *F. hepatica* in rats. Shown are artemether (A and D), artesunate (B and E), and OZ78 (C and F). Box plots are as described in the legend to Fig. 1. The dosages are arranged on a square-root scale.

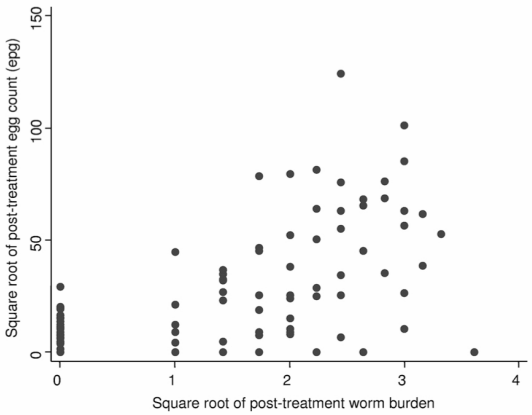



FIG. 5. Association of posttreatment adult worm burdens and egg counts of *F. hepatica* in rats. The worm burdens and egg counts are arranged on a square-root scale.

*hepatica*. OZ78 at 50 mg/kg achieved a modest worm burden reduction of 50%.

The combinations with 2.5 mg/kg triclabendazole plus 25 mg/kg of the peroxidic drugs did not show higher worm burden reductions compared to those of the single-drug treatments. Higher worm burden reductions compared to those of monotherapy were observed when 5 mg/kg triclabendazole was combined with 50 mg/kg artemether, artesunate, or OZ78. Nevertheless, there were no significant interactions between the triclabendazole effect and the effects of the other partner drugs on juvenile worms, since the numbers of rats tested were small and low doses of triclabendazole were used, but this does not mean that interactions would be detected in larger experiments with higher doses.

**Egg excretion analysis.** Egg counts were recorded from 132 rats harboring adult *F. hepatica* infections 1 day prior to drug treatment and 7 days posttreatment. Each of these rats tested positive in shedding *F. hepatica* eggs 1 day before treatment. Additionally, the egg excretion of 85 of the 132 rats were sampled 2 months prior drug treatment and also at the time of treatment. The median egg count of 2 months prior to treatment was 6,600 EPG (interquartile range [IQR], 3,780 to 9,880), and the median EPG the day before treatment was 6,014 (IQR, 3,674 to 8,962), indicating very little change in average egg counts during this period, with a statistically nonsignificant difference ($P = 0.4$, two-sided Wilcoxon test). It follows from this that average reductions in egg load during this 2-month period are negligible and can be ignored relative to the drug effects.

Posttreatment egg counts were clearly correlated with surviving worms (Spearman correlation, 0.58; $P < 0.0001$) (Fig. 5), but there was considerable scatter, with many rats with surviving worms producing very low or no egg counts. Thirty-six (27.3%) of the rats tested had negative posttreatment egg counts, while 64 (48.4%) had 0 worms; however, only 25 had neither eggs nor worms.

The overall egg count distributions of pre- and posttreatment egg counts of the 132 rats are given in Table 2. The median of pretreatment counts was 4,902.9 (IQR, 2,929.8 to 7,798.8). A median of 83.5 EPG (IQR, 0 to 844.4) was calcu-

TABLE 2. Overall egg count distributions[a]

| Treatment status | Median | Lower quartile | Upper quartile |
|---|---|---|---|
| Pretreatment | 4,902.9 | 2,929.8 | 7,798.8 |
| Posttreatment | 83.5 | 0 | 844.4 |
| Ratio | 0.016 | 0 | 0.475 |

[a] Units are eggs per gram of feces.

lated posttreatment. Each drug was able to reduce the egg excretion. The ratios of posttreatment to pretreatment egg counts reached from 0.001 to 1.951 (99.9 to −95.1% egg burden reduction). An EPG ratio of 1.951 (standard deviation [SD], 0.627), 0.01 (SD, 0.012), 0.032 (SD, 0.062), and 0.007 (SD, 0.014) was recorded for triclabendazole administered at 1.25, 2.5, 5, and 10 mg/kg. The treatment of rats with 100 and 200 mg/kg artesunate or artemether resulted in a posttreatment-to-pretreatment egg count ratio of 0.017 to 0.013 (SD, 0.023 to 0.021) and 0.351 to 0.066 (SD, 0.292 to 0.104), respectively. The EPG ratio decreases from 1.454 (SD, 0.598) to 0.229 (SD, 0.376) to 0.003 (SD, 0.003) with increasing OZ78 dosages of 25, 50, and 100 mg/kg, respectively.

The $ED_{50}$s of triclabendazole, artesunate, artemether, and OZ78 calculated on the basis of the egg counts are 1.3 mg/kg (95% CL, 0.8 to 3.0 mg/kg), 65.8 mg/kg (95% CL, 30.1 to ∞ mg/kg), 102.7 mg/kg (95% CL, 36.9 to ∞ mg/kg), and 16.6 mg/kg (95% CL, 8.5 to 356.2 mg/kg). For all drugs, estimates of $ED_{50}$ on egg counts were similar to those for the effect on worm burdens (e.g., $ED_{50}$ of 2.7 mg/kg for triclabendazole and 22.9 mg/kg for OZ78). Significance tests of the linear effect of drug dose on egg counts demonstrated dose dependence for OZ78 and triclabendazole but not for artemether or artesunate (Table 1), despite the very low ratios of posttreatment-to-pretreatment egg counts in many of the rats treated with artemether or artesunate.

The combination of 2.5 mg/kg triclabendazole plus the peroxidic drugs achieved a posttreatment/pretreatment egg count ratio mean of 0.025 (egg burden reduction [EBR], 97.5%) with a range of 0.001 (EBR, 99.9%) to 0.138 (EBR, 86.2%), which is in line with 2.5 mg/kg triclabendazole single dosage (EBR, 99.0%). In more detail, triclabendazole (2.5 mg/kg) applied together with artesunate (6.25 to 200 mg/kg), artemether (6.25 to 100 mg/kg), or OZ78 (12.5 to 50 mg/kg) reduced the mean *F. hepatica* egg output to about 96.7, 98.7, and 97.0%, respectively. The effectiveness of the egg reduction of combinations with a triclabendazole dosage of 1.25 mg diminished the mean ratio to 0.67 (EBR, 33%) with a range of 0.007 (EBR, 99.3%) to 1.266 (EBR, −26.6%), which is considerably lower than the ratio of 1.951 at the 1.25-mg/kg dosage of triclabendazole alone. On the other hand, artesunate and artemether treatments (100 mg/kg) were more effective than in combination with 1.25 mg/kg triclabendazole. Tests of deviation from linearity were significant for OZ78 and for triclabendazole, indicating that the dose-response relationship for these drugs was not well modeled by the assumption of log linearity, with a substantial difference between egg counts in rats treated with 1.25 mg/kg triclabendazole combinations (which had little effect on egg counts) and those receiving combinations with 2.5

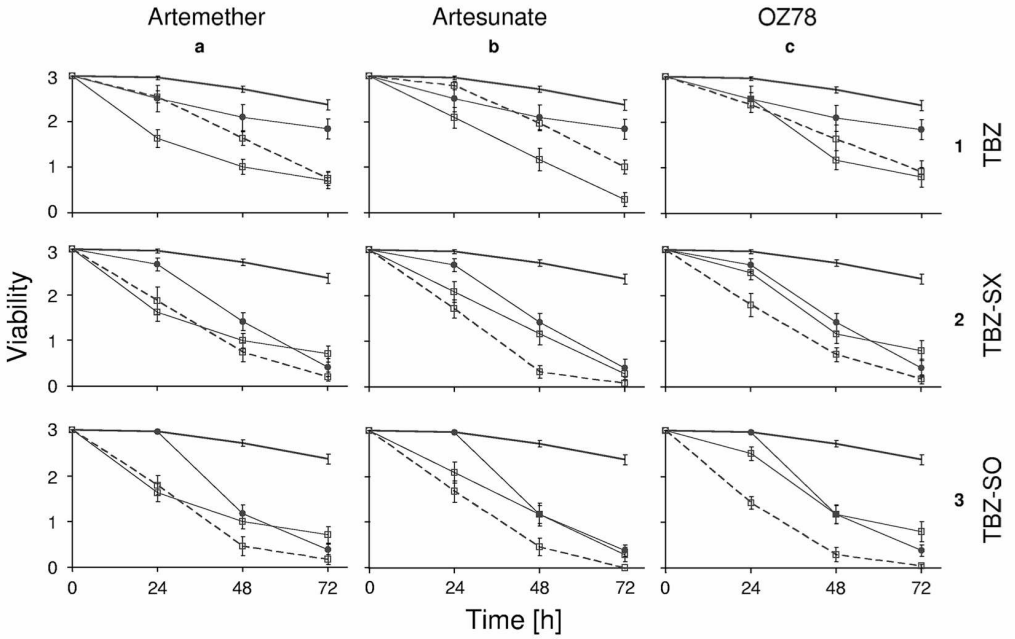



FIG. 6. *In vitro* effects of monotherapy and combination chemotherapy on adult *F. hepatica* flukes. Single-drug incubation (continuous lines): 15 μg/ml benzimidazole derivatives (●); triclabendazole (1; TBZ), triclabendazole-sulfoxide (2; TBZ-SX), and triclabendazole-sulfone (3; TBZ-SO); 50 μg/ml peroxidic drugs (□); artemether (a), artesunate (b), and OZ78 (c). Combinations (dotted lines): 15 μg/ml triclabendazole (1), triclabendazole-sulfoxide (2), or triclabendazole-sulfone (3) plus either 50 μg/ml artemether (a), artesunate (b), or OZ78 (c). The adult control worms ($n$ = 26) are described with a continuous line without symbols. The limits of the whiskers correspond to the standard error of the mean values per time point.

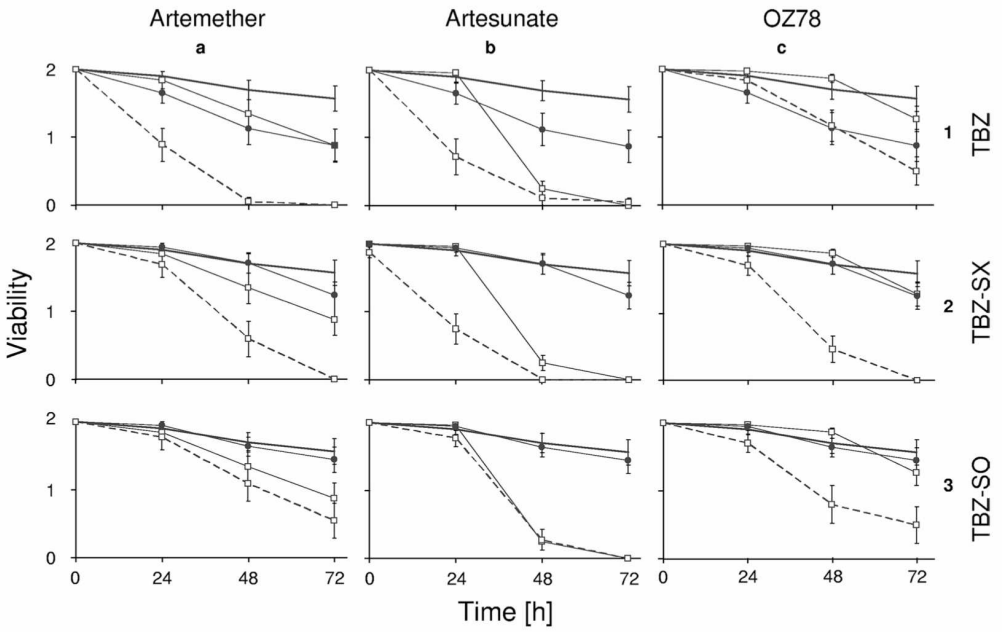



FIG. 7. *In vitro* effects of monotherapy and combination chemotherapy on juvenile *F. hepatica* flukes. Single-drug incubation (continuous lines): 30 μg/ml benzimidazole derivatives (●); triclabendazole (1; TBZ), triclabendazole-sulfoxide (2; TBZ-SX), and triclabendazole-sulfone (3; TBZ-SO); 100 μg/ml peroxidic drugs (□); artemether (a), artesunate (b), and OZ78 (c). Combinations (dotted lines): 30 μg/ml triclabendazole (1), triclabendazole-sulfoxide (2), or triclabendazole-sulfone (3) plus either 100 μg/ml artemether (a), artesunate (b), or OZ78 (c). The juvenile control worms ($n$ = 15) are described with a continuous line without symbols. The limits of the whiskers correspond to the standard error of the mean values per time point.

mg/kg triclabendazole, which had very low posttreatment egg counts.

**Effects of *in vitro* chemotherapy.** Temporal drug effects (monotherapy and combination chemotherapy) on the viability on adult *F. hepatica in vitro* are presented in Fig. 6. Adult *F. hepatica* controls ($n$ = 26) showed normal activities during the entire observation period. Flukes incubated in the presence of peroxides showed a fast decrease of movements, and after 72 h 33% (artemether) to 75% (artesunate) of worms had died. Differences in the effect of the triclabendazole derivatives on *F. hepatica* were observed. While triclabendazole showed only moderate activity on adult worms *in vitro*, incubation with the sulfone and sulfoxide metabolite resulted in decreased movement and the death of 50 to 67% of flukes.

Flukes incubated with the combination of triclabendazole plus artesunate, artemether, or OZ78 were slightly less affected than adult *F. hepatica* exposed to the peroxidic drugs alone (Fig. 6, row 1, graphs a to c). Lower mortality rates were calculated for the triclabendazole combinations (mortality rates of 8 to 25%) than for the single peroxidic drugs.

*F. hepatica* exposed to triclabendazole-sulfoxide and triclabendazole-sulfone combinations showed a rapid decrease in viability, and after 48 h only minimal activities were recorded. Adult flukes incubated with triclabendazole-sulfoxide and triclabendazole-sulfone combinations showed significantly less movement than worms exposed to single drugs, and the majority of these worms (67 to 100%) were killed 72 h postincubation (Fig. 6, rows 2 and 3, graphs a to c).

In Fig. 7, the effects of combination chemotherapy and single drugs on juvenile *F. hepatica* are depicted. Juvenile control flukes showed a viability 72 h postincubation similar to that at the beginning of the assay. Incubation with single drugs revealed clear differences in the activities on juvenile *F. hepatica*. Artesunate showed the highest activity, resulting in the death of all juvenile flukes after 72 h (Fig. 7, rows 1 to 3, graph b). A moderate effect (decreased viability) was observed with artemether, OZ78, and triclabendazole (Fig. 7, row 1, graphs a and c). On the other hand, triclabendazole-sulfone- and triclabendazole-sulfoxide-incubated worms behaved like controls (Fig. 7, rows 2 and 3, graphs a to c). Figure 7 illustrates that with the exception of a triclabendazole-sulfone–artesunate combination (which had an activity similar to that of artesunate alone [Fig. 7, row 3, graph b]), all combinations tested showed a superior activity compared to that of incubation with single drugs. For example, 24 h postincubation with artesunate and artemether in combination with triclabendazole, flukes showed only minimal activities, and approximately 90% of the flukes had died after 48 h (Fig. 7, row 1, graphs a and b). None of the juvenile *F. hepatica* survived in the presence of any of the triclabendazole-sulfoxide combinations tested (Fig. 7, row 2, graphs a to c).

## DISCUSSION

Cases of triclabendazole resistance are continuously documented from livestock, and hence new strategies for the treatment of *F. hepatica* infections are needed (10). Today, drug combinations are a popular tool to decrease the selection pressure and increase parasitological cure rates to reduce toxicity (11). In addition, drug combinations are less likely to produce

adverse events as reduced dosages are used. Artemisinin combinations therefore might have a better safety profile than monotherapies. For example, toxicological effects were observed with artesunate in *F. hepatica*-infected rats at doses of 200 mg/kg and above, and neurotoxicity or embryotoxicity have been described in rodents, dogs, and monkeys (9, 19).

The present study investigated the effect on worm and egg burden of triclabendazole, artesunate, artemether, and OZ78 monotherapy and combinations in rats harboring acute and chronic *F. hepatica* infections. In addition, drug effects on juvenile and adult *F. hepatica* organisms were monitored *in vitro*.

This study confirmed that single oral doses of artesunate, artemether, OZ78, and triclabendazole are active against *F. hepatica* in rats, with the lowest $ED_{50/95}$ values observed for the reference drug triclabendazole. Somewhat higher activities were observed with OZ78 compared to those of the artemisinins *in vivo*, but an opposite tendency was observed *in vitro*, which might be explained by the low bioavailability and half-lives of artemether and artesunate (15, 37). *In vivo*, OZ78, artemether, and artesunate all showed reductions in burdens of juvenile flukes similar to those of adults, while *in vitro* the concentrations of these drugs needed to kill the juvenile flukes were about twice those that killed the adults. On the other hand, triclabendazole was more effective against adult than juvenile *F. hepatica* flukes *in vivo*; for instance, at a dosage of 5 mg/kg it achieved worm burden reductions of 56.8 and 12.8% against adult and juvenile flukes, respectively. Although triclabendazole has been well studied against immature and mature *F. hepatica* in sheep and cattle (2, 14, 32, 38), to our knowledge thorough stage specifity studies of rats have not been carried out to date. This stage specificity appears to arise because of the reduced *in vitro* sensitivity of the juvenile flukes to the metabolites of triclabendazole, into which the drug is converted rapidly *in vivo* (10). *In vitro*, triclabendazole sulfoxide- and sulfone-treated adult flukes were killed, while juveniles behaved like the controls. In contrast, triclabendazole was much more effective against juvenile than adult *F. hepatica* flukes. In a previous study, immature flukes also were found to be more sensitive to triclabendazole than adults *in vitro*, while triclabendazole-sulfoxide exhibited a delayed effect on juvenile flukes (1).

Our preliminary findings obtained with drug combinations on adult flukes *in vitro* somewhat correspond to the findings observed with adult infections *in vivo*. The combinations with the triclabendazole metabolites plus peroxidic drug (Fig. 6, rows 2 and 3, graphs a to c) against mature *F. hepatica in vitro* were more effective than the single drugs, and this was supported by *in vivo* experiments combining 2.5 mg/kg triclabendazole with artesunate or artemether. The almost-complete elimination of worms was achieved with dosages of these two semisynthetic artemisinins as low as 12.5 mg/kg when combined with 2.5 mg/kg triclabendazole (Fig. 4D and E). This finding might be explained by the independent mechanism of action of the drugs. While artemether enters the parasite through oral ingestion and causes substantial disruption to the gut, triclabendazole mainly affects the tegument (29, 35). Furthermore, artemether and OZ78 also act against triclabendazole-resistant *F. hepatica* (22).

On the other hand, combinations with 1.25 mg/kg triclabendazole appeared to slightly inhibit the effect of artemether or artesunate; i.e., somewhat higher worm burdens were observed than those achieved with artemisinin monotherapy (Fig. 4A and B). Similar antagonistic effects have been observed when the artemisinins were combined with low doses of praziquantel (75 mg/kg) and administered to *Clonorchis sinensis*-infected rats (24). The rather contradictory results obtained following small modifications in the triclabendazole dose (1.25 versus 2.5 mg/kg) cannot be explained at the moment and warrant further investigation, such as pharmacokinetic studies.

Interestingly, we observed no overall significant interaction between triclabendazole and OZ78 when administered to rats infected with mature flukes. Unlike the artemisinins, OZ78 effects did not depend on the dosage of triclabendazole, and it also could cure rats in combination with 1.25 mg/kg triclabendazole with a dose response similar to that of monotherapy with OZ78. Combinations with OZ78 showed a trend toward a synergistic effect (however, likelihood ratio statistics showed no significance; $P = 0.07$) at lower dosages of OZ78; for example, 25 mg/kg of OZ78 was more effective in combination with both dosages of triclabendazole used than when applied alone.

*In vitro*, all drug combinations had more pronounced effects against immature than adult flukes (Fig. 6 and 7), and the *in vitro* combination chemotherapy data suggest synergistic effects on juveniles. However, serial drug dilutions would have been necessary to construct isobolograms to detect true synergistic or antagonistic effects (3). Furthermore, while our *in vitro* study was based on periodic phenotypic evaluation, more sophisticated approaches such as calorimetric measurement (25), which continuously measures the energy release of the worm, are required to evaluate drug-worm interactions in more detail. On the other hand, *in vivo* most of the combinations, like the monotherapies, were less effective against juveniles than against mature flukes, and we found little evidence for synergy. Exceptions were combinations with 5 mg/kg triclabendazole and 50 mg/kg peroxidic drug, which killed juvenile worms, despite the ineffectiveness of monotherapies at these doses against them. We did not use dosages higher than 5 mg/kg in our studies, since a 10-mg/kg triclabendazole dose has been shown to achieve worm burden reductions of 85 to 100% (14 and unpublished observations). Nonetheless, it would be interesting to evaluate intermediate triclabendazole doses.

Egg excretion analyses are helpful to confirm the presence of *F. hepatica* and other helminth infections and to monitor infection intensities. *Fasciola hepatica* eggs were detected in all 132 fecal samples examined prior to drug treatment, and post-treatment egg counts correlated significantly with surviving worms. It appears that the treatment reduced the worm burden and therefore minimized the influence of the crowding effect (a decreased egg output as a function of higher worm burden), which was previously documented in *F. hepatica*-infected rats (36).

However, there is a substantial loss of precision in using egg counts as indicators of worm burdens. There are several reasons why the direct counting of worms gives more-precise estimates of dose-response relationships. Some rats with surviving worms produced very few or no eggs, probably because drug treatment can inhibit egg production even when it does not kill the worms. It has been demonstrated that triclabendazole affects spermatogenic and vitelline cells of *F. hepatica* and

artemether inhibits the egg production of *F. gigantica in vitro* (6, 33, 34). We also recorded rats characterized by no worms but egg-positive fecal samples. This could be because the period of 7 days between treatment and dissection was too short to eliminate all eggs from the rats' bodies. In addition, since rats were housed in groups of five, we might have detected transient eggs, which were orally taken up from egg-positive fecal pellets present in the cage.

In conclusion, we confirmed the promising fasciocidal properties of the peroxidic drugs artesunate, artemether, and OZ78. It is encouraging that enhanced drug effects were observed using combinations of artesunate, artemether, and OZ78 plus triclabendazole in *F. hepatica*-infected rats. However, the great variations in dose response following slight titrations in the doses are striking. Further experiments, such as pharmacokinetic and pharmacodynamic studies following combination chemotherapy in rats, studies using triclabendazole-resistant *F. hepatica* strains, or combination trials in larger animals (e.g., sheep), are warranted, which might further strengthen our knowledge of the fasciocidal properties of triclabendazole-peroxide drug combinations.

## ACKNOWLEDGMENTS

We thank Mireille Vargas for her excellent technical assistance with the treatment of rats and the worm and egg count analyses.

U.D. and J.K. are financially supported by the Swiss National Science Foundation (project no. PPOOA-114941).

## REFERENCES

1. **Bennett, J. L., and P. Kohler.** 1987. Fasciola hepatica: action in vitro of triclabendazole on immature and adult stages. Exp. Parasitol. **63:**49–57.
2. **Boray, J. C., P. D. Crowfoot, M. B. Strong, J. R. Allison, M. Schellenbaum, M. Von Orelli, and G. Sarasin.** 1983. Treatment of immature and mature Fasciola hepatica infections in sheep with triclabendazole. Vet. Rec. **113:** 315–317.
3. **Chou, T. C.** 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. **58:**621–681.
4. **Cringoli, G., L. Rinaldi, M. P. Maurelli, and J. Utzinger.** 2010. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat. Protoc. **5:**503–515.
5. **DeFronzo, R. A.** 2010. Current issues in the treatment of type 2 diabetes. Overview of newer agents: where treatment is going. Am. J. Med. **123:**S38–S48.
6. **Diab, T. M., H. H. Mansour, and S. S. Mahmoud.** 2010. Fasciola gigantica: parasitological and scanning electron microscopy study of the in vitro effects of ivermectin and/or artemether. Exp. Parasitol. **124:**279–284.
7. **Duthaler, U., L. Rinaldi, M. P. Maurelli, M. Vargas, J. Utzinger, G. Cringoli, and J. Keiser.** 2010. Fasciola hepatica: comparison of the sedimentation and FLOTAC techniques for the detection and quantification of faecal egg counts in rats. Exp. Parasitol. **126:**161–166.
8. **Edwards, G., and S. Krishna.** 2004. Pharmacokinetic and pharmacodynamic issues in the treatment of parasitic infections. Eur. J. Clin. Microbiol. Infect. Dis. **23:**233–242.
9. **Efferth, T., and B. Kaina.** 2010. Toxicity of the antimalarial artemisinin and its dervatives. Crit. Rev. Toxicol. **40:**405–421.
10. **Fairweather, I.** 2009. Triclabendazole progress report, 2005–2009: an advancement of learning? J. Helminthol. **83:**139–150.
11. **Fairweather, I., and J. C. Boray.** 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet. J. **158:**81–112.
12. **Gupta, A. K., S. Arshad, and N. R. Poulter.** 2010. Compliance, safety, and effectiveness of fixed-dose combinations of antihypertensive agents: a meta-analysis. Hypertension **55:**399–407.
13. **Keiser, J.** 2010. In vitro and in vivo trematode models for chemotherapeutic studies. Parasitology **137:**589–603.
14. **Keiser, J., D. Engels, G. Buscher, and J. Utzinger.** 2005. Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin. Investig. Drugs **14:**1513–1526.
15. **Keiser, J., M. S. Gruyer, N. Perrottet, B. Zanolari, T. Mercier, and L. Decosterd.** 2009. Pharmacokinetic parameters of artesunate and dihydroartemisinin in rats infected with Fasciola hepatica. J. Antimicrob. Chemother. **63:**543–549.
16. **Keiser, J., C. Kirchhofer, M. Haschke, J. Huwyler, Y. Dong, J. L. Vennerstrom, K. Vanhoff, R. Kaminsky, and N. Malikides.** 2010. Efficacy, safety and pharmacokinetics of 1,2,4-trioxolane OZ78 against an experimental infection with Fasciola hepatica in sheep. Vet. Parasitol. doi: 10.1016/j.vetpar.2010.06.031.
17. **Keiser, J., and G. Morson.** 2008. Fasciola hepatica: tegumental alterations in adult flukes following in vitro and in vivo administration of artesunate and artemether. Exp. Parasitol. **118:**228–237.
18. **Keiser, J., L. Rinaldi, V. Veneziano, L. Mezzino, M. Tanner, J. Utzinger, and G. Cringoli.** 2008. Efficacy and safety of artemether against a natural Fasciola hepatica infection in sheep. Parasitol. Res. **103:**517–522.
19. **Keiser, J., X. Shu-Hua, M. Tanner, and J. Utzinger.** 2006. Artesunate and artemether are effective fasciolicides in the rat model and in vitro. J. Antimicrob. Chemother. **57:**1139–1145.
20. **Keiser, J., and J. Utzinger.** 2009. Food-borne trematodiases. Clin. Microbiol. Rev. **22:**466–483.
21. **Keiser, J., J. Utzinger, M. Tanner, Y. Dong, and J. L. Vennerstrom.** 2006. The synthetic peroxide OZ78 is effective against Echinostoma caproni and Fasciola hepatica. J. Antimicrob. Chemother. **58:**1193–1197.
22. **Keiser, J., J. Utzinger, J. L. Vennerstrom, Y. Dong, G. Brennan, and I. Fairweather.** 2007. Activity of artemether and OZ78 against triclabendazole-resistant Fasciola hepatica. Trans. R. Soc. Trop. Med. Hyg. **101:**1219–1222.
23. **Keiser, J., V. Veneziano, L. Rinaldi, L. Mezzino, U. Duthaler, and G. Cringoli.** 2010. Anthelmintic activity of artesunate against Fasciola hepatica in naturally infected sheep. Res. Vet. Sci. **88:**107–110.
24. **Keiser, J., S. H. Xiao, T. A. Smith, and J. Utzinger.** 2009. Combination chemotherapy against Clonorchis sinensis: experiments with artemether, artesunate, OZ78, praziquantel, and tribendimidine in a rat model. Antimicrob. Agents Chemother. **53:**3770–3776.
25. **Manneck, T., O. Braissant, W. Ellis, and J. Keiser.** 2010. Schistosoma mansoni: antischistosomal activity of the four optical isomers and the two racemates of mefloquine on schistosomula and adult worms in vitro and in vivo. Exp. Parasitol. doi:10.1016/j.exppara.2010.08.011.
26. **Mas-Coma, S., M. D. Bargues, and M. A. Valero.** 2005. Fascioliasis and other plant-borne trematode zoonoses. Int. J. Parasitol. **35:**1255–1278.
27. **McManus, D. P., and J. P. Dalton.** 2006. Vaccines against the zoonotic trematodes Schistosoma japonicum, Fasciola hepatica and Fasciola gigantica. Parasitology **133**(Suppl.)**:**S43–S61.
28. **Meaney, M., J. Allister, B. McKinstry, K. McLaughlin, G. P. Brennan, A. B. Forbes, and I. Fairweather.** 2006. Fasciola hepatica: morphological effects of a combination of triclabendazole and clorsulon against mature fluke. Parasitol. Res. **99:**609–621.
29. **O'Neill, J. F., R. C. Johnston, L. Halferty, G. P. Brennan, J. Keiser, and I. Fairweather.** 2009. Adult triclabendazole-resistant Fasciola hepatica: morphological changes in the tegument and gut following in vivo treatment with artemether in the rat model. J. Helminthol. **83:**151–163.
30. **Raffa, R. B., R. Clark-Vetri, R. J. Tallarida, and A. I. Wertheimer.** 2003. Combination strategies for pain management. Expert Opin. Pharmacother. **4:**1697–1708.
31. **Robinson, M. W., and J. P. Dalton.** 2009. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos. Trans. R. Soc. Lond. B Biol. Sci. **364:**2763–2776.
32. **Smeal, M. G., and C. A. Hall.** 1983. The activity of triclabendazole against immature and adult Fasciola hepatica infections in sheep. Aust. Vet. J. **60:**329–331.
33. **Stitt, A. W., and I. Fairweather.** 1996. Fasciola hepatica: disruption of the vitelline cells in vitro by the sulphoxide metabolite of triclabendazole. Parasitol. Res. **82:**333–339.
34. **Stitt, A. W., and I. Fairweather.** 1992. Spermatogenesis in Fasciola hepatica–an ultrastructural comparison of the effects of the anthelmintic, triclabendazole (fasinex) and the microtubule inhibitor, tubulozole. Invertebr. Reprod. Dev. **22:**139–150.
35. **Toner, E., F. McConvery, G. P. Brennan, M. Meaney, and I. Fairweather.** 2009. A scanning electron microscope study on the route of entry of triclabendazole into the liver fluke, Fasciola hepatica. Parasitology **136:**523–535.
36. **Valero, M. A., M. De Renzi, M. Panova, M. A. Garcia-Bodelon, M. V. Periago, D. Ordonez, and S. Mas-Coma.** 2006. Crowding effect on adult growth, pre-patent period and egg shedding of Fasciola hepatica. Parasitology **133:**453–463.
37. **Vennerstrom, J. L., S. Arbe-Barnes, R. Brun, S. A. Charman, F. C. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. Santo Tomas, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin, and W. N. Charman.** 2004. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature **430:**900–904.
38. **Walker, S. M., B. McKinstry, J. C. Boray, G. P. Brennan, A. Trudgett, E. M. Hoey, H. Fletcher, and I. Fairweather.** 2004. Response of two isolates of Fasciola hepatica to treatment with triclabendazole in vivo and in vitro. Parasitol. Res. **94:**427–438.
39. **Zhang, Y.** 2007. Advances in the treatment of tuberculosis. Clin. Pharmacol. Ther. **82:**595–600.

# *Chapter 4*

# Anthelmintic Activity of Artesunate against *Fasciola hepatica* in Naturally Infected Sheep

Jennifer Keiser [a], Vincenzo Veneziano [b], Laura Rinaldi [b], Laura Mezzino [b], Urs Duthaler [a], Giuseppe Cringoli [b]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

[b] Department of Pathology and Animal Health, CREMOPAR Regione Campania, University of Naples Federico II, Naples, Italy

**Published in Research in Veterinary Science 88 (2010): p. 107-110**

Research in Veterinary Science 88 (2010) 107–110

Contents lists available at ScienceDirect

Research in Veterinary Science

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

# Anthelmintic activity of artesunate against *Fasciola hepatica* in naturally infected sheep

Jennifer Keiser [a,*], Vincenzo Veneziano [b], Laura Rinaldi [b], Laura Mezzino [b], Urs Duthaler [a], Giuseppe Cringoli [b]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, CH-4002 Basel, Switzerland
[b] Department of Pathology and Animal Health, University of Naples "Federico II", CREMOPAR Regione Campania, Naples, Italy

ARTICLE INFO

*Article history:*
Accepted 11 May 2009

*Keywords:*
*Fasciola hepatica*
Fascioliasis
Artesunate
Sheep
Natural infection
Worm burden
Egg count
Safety

ABSTRACT

In light of rapidly spreading triclabendazole resistance alternative fasciocidal drugs are urgently needed. Following up on promising results obtained with artemether in *Fasciola hepatica* infected sheep, we here report the efficacy and safety of artesunate in sheep with a natural *F. hepatica* infection. Artesunate was administered intravenously and intramuscularly, adverse events were monitored and drug efficacy was elucidated by means of faecal egg and worm burden reductions. A single 40 mg/kg intravenous dose of artesunate induced an egg count reduction of 68.9% and a worm burden reduction of 77.4%. Intramuscular artesunate at 40 mg/kg reduced faecal egg count and worm burden by 97.6% and 91.9%, respectively; whereas at 60 mg/kg it caused 93.2% and 87.1% reduction in faecal egg count and worm burden, respectively. Three sheep died 24–72 h post-treatment with a double dose of 40 mg/kg intramuscular artesunate, showing lethargy, sialorrhoea, reduced rumination and tremors. Egg and worm burden reductions of 93.3% and 83.9%, respectively, were calculated in the three surviving sheep. In conclusion, the interesting fasciocidal properties of artesunate in sheep warrant further investigations with an emphasis on toxicity studies.



## 1. Introduction

Fascioliasis (fasciolosis), caused by the digenetic trematode flatworms *Fasciola hepatica* and *F. gigantica* is a serious public health problem and a major problem in ruminant livestock production (Keiser and Utzinger, 2007a; McManus and Dalton, 2006). It is estimated that there are from 2.4 to 17 million people infected throughout the world (Keiser and Utzinger, 2005). In livestock mainly cattle and sheep are affected and significant economic losses occur each year, due to reduced fertility, lower milk yield and decreased meat production (Schweizer et al., 2005; Torgerson and Claxton, 1999). Suitable control strategies are necessary for the health and productivity of affected livestock and to avoid some of the economic losses.

A vaccine for the prevention of fascioliasis is presently not available (McManus and Dalton, 2006). Control strategies include the use of molluscicides, biological control, feeding and grazing control and environmental management as lowering the ground water level, good drainage, cleaning of ditches or pasture management (De et al., 2008; Martindah et al., 2003; Over, 1982). The benzimidazole derivative triclabendazole is the current treatment of choice for fascioliasis as this drug is effective against juvenile and adult flukes (Keiser et al., 2005). Nonetheless, the need for a novel fasciocidal drug has been emphasized since alternative drugs are not available and triclabendazole resistance has been reported from different continents (Keiser et al., 2005).

The semisynthetic artemisinin derivatives artemether and artesunate (Fig. 1), essential components of malaria treatment (White, 2008), have been reported to have a broad spectrum of activity against trematodes: they are effective in the treatment of schistosomiasis (Utzinger et al., 2007) and have recently also been used for the treatment of acute fascioliasis (Hien et al., 2008). High worm burden reductions were obtained with single oral doses of artemether and artesunate in rodents infected with *Clonorchis sinensis*, *F. hepatica* and *Opisthorchis viverrini* (Keiser and Utzinger, 2007b). Importantly, treatment of rats harboring a triclabendazole resistant *Fasciola* isolate with artemether resulted in complete elimination of the worm burden (Keiser et al., 2007). First investigations in sheep infected with a natural *F. hepatica* infection have shown that artemether achieved a worm burden reduction of 91% when a single intramuscular dose of 160 mg/kg was given (Keiser et al., 2008). The mechanism of action of the artemisinins on trematodes is not known, but cleavage of the peroxide bridge resulting in the generation of free radicals might play a role as a greater disruption to the tegument of *F. hepatica* was observed following incubation with the artemisinins *in vitro* when haemin was added to the culture medium (Keiser and Morson, 2008).

The purpose of this study was to further elucidate the fasciocidal properties of the artemisinins in sheep. Here, we studied the

* Corresponding author. Tel.: +41 61 284 8218; fax: +41 61 284 8105.
*E-mail address:* jennifer.keiser@unibas.ch (J. Keiser).

0034-5288/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2009.05.007

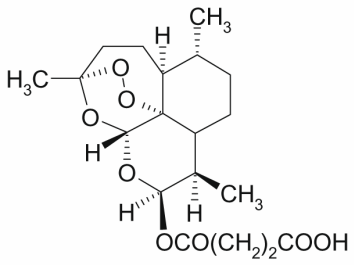



**Fig. 1.** Chemical structure of artesunate.

effect of artesunate in sheep harboring a natural *F. hepatica* infection. We first comparatively assessed two routes of artesunate administration, a 40 mg/kg single intravenous and a 40 mg/kg single intramuscular drug administration. We then tested a single 60 mg/kg intramuscular dosage and one double (2 × 40 mg/kg) intramuscular treatment regimen. Adverse events were monitored and drug efficacy was elucidated by means of faecal egg count reduction and worm burden reductions following the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) for evaluation of the efficacy of anthelminthic drugs in ruminants (Wood et al., 1995).

## 2. Materials and methods

### 2.1. Study site and ethical clearance

The study was conducted on a meat/dairy sheep farm located in the Salerno province of southern Italy (geographical coordinates: 40° 21′ 29″ N latitude and 14° 59′ 12″ E longitude). The flock contains up to 100 cross-breed dairy and meat sheep and other ruminants. The study animals were separated from the rest of the animals and maintained on a designated paddock.

Ethical clearance was obtained from the Centre for Veterinary Service of the University of Naples Federico II (Ref. no. 98/08).

### 2.2. Drug

Artesunate (Fig. 1) was kindly provided by Dafra Pharma (Turnhout, Belgium) and Mepha AG (Aesch, Switzerland). Artesunate was dissolved in a 40:60 mixture of 5% $NaHCO_3$ (Merck, Darmstadt, Germany) and polyethylene glycol (PEG) 400 (Fluka, Buchs, Switzerland). PEG 400 was added to increase physical stability, solubility and to decrease the pH towards physiological conditions.

### 2.3. Study design

Faecal samples from 100 sheep from the above mentioned farm were examined for the presence of *F. hepatica* eggs by the Flotac basic technique having an analytic sensitivity of 1 egg per gram of faeces (Cringoli, 2006,2009) as described in our previous study (Keiser et al., 2008). A zinc sulphate plus potassium iodomercurate solution was employed as flotation solution (specific gravity = 1.450) (Cringoli et al., 2004).Two additional faecal samples were collected from all *F. hepatica*-positive sheep on two consecutive days and the *F. hepatica* egg burden was determined. Sheep were randomly allocated to four treatment groups and one control group, according to the geometric mean egg count based on three faecal samples. In the first round of treatment group 1 ($n = 10$) served as controls, hence remained untreated. Group 2 ($n = 5$) was administered a single 40 mg/kg artesunate intravenously and group 3 ($n = 6$) received a single intramuscular dose of 40 mg/kg artesunate. In the second round of treatment group 4 ($n = 6$) was given a double dose of 40 mg/kg intramuscular artesunate spaced by 13 h (evening and morning). Group 5 ($n = 6$) was administered a single 60 mg/kg intramuscular dose of artesunate. We monitored sheep for the occurrence of adverse events for 8 h (sheep of group 4 were monitored for 72 h following the second dose) and then once daily following drug administration. Starting 18 days post-treatment three consecutive faecal samples were collected from each sheep and analyzed by the above cited Flotac technique for the *F. hepatica* egg burden. All sheep were slaughtered 21 days post-treatment. Livers were removed, and transferred to the laboratory in thermal containers. All *F. hepatica* flukes were collected from the gall bladder and excised bile ducts, counted and the morphology and movement examined and recorded.

### 2.4. Statistical analysis

Statsdirect statistical software (version 2.4.5 of (Statsdirect Ltd., Cheshire, UK) was used for the statistical analyses. The reduction of the geometric mean of faecal egg counts pre-treatment and post-treatment was examined. EPG values were logarithmically transformed (log (count +1)) and pre-treatment and post-treatment data were analyzed by using an unpaired two-tailed Student's *t*-test, allowing for unequal variance. Data were considered significant with a significance level of 0.05.

The Kruskal–Wallis (KW) test was used to compare the medians of the *F. hepatica* counts of the treatment and control groups. Differences in medians were considered to be significant at a significance level of 0.05.

## 3. Results

### 3.1. Safety assessment

Both intramuscular and intravenous administration of single doses of artesunate at 40 and 60 mg/kg were well tolerated by the sheep and no physical clinical sign of toxicity were observed. However, three sheep died 24–72 h post-treatment with a double dose (2 × 40 mg/kg) of artesunate, showing signs of clinical toxicosis. The clinical signs observed before the death of sheep were lethargy, sialorrhoea, teeth grinding, hypothermia, reduced rumination and tremors. The gross necropsies revealed hepatomegaly and nephrosis.

Local injection site reactions were not observed.

### 3.2. Effect of artesunate on F. hepatica faecal egg burden

The effect of artesunate treatment on the *F. hepatica* egg burden is summarized in Table 1. Before treatment sheep passed a mean of 30.6–55.1 EPG. A single 40 mg/kg intravenous dose of artesunate achieved an egg count reduction of 68.9%. At doses of 40 and 60 mg/kg artesunate administered intramuscularly we found a significant egg count reduction of 97.6% and 93.2% ($P < 0.001$), respectively. When two doses of artesunate, spaced by 13 h, were given a faecal egg count reduction of 93.3% ($P < 0.001$) was calculated in the three surviving sheep.

### 3.3. Effect of artesunate on F. hepatica worm burden

Table 2 presents the effect of artesunate treatment on *F. hepatica* worm counts in sheep. The untreated control group harbored a mean of six flukes in their bile ducts. A worm burden reduction of 77.4% was achieved when artesunate (40 mg/kg) was given intravenously, which was not statistically significant (KW = 3.03; $P = 0.08$). A single 40 mg/kg intramuscular dose of artesunate resulted in a significant worm burden reduction of 91.9%

**Table 1**
Effect of intravenous and intramuscular artesunate on *F. hepatica* faecal egg counts in sheep, expressed as geometric mean eggs per gram of faeces (EPG).

| Treatment group | Dose (mg/kg) | Route of administration | Number of animals | Pre-treatment (EPG) Geometric mean | Post-treatment (EPG) Geometric mean | % Reduction |
|---|---|---|---|---|---|---|
| 1 (Control) | – | – | 10 | 49.0 | 42.6 | – |
| 2 | 40 | Iv | 5 | 30.6 | 9.5 | 68.9 |
| 3 | 40 | Im | 6 | 55.1 | 1.3 | 97.6 |
| 4 | 2 × 40* | Im | 6 | 36.0 | 2.4 | 93.3 |
| 5 | 60 | Im | 6 | 33.8 | 2.3 | 93.2 |

* Three sheep died 24–72 h following the second artesunate dose.

**Table 2**
Worm burden reductions of *F. hepatica* in sheep after treatment with artesunate.

| Treatment group | Dose (mg/kg) | Route of administration | Animals cured[a] | Mean worm burden (SD) | Total worm burden reduction (%) | KW | *P* |
|---|---|---|---|---|---|---|---|
| 1 (Control) | – | – | 0 | 6.2 (9.4) | | | |
| 2 | 40 | Iv | 2 | 1.4 (1.3) | 77.4 | 3.026 | 0.081 |
| 3 | 40 | Im | 4 | 0.5 (0.8) | 91.9 | 8.181 | 0.004 |
| 4 | 2 × 40* | Im | 1 | 1.0 (1.0) | 83.9 | 3.544 | 0.059 |
| 5 | 60 | Im | 3 | 0.8 (1.2) | 87.1 | 6.402 | 0.011 |

* Three sheep died 24–72 h following the second artesunate dose.
[a] Number of animals without flukes. SD, standard deviation.

(KW = 8.18; $P = 0.004$). At a slightly higher intramuscular dose (60 mg/kg) a similar worm burden reduction of 87.1% was observed (KW = 6.40; $P = 0.01$). Analysis of the worm burden of the three remaining sheep treated with a double dose of artesunate revealed a worm burden reduction of 83.9% (KW = 3.54; $P = 0.06$). Examination of the *Fasciola* flukes collected from the excised bile ducts of treated sheep revealed no morphological alterations and *Fasciola* showed a similar activity when compared to flukes recovered from non-treated sheep.

## 4. Discussion

We have previously reported that artemether exhibited activity in sheep harboring a natural *F. hepatica* infection. Briefly, oral artemether showed no effect on the *F. hepatica* egg and worm burden. However, treatment with a single 160 mg/kg intramuscular dose of artemether yielded a worm burden reduction of 91.3% (Keiser et al., 2008). In view of these results, we were motivated to extend our investigations to the structurally related semisynthetic artemisinin derivative artesunate. Based on our previous experiences with artemether in sheep only parenteral treatment was used in the present investigation. Since artesunate is – in contrast to artemether – characterized by high water solubility, in a first step both an intravenous and an intramuscular treatment regimen were tested. In practical terms, intramuscular drug administration is preferred over intravenous application, as most farmers need to rely on veterinarians for intravenous injections (G. Cringoli, personal communication). Nonetheless, there are few comparative studies on the efficacy, bioavailability and disposition of artesunate available, and to our knowledge no such study has ever been carried out in *F. hepatica* infected sheep. We found that intramuscular artesunate was slightly superior to intravenous artesunate (worm burden reduction of 91.9% versus 77.4%). A previous study which has analyzed pharmacokinetic (PK) parameters of different administration routes of all artemisinin derivatives in the rat model found very similar PK parameters following intravenous and intramuscular artesunate, with the exception of the half life, which was slightly longer (54 versus 35 min) following intramuscular treatment (Li et al., 1998). Hence, one might speculate that the somewhat increased worm burden reduction following intramuscular artesunate treatment might be due to a longer exposure of the parasite to the drug. Analysis of the PK parameters of intramuscular and intravenous artesunate in *F. hepatica* infected sheep is ongoing in our laboratories and might help to answer these questions.

Interestingly, artesunate achieved a similar worm burden reduction as artemether in *F. hepatica* infected sheep with a fourfold lower dose administered. This finding might be explained with the above mentioned better biopharmaceutical properties of the water soluble artesunate when compared to artemether. Artemether is characterized by a low bioavailability following intramuscular application, which might be due to the slow, prolonged absorption of the oil formulation (Li et al., 1998). It is interesting to note, when we increased the intramuscular artesunate dosage from 40 to 60 mg/kg, we observed a comparable effect on the egg count and the worm burden. The lack of dose dependent increase in effect might be explained with the lower (40 mg/kg) artesunate dose being already at the top of the dose–response curve, hence lower artesunate doses (e.g., 20 mg/kg) should also be tested.

While single artesunate treatments were well tolerated by sheep, half of the six sheep died following administration of a double dose of artesunate. The death was preceded by clinical symptoms as lethargy, sialorrhoea, reduced rumination and tremors, which point to an impairment of the hepatic function of these sheep.

Pharmacokinetic data has shown that drug accumulation occurs following multiple artemether doses for example in dogs (Classen et al., 1999). However, drug accumulation was only seen after repeated dosing and appeared to be associated with a slow absorption of the oil formulation (Classen et al., 1999). To our knowledge a toxicity of artesunate related to drug accumulation has not been described. Toxicity evaluations of single oral artesunate found an LD50 value of 351 mg/kg in healthy rats (Li et al., 2007). A single oral dose of 240 mg/kg in healthy rats showed moderate signs of renal failure and histopathological evaluation demonstrated mild to moderate tubular necrosis (Li et al., 2007). In mice LD50 values of 520 and 475 mg/kg were reported following intravenous and intramuscular administration (China Cooperative Research Group, 1982). Increasing intravenous doses of artesunate (37.5, 75 and 100 mg/kg) in dogs caused vomiting and sign of exhaustion but none of the animals died (China Cooperative Research Group, 1982). It cannot be excluded that toxicity in sheep observed in the present study following a double dose of artesunate might be related to an impaired metabolism of artesunate due to liver damage caused by *Fasciola* flukes. In a previous study in *F. hepatica* infected rats toxicity was observed following single

oral artesunate doses of 200 mg/kg and above (Keiser et al., 2006a). Subsequent pharmacokinetic studies revealed a strong influence of the infection on the drug disposition of artesunate. For example, following oral administration the areas under the curve and maximum plasma concentrations of artesunate and its main metabolite dihydroartemisinin were 1.7–4.4-fold higher in infected rats (Keiser et al., 2009). Further studies have been planned to elucidate the PK parameters in healthy and *F. hepatica* infected sheep following single and multiple artesunate administration.

In conclusion, we have documented that artesunate possesses interesting fasciocidal properties in sheep. Intramuscular artesunate (40 mg/kg) fulfils WAAVP's effectiveness standard of drugs (worm burden reductions of 90% or higher). In light of rapidly spreading triclabendazole resistance alternative fasciocidal drugs are urgently needed (Keiser et al., 2005). Hence, follow up studies in sheep, ideally experimental *F. hepatica* infections, are necessary to elucidate the efficacy of artesunate also against higher infection intensities and against juvenile infections. In addition, the cause of toxicity, which we have observed following a double dose of artesunate in *F. hepatica* infected sheep should be elucidated carefully. Synthetic peroxides as for example the 1,2,4 trioxolane OZ78, which displayed a more favourable selectivity index (efficacy/toxicity ratio) than the artemisinins in the *F. hepatica* rat model (Keiser et al., 2006b; Vennerstrom et al., 2004) might be a valuable alternative and should also be tested in *F. hepatica* infected sheep.

## Conflict of interest

We have no conflict of interest to declare.

## Role of the funding source

The study sponsors had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

## Acknowledgements

We are grateful to the farmer Alessandro Mario for his kind collaboration, and thank Dafra Pharma and Mepha for the supply of artesunate. This investigation received financial support from the Swiss National Science Foundation via project PPOOA-114941 (to J. Keiser and U. Duthaler).

## References

China Cooperative Research Group on qinghaosu and its derivatives as antimalarials 1982. Studies on the toxicity of qinghaosu and its derivatives. J. Tradit. Chin. Med. 2, 31–38.

Classen, W., Altmann, B., Gretener, P., Souppart, C., Skelton-Stroud, P., Krinke, G., 1999. Differential effects of orally versus parenterally administered qinghaosu derivative artemether in dogs. Exp. Toxicol. Pathol. 51, 507–516.

Cringoli, G., 2006. FLOTAC, a novel apparatus for a multivalent faecal egg count technique. Parassitologia 48, 381–384.

Cringoli, G. (Ed.), 2009. FLOTAC® Manual – Flotac techniques – Herbivores, 80p. ISBN 978-88-89132-27-2.

Cringoli, G., Rinaldi, L., Veneziano, V., Capelli, G., Scala, A., 2004. The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and *Dicrocoelium dendriticum* in sheep. Vet. Parasitol. 123, 121–131.

De, S., Sanyal, P.K., Sarkar, A.K., Patel, N.K., Pal, S., Mandal, S.C., 2008. Screening for Indian isolates of egg-parasitic fungi for use in biological control of fascioliasis and amphistomiasis in ruminant livestock. J. Helminthol. 82, 271–277.

Hien, T.T., Truong, N.T., Minh, N.H., Dat, H.D., Dung, N.T., Hue, N.T., Dung, T.K., Tuan, P.Q., Campbell, J.I., Farrar, J.J., Day, J.N., 2008. A randomized controlled pilot study of artesunate versus triclabendazole for human fascioliasis in central Vietnam. Am. J. Trop. Med. Hyg. 78, 388–392.

Keiser, J., Morson, G., 2008. *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. Exp. Parasitol. 119, 87–93.

Keiser, J., Utzinger, J., 2005. Emerging foodborne trematodiasis. Emerg. Infect. Dis. 11, 1507–1514.

Keiser, J., Utzinger, J., 2007a. Advances in the discovery and development of novel trematocidal drugs. Expert Opin. Drug Discov. 2, 9–23.

Keiser, J., Utzinger, J., 2007b. Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol. 23, 555–562.

Keiser, J., Engels, D., Büscher, G., Utzinger, J., 2005. Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin. Invest. Drugs 14, 1513–1526.

Keiser, J., Xiao, S.H., Tanner, M., Utzinger, J., 2006a. Artesunate and artemether are effective fasciolicides in the rat model and in vitro. J. Antimicrob. Chemother. 57, 1139–1145.

Keiser, J., Utzinger, J., Tanner, M., Dong, Y., Vennerstrom, J.L., 2006b. The synthetic peroxide OZ78 is effective against *Echinostoma caproni* and *Fasciola hepatica*. J. Antimicrob. Chemother. 58, 1193–1197.

Keiser, J., Utzinger, J., Vennerstrom, J.L., Dong, Y., Brennan, G., Fairweather, I., 2007. Activity of artemether and OZ78 against triclabendazole-resistant *Fasciola hepatica*. Trans. R. Soc. Trop. Med. Hyg. 101, 1219–1222.

Keiser, J., Rinaldi, L., Veneziano, V., Mezzino, L., Tanner, M., Utzinger, J., Cringoli, G., 2008. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol. Res. 103, 517–522.

Keiser, J., Gruyer, M., Perrottet, N., Zanolari, B., Mercier, T., Decosterd, L., 2009. Pharmacokinetic parameters of artesunate and dihydroartemisinin in *Fasciola hepatica*-infected rats. J. Antimicrob. Chemother. 63, 543–549.

Li, Q.G., Peggins, J.O., Fleckenstein, L.L., Masonic, K., Heiffer, M.H., Brewer, T.G., 1998. The pharmacokinetics and bioavailability of dihydroartemisinin, arteether, artemether, artesunic acid and artelinic acid in rats. J. Pharm. Pharmacol. 50, 173–182.

Li, Q., Xie, L.H., Johnson, T.O., Si, Y., Haeberle, A.S., Weina, P.J., 2007. Toxicity evaluation of artesunate and artelinate in *Plasmodium berghei*-infected and uninfected rats. Trans. R. Soc. Trop. Med. Hyg. 101, 104–112.

Martindah, E., Cameron, D., Frank, B., 2003. Participative approaches to enhance adoption of fascioliasis control strategies in cattle in Yokyakarta province, Indonesia. International Farm Management Congress.

McManus, D.P., Dalton, J.P., 2006. Vaccines against the zoonotic trematodes *Schistosoma japonicum*, *Fasciola hepatica* and *Fasciola gigantica*. Parasitology 133 (Suppl.), 43–61.

Over, H.J., 1982. Ecological basis of parasite control: trematodes with special reference to fascioliasis. Vet. Parasitol. 11, 85–97.

Schweizer, G., Braun, U., Deplazes, P., Torgerson, P.R., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet. Rec. 157, 188–193.

Torgerson, P.R., Claxton, J., 1999. Epidemiology and control. In: Dalton, J.P. (Ed.), Fasciolosis. CAB International, London, pp. 113–149.

Utzinger, J., Xiao, S.H., Tanner, M., Keiser, J., 2007. Artemisinins for schistosomiasis and beyond. Curr. Opin. Investig. Drugs 8, 105–116.

Vennerstrom, J.L., Arbe-Barnes, S., Brun, R., Charman, S.A., Chiu, F.C., Chollet, J., Dong, Y., Dorn, A., Hunziker, D., Matile, H., McIntosh, K., Padmanilayam, M., Santo Tomas, J., Scheurer, C., Scorneaux, B., Tang, Y., Urwyler, H., Wittlin, S., Charman, W.N., 2004. Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 430, 900–904.

White, N.J., 2008. Qinghaosu (artemisinin): the price of success. Science 320, 330–334.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone Jr., J.B., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M., et al., 1995. World Association for the advancement of veterinary parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet. Parasitol. 58, 181–213.

# *Chapter 5*

# Development and Validation of a LC-MS/MS Method for the Quantification of Artesunate, Artemether, and their Major Metabolites in Sheep Plasma

Urs Duthaler [a], Jennifer Keiser [a], Jörg Huwyler [b]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

[b] Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Basel, Switzerland.

**Published in Journal of Mass Spectrometry 46 (2011): p. 172-181**

Research Article

Journal of MASS SPECTROMETRY

Received: 6 October 2010 Accepted: 11 December 2010 Published online in Wiley Online Library: 24 January 2011

(wileyonlinelibrary.com) DOI 10.1002/jms.1883

# Development and validation of a liquid chromatography and ion spray tandem mass spectrometry method for the quantification of artesunate, artemether and their major metabolites dihydroartemisinin and dihydroartemisinin-glucuronide in sheep plasma

**Urs Duthaler,[a,b] Jennifer Keiser[a,b]* and Jörg Huwyler[c]**

**Recently, promising fasciocidal activities of artesunate and artemether were described in rats and sheep. Therefore, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed to quantify artesunate, artemether and their metabolites dihydroartemisinin and dihydroartemisinin-glucuronide in sheep plasma. Protein precipitation with methanol was used for sample workup. Reversed-phase high-performance liquid chromatography (HPLC) was performed using an Atlantis C18 analytical column with a mobile phase gradient system of ammonium formate and acetonitrile. The analytes were detected by MS/MS using selected reaction monitoring (SRM) with electrospray ionisation in the positive mode (transition *m/z* 267.4 → 163.0). The analytical range for dihydroartemisinin, dihydroartemisinin-glucuronide and artesunate was 10–1000 ng/ml and for artemether 90–3000 ng/ml with a lower limit of quantification of 10 and 90 ng/ml, respectively. Inter- and intra-day accuracy and precision deviations were <10%. Consistent relative recoveries (60–80%) were observed over the investigated calibration range for all analytes. All analytes were stable in the autosampler for at least 30 h (6 °C) and after three freeze and thaw cycles. The validation results demonstrated that the LC–MS/MS method is precise, accurate and selective and can be used for the determination of the artemisinins in sheep plasma. The method was applied successfully to determine the pharmacokinetic parameters of artesunate and its metabolites in plasma of intramuscularly treated sheep. Copyright © 2011 John Wiley & Sons, Ltd.**

**Keywords:** artemisinins; metabolites; glucuronide; fascioliasis; liquid chromatography–tandem mass spectrometry

## Introduction

The endoperoxide artemisinin (qinghaosu), a secondary plant compound of the herb *Artemisia annua* (Chinese wormwood), is an efficacious and fast acting antimalarial drug.[1] Because artemisinin itself has biopharmaceutical shortcomings such as a poor bioavailability that limits its effectiveness, the sesquiterpene lactone scaffold of artemisinin was modified and the semi-synthetic derivatives have been developed. For example, the methyl ether derivative artemether (AM, Fig. 1) is characterised by a higher antiplasmodial activity than artemisinin.[1] The succinate derivative, artesunate (AS, Fig. 1), is water soluble and can be applied intravenously and is therefore indispensable for the treatment of cerebral malaria.[2] The semi-synthetic artemisinins are converted rapidly to dihydroartemisinin (DHA, Fig. 1) and are eliminated primary over bile by glucuronidation *in vivo* (Fig. 1).[1,3] Importantly, DHA and partly also the glucuronide adducts possess high-to-moderate antimalarial activity.[4]

The artemisinins do not only exhibit antiplasmodial but also trematocidal activities, since haemoglobin metabolism is common in *Plasmodia* and several trematodes including *Fasciola* spp.[5] The liver and bile parasitising food-borne trematodes *Fasciola hepatica* and *F. gigantica* are the causative agents of fascioliasis (fasciolosis). As many as 17 million people might be infected with *Fasciola* species. In addition, the veterinary impact is considerable as sheep, cattle and bovine are globally affected by the disease.[6,7] Currently, triclabendazole is the sole drug for the treatment of human fascioliasis. Additional drugs are commonly used in the treatment of veterinary infections with *Fasciola* spp. including albendazole, closantel, hexachlorophene, nitroxynil and rafoxanide. Resistance

* *Correspondence to: Jennifer Keiser, Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. E-mail: jennifer.keiser@unibas.ch*

a *Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, Switzerland*

b *University of Basel, P.O. Box, CH-4003 Basel, Switzerland*

c *Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland*

Journal of MASS SPECTROMETRY

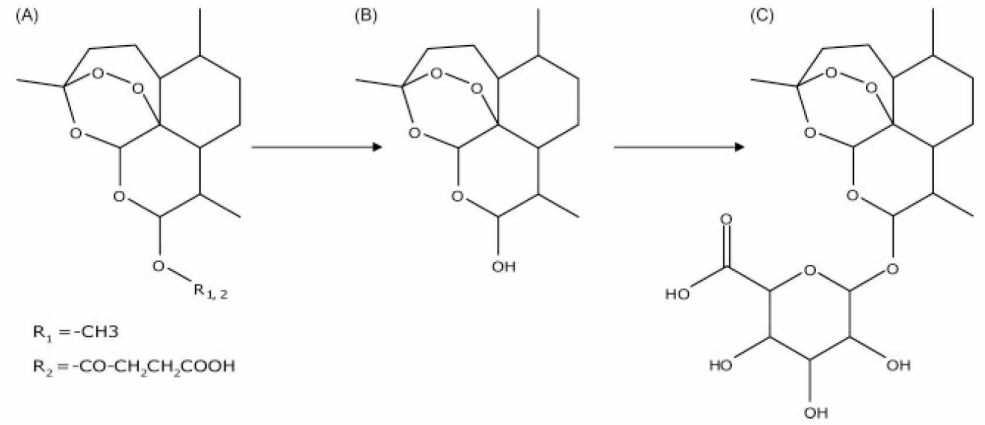



**Figure 1.** Hypothetical metabolic pathway of artemether and artesunate. (A) Parent drug: artemether ($R_1$, AM, molecular weight (MW): 298.37 g/mol) or artesunate ($R_2$, AS, MW: 384.42 g/mol). (B) Primary metabolite: dihydroartemisinin (DHA, MW: 284.35 g/mol). (C) Phase II metabolite: dihydroartemisinin-glucuronide (DHA-G, MW: 460.47 g/mol).

of triclabendazole and other trematocidals is widely spreading in livestock, but fortunately it has not been demonstrated in humans to date.[8] Promisingly, AM was effective against a triclabendazole-resistant *F. hepatica* strain in rats.[9]

Various analytical methods have been established in order to quantify the artemisinin derivatives in biological fluids and tissues. In the recent past, liquid chromatography–mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS) methods were developed for experimental pharmacokinetic (PK) studies of $\alpha,\beta$-arteether and DHA in rat plasma and serum.[10,11] In addition, different high performance liquid chromatography (HPLC) methods with mass spectrometric or electrochemical detection (HPLC-ECD) were developed for the measurement of AS or AM and DHA plasma levels in humans including a range of different sample procession methods (e.g. solid-phase extraction, liquid–liquid extraction and protein precipitation).[12–16] Lately, analytical methods were also developed for the simultaneous determination of up to 14 antimalarial drugs in human plasma since the artemisinins are nowadays routinely applied in combination with other antimalarial drugs to prevent resistance development.[17–20] However, to date no analytical method exists for the concurrent analysis of the semi-synthetic artemisinins, DHA and dihydroartemisinin-glucuronide (DHA-G).

Recently, we studied the efficacy and safety of single oral, intramuscular and intravenous dosages of AS and AM in *F. hepatica* infected sheep.[21,22] Plasma samples were collected at selected time points to support the drug efficacy study with PK data. We were interested to monitor the disposition of AS, AM as well as the two metabolites DHA and DHA-G, since experimental studies of humans and rats exposed to the artemisinins showed the presence of glucuronide metabolites in urine, bile and plasma.[23,24]

The aim of the present study was to develop and validate a LC–MS/MS method to quantify AS, AM and their metabolites DHA and DHA-G in sheep plasma for the prospective application to PK studies.

## Experimental

### Chemicals and reagents

AM and $\alpha,\beta$-DHA were kindly provided from Dafra Pharma (Turnhout, Belgium). AS was obtained from Mepha AG (Aesch, Switzerland). Dihydroartemisinin-12-$\alpha$-o-$\beta$-d-glucuronide (DHA-G) was purchased from Clearsynth (Mumbai, India). The chemical structures of AS, AM, DHA and DHA-G are depicted in Fig. 1. HPLC-grade acetonitrile and methanol were products of Bisolve (Valkenswaard, The Netherlands) and J.T. Baker (Deventer, The Netherlands), respectively. Ammonium formate (LC-MS grade), sodium nitrite, formic acid (98%, LC–MS grade) and acetic acid (glacial) were purchased from Fluka (Buchs, Switzerland). Potassium phosphate ($KH_2PO_4$), potassium hydroxide (reagent grade, >90%) and $\beta$-glucuronidase type IX-A from Escherichia coli (1 000 000–5 000 000 U/g protein) were obtained from Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was produced with an Arium 61 215 water purification system (Sartorius Stedim Biotech, Göttingen, Germany) and applied for the preparation of mobile phase. Plasma from untreated sheep was collected in the framework of our PK studies in the Campania region of Italy.[21,22]

### LC–MS/MS equipment and conditions

The high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD pumps, a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), a thermostated Jones chromatography column oven (Omnilab, Mettmenstetten, Switzerland) and an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan). Chromatographic separation was performed at 25 °C using a 2.1 mm × 20 mm Atlantis T3 3-µm analytical column (Waters, Milford, MA, USA). A 2.1 mm × 10 mm Atlantis 3-µm guard cartridge (Waters) was connected to the analytical column.

The mobile phase consisted of a mixture of 5 mM ammonium formate plus 0.15% (v/v) formic acid in ultra pure water (mobile A) and 0.15% (v/v) formic acid in acetonitrile (mobile B). The following stepwise gradient elution program was used: 0–1 min, B 2%; 1–10 min, B 2–45%; 10–13 min, B 45–50%; 13–15 min, B 50–95%; 15–20 min, B 95%; 20–21 min, B 95–2%; 21–22 min, B 2%. The gradient program allowed for a baseline separation of all analytes. Carryover (<0.1% carryover between subsequent analytical cycles) was managed by adding a wash cycle (95% mobile B for 5 min) at the end of each run. The flow rate of the mobile phases was set at 0.3 ml/min. Conditioning of the column was necessary to avoid shifts in retention time of certain analytes, i.e. AS, after a prolonged standby time of the instrument.

Journal of MASS SPECTROMETRY



In more detail, the analytical column was conditioned with a replicate injection ($n = 6$) of the artemisinins standards (10 µg/ml in 1 : 1 methanol/mobile A) at the beginning of each series of experiments. The HPLC system was coupled to an API 365 triple-quadrupole mass spectrometer (PE Biosystems, Foster City, CA, USA) equipped with a turbo ion spray interface, operated in positive ionisation mode. A six-port switching valve (VICI Valco Instruments, Schenkon, Switzerland) was used to divert the effluent from the analytical column to the ion-spray interface of the mass spectrometer during 0–8 and 18–22 min of each run to avoid contamination of the mass spectrometer. The mass spectrometer was tuned by direct infusion (Harvard apparatus infusion pump 11, Massachusetts, USA) of 10 µg/ml of each artemisinin derivative solved in acetonitrile plus 0.1% (v/v) formic acid. All analytes were detected by selected reaction monitoring (SRM) with a transition of $m/z$ 267.4 → 163.0. DHA-G, DHA and AS were analysed with a scan width and scan time of 3 amu and 2 s, respectively. AM was measured with a scan width and scan time of 5 amu and 3 sec, respectively. The major MS instrumentation settings are summarised in Table 1. Instrumentation control and data analyses were performed with Analyst 1.4.2. software (PE Biosystems) and Microsoft Excel 2003.

### Standard, quality control and internal standard preparation

Standard stock solutions (1 mg/ml) were prepared in methanol. Appropriate volumes of stock solutions were serially diluted in a 1 : 1 mixture of methanol and mobile phase A to obtain working solutions of 0.3–30.0 µg/ml for DHA-G, DHA and AS and 2.8–90.0 µg/ml for AM. Plasma calibration samples were freshly prepared by diluting working solutions with blank sheep plasma (1 : 30, total volume of 300 µl), resulting in final concentrations of 1000.0, 400.0, 160.0, 64.0, 25.6 and 10.2 ng/ml for DHA-G, DHA and AS and 3000.0, 1500.0, 750.0, 375.0, 187.5 and 93.8 ng/ml for AM. Quality control (QC) samples were prepared with blank sheep plasma at low, medium and high concentrations (DHA-G, DHA, AS: 10.2, 160.0 and 1000.0 ng/ml; AM: 93.8, 750.0 and 3000.0 ng/ml). AS or alternatively AM was used as the internal standard (IS). Internal standard working solutions of 7.5 µg/ml AS or 150.0 µg/ml AM were diluted with blank sheep plasma (1 : 30, final concentration: 250.0 ng/ml or 5.0 µg/ml, respectively).

**Table 1.** LC–MS/MS instrumentation settings

| Parameter | Value |
|---|---|
| Source temperature | 400 °C |
| Nebuliser gas (NEB) | 15 l/min |
| Curtain gas (CUR) | 15 l/min |
| Declustering potential (DP) | 26 V |
| Focusing potential (FP) | 230 V |
| Entrance potential (EP) | 6 V |
| Collision cell exit potential (CXP) | 6 V |
| Ion spray voltage (IS) | 5500 V |
| Collision gas $N_2$ (CAD) | 3 l/min |
| Collision energy (CE) | 13 eV |
| Polarity of analysis | Positive |
| Mass transition for the artemisinins | 267.4 → 163.0 $m/z$ |

### Plasma sample extraction procedure

An aliquot of 90 µl sodium nitrite solution (3 M) containing 1% (v/v) acetic acid was added to 300 µl of the sheep plasma samples and stirred (75 rpm) for 30 min at 37 °C in a TH/KS 15 incubator (Edmund Bühler, Hechingen, Germany). The addition of sodium nitrite was shown to prevent the degradation of the artemisinins in the presence of haemoglobin ($Fe^{2+}$-haeme) in haemolysed plasma samples.[19,25] The plasma–sodium nitrite mixture was vortex-mixed (Vortex Genius 3, IKA, Staufen, Germany) with 10 µl of IS working solution. For protein precipitation, 1000 µl of ice-cooled methanol was added to each sample and mixed for 1 min. The precipitated samples were cooled on ice for 10 min and subsequently centrifuged for 15 min at 16 100$g$ (Eppendorf centrifuge 5415 R, Hamburg, Germany) at 4 °C. The supernatant was transferred to a 2-ml microtube and stored on ice. The pellet was vortex mixed with 500 µl of methanol for another minute and centrifuged as described above. The methanol extracts were combined, mixed and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 250 µl of methanol–mobile phase A (1 : 1, v/v), vortex mixed, centrifuged (15 min, 16 100$g$, 4 °C) and transferred to an autosampler vial. The autosampler rack was cooled at 6 °C. A 30-µl aliquot of each sample was injected into the LC–MS/MS system for analysis.

### Calibration curves

Calibration curves were established using the internal standard method for the quantification of artemisinins plasma concentrations. Concentrations were plotted *versus* the peak-area ratios of the analytes to the IS. For $\alpha,\beta$-DHA, the sum of the area under the curve of both anomers was used. Each calibration set consisted of one blank plasma sample (matrix sample processed without internal standard), one zero sample (matrix sample processed with internal standard) and six calibration samples including the lower limit of quantification (LLOQ). Concentrations chosen for the calibration curves ranged from 10.2 to 1000.0 ng/ml (DHA-G, DHA and AS) and 93.8–3000.0 ng/ml (AM). The six-point calibration standard curves were calculated and fitted either by linear (DHA-G and DHA) or quadratic regression (AS and AM). The Akaike information criterion (AIC) was used to select the most favourable type of regression for each calibration.[26] The quadratic regression model was applied because an increased signal strength was observed at higher concentration (fit by $y = ax^2 + bx + c$) and not because of saturation of the signal (i.e. fit by $y = -ax^2 + bx + c$). To determine the best weighting factor (none, $1/x$ or $1/x^2$), concentrations were back-calculated and the model with the lowest total bias across the concentration range was selected.

### Glucuronide identification

A mass spectrometric approach followed by verification with an enzyme assay was conducted to assign and identify the glucuronide metabolite (DHA-G) in our sheep samples.

Plasma samples (10 × 300 µl) of AS or AM-treated sheep[21,22] were subjected to protein precipitation by the addition of 1000 µl ice-cooled methanol followed by centrifugation for 15 min at 16 100$g$ at 4 °C. The supernatants were collected, stored at 4 °C and DHA-G was isolated by HPLC using the same chromatography program as described above (100 µl injection volume). Chromatography peaks representing DHA-G were collected on dry ice (retention time: 8.0–10.5 min). Collected samples were taken to dryness under nitrogen, resuspended in

Journal of MASS SPECTROMETRY

acetonitrile/formic acid 0.1% (v/v) and introduced into the MS/MS by infusion (10 µl/min). First, a Q1 single MS scan from *m/z* 100 to 1000 was carried out. MS conditions are described in Table 1 (CAD and CE were 0 l/min and 0 eV, respectively). Second, each signal with a relative intensity >30% was further examined using the artemisinin-specific transition *m/z* 267.4 → 163.0 in the SRM mode (CAD and CE were 3 l/min and 10–20 eV, respectively). Third, results were compared with reference spectra of DHA-G (10 µg/ml) obtained under identical conditions.

For the enzyme assay, the collected eluent was taken to dryness under nitrogen and resuspended in 1000 µl of 75 mM phosphate buffer, pH of 6.8, containing 1 mg/ml $\beta$-glucuronidase. This mixture was incubated for 120 min at 37 °C and agitated at 600 rpm using a Thermomixer (Eppendorf Thermomixer Comfort, Hamburg, Germany). The $\beta$-glucuronidase was inactivated by the addition of 1000 µl acetonitrile and the incubation mixture was analysed by LC–MS/MS. A 30-µl aliquot was measured before ($t = 0$) and after 120 min exposure to $\beta$-glucuronidase.

### Method validation

Our method was validated for selectivity, precision, accuracy, recovery and stability according to the bioanalytical method validation guidance for industry 2001 by the Food and Drug Administration (FDA).[27]

#### *Selectivity*

Blank plasma obtained from sheep of different origin ($n = 6$) was examined for interferences with endogenous substances using the above-described extraction procedure but without adding the IS working solution.

#### *Lower limit of quantification*

The LLOQ was selected as the minimal concentration in plasma samples, which could be analysed with a precision of ≤20% and accuracy between 80% and 120%. In addition, the analyte response was at least five times above the noise level of the blank response.

#### *Accuracy and precision*

Accuracy and precision of the method was evaluated by analysing QC samples ($n = 5$) at the LLOQ, a medium concentration (middle QC) and at the upper limit of quantification (ULOQ). The intra- and inter-day accuracy/precision was determined within a single run and between different assays ($n = 3$), respectively. The following concentrations were selected for QC samples, covering the entire range of the calibration curve: 93.8, 750.0 and 3000.0 ng/ml for AM and 10.2, 160.0 and 1000.0 ng/ml for DHA-G, DHA and AS. Freshly prepared calibration standards were used for analyses. The precision was calculated using the coefficient of variation (CV [%]). The accuracy was determined as the percentage ratio of the measured concentration to the nominal concentration. A precision of ±15% (LLOQ: ±20%) and accuracy between 85% and 115% (LLOQ: 80–120%) was accepted in our study.

#### *Recovery and matrix effect*

Relative recoveries (RRE) of the artemisinin derivatives were determined by comparing the absolute peak areas of blank plasma samples spiked before and after the extraction at three different concentrations ($n = 3$ per concentration). The recovery of AM was investigated at 93.8, 750.0 and 3000.0 ng/ml and the recovery of DHA-G, DHA and AS at 10.2, 160.0 and 1000.0 ng/ml.

Matrix effects of the artemisinins were assessed as the ratio of the absolute peak areas of blank plasma samples spiked after the extraction to the absolute peak areas of the analytes solved in a mixture of methanol–mobile A (1 : 1, v/v).

#### *Stability: autosampler and freeze–thaw stability*

Autosampler stability and freeze–thaw stability studies were included in our method validation. QC samples at low, medium and high concentration (as described above) were used to test stabilities under different conditions. Autosampler stability was evaluated by the analysis of QC samples ($n = 3$ per concentration) over a period of 30 h. For the freeze–thaw stability, QC samples were frozen at −80 °C (1 h) and thawed at room temperature (1 h). This cycle was repeated three times before analysis. The concentrations of these samples were compared with concentrations of freshly prepared QC samples. The drug solutions were considered as stable with a deviation of not more than ±15% and ±20% at the LLOQ.

#### *Sample dilution*

PK samples with drug concentrations exceeding the ULOQ were diluted using blank sheep plasma. The dilution effect was evaluated as described by Hodel *et al.*[19] to assure that the accuracy of the analysis was not affected. In brief, blank sheep plasma ($n = 3$) was spiked with a concentration exceeding two times the ULOQ. The samples were then fourfold diluted with blank plasma to achieve a concentration within the calibration range. The accuracy of drug quantification was defined as the percentage ratio of the measured concentration to the nominal concentration. A deviation of ±15% of the measured concentration from the nominal value was accepted in our study.

### Pharmacokinetic study

The PK studies were carried out in the framework of two efficacy studies published recently.[21,22] Here, we present the PK profile of one sheep treated with 60 mg/kg AS intramuscularly to demonstrate the usefulness of the developed and validated LC–MS/MS method. Blood samples were withdrawn from the jugular vein into lithium-heparin-coated vacutainer tubes (BD, Franklin Lakes, NJ, USA) at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8 and 24 h posttreatment. Plasma was produced by centrifugation and stored at −80 °C. AM (5.0 µg/ml) was used as IS for the analysis. PK samples with drug concentrations exceeding the ULOQ were diluted with blank sheep plasma as described above.

## Results and Discussion

### Method development

To date, no validated analytical method exists for the simultaneous quantification of the antimalarials AS, AM and their metabolites DHA and DHA-G for the analysis of plasma samples from PK studies. We, therefore, decided to develop a LC–MS/MS method for the quantification of these drugs and metabolites in plasma samples obtained from *F. hepatica*-infected sheep. Importantly, DHA-G seems to be the major glucuronide metabolite of DHA and the primary elimination occurs over the bile.[3] Furthermore, since adult

Journal of MASS SPECTROMETRY



*F. hepatica* live in the bile ducts, biliary elimination of potentially active drug metabolites might even enhance drug activity.

In a first step, MS/MS conditions were defined and optimised. Stock solutions in acetonitrile of each analyte were directly infused and single MS scans were carried out in the positive ion detection mode. Base peaks with good responses were observed at *m/z* 221.5, 267.4 and 284.4, corresponding to protonated fragments of the parent molecules of AS, AM and DHA. In order to increase the sensitivity and selectivity of the MS signal, the common fragment with the best response (*m/z* 267.4) was selected and subjected to further fragmentation. Best results for all analytes of interest were obtained using the transition *m/z* 267.4 → 163.0. The MS/MS parameters were adjusted to maximise the amount of protonated fragment ion (163 *m/z*) to achieve the highest sensitivity (Table 1).

Next, the LC conditions were refined. Different analytical columns, which had been used previously for the separation of the artemisinins and synthetic peroxides,[15,19,28] were tested. A major challenge was the baseline separation of DHA and AS. This could be achieved using all tested columns, i.e. the X Terra (C18, 2.1 mm × 150 mm, 5 µm), the Phenomenex (C8 (2), 2.0 mm × 50 mm, 5 µm) and the Atlantis (C18, 2.1 × 20, 50 and 100 mm, 3 µm). However, an additional separation of the two DHA anomers ($\alpha$,$\beta$-DHA) was best achieved using the Atlantis column (C18, 2.1 mm × 20 mm, 3 µm). In addition, good peak symmetry of $\alpha$,$\beta$-DHA was obtained with this column (Fig. 2(F)). An increase in the column length from 20 to 100 mm did not offer additional advantages. A slow gradient of mobile B (acetonitrile plus 0.15% (v/v) formic acid) from 2 to 95% over 15 min was required for the separation of $\beta$-DHA and AS. A wash cycle of 5 min at 95% mobile B and an additional equilibration step to 2% mobile B was necessary to avoid carryover effects (<0.1%). It might be possible to shorten the run time of 23 min using chromatography columns with smaller particles (e.g. pellicular silica columns) or column switching,[29,30] in case a need of high-throughput analysis would arise. Variations in the ammonium formate concentration (0, 5, 10, 20 and 50 mM) of mobile A did not affect the separation; hence, 5 mM ammonium formate plus 0.15% formic acid was used, which

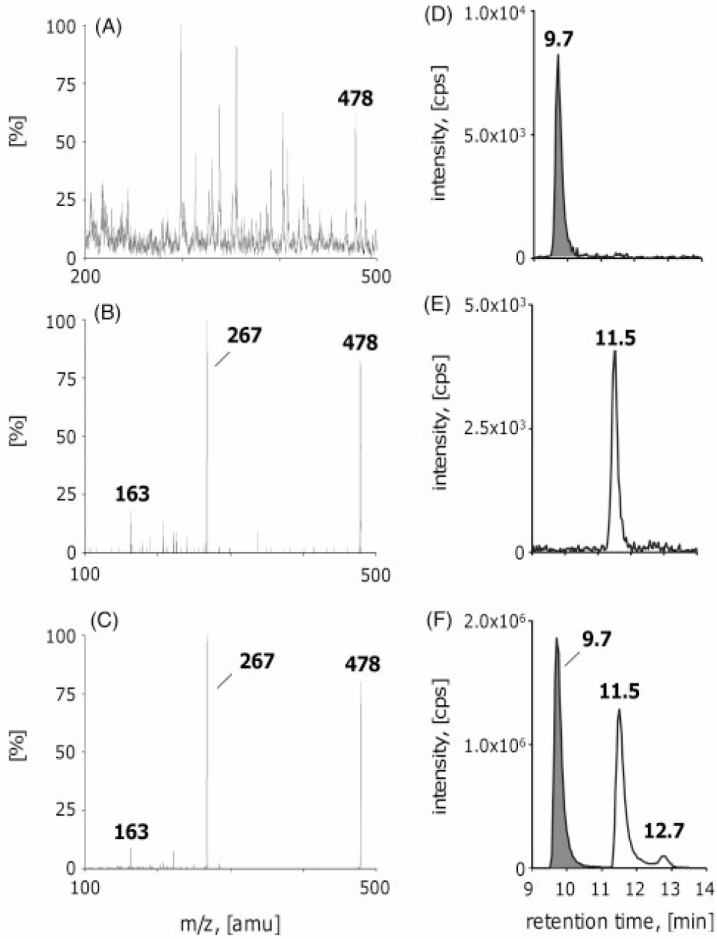



**Figure 2.** Chromatographic separation of DHA-G and $\alpha$,$\beta$-DHA and confirmation of the identity of DHA-G in sheep plasma. (A) Single MS (Q1 positive mode) spectrum of the collected DHA-G chromatographic peak. The ammonium ion adduct of DHA-G (DHA-G-$NH_4^+$) is characterised by *m/z* 478. (B) Tandem MS (MS/MS positive mode) selected reaction monitoring of parent *m/z* 478. Characteristic artemisinins-scaffold fragments *m/z* 267 and 163 are shown. (C) Selected reaction monitoring of DHA-G (*m/z* 478) reference compound leads to an identical fragmentation pattern as shown in (B). (D) Chromatogram of isolated metabolite DHA-G (retention time: 9.7 min; grey peak). (E) Chromatogram of isolated metabolite DHA-G after incubation with $\beta$-glucuronidase giving rise to DHA (retention time: 11.5 min; white peak). (F) Chromatogram of reference compounds. DHA-G: retention time = 9.7 min, grey peak. $\alpha$-DHA: retention time = 11.5 min, white peak. $\beta$-DHA: retention time = 12.7 min, white peak.

achieved the best peak symmetry and a good ratio of height to width.

Finally, different sample workup methods were tested for simplicity, robustness and recovery of analytes from plasma. A liquid–liquid extraction method with ethyl acetate or ether[15,31] was found to be laborious and recovery rates were not superior to a plasma protein precipitation with methanol or acetonitrile. A precipitation with methanol at a ratio of 3 : 1 (precipitant to plasma) provided the highest recovery rates of all analytes. An improved sensitivity and signal-to-noise ratio was achieved when the supernatant was evaporated under a stream of nitrogen and resuspended in a smaller volume of methanol and mobile phase A (50 : 50, v/v).[19]

### Glucuronide identification

We identified an unknown metabolite in plasma samples of AS and AM-treated sheep, which was suspected to represent the phase II metabolite DHA-G (Fig. 1). The collected metabolite fraction contained neither DHA, AS nor AM as demonstrated using LC–MS/MS. The absence of DHA was a prerequisite to be able to carry out $\beta$-glucuronidase biotransformation experiments *in vitro*. The retention time of the metabolite was 9.7 min (Fig. 2(D)) and was characterised by *m/z* 478 (Fig. 2(A)), corresponding to the ammonium ion (*m/z* 18) adduct of DHA-O-glucuronide (DHA-G, *m/z* 460). In Q1 negative mode, the parent molecule (free acid of DHA-G) was apparent with *m/z* 459. Fragmentation of the isolated metabolite gave rise to two characteristic fragments with *m/z* 163.0 and 267.4 (Fig. 2(B)). These fragments were identical to the typical artemisinins scaffold fragments identified during method setup (see above). It is important to note that only mass *m/z* 478 (out of all signals with a relative intensity >30%) was characterised by the transition *m/z* 267 → 163. The identity of the collected metabolite DHA-G was further confirmed by enzymatic digestion using $\beta$-glucuronidase. By this procedure, DHA-G was converted completely to DHA (Fig. 2(D and E)). This conversion was observed in the presence of $\beta$-glucuronidase only, but not in control incubations, which were carried out in absence of the enzyme. Finally, DHA-G reference compound was obtained and used as an additional control. The fragmentation pattern of this compound was identical to the fragmentation pattern of the isolated metabolite (Fig. 2(C)). Furthermore, retention times of DHA-G and DHA (Fig. 2(F)) matched the retention times of the isolated metabolite or DHA-G reference compound prior (Fig. 2(D)) and after (Fig. 2(E)) enzymatic digestion. We conclude that DHA-G is a major metabolite of AS and AM in sheep.

### Method validation

#### *Selectivity and LLOQ*

Blank sheep plasma samples ($n = 6$) of different origin were analysed for the presence of interfering endogenous matrix components. No endogenous peaks were detected in any of the six blank plasma samples at the retention time of DHA-G (9.7 ± 0.5 min), DHA (11.5 ± 0.5 min), AS (13.7 ± 0.5 min) or AM (16.0 ± 0.5 min) as demonstrated in Fig. 3(A). We conclude that the present method is selective.

The LLOQ for DHA-G, DHA and AS was set at 10.2 ng/ml. The obtained LLOQ for AM was 93.8 ng/ml, which was considered to be acceptable. The signal-to-noise ratio at LLOQ was 5 : 1. Representative chromatograms of processed blank sheep plasma spiked with the four artemisinins at LLOQ are depicted in Fig. 3(B and C).

#### *Calibration curve*

The AIC was used to select the most favourable type of regression for each analyte.[26] The calibration curves of DHA-G and DHA were best fitted with a linear model over a concentration range from 10.2 to 1000.0 ng/ml in sheep plasma. The coefficients of correlation ($r^2$) were >0.998. The calibration standards of AS and AM were analysed using a quadratic regression model over a range of 10.2–1000.0 and 93.8–3000.0 ng/ml, respectively ($r^2 > 0.998$). According to the regressions used, the accuracy of the back-calculated concentration standards was better than 100 ± 10% of the nominal concentration in each experiment. This value is within the recommended limit of 100 ± 15%.[27]

#### *Accuracy and precision*

Inter- and intra-day precision and accuracy were calculated by analysing five QC samples at three concentrations on three different days. For the intra-day precision and accuracy, a representative experiment is shown ($n = 5$ measurements, Table 2). For the inter-day precision and accuracy, three independent sets of experiments are summarised in Table 2 ($n = 15$ measurements). The mean relative standard deviation (RSD) values in the intra- and inter-day

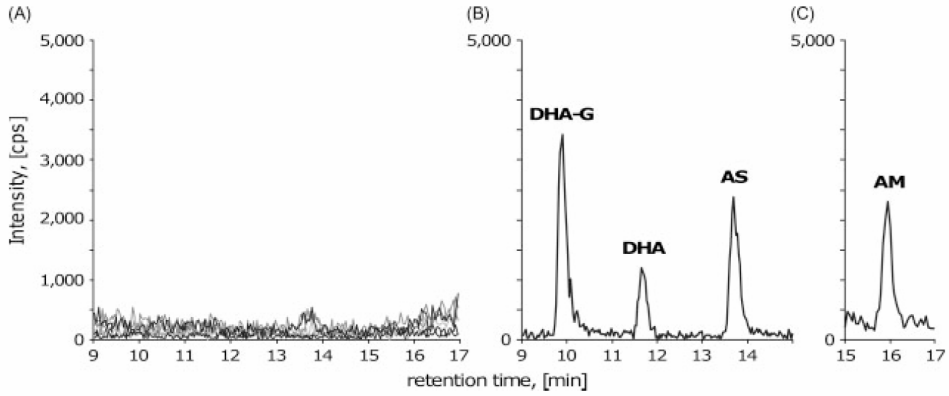



**Figure 3.** Chromatograms illustrate the selectivity and the analytes LLOQ of the analytical method. (A) Chromatograms of extracted blank sheep plasma samples ($n = 6$). (B) Chromatogram of the LLOQ of DHA-G, DHA and AS (10.2 ng/ml). (C) Chromatogram of the LLOQ of AM (93.8 ng/ml).

Journal of MASS SPECTROMETRY



**Table 2.** Intra-day and inter-day accuracy and precision

| Analytes (regression model) | Nominal concentration (ng/ml) | Intra-day[a] Mean concentration found (ng/ml) | Intra-day[a] RSD (%) | Intra-day[a] Accuracy (%) | Inter-day[b] Mean concentration found (ng/ml) | Inter-day[b] RSD (%) | Inter-day[b] Accuracy (%) |
|---|---|---|---|---|---|---|---|
| Artemether (quadratic, $R^2 = 0.9998$) | 93.8 | 93.0 | 5.2 | 99.1 | 94.8 | 9.2 | 101.1 |
| | 750.0 | 751.0 | 3.0 | 100.1 | 735.4 | 7.5 | 98.0 |
| | 3000.0 | 3075.7 | 3.3 | 102.5 | 2898.7 | 7.4 | 96.6 |
| Artesunate (quadratic, $R^2 = 0.9999$) | 10.2 | 10.5 | 4.0 | 103.4 | 10.6 | 6.0 | 103.6 |
| | 160.0 | 155.2 | 3.2 | 97.0 | 163.6 | 6.3 | 102.3 |
| | 1000.0 | 942.1 | 2.6 | 94.2 | 971.8 | 3.8 | 97.2 |
| Dihydroartemisinin (linear, $R^2 = 0.9993$) | 10.2 | 10.8 | 3.2 | 106.1 | 10.6 | 5.3 | 104.2 |
| | 160.0 | 159.9 | 4.7 | 99.9 | 161.4 | 4.3 | 100.9 |
| | 1000.0 | 1011.0 | 3.8 | 101.1 | 998.7 | 4.7 | 99.9 |
| DHA-glucuronide (linear, $R^2 = 0.9991$) | 10.2 | 10.3 | 8.6 | 100.5 | 10.3 | 7.7 | 100.6 |
| | 160.0 | 161.2 | 2.6 | 100.7 | 165.7 | 6.0 | 103.6 |
| | 1000.0 | 993.8 | 3.4 | 99.4 | 997.1 | 3.4 | 99.7 |

[a] Values are means of $n = 5$ samples. A representative experiment is shown.
[b] Values are means of $n = 3$ independent sets of experiments.

precision runs of all analytes were 4.0% (range: 2.6–8.6%) and 6.0% (range: 3.4–9.2%), respectively. The intra- and inter-day accuracies ranged from 94.2% to 106.1% and 96.6% to 104.2%, respectively. These data indicate that the developed method is very reliable.

*Extraction recovery and matrix effect*

RRE of the artemisinins were determined by comparing the absolute signal of blank plasma samples spiked before and after extraction at low, medium and high concentrations ($n = 3$). As shown in Table 3, mean recoveries of 83.9%, 77.7% and 78.9% were observed for DHA-G, DHA and AS, respectively. The mean recovery of AM (57.8%) was about 20% lower compared with the other analytes. The CV of the recoveries over the entire concentration range was <6.7%, indicating consistency in our sample processing method.

Matrix effects were assessed as the ratio of the absolute peak areas of blank plasma samples spiked after extraction to the absolute peak areas of the analytes solved in a mixture of mobile A and methanol (1 : 1, v/v) (Table 3). The matrix effects were not significant for DHA-G (83.9%) and AS (93.3%). On the other hand, a considerable loss of signal was seen for DHA and AM in sheep plasma (matrix effect of 57.7% and 63.2%, respectively). The matrix effect of DHA was independent of the analyte concentration with a CV of 4.4% over the entire concentration range and of the plasma batches. In contrast, the matrix effect was highly dependent on analyte concentrations of AM (CV 19.62%), with a particularly high suppression observed at low AM concentrations but was not affected by the plasma source. Different operational strategies have been suggested such as extensive cleanup procedures (solid phase or liquid–liquid extraction) or more efficient chromatographic separations to minimise the interferences of coeluting matrix compounds.[32] However, our method achieved consistent results over the course of several experiments. Since the sensitivity of our method was considered to be sufficiently high, no additional sample workup procedures were implemented.

**Table 3.** Relative recovery (RRE) and matrix effect of artesunate, artemether and its metabolites dihydroartemisinin and DHA-glucuronide

| Analytes | Nominal concentration (ng/ml) | RRE (%)[a] | Mean ± CV (%) | Matrix effect (%)[a] |
|---|---|---|---|---|
| Artemether | 93.8 | 61.8 | 57.8 ± 6.7 | 51.5 |
| | 750.0 | 57.4 | | 61.9 |
| | 3000.0 | 54.1 | | 76.2 |
| Artesunate | 10.2 | 79.3 | 78.9 ± 0.5 | 111.9 |
| | 160.0 | 78.5 | | 89.6 |
| | 1000.0 | 78.9 | | 78.3 |
| Dihydroartemisinin | 10.2 | 74.6 | 77.7 ± 5.2 | 55.6 |
| | 160.0 | 76.3 | | 60.3 |
| | 1000.0 | 82.4 | | 57.2 |
| DHA-Glucuronide | 10.2 | 83.3 | 83.9 ± 2.7 | 91.8 |
| | 160.0 | 82.0 | | 79.9 |
| | 1000.0 | 86.3 | | 80.0 |

[a] Values are means of $n = 3$ samples.

*Stability*

Results obtained from autosampler and freeze–thaw stability studies demonstrated that the samples were stable under these conditions (Table 4). The variations of all target drugs in processed plasma samples kept at 6 °C for 30 h were below 9.8%. The accuracies under these conditions ranged from 88.1% to 111.2%. The samples were highly stable following several complete freeze/thaw cycles (accuracies: 91.0–107.5%; RSD <13.4%). Previous studies have shown that AS and DHA are not stable at room temperature.[19] Similarly, in our experiments a substantial variability of DHA-G precision was observed if samples were kept at room temperature. These effects were particularly pronounced at low concentrations (data not shown). In light of these findings, samples were processed on ice and stored at low temperatures.

The succinate functional group of AS is vulnerable to chemical hydrolysis or enzymatic degradation by means of plasma

Journal of MASS SPECTROMETRY

**Table 4.** Stability analysis of quality control samples for artesunate, artemether, dihydroartemisinin and DHA-glucuronide in sheep plasma ($n = 3$)

| Experiment | Analytes | Nominal concentration (ng/ml) | Mean concentration found (ng/ml) | RSD (%) | Accuracy (%) |
|---|---|---|---|---|---|
| Autosampler | Artemether | 93.8 | 88.5 | 3.4 | 94.4 |
| | | 750.0 | 809.4 | 4.4 | 107.9 |
| | | 3000.0 | 3021.8 | 7.2 | 100.7 |
| Autosampler | Artesunate | 10.2 | 11.3 | 2.4 | 111.2 |
| | | 160.0 | 168.5 | 5.4 | 105.3 |
| | | 1000.0 | 1007.7 | 3.0 | 100.8 |
| Autosampler | Dihydroartemisinin | 10.2 | 9.0 | 2.1 | 88.1 |
| | | 160.0 | 166.8 | 1.4 | 104.3 |
| | | 1000.0 | 1107.5 | 6.2 | 110.7 |
| Autosampler | DHA-glucuronide | 10.2 | 11.2 | 9.8 | 109.8 |
| | | 160.0 | 159.6 | 4.0 | 99.7 |
| | | 1000.0 | 998.3 | 4.8 | 99.8 |
| F/T | Artemether | 93.8 | 89.9 | 5.2 | 95.8 |
| | | 750.0 | 682.6 | 7.4 | 91.0 |
| | | 3000.0 | 2736.3 | 6.9 | 91.2 |
| F/T | Artesunate | 10.2 | 9.2 | 7.7 | 90.0 |
| | | 160.0 | 167.7 | 13.4 | 104.8 |
| | | 1000.0 | 1075.1 | 4.4 | 107.5 |
| F/T | Dihydroartemisinin | 10.2 | 10.1 | 0.3 | 99.2 |
| | | 160.0 | 152.5 | 1.4 | 95.3 |
| | | 1000.0 | 988.1 | 6.4 | 98.8 |
| F/T | DHA-glucuronide | 10.2 | 10.5 | 8.4 | 103.3 |
| | | 160.0 | 158.4 | 2.5 | 99.0 |
| | | 1000.0 | 975.8 | 6.4 | 97.6 |

Autosampler, samples were kept in the autosampler at 6 °C for 30 h after processing; F/T, stability after three freeze–thaw cycles.

esterases[33] (Fig. 1). We observed that on average 5.5% (95% CI: 5.2–5.8%) of AS reacted to DHA during sample workup. This degradation should be kept in mind, in particular, if in an analysed specimen the ratio between the AS signal and DHA signal is very high. Note that the endoperoxidic artemisinins degrade in the presence of $Fe^{2+}$ in haemolysed plasma samples. The addition of sodium nitrite, a known methaemoglobin-forming agent, to all plasma samples successfully prevented this reaction in recent studies and had no impact on the results in non-haemolysed plasma samples.[19,25,34]

*Sample dilution*

Spiked plasma samples ($n = 3$) at a concentration twofold of the ULOQ were diluted fourfold with blank plasma. The mean deviations (bias) from the nominal concentrations were 7.7%, 5.4%, 7.5% and 7.2% for AS, AM, DHA and DHA-G, respectively. In conclusion, plasma samples containing high artemisinin concentrations (above the ULOQ) can be diluted with blank plasma without affecting the accuracy of the measurement.

### Method application

The validated method was applied to analyse plasma samples from one *F. hepatica*-infected sheep treated intramuscularly with 60 mg/kg of AS.[22] AM (5 µg/ml) was used as the internal standard. DHA-G eluted at 9.9 min, followed by DHA at 11.7 min, AS at 13.8 and AM at 16.0 min, which is in line with spiked reference plasma samples (Fig. 4(A and B)). The concentration *versus* time profile of one AS-treated sheep is depicted in Fig. 4(C). In this sheep AS reached a maximal plasma concentration ($C_{max}$) of 6669 ng/ml 15 min posttreatment, followed by DHA ($C_{max}$ = 1525 ng/ml) and DHA-G ($C_{max}$ = 18 526 ng/ml) at 30 and 45 min posttreatment, respectively. This result reflects the suggested metabolism pathway of AS (Fig. 1): AS is converted to DHA by ester cleavage and subsequently metabolised by *o*-glucuronidation to DHA-G. Glucuronidated DHA was found to be a prominent main metabolite, which showed a three times higher $C_{max}$ than the parent compound AS. Based on these results, the trematocidal activity of the main metabolite DHA-G will be explored in more detail.

## Conclusion

A LC–MS/MS method was developed and validated for the simultaneous quantification of AS or AM and their metabolites DHA and DHA-G. The method has proven to be selective, precise, accurate and simple to perform. The sensitivity for all analytes was in the range of 10–90 ng/ml. The method was applied successfully to analyse plasma samples of AS-treated sheep and will be used in the future for detailed PK studies. For the first time, *o*-glucuronidated DHA (DHA-G) was described to be a main phase II metabolite of AS in sheep. This method will be adapted to analyse additional biofluids (e.g. bile) or tissues (e.g. liver) in different species including humans to explore pharmacological effects of artemisinins in *F. hepatica*-infected individuals.

### Acknowledgements

U.D. and J.K. are grateful to the Swiss National Science Foundation for financial support (project no.: PPOOA-114941). We thank

Journal of MASS SPECTROMETRY



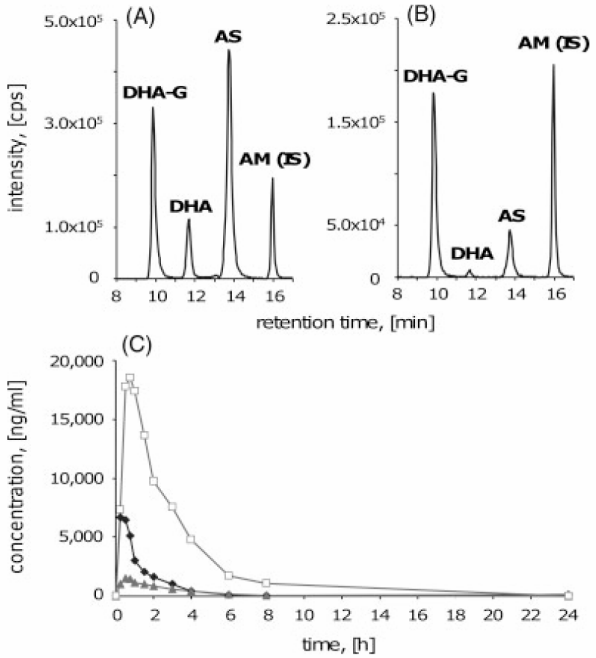



**Figure 4.** Plasma-concentration profile of an AS-treated sheep infected with *F. hepatica*, illustrated as an application of the method. (A) Reference chromatogram of DHA-G, DHA and AS 1000.0 ng/ml and AM (IS) 5.0 µg/ml spiked blank sheep plasma. (B) Chromatogram of a plasma sample 60 min after intramuscular administration of AS 60 mg/kg (diluted with blank plasma 1 : 30). (C) Plasma concentration–time profile of AS (□), DHA (♦) and DHA-G (▲) in sheep following intramuscular administration of 60 mg/kg AS.

Massimilliano Donzelli, Dr Sabine Meyer, Dr Manfred Zell and Dr Philippe Coassolo for their assistance and helpful suggestions. We are thankful to Prof. Dr G. Imanidis and Prof. Dr S. Krähenbühl for their continuous support. Part of this project was carried out in the laboratories of the University of Applied Sciences, Northwestern Switzerland.

## References

[1] N. J. White. Qinghaosu (artemisinin): the price of success. *Science* **2008**, *320*, 330.
[2] S. K. Mishra, S. Mohanty, A. Mohanty, B. S. Das. Management of severe and complicated malaria. *J. Postgrad. Med.* **2006**, *52*, 281.
[3] J. L. Maggs, S. Madden, L. P. Bishop, P. M. O'Neill, B. K. Park. The rat biliary metabolites of dihydroartemisinin, an antimalarial endoperoxide. *Drug Metab. Dispos.* **1997**, *25*, 1200.
[4] K. Ramu, J. K. Baker. Synthesis, characterization, and antimalarial activity of the glucuronides of the hydroxylated metabolites of arteether. *J. Med. Chem.* **1995**, *38*, 1911.
[5] J. Keiser, S. H. Xiao, M. Tanner, J. Utzinger. Artesunate and artemether are effective fasciolicides in the rat model and *in vitro*. *J. Antimicrob. Chemother.* **2006**, *57*, 1139.
[6] J. Keiser, J. Utzinger. Food-borne trematodiases. *Clin. Microbiol. Rev.* **2009**, *22*, 466.
[7] M. W. Robinson, J. P. Dalton. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2009**, *364*, 2763.
[8] I. Fairweather, J. C. Boray. Fasciolicides: efficacy, actions, resistance and its management. *Vet. J.* **1999**, *158*, 81.
[9] J. Keiser, J. Utzinger, J. L. Vennerstrom, Y. Dong, G. Brennan, I. Fairweather. Activity of artemether and OZ78 against triclabendazole-resistant *Fasciola hepatica*. *Trans. R. Soc. Trop. Med. Hyg.* **2007**, *101*, 1219.
[10] M. Rajanikanth, K. P. Madhusudanan, R. C. Gupta. An HPLC-MS method for simultaneous estimation of alpha,beta-arteether and its metabolite dihydroartemisinin, in rat plasma for application to pharmacokinetic study. *Biomed. Chromatogr.* **2003**, *17*, 440.
[11] M. Rajanikanth, K. P. Madhusudanan, R. C. Gupta. Liquid chromatographic-mass spectrometric method for the determination of alpha-,beta-arteether in rat serum. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2003**, *783*, 391.
[12] C. Souppart, N. Gauducheau, N. Sandrenan, F. Richard. Development and validation of a high-performance liquid chromatography–mass spectrometry assay for the determination of artemether and its metabolite dihydroartemisinin in human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2002**, *774*, 195.
[13] Y. Gu, Q. Li, V. Melendez, P. Weina. Comparison of HPLC with electrochemical detection and LC–MS/MS for the separation and validation of artesunate and dihydroartemisinin in animal and human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2008**, *867*, 213.
[14] W. Hanpithakpong, B. Kamanikom, A. M. Dondorp, P. Singhasivanon, N. J. White, N. P. Day, N. Lindegardh. A liquid chromatographic-tandem mass spectrometric method for determination of artesunate and its metabolite dihydroartemisinin in human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2008**, *876*, 61.
[15] S. A. Van Quekelberghe, S. A. Soomro, J. A. Cordonnier, F. H. Jansen. Optimization of an LC-MS method for the determination of artesunate and dihydroartemisinin plasma levels using liquid–liquid extraction. *J. Anal. Toxicol.* **2008**, *32*, 133.
[16] H. Naik, D. J. Murry, L. E. Kirsch, L. Fleckenstein. Development and validation of a high-performance liquid chromatography–mass spectroscopy assay for determination of artesunate and dihydroartemisinin in human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2005**, *816*, 233.
[17] C. S. Lai, N. K. Nair, S. M. Mansor, P. L. Olliaro, V. Navaratnam. An analytical method with a single extraction procedure and two separate high performance liquid chromatographic systems for the determination of artesunate, dihydroartemisinin and mefloquine in human plasma for application in clinical pharmacological studies of the drug combination. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2007**, *857*, 308.
[18] C. S. Lai, N. K. Nair, A. Muniandy, S. M. Mansor, P. L. Olliaro, V. Navaratnam. Validation of high performance liquid chromatography-electrochemical detection methods with simultaneous extraction procedure for the determination of artesunate, dihydroartemisinin, amodiaquine and desethylamodiaquine in human plasma for application in clinical pharmacological studies of artesunate-amodiaquine drug combination. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2009**, *877*, 558.
[19] E. M. Hodel, B. Zanolari, T. Mercier, J. Biollaz, J. Keiser, P. Olliaro, B. Genton, L. A. Decosterd. A single LC–tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2009**, *877*, 867.
[20] I. C. Cesar, J. A. de Aquino Ribeiro, L. de Souza Teixeira, K. B. Bellorio, F. C. de Abreu, J. M. Moreira, P. R. Chellini, G. A. Pianetti. Liquid chromatography–tandem mass spectrometry for the simultaneous quantitation of artemether and lumefantrine in human plasma: application for a pharmacokinetic study. *J. Pharm. Biomed. Anal.* **2011**, *54*, 114.
[21] J. Keiser, L. Rinaldi, V. Veneziano, L. Mezzino, M. Tanner, J. Utzinger, G. Cringoli. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. *Parasitol. Res.* **2008**, *103*, 517.
[22] J. Keiser, V. Veneziano, L. Rinaldi, L. Mezzino, U. Duthaler, G. Cringoli. Anthelmintic activity of artesunate against *Fasciola hepatica* in naturally infected sheep. *Res. Vet. Sci.* **2010**, *88*, 107.
[23] K. F. Ilett, B. T. Ethell, J. L. Maggs, T. M. Davis, K. T. Batty, B. Burchell, T. Q. Binh, T. A. Thu le, N. C. Hung, M. Pirmohamed, B. K. Park, G. Edwards. Glucuronidation of dihydroartemisinin *in vivo* and by human liver microsomes and expressed UDP-glucuronosyltransferases. *Drug Metab. Dispos.* **2002**, *30*, 1005.
[24] K. Ramu, J. K. Baker. Identification of the glucuronides of the hydroxylated metabolites of the antimalarial arteether in rat plasma and urine by thermospray high-performance liquid chromatography/mass spectrometry. *J. Pharm. Sci.* **1997**, *86*, 915.
[25] J. Keiser, M. S. Gruyer, N. Perrottet, B. Zanolari, T. Mercier, L. Decosterd. Pharmacokinetic parameters of artesunate and

dihydroartemisinin in rats infected with *Fasciola hepatica*. *J. Antimicrob. Chemother.* **2009**, *63*, 543.
[26] B. P. Imbimbo, E. Imbimbo, S. Daniotti, D. Verotta, G. Bassotti. A new criterion for selection of pharmacokinetic multiexponential equations. *J. Pharm. Sci.* **1988**, *77*, 784.
[27] US Department of Health and Human Services. *FDA guidance for industry bioanalytical method validation*, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine: **2001**.
[28] J. Keiser, C. Kirchhofer, M. Haschke, J. Huwyler, Y. Dong, J. L. Vennerstrom, K. Vanhoff, R. Kaminsky, N. Malikides. Efficacy, safety and pharmacokinetics of 1,2,4-trioxolane OZ78 against an experimental infection with *Fasciola hepatica* in sheep. *Vet. Parasitol.* **2010**, *173*, 228.
[29] S. Fekete, J. Fekete, K. Ganzler. Characterization of new types of stationary phases for fast liquid chromatographic applications. *J. Pharm. Biomed. Anal.* **2009**, *50*, 703.
[30] G. Hopfgartner, C. Husser, M. Zell. High-throughput quantification of drugs and their metabolites in biosamples by LC–MS/MS and CE-MS/MS: possibilities and limitations. *Ther. Drug. Monit.* **2002**, *24*, 134.
[31] J. Xing, H. Yan, S. Zhang, G. Ren, Y. Gao. A high-performance liquid chromatography/tandem mass spectrometry method for the determination of artemisinin in rat plasma. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1463.
[32] A. Cappiello, G. Famiglini, P. Palma, E. Pierini, V. Termopoli, H. Trufelli. Overcoming matrix effects in liquid chromatography–mass spectrometry. *Anal. Chem.* **2008**, *80*, 9343.
[33] N. Lindegardh, W. Hanpithakpong, B. Kamanikom, P. Singhasivanon, D. Socheat, P. Yi, A. M. Dondorp, R. McGready, F. Nosten, N. J. White, N. P. Day. Major pitfalls in the measurement of artemisinin derivatives in plasma in clinical studies. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2008**, *876*, 54.
[34] C. L. French, S. S. Yaun, L. A. Baldwin, D. A. Leonard, X. Q. Zhao, E. J. Calabrese. Potency ranking of methemoglobin-forming agents. *J. Appl. Toxicol.* **1995**, *15*, 167.

# *Chapter 6*

# Evaluation of the Pharmacokinetic Profile of Artesunate, Artemether and their Metabolites in Sheep Naturally Infected with *Fasciola hepatica*

Urs Duthaler [a], Jörg Huwyler [b], Laura Rinaldi [c], Giuseppe Cringoli [c], Jennifer Keiser [a]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

[b] Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Klingelbergstrasse 50, Basel, Switzerland.

[c] Department of Pathology and Animal Health, CREMOPAR Regione Campania, University of Naples Federico II, Naples, Italy

**Published in Veterinary Parasitology: in press**

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS

Veterinary Parasitology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

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

# Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*

Urs Duthaler[a,b], Jörg Huwyler[c], Laura Rinaldi[d], Giuseppe Cringoli[d], Jennifer Keiser[a,b,*]

[a] *Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, Switzerland*
[b] *University of Basel, P.O. Box, CH-4003 Basel, Switzerland*
[c] *Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland*
[d] *Department of Pathology and Animal Health, CREMOPAR Regione Campania, University of Naples Federico II, Via della Veterinaria 1, 80137 Naples, Italy*

ARTICLE INFO

*Article history:*
Received 3 October 2011
Received in revised form
18 November 2011
Accepted 29 November 2011

*Keywords:*
Pharmacokinetics
Fascioliasis
Sheep
Artemisinins
Activity

ABSTRACT

The pharmacokinetic (PK) parameters of artesunate, artemether and their metabolites dihydroartemisinin (DHA) and dihydroartemisinin-glucuronide (DHA-glucuronide) were determined in sheep naturally infected with *Fasciola hepatica*.

Sheep were treated either with artesunate (intramuscular (i.m.): 40 and 60 mg/kg) or artemether (i.m.: 40 and 160 mg/kg; oral: 80 mg/kg). Blood samples were withdrawn at selected time points post treatment and the artemisinins were quantified in plasma by liquid chromatography and tandem mass spectrometry (LC–MS/MS). The *in vitro* effect of the metabolites against *F. hepatica* was investigated using a phenotype-based assay and scanning electron microscopy (SEM).

Following artesunate applications (40 and 60 mg/kg), comparable $C_{max}$ (maximal plasma concentration) and AUCs (area under the plasma concentration–time curve) were observed for artesunate ($C_{max}$: $8.4 \times 10^3$ and $9.4 \times 10^3$ ng/ml; AUC: $6.9 \times 10^5$ and $9.7 \times 10^5$ ng min/ml), DHA ($C_{max}$: both $2.4 \times 10^3$ ng/ml; AUC: $3.7 \times 10^5$ and $5.0 \times 10^5$ ng min/ml), and DHA-glucuronide ($C_{max}$: $1.7 \times 10^4$ and $1.6 \times 10^4$ ng/ml; AUC: $2.6 \times 10^6$ and $3.3 \times 10^6$ ng min/ml). Mean elimination half-lifes ($t_{1/2}$) of artesunate, DHA and DHA-glucuronide ranged between 58 and 63 min, 94 and 113 min, and 89 and 98 min, respectively. The i.m. oil-based drug formulation liberated artemether slowly and constant levels of artemether and its metabolites were observed during the entire sampling period (24 h). The AUCs of all analytes were significantly higher for the i.m. 160 mg/kg dose compared to i.m. 40 and oral 80 mg/kg doses ($P = 0.018$). Mean $C_{max}$ of artemether (2126 and 426 ng/ml) and DHA-glucuronide (3477 and 1587 ng/ml) were higher following oral compared to i.m. (160 mg/kg) treatments ($P > 0.068$), whereas $C_{max}$ of DHA was significantly higher following i.m. applications ($P = 0.0062$). DHA rapidly reduced the viability of *F. hepatica in vitro*, whereas DHA-glucuronide showed no activity. SEM observations revealed only minor and focal tegumental alterations in few of the DHA treated worms.

The calculated PK parameters reflect the anthelmintic activity of artesunate and artemether following different routes of application and will aid in the design of future studies with these drugs.



## 1. Introduction

The semisynthetic artemisinin derivatives artesunate and artemether are highly effective antimalarial drugs and are recommended in combination with other antimalarials

* Corresponding author at: Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland. Tel.: +41 61 284 8218; fax: +41 61 284 8105.
*E-mail address:* jennifer.keiser@unibas.ch (J. Keiser).

0304-4017/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2011.11.076

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS



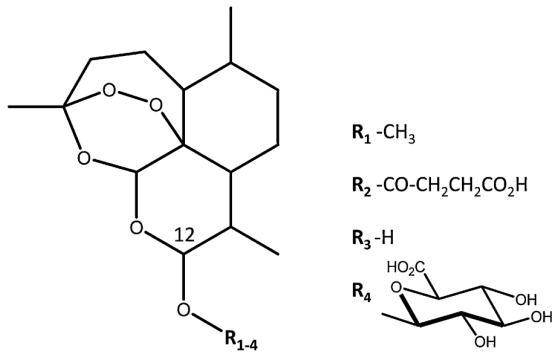



**Fig. 1.** Molecular structure of the artemisinins. The parent drugs and metabolites differ at position 12 of the sesquiterpene lactone scaffold. Parent drugs: $R_1$, = artemether (molecular weight (MW): 298.37 g/mol) and $R_2$, = artesunate (MW: 384.42 g/mol). Phase-I metabolite: $R_3$, = dihydroartemisinin (MW: 284.35 g/mol). Phase-II metabolite: $R_4$, = dihydroartemisinin-glucuronide (MW: 460.47 g/mol).

as first line therapy (Fig. 1) (White, 2008). The antiparasitic activity of the artemisinins is not limited to *Plasmodium*, with among others, trematode worms including *Schistosoma* and *Fasciola* spp. being also affected by these drugs (Keiser and Utzinger, 2009; Utzinger et al., 2007). The foodborne trematodes *Fasciola hepatica* and *F. gigantica* infect humans, farm animals including sheep and cattle as well as a broad range of wild animals (Robinson and Dalton, 2009). The flukes damage the liver and biliary system of the host, may cause gastrointestinal disorders, severe anaemia, weight loss, bile stones, as well as inflammation and fibrosis of bile and liver (e.g. cholangitis and cholecystitis) (Chen and Mott, 1990). Worldwide as many as 17 million humans might be infected with *Fasciola* spp. (Keiser and Utzinger, 2009). Moreover, an estimated 250–300 million sheep and cattle are affected by fascioliasis (fasciolosis) resulting in an estimated annual financial loss of around US$ 3 billion to the agricultural sector (Robinson and Dalton, 2009; Spithill et al., 1999).

Artesunate and artemether kill *F. hepatica in vitro*, by inflicting damage to tegument and the gastrointestinal system of the worms (Keiser and Morson, 2008; O'Neill et al., 2009). *In vivo*, rats harbouring an experimental *F. hepatica* infection were cured with both compounds, with 50% effective dosages ($ED_{50}$) of about 80 mg/kg and 115 mg/kg estimated for artemether and artesunate, respectively (Duthaler et al., 2010). Moreover, artemether was also active against a triclabendazole resistant *F. hepatica* strain in the rat model (Keiser et al., 2007).

Considering the veterinary importance of this parasite and the promising *in vitro* and *in vivo* activities of the artemisinins, their efficacy was assessed in sheep naturally infected with *F. hepatica*. Two studies have been recently carried out in the Campania region in Southern Italy, where the prevalence of fascioliasis in sheep is approximately 4–12% on farms (Cringoli et al., 2002; Keiser et al., 2008, 2010; Musella et al., 2011). Interestingly, large differences in fasciocidal activity were observed in sheep following different routes of drug administration, as only parenteral treatments were effective (Keiser et al., 2008). Furthermore, artesunate showed a higher activity than artemether in the treatment of infected sheep, which is in contrast to the results obtained in the *F. hepatica* rat model (Keiser et al., 2006, 2008, 2010). An evaluation of the pharmacokinetic (PK) behaviour of artesunate and artemether in sheep might help to understand these differences. Moreover, the analysis of phase-I/II metabolites possibly will further enhance our knowledge on these drugs, since a preliminary investigation of one treated sheep revealed high plasma levels of dihydroartemisinin (DHA, Fig. 1) and its glucuronide conjugate dihydroartemisinin-glucuronide (DHA-glucuronide, Fig. 1) (Duthaler et al., 2011). Finally, although, the disposition of the artemisinins has been well studied in rodents and humans, to our knowledge their PK parameters have not been determined in sheep (Medhi et al., 2009).

The aim of this study was to determine the PK parameters of artesunate and artemether and their metabolites DHA and DHA-glucuronide in sheep naturally infected with *F. hepatica*, using a recently developed high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method (Duthaler et al., 2011). In addition, we assessed the *in vitro* activity of DHA and DHA-glucuronide, since (according to the PK profiles obtained) these metabolites might contribute to the fasciocidal effect of the artemisinins. Finally, obtained drug disposition parameters were compared with previously determined efficacy data (Keiser et al., 2008, 2010).

## 2. Materials and methods

### 2.1. Pharmacokinetic studies

Two studies were carried out on two cross-breed meat/dairy sheep farms located in the Salerno province of Campania in southern Italy (Keiser et al., 2008, 2010). Ethical permission was obtained from the Centre for Veterinary Service of the University of Naples Federico II (Ref. no. 98/08 and 116/07). Briefly, in both studies, stool samples were collected of randomly selected sheep. The FLOTAC double technique was used to identify infected animals and to quantify the egg load per animal (Cringoli et al., 2010). Sheep were assigned randomly to treatment groups according to their weight and the mean *F. hepatica* egg count. Treatment efficacy was evaluated based on the worm count reduction after treatment and the faecal egg count reduction test (FECRT) as described by Keiser et al. (2008, 2010). In brief, *F. hepatica* eggs were quantified in at least three stool samples, which had been collected on consecutive days, before treatment and between 10 and 28 days post treatment. The *F. hepatica* worm count was determined in treated and untreated sheep 3–4 weeks post-treatment.

In the framework of the above mentioned efficacy trials, drug disposition was studied from the following treatments: 40 mg/kg ($n = 6$) and 60 mg/kg ($n = 6$) intramuscular (i.m.) artesunate, 40 mg/kg ($n = 5$) and 160 mg/kg ($n = 5$) i.m. administered artemether and 80 mg/kg ($n = 6$) oral artemether. These dosages had been calculated based on activities observed in rats.

Blood samples were withdrawn from the jugular vein of the sheep into lithium–heparin coated Vacutainer® tubes

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS
U. Duthaler et al. / Veterinary Parasitology xxx (2011) xxx–xxx 

(BD, Franklin Lakes, NJ, USA). Blood samples were obtained from all study animals prior to treatment to determine pre-treatment PK data ($T_{0,min}$) and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, (8), and 24 h post-treatment. Immediately after the blood withdrawal, the samples were cooled on ice and subsequently centrifuged to obtain plasma.

### 2.2. Drugs, metabolites and drug formulation

Artesunate was a kind gift from Mepha AG (Aesch, Switzerland). Artemether was kindly provided by Dafra Pharma (Turnhout, Belgium) and Kunming Pharmaceutical Cooperation (Kunming, People's Republic of China). The latter product was used for treating *F. hepatica* infected sheep. Artesunate was dissolved in an aqueous formulation of 5% $NaHCO_3$ (Merck, Darmstadt, Germany) and polyethylene glycol 400 (PEG; Fluka, Buchs, Switzerland) in a ratio of 40:60 (v/v). Orally applied artemether was given as a suspension of 7% (v/v) Tween 80 (Sigma–Aldrich, Buchs, Switzerland), 3% (v/v) ethanol 96% (Merck, Darmstadt, Germany) in tap water. Artemether was dissolved in 10–30 ml peanut oil (Roth, Basel, Switzerland) for the i.m. application. All drugs were prepared shortly before administration.

For the *in vitro* studies, dihydroartemisinin-12-α-o-β-D-glucuronide (DHA-glucuronide) was purchased from Clearsynth (Mumbai, India) and α,β-dihydroartemisinin (DHA) was a gift from Dafra Pharma (Turnhout, Belgium).

### 2.3. Liquid chromatography tandem mass spectrometry analysis

Plasma samples stored at −80 °C were analysed using a recently developed and validated LC–MS/MS method for the simultaneous quantification of artesunate, artemether, and their metabolites, DHA and DHA-glucuronide in sheep plasma (Duthaler et al., 2011). In brief, the analysis was performed with a high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) connected to an API 365 triple-quadrupole mass spectrometer (PE Biosystems, Foster City, CA, USA) with a turbo ion spray interface. Nitrogen was used as nebulizer, curtain, and collision gas. All artemisinins were detected in the positive mode by selected reaction monitoring with a transition of $m/z$ 267.4 → 163.0.

Chromatographic baseline separation of the analytes was performed at 25 °C (column oven: Omnilab, Mettmenstetten, Switzerland) using a 2.1 mm × 10 mm guard cartridge (3 μm) connected upstream to a 2.1 mm × 20 mm Atlantis T3 3 μm analytical column (Waters, Milford MA, USA). A stepwise gradient elution program of mobile phase A (A: 5 mM ammonium formate plus 0.15% (v/v) formic acid in ultra pure water) and mobile phase B (B: 0.15% (v/v) formic acid in acetonitrile) at a flow rate of 0.3 ml/min was used: 0–1 min, B 2%; 1–10 min, B 2–45%; 10–13 min, B 45–50%; 13–15 min, B 50–95%; 15–20 min, B 95%; 20–21 min, B 95–2%; 21–22 min, B 2%.

Calibration curves of the analytes were prepared by diluting analyte working solutions with blank sheep plasma (1:30, $V_{total}$ = 300 μl). Each calibration set consisted of one blank plasma sample (plasma sample processed without internal standard (IS)), one zero sample (plasma sample spiked with IS) and at least 6 calibration samples.

For the artesunate treatment samples, calibration curves ranged from 4 to 900 ng/ml, 25 to 1500 ng/ml and 100 to 3000 ng/ml for artesunate, DHA, and DHA-glucuronide, respectively. For the artemether samples, calibration ranges were 90–6000 ng/ml for artemether and 25–3000 ng/ml for DHA and DHA-glucuronide. The lowest and highest calibrator corresponded to the lower (LLOQ) and upper limit (ULOQ) of quantification, respectively. In addition, quality control (QC) samples were prepared at low, medium, and high (=ULOQ) concentrations covering the whole calibration range. The following QC samples were measured in the PK study of artesunate treated sheep: artesunate; 900, 90, and 9 ng/ml, DHA; 1500, 500 and 50 ng/ml, DHA-glucuronide; 3000, 750, and 93.8 ng/ml. For the artemether PK, QC samples at 6000, 1500, and 93.8 ng/ml were selected for artemether and 3000, 750, and 46.9 ng/ml for both DHA and DHA-glucuronide. PK plasma samples with concentrations above the ULOQ were diluted with blank plasma to obtain concentrations within the respective calibration range. Artemether (5 μg/ml) or otherwise artesunate (0.25 μg/ml) was used as IS depending on the analysed drug.

A 300 μl sheep plasma aliquot was spiked with 90 μl sodium nitrite solution (3 M) containing 1% (v/v) acetic acid and gently agitated for 30 min at 37 °C (Hodel et al., 2009; Keiser et al., 2009). In a next step, 10 μl of IS working solution (artesunate: 7.5 μg/ml or artemether: 150 μg/ml) was added to each plasma aliquot. The plasma proteins were precipitated with 1000 μl ice-cold methanol, vortex mixed and centrifuged (Eppendorf centrifuge 5415 R, Hamburg, Germany) at 16,100 × $g$ and 4 °C for 15 min. The supernatant was transferred to a 2 ml microtube and the protein pellet was washed with 500 μl methanol. The sample was then centrifuged as described before and the combined extracts were evaporated to dryness under a stream of nitrogen. The residue was reconstituted in a mixture of mobile phase A – methanol (250 μl, 1:1, v/v), mixed, centrifuged (15 min, 16,100 × $g$, 4 °C), and transferred to an autosampler vial. The prepared samples were stored at 6 °C in the CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) prior to analysis. A 30 μl aliquot of each sample was injected into the LC–MS/MS system.

### 2.4. In vitro studies of the artemisinin metabolites

#### 2.4.1. Phenotypic assay

Adult *F. hepatica* were recovered from bovine livers obtained from the local slaughterhouse (Basel, Switzerland) or from rats experimentally infected with *F. hepatica*. The flukes were pre-incubated (<24 h) in RPMI 1640 culture medium (Gibco, NY, USA) at 37 °C in an atmosphere of 5% $CO_2$. A single adult worm was placed per well (6-well plate, Costar, MA, USA) containing 4 ml RPMI 1640, supplemented with 1% (v/v) antibiotics (50 μg/ml streptomycin and 50 U/ml penicillin; Sigma, Buchs, Switzerland) and 80 μg/ml haemin (Keiser and Morson, 2008). Adult *F. hepatica* were incubated in the presence of 50 μg/ml DHA ($n$ = 9) or DHA-glucuronide ($n$ = 6). Drug stock solutions (5 mg/ml) were prepared in dimethyl sulfoxide (DMSO;

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS



Sigma–Aldrich, Buchs, Switzerland) and stored at −20 °C. Control worms ($n = 12$) were incubated in 1% (v/v) DMSO, in line with the amount of DMSO added to the drug assays. The viabilities of treated and untreated *F. hepatica* flukes were monitored for 72 h at 37 °C and 5% $CO_2$ and scored after 24, 48 and 72 h using the following scale: 2 = normal activity; movements were visible without microscope, 1 = reduced activity; movements could be detected only by means of microscopic magnification (20×), and 0 = death of worm absence of movements for 2 min using a microscope (20×).

#### 2.4.2. Scanning electron microscopy observations

*F. hepatica* flukes isolated from bovine or rat bile ducts were incubated in the presence of 50 μg/ml DHA-glucuronide ($n = 4$) or 50 μg/ml DHA ($n = 9$) over a period of 72 h under conditions as described in Section 2.4.1. Control worms were incubated in presence of DMSO. The flukes were collected after 72 h. The specimen were fixed for several hours in 2.5% glutaraldehyde (Alfa Aesar, Karlsruhe, Germany) in phosphate buffer saline (PBS, pH 7.4) at room temperature. The flukes were washed with PBS buffer, dehydrated in ascending ethanol concentrations and processed as described previously (Keiser and Morson, 2008).

### 2.5. Data analysis and statistics

Data analysis was performed with Analyst 1.4.2. software (PE Biosystems). Statsdirect statistical software (Statsdirect Ltd., Cheshire, UK) and Microsoft Excel 2003 were used for the statistical analysis. The Kruskal–Wallis test was applied to compare PK parameters of different treatment groups. A $P$ value below 0.05 was considered to be statistically significant.

PK parameters of artesunate, artemether, DHA and DHA-glucuronide were determined by non-compartmental analysis using WinNonLin (Version 5.2, Pharsight Corporation, USA). The following PK parameters were included in the calculation: maximal plasma concentration, $C_{max}$ (ng/ml); time to achieve maximal plasma concentration, $T_{max}$ (min); area under the plasma concentration–time curve, AUC (ng min/ml); and elimination half-life, $t_{1/2}$ (min). $C_{max}$ and $T_{max}$ were observed values of the plasma time course of the analytes. The AUC was estimated from time point zero to the time point of last quantifiable concentration using the linear trapezoidal rule. $AUC_{DOSE}$ corresponds to the AUC normalised to the applied dosage (AUC/mg kg). Whenever possible, the elimination half-life ($t_{1/2}$) was calculated using $t_{1/2} = \ln(2)/\lambda$, where the elimination rate constant of the analyte ($\lambda$) is determined by linear regression of the natural logarithm of the concentration values in the elimination phase. These parameters were calculated for each sheep and the arithmetic mean and the standard error of the mean ($SE = \text{standard deviation}/\sqrt{\text{number of observations}}$) were determined. For treatments for which only few plasma concentrations ($n < 4$ per time point) could be quantified (since these were below the LLOQ) the AUC was calculated from the mean curve of the pooled data. In this case, the sparse analysis command in WinNonLin was used for the AUC estimation.

## 3. Results

### 3.1. LC–MS/MS method

All analytes were quantified by linear regression of the respective calibration curve with correlation coefficients ($r^2$) > 0.99. The LLOQ of the parent drugs artesunate and artemether was set at 3.7 ng/ml and 93.8 ng/ml, respectively. The metabolites, DHA and DHA-glucuronide were quantifiable down to 23.4 ng/ml.

The inter-day precisions and accuracies of the QC samples were analysed separately for artesunate and artemether treated sheep. The inter-day precision was ≤15% (range: 0.7–15%) for all analytes. The corresponding inter-day accuracies ranged from 96.4% to 112.6%. These data are summarized in Table 1.

### 3.2. Pharmacokinetics of artesunate intramuscular treated sheep

Artesunate, DHA and DHA-glucuronide were observed in plasma of all sheep over 8 h. Only traces of artesunate were present 24 h post-treatment. The mean concentration versus time profiles of artesunate, DHA and DHA-glucuronide of both treatment dosages are depicted in Fig. 2A and B and the corresponding PK parameters are summarized in Table 2.

Maximal plasma concentrations of artesunate, DHA, and DHA-glucuronide were detected at 15, 45, and 60 min, respectively after drug application for both dosages ($T_{max}$). Treatment with 40 and 60 mg/kg showed comparable mean $C_{max}$ for the parent drug and its metabolites, namely $1.7 \times 10^4$ and $1.6 \times 10^4$ ng/ml for DHA-glucuronide, respectively, $8.4 \times 10^3$ and $9.4 \times 10^3$ ng/ml for artesunate, respectively, and $2.4 \times 10^3$ ng/ml for DHA for both dosages (Table 2). The dose normalised mean $AUC_{DOSE}$ of artesunate, DHA, and DHA-glucuronide were 17,294, 9152, and 64,910 (ng min/ml)/mg, respectively for 40 mg/kg and 16,114, 8328, and 55,037 (ng min/ml)/mg, respectively for the 60 mg/kg dosage, hence the dose normalised AUCs were 7–15% lower for the 60 mg/kg than for the 40 mg/kg group. The terminal $t_{1/2}$ was independent of the dose for all analytes ($P > 0.26$). Mean $t_{1/2}$ of 63 ± 8 min and 58 ± 4 min were calculated for artesunate 40 and 60 mg/kg treatments, respectively. Slightly higher $t_{1/2}$ values were estimated for DHA (94 ± 9 and 113 ± 14 min) and DHA-glucuronide (89 ± 9 and 98 ± 5 min) following treatment with 40 and 60 mg/kg artesunate, respectively. Overall, the fitting ($r^2$) of the drug elimination parameter, $t_{1/2}$ was always >0.94 for each analyte and all sheep (mean: 0.988, 95% CI: 0.982–0.993).

### 3.3. Pharmacokinetics of artemether treated sheep

#### 3.3.1. Intramuscular drug administration

The i.m. applied oily drug formulation, 40 and 160 mg/kg, liberated artemether during the entire sampling period of 24 h. Artemether, DHA, and DHA-glucuronide were detected in all treated sheep. Mean plasma concentration curves of both i.m. artemether treatments over 24 h are presented in Fig. 3A and B and the

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS
U. Duthaler et al. / Veterinary Parasitology xxx (2011) xxx–xxx 

**Table 1**
Quality control samples of the liquid chromatography tandem mass spectrometry method: inter-day accuracy and precision.

| PK study | Analyte | Nominal concentration [ng/ml] | Mean concentration found [ng/ml] | Accuracy [%][a] | Precision [%][b] | Sample size [n] |
|---|---|---|---|---|---|---|
| Artesunate | Artesunate | 900.0 | 913.7 | 101.5 | 9.4 | 33 |
| | | 90.0 | 88.1 | 97.9 | 9.6 | 12 |
| | | 9.0 | 9.1 | 101.4 | 12.9 | 12 |
| | Dihydroartemisinin | 1500.0 | 1472.7 | 98.2 | 8.4 | 33 |
| | | 500.0 | 486.8 | 97.4 | 10.1 | 11 |
| | | 50.0 | 49.5 | 99.1 | 15.0 | 10 |
| | DHA-glucuronide | 3000.0 | 3143.4 | 104.8 | 0.7 | 4 |
| | | 750.0 | 732.5 | 97.7 | 2.9 | 4 |
| | | 93.8 | 98.2 | 104.7 | 2.4 | 4 |
| Artemether | Artemether | 6000.0 | 6756.2 | 112.6 | 5.6 | 9 |
| | | 1500.0 | 1513.9 | 100.9 | 5.9 | 9 |
| | | 93.8 | 96.3 | 102.7 | 4.6 | 8 |
| | Dihydroartemisinin | 3000.0 | 3122.0 | 104.1 | 5.4 | 9 |
| | | 750.0 | 756.7 | 100.9 | 3.9 | 9 |
| | | 46.9 | 45.7 | 97.5 | 4.5 | 8 |
| | DHA-glucuronide | 3000.0 | 3036.3 | 101.2 | 4.3 | 9 |
| | | 750.0 | 760.5 | 101.4 | 3.5 | 9 |
| | | 46.9 | 45.2 | 96.4 | 6.6 | 8 |

[a] The accuracy is the percentage ratio of the measured concentration to the nominal concentration.
[b] The precision is calculated using the relative standard deviation ([standard deviation/mean] × 100).

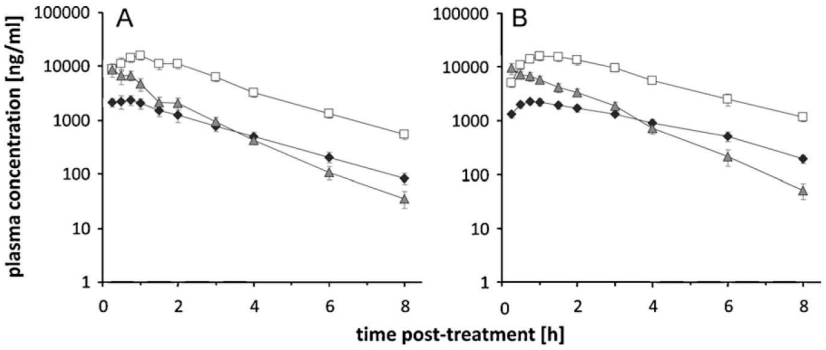



**Fig. 2.** Mean plasma concentration–time profiles of artesunate (▲), dihydroartemisinin (♦), and dihydroartemisinin-glucuronide (□) following a single intramuscular (i.m.) dose of artesunate. (A) I.m. administration of artesunate 40 mg/kg ($n = 6$). (B) I.m. administration of artesunate 60 mg/kg. None of the samples were quantifiable at time point 24 h. Data points are means ± standard error.

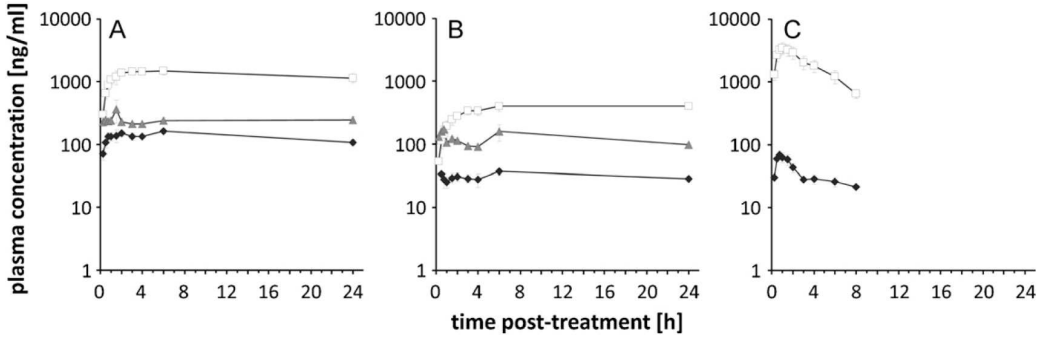



**Fig. 3.** Mean plasma concentration–time profiles of artemether (▲), dihydroartemisinin (DHA) (♦), and dihydroartemisinin-glucuronide (□) following a single intramuscular (i.m.) or oral dose of artemether. (A) Artemether 160 mg/kg i.m. ($n = 5$). (B) Artemether 40 mg/kg i.m. ($n = 5$): $n \leq 3$ plasma samples of artemether were quantifiable at time point 2 h, 4 h, and 24 h post treatment. None of the samples of DHA were quantifiable at time point 0.25 h and $n \leq 3$ at time point 0.5–2 h and 4 h. (C) Artemether 80 mg/kg oral ($n = 6$): $n \leq 3$ plasma samples of DHA were quantifiable from time point 4 h to 8 h and none of the analytes were quantifiable at 24 h post treatment. Data points are means ± standard error.

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS



**Table 2**
Pharmacokinetic parameters in sheep determined after administration of artesunate or artemether. Parent drugs as well as the phase I metabolite dihydroartemisinin (DHA) and the phase II metabolite dihydroartemisinin-glucuronide (DHA-glucuronide) were monitored. Values are means ± standard error ($n \geq 5$).

| Treatment | Artesunate/artemether (parent drugs) | | | | DHA (phase I metabolite) | | | | DHA-glucuronide (phase II metabolite) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C_{max}$ [ng/ml] | AUC × $10^5$ [ng min/ml] | $t_{1/2}$ [min] | $T_{max}$ [min] | $C_{max}$ [ng/ml] | AUC × $10^5$ [ng min/ml] | $t_{1/2}$ [min] | $T_{max}$ [min] | $C_{max}$ [ng/ml] | AUC × $10^5$ [ng min/ml] | $t_{1/2}$ [min] | $T_{max}$ [min] |
| Artesunate 40 mg/kg i.m. | 8391 ± 1913 | 6.9 ± 1.4 | 63.4 ± 8.3 | 15 | 2425 ± 465.7 | 3.7 ± 0.6 | 93.8 ± 9.0 | 45 | 16,683 ± 3057 | 26.0 ± 3.3 | 88.8 ± 9.2 | 60 |
| Artesunate 60 mg/kg i.m. | 9421 ± 2158 | 9.7 ± 1.6 | 57.8 ± 3.7 | 15 | 2414 ± 288.8 | 5.0 ± 0.5 | 112.8 ± 13.8 | 45 | 16,055 ± 2677 | 33.0 ± 4.9 | 98.1 ± 4.7 | 60 |
| Artemether 40 mg/kg i.m. | 225.4 ± 30.8 | 1.8 ± 0.3[a] | n.d. | 45 | 39.5 ± 3.8 | 0.46 ± 0.04[a] | n.d. | 360 | 420.2 ± 61.7 | 5.3 ± 0.7 | n.d. | 360 |
| Artemether 160 mg/kg i.m. | 426.0 ± 119.1 | 3.4 ± 0.3 | n.d. | 90 | 174.6 ± 10.0 | 1.9 ± 0.1 | n.d. | 120 | 1587 ± 189.9 | 18.6 ± 2.6 | n.d. | 120 |
| Artemether 80 mg/kg oral | 2126 ± 915.2 | 1.7 ± 0.6[a] | n.d. | n.d. | 66.1 ± 11.6 | 0.16 ± 0.01[a] | 95.4 ± 18.4 | 45 | 3477 ± 718.8 | 8.7 ± 1.6 | 123.3 ± 9.7 | 60 |

$C_{max}$, maximal plasma concentration; AUC, area under the plasma concentration–time curve; $t_{1/2}$, elimination half-life; $T_{max}$, time to achieve maximal plasma concentration; i.m., intramuscular.
[a] The AUC was calculated from the mean curve of the pooled data, since only few plasma concentrations per time point ($n < 4$) were quantifiable.

PK parameters are presented in Table 2. Due to slow drug liberation, $t_{1/2}$ were not calculated for artemether and its metabolites, since the terminal elimination phase could not be determined with sufficient accuracy within the applied sampling period of 24 h.

Sheep treated with a dose of 160 mg/kg showed constant levels of artemether over 24 h, with a $T_{max}$ observed after 90 min ($C_{max}$: 426 ng/ml). DHA and DHA-glucuronide reached the $C_{max}$ at 2 h post-treatment and showed constant drug levels until 24 h post treatment ($C_{max}$: DHA: 175 ng/ml; DHA-glucuronide: 1587 ng/ml) (Fig. 3A).

For sheep treated with 40 mg/kg artemether, only DHA-glucuronide could be quantified accurately for each sheep at all time points ($n \geq 4$). In the case of DHA none or only one to three sheep exhibited concentrations within the calibration range between 15 and 120 min post treatment. Similarly, for artemether many observed plasma concentrations were below the LLOQ (93.8 ng/ml). For example, only two sheep showed plasma concentrations above the LLOQ after 24 h. $T_{max}$ was observed 45 min post treatment for artemether, and 6 h post treatment for DHA and DHA-glucuronide. Mean $C_{max}$ were 225 ng/ml, 40 ng/ml and 420 ng/ml for artemether, DHA and DHA-glucuronide, respectively.

Dose proportionality was assessed after normalizing the AUC by the theoretical doses (40 and 160 mg/kg). For artemether the dose normalised $AUC_{DOSE}$ was 52% higher for the 40 mg/kg dose than for the 160 mg/kg dose (4479 versus 2137 (ng min/ml)/mg). However, note that the AUC of 40 mg/kg treated sheep was calculated using the pooled data (only concentrations above the LLOQ (e.g. 24 h time point $n = 2$) were included in the analysis), hence might be overestimated. For the metabolites, there were only minor differences of the $AUC_{DOSE}$ (1140 versus 1206 (ng min/ml)/mg for DHA, and 13,275 versus 11,641 (ng min/ml)/mg for DHA-glucuronide) observed between the dose levels.

### 3.3.2. Oral drug administration

Artemether and the metabolites were detected in sheep plasma up to 8 h post treatment with oral artemether. 24 h after drug administration, no drug or metabolite could be detected in any of the sheep. Fig. 3C illustrates the plasma concentration–time profiles of the metabolites DHA and DHA-glucuronide following oral treatment of artemether. PK data of orally given artemether are summarized in Table 2.

High variations in the plasma concentrations of artemether were observed within sheep and between different sheep. For example, in the plasma samples of two of the sheep only very low artemether concentrations could be detected ($C_{max}$ of 222 and 337 ng/ml). In these sheep few concentrations ($n = 7$ and 4) were above the LLOQ (93.8 ng/ml). On the other hand, in three other sheep very high artemether concentrations compared to all other values were observed at 0.25 h, 2 h, and 8 h post treatment, respectively. Overall, the $C_{max}$ of the sheep were highly variable and ranged from 222 to 4983 ng/ml (mean 2126 ng/ml) with an estimated AUC and dose normalised $AUC_{DOSE}$ of $1.7 \times 10^5$ ng min/ml and 2073 (ng min/ml)/mg, respectively. For these reasons, it was not

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS
U. Duthaler et al. / Veterinary Parasitology xxx (2011) xxx–xxx 

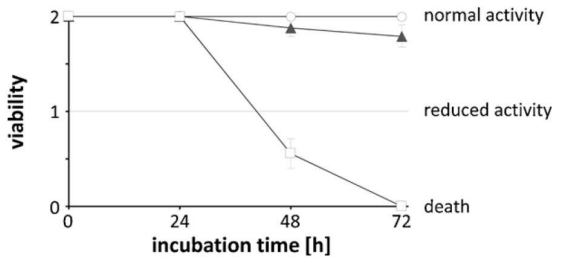



**Fig. 4.** Phenotype-based *in vitro* viability assay of adult *F. hepatica*. Viability of adult *F. hepatica* was determined in the presence of 50 μg/ml dihydroartemisinin ($n = 9$) (□), 50 μg/ml DHA-glucuronide (o), and of untreated controls ($n = 12$) (▲). Incubation of adult *F. hepatica* ($n = 6$) with dihydroartemisinin-glucuronide (o) showed no *in vitro* activity and the viability of the flukes behaved similar to untreated controls. Error bars represent the standard error of the mean.

possible to estimate an accurate $t_{1/2}$ of artemether and to graphically present the dose–response curve (plasma concentration–time profile) (Fig. 3C).

DHA and DHA-glucuronide reached a $C_{max}$ of 66 and 3477 ng/ml 45 and 60 min post treatment, respectively. DHA concentrations declined rapidly and 4–8 h post treatment only one to three samples per time point exhibited concentrations within the calibration range and could be included in the analysis. The calculated dose normalised $AUC_{DOSE}$ of DHA and DHA-glucuronide was 201 and 10,937 (ng min/ml)/mg, respectively. A $t_{1/2}$ of 95 ± 18 min was calculated for DHA and the estimated $t_{1/2}$ is 123 ± 10 min for DHA-glucuronide. The fitting ($r^2$) was >0.94 for DHA and DHA-glucuronide with a mean of 0.984 (95% CI: 0.972–0.996).

### 3.4. *In vitro* activity of the artemisinin metabolites

#### 3.4.1. Phenotype-based assay

The temporal *in vitro* effects of DHA and DHA-glucuronide against adult *F. hepatica* were evaluated using a phenotype-based assay. The viabilities of DHA treated *F. hepatica* ($n = 9$) decreased rapidly between 24 and 48 h post exposure and all flukes died within 72 h of incubation. Only slight decreases of viabilities were observed for adult *F. hepatica* controls over 72 h. *F. hepatica* flukes ($n = 6$) incubated in the presence of DHA-glucuronide were not affected by the metabolite and behaved similar to untreated controls during the entire incubation period of 72 h. The viability courses of DHA and DHA-glucuronide treated and untreated flukes over a period of 72 h are presented in Fig. 4.

#### 3.4.2. Scanning electron microscopy observations

No alterations of the tegument could be observed in DHA-glucuronide treated (50 μg/ml) as well as untreated *F. hepatica*. On the other hand, scanning electron microscopy (SEM) observations of DHA treated *F. hepatica* revealed slight alterations of the tegument primarily around the ventral sucker. Blebs, sloughing and swelling of minor intensity were monitored in some worms, whereas others appeared not be affected by DHA. A representative image of a fluke treated with DHA-glucuronide, DHA, and of an untreated control worm is illustrated in Fig. 5.

## 4. Discussion

*F. hepatica* infections are frequently observed in livestock and humans can as well be affected by fascioliasis (Mas-Coma et al., 2005; Robinson and Dalton, 2009). Additional drugs ideally from novel compound classes are needed, since drug resistant *F. hepatica* strains have been documented for several fasciocidals and to date no vaccines are available (Fairweather and Boray, 1999; McManus and Dalton, 2006). The sesquiterpenlactons, artesunate and artemether show interesting activities against *F. hepatica in vitro* and in the rat model (Keiser et al., 2006). Treatment outcome in naturally *F. hepatica* infected sheep was dependent on the route of drug administration and surprisingly artesunate was more active than artemether, which is different to observations in rats (Keiser et al., 2008, 2010). Therefore, we were motivated to assess the pharmacokinetics of the artemisinins in sheep.

The metabolites DHA and DHA-glucuronide were included in the PK analysis, since high plasma levels were observed in a preliminary analysis in one sheep (Duthaler et al., 2011). In addition, the primary elimination of artemisinin metabolites occurs over bile and even enterohepatic circulation was documented in rats (Li et al., 2008; Maggs et al., 1997). This underlines that the metabolites might significantly contribute to the activity of the treatments, in particular since adult *F. hepatica* lodge in the bile ducts of the host organism. However, DHA-glucuronide affected neither the viability nor the tegument of adult *F. hepatica* flukes *in vitro* and it is most likely that glucuronidation inactivates the parent drugs *in vivo*. On the other hand, DHA clearly impaired the viability of adult worms *in vitro*, which is in line with previous observations (Keiser et al., 2006). The observed activity of DHA was comparable with the activity of artesunate and artemether documented against *F. hepatica in vitro* (Duthaler et al., 2010). However, the use of equimolar concentrations of DHA and DHA-glucuronide as well as assessing different concentrations would have been required to comprehensively compare the activity of both metabolites. Interestingly only marginal focal damages on the tegument including sloughing, swelling, and blebs were recorded after DHA exposure. In contrast, artemether and artesunate caused extensive tegumental alterations after *in vitro* and *in vivo* exposition (Keiser and Morson, 2008; Keiser et al., 2006). The unaffectedness of the *F. hepatica* tegument following exposure to the metabolites might be explained with their physicochemical properties (hydrophilicity, polarity, and molecular weight), which are different from the ones of the parent drugs. Oral ingestion of the drugs followed by disruption of the gastrointestinal system of the parasite, as it was already observed for artemether (O'Neill et al., 2009), might even be more likely for the metabolites than for the parents. Hence, it would be interesting to study the effect of the metabolites on the *F. hepatica* gut using transmission electron microscopy.

Both i.m. artesunate treatments (40 and 60 mg/kg) showed good activity against *F. hepatica* in sheep (Keiser

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS



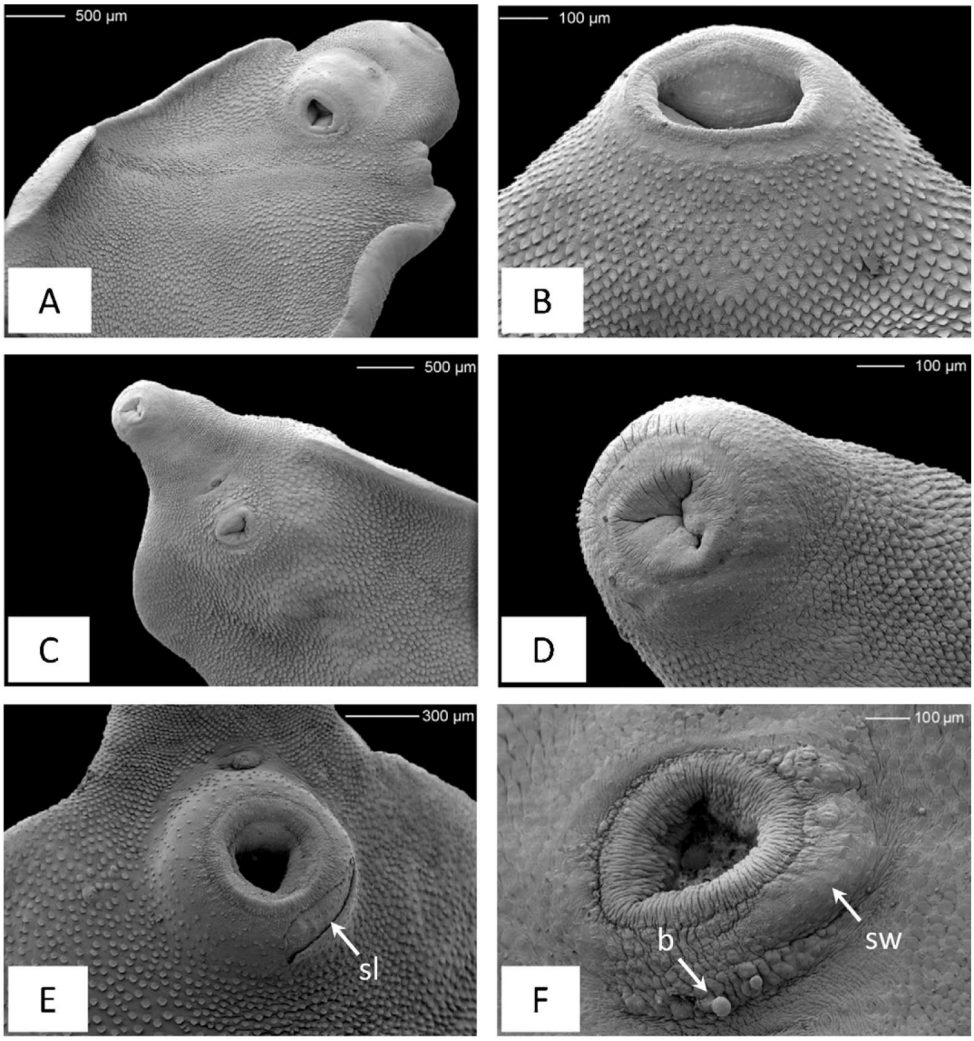



**Fig. 5.** Scanning electron microscopy (SEM) pictures of treated and untreated adult *F. hepatica*. (A and B) SEM pictures of *F. hepatica* controls incubated in RPMI medium for 72 h. (C and D) SEM pictures of *F. hepatica* incubated for 72 h in presence of dihydroartemisinin-glucuronide 50 μg/ml. No alterations of the tegument were visible. (E and F) SEM pictures of *F. hepatica* incubated for 72 h in presence of dihydroartemisinin 50 μg/ml. Sloughing (sl), swelling (sw) and blebbing (b) of minor intensity were present in some but not all of the treated flukes (white arrows).

et al., 2010), which is in line with the observed drug disposition. In Fig. 6 we compare the two main PK parameters, $C_{max}$ and AUC, obtained in the present work and the effectiveness of the treatments presented previously (Keiser et al., 2008, 2010). Both artesunate dosages resulted in high, not significantly different, AUC and $C_{max}$ values of the parent and its metabolites (Fig. 6). Artesunate at 40 mg/kg might already be at the peak of the dose–response curve and it would be interesting to test also lower dosages (e.g. 20 mg/kg). A comparable drug absorption process with similar $T_{max}$ was observed following both artesunate doses (Table 2). However, the $T_{max}$ of artesunate might be slightly overestimated, since the first plasma sample was only taken 15 min post treatment. Nonetheless, it might be interesting to note that a PK study done in humans calculated similar $T_{max}$s for artesunate and DHA after i.m. treatment (Ilett et al., 2002). In our study the mean estimated AUC of artesunate were larger than AUCs observed for DHA, which contrasts results of various studies in humans and one study undertaken in pigs following parenteral artesunate application (Li et al., 2009; Newton et al., 2000; Sinou et al., 2008). These differences might be explained by species specific differences in ADME processes including e.g. extensive glucuronidation of DHA in sheep or the high dosages used in our study and therefore a probable saturation of metabolism. In addition, the applied PEG-based drug formulation might have lead to a drug depot effect and a retarded drug invasion. Hence, the apparent $t_{1/2}$ of artesunate following i.m. administration does likely reflect an absorption rate limited process

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS
U. Duthaler et al. / Veterinary Parasitology xxx (2011) xxx–xxx 

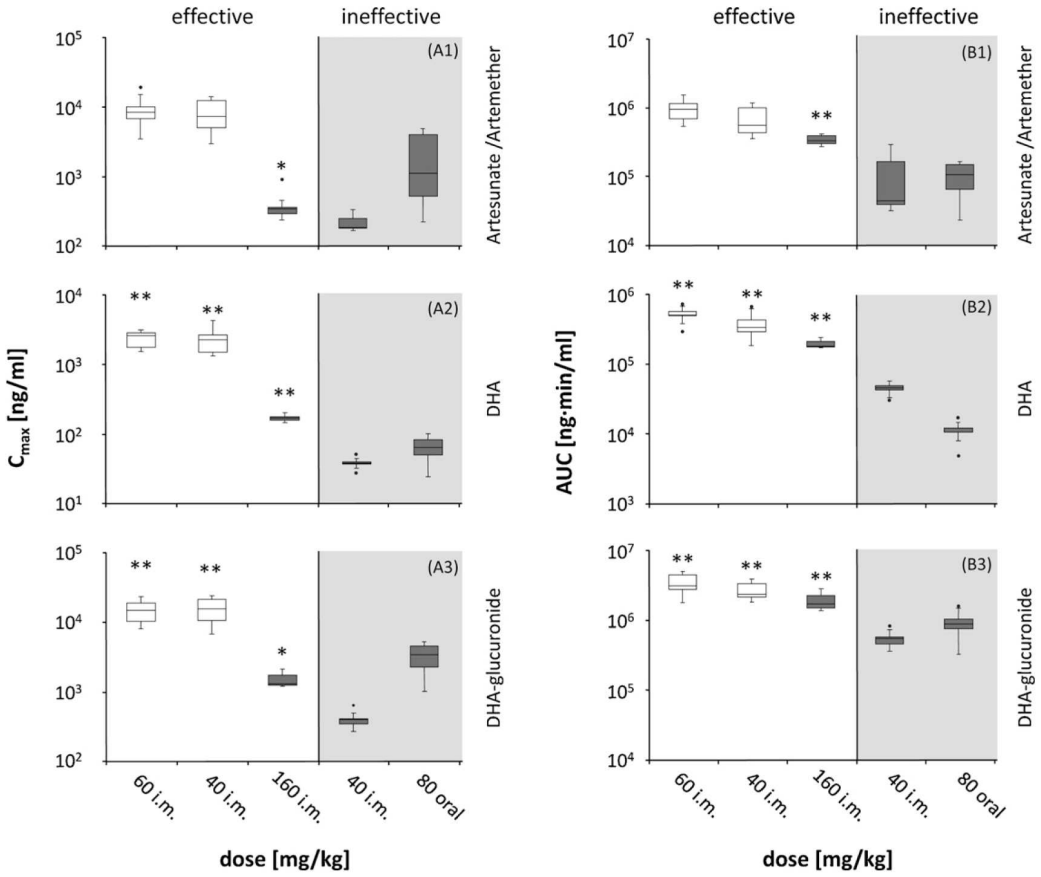



**Fig. 6.** Comparison of the observed drug effect against *F. hepatica* in sheep (Keiser et al., 2008, 2010) and the measured drug exposition parameters maximal plasma concentration ($C_{max}$) and area under the plasma concentration–time curve (AUC). Artesunate (40/60 mg/kg intramuscular (i.m.)) and artemether (40/160 mg/kg i.m. and 80 mg/kg oral) treatments are shown as white and grey box plots, respectively. A and B present $C_{max}$ (A) and AUC (B) values of the parent drugs (A1 and B1) as well as the phase-I and II metabolites (A2–A3 and B2–B3) following artesunate or artemether administration. The upper and lower limits of the boxes correspond to the interquartile range (IQR). The value in the middle is the median of the data set ($n \geq 5$). The limits of the whiskers are set at 1.5·IQR above the third quartile and 1.5·IQR below the first quartile. Values are shown as outliners (•), if the maximum or minimum values are beyond this range. *$C_{max}$/AUC is significantly different compared to the ineffective artemether i.m. 40 mg/kg treatment ($P < 0.05$). **$C_{max}$/AUC is significantly different from the ineffective artemether treatments i.m. 40 mg/kg and oral 80 mg/kg, respectively ($P < 0.05$).

rather than true elimination, since it has also been shown that artesunate is rapidly converted to DHA *in vivo* (Ilett et al., 2002).

Intramuscularly applied artemether showed fasciocidal efficacy of >90% at 160 mg/kg, while a 40 mg/kg i.m. dose lacked activity (Keiser et al., 2008). In accordance with this finding, the estimated $C_{max}$ as well as the AUC of all investigated analytes following 40 mg/kg i.m. artemether were significantly smaller compared to artemether given at 160 mg/kg (Fig. 6). It is interesting to note that a 4-fold lower artesunate i.m. dosage achieved a similar efficacy as artemether 160 mg/kg in sheep (Keiser et al., 2008, 2010). Contrary results were obtained in the *F. hepatica* rat model, with higher doses of artesunate required to achieve cure of animals (Keiser et al., 2006). Most PK parameters measured following artesunate treatment were significantly higher when compared to artemether 160 mg/kg (Fig. 6). However, while drug elimination of artesunate and its metabolites was completed after the last sampling point of 24 h it was not finished for artemether and the respective metabolites. Poor liberation of the drug from the oily vehicle and consequently decreased peak concentrations ($C_{max}$) might be the reason for the reduced efficacy of artemether i.m. treatments. In addition, little conversion to the active metabolite DHA was observed after artemether treatment (e.g. $C_{max}$ of the active metabolite DHA is about 10 times higher after artesunate (40 mg/kg) compared to artemether (160 mg/kg) application). Poor liberation, low $C_{max}$ and minor biotransformation of i.m. artemether have also been demonstrated in other studies (Li et al., 1998; Silamut et al., 2003).

A single oral dose (80 mg/kg) of artemether had no effect on *F. hepatica* worm and egg counts (Keiser et al., 2008). In line with this result, AUCs of all analytes following oral administration were significantly lower compared to the effective 160 mg/kg i.m. artemether dose (Fig. 6). However,

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS



this result has to be interpreted with caution because a diverse plasma concentration–time profile was observed for artemether given orally with somewhat erratic and inconsistent plasma levels between the investigated sheep. To our knowledge, an erratic absorption of artemether is not uncommon and has for example also been observed after i.m. treatments in humans (Silamut et al., 2003). The variations in the plasma profile of artemether following oral treatment might be explained as follows. First, artemether was applied as a suspension with a dissolved and dispersed phase and therefore it is conceivable that the absorption process might be inconstant, with diverse dissolution rate and only the solved molecules being capable to be absorbed (Porter et al., 2008). Second, degradation of artemether might have occurred in the digestive tract of the ruminants (Vandamme and Ellis, 2004). Third, food intake might have altered drug adsorption or changed the gastric transit time, which has been shown to affect rate and extent of absorption of orally given anthelmintics (Hennessy, 1993; Lanusse and Prichard, 1993). In addition, the oesophageal groove closure of the sheep followed by oral drench might have influenced the PK disposition of artemether differently (Prichard and Hennessy, 1981). Finally, an analytical problem cannot be excluded, since several plasma samples obtained following the oral treatment showed haemolysis and might have degraded artemether or somewhat influenced the analysis (Keiser et al., 2009). We offer the following explanations for the ineffectiveness of the oral treatment (80 mg/kg) compared to i.m. artemether treatment (160 mg/kg). As mentioned above, the observed AUC for all analytes were significantly higher following i.m. compared to oral treatment with an especially large difference observed for DHA (~10 times larger AUC). Hence, the relative bioavailability of oral treatments appears to be extensively reduced, a finding which contrasts other studies, where a larger DHA disposition was recorded after oral compared to i.m. application (Classen et al., 1999; Silamut et al., 2003). Degradation of artemether in the complex digestive tract of ruminants (Vandamme and Ellis, 2004), different drug formulations (solid versus suspension), and species dependent variability in the absorption process and metabolism may be the reasons for the observed differences.

In conclusion, we determined the PK parameters of artesunate, artemether and their metabolites DHA and DHA-glucuronide in sheep. We confirmed that our recently developed LC/MS–MS method is capable to adequately quantify the artemisinins in sheep plasma. The main disposition parameters satisfactorily reflect the observed activities of artesunate and artemether following different routes of application against *F. hepatica* in naturally infected sheep. *In vitro* studies of DHA and DHA-glucuronide suggest that DHA might contribute to the activity of the treatments while DHA-glucuronide possesses no activity. The obtained PK data might aid in designing additional studies with peroxides in the treatment of *F. hepatica* infections in sheep.

## Conflict of interest

There is no conflict of interest to declare.

## Acknowledgments

We thank Massimilliano Donzelli, Dr. Sabine Meyer, Dr. Manuel Haschke and Dr. Manfred Zell for their support and helpful suggestions concerning analytical and pharmacokinetic questions. We thank Laura Mezzino and Vincenzo Veneziano for their excellent technical assistance with the treatments and plasma sampling. A special thank goes to the farmers Allesandro Mario and Massimo Lullo for their kind collaboration. We are thankful to Prof. Dr. G. Imanidis for his continuous support and that we could carry out part of this project in his laboratories of the University of Applied Sciences, Northwestern Switzerland. Our sincerest thanks are addressed to Gianni Morson for the assistance with the SEM observations. We are grateful to Dafra Pharma, Kunming Pharmaceutical Cooperation, and Mepha for the supply of artesunate and artemether. UD and JK are financially supported by the Swiss National Science Foundation (project number: PPOOA-114941).

## References

Chen, M.G., Mott, K., 1990. Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. Trop. Dis. Bull. 4, R1–R38.
Classen, W., Altmann, B., Gretener, P., Souppart, C., Skelton-Stroud, P., Krinke, G., 1999. Differential effects of orally versus parenterally administered qinghaosu derivative artemether in dogs. Exp. Toxicol. Pathol. 51, 507–516.
Cringoli, G., Rinaldi, L., Maurelli, M.P., Utzinger, J., 2010. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat. Protoc. 5, 503–515.
Cringoli, G., Rinaldi, L., Veneziano, V., Capelli, G., Malone, J.B., 2002. A cross-sectional coprological survey of liver flukes in cattle and sheep from an area of the southern Italian Apennines. Vet. Parasitol. 108, 137–143.
Duthaler, U., Keiser, J., Huwyler, J., 2011. Development and validation of a liquid chromatography and ion spray tandem mass spectrometry method for the quantification of artesunate, artemether and their major metabolites dihydroartemisinin and dihydroartemisinin-glucuronide in sheep plasma. J. Mass Spectrom. 46, 172–181.
Duthaler, U., Smith, T.A., Keiser, J., 2010. *In vivo* and *in vitro* sensitivity of *Fasciola hepatica* to triclabendazole combined with artesunate, artemether, or OZ78. Antimicrob. Agents Chemother. 54, 4596–4604.
Fairweather, I., Boray, J.C., 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet. J. 158, 81–112.
Hennessy, D.R., 1993. Pharmacokinetic disposition of benzimidazole drugs in the ruminant gastrointestinal tract. Parasitol. Today 9, 329–333.
Hodel, E.M., Zanolari, B., Mercier, T., Biollaz, J., Keiser, J., Olliaro, P., Genton, B., Decosterd, L.A., 2009. A single LC–tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877, 867–886.
Ilett, K.F., Batty, K.T., Powell, S.M., Binh, T.Q., Thu, L.T.A., Phuong, H.L., Hung, N.C., Davis, T.M.E., 2002. The pharmacokinetic properties of intramuscular artesunate and rectal dihydroartemisinin in uncomplicated falciparum malaria. Brit. J. Clin. Pharmacol. 53, 23–30.
Keiser, J., Gruyer, M.S., Perrottet, N., Zanolari, B., Mercier, T., Decosterd, L., 2009. Pharmacokinetic parameters of artesunate and dihydroartemisinin in rats infected with *Fasciola hepatica*. J. Antimicrob. Chemother. 63, 543–549.
Keiser, J., Morson, G., 2008. *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. Exp. Parasitol. 118, 228–237.
Keiser, J., Rinaldi, L., Veneziano, V., Mezzino, L., Tanner, M., Utzinger, J., Cringoli, G., 2008. Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol. Res. 103, 517–522.
Keiser, J., Xiao, S.H., Tanner, M., Utzinger, J., 2006. Artesunate and artemether are effective fasciolicides in the rat model and *in vitro*. J. Antimicrob. Chemother. 57, 1139–1145.
Keiser, J., Utzinger, J., 2009. Food-borne trematodiases. Clin. Microbiol. Rev. 22, 466–483.
Keiser, J., Utzinger, J., Vennerstrom, J.L., Dong, Y., Brennan, G., Fairweather, I., 2007. Activity of artemether and OZ78 against

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

G Model
VETPAR-6173; No. of Pages 11
ARTICLE IN PRESS



triclabendazole-resistant *Fasciola hepatica*. Trans. R. Soc. Trop. Med. Hyg. 101, 1219–1222.
Keiser, J., Veneziano, V., Rinaldi, L., Mezzino, L., Duthaler, U., Cringoli, G., 2010. Anthelmintic activity of artesunate against *Fasciola hepatica* in naturally infected sheep. Res. Vet. Sci. 88, 107–110.
Lanusse, C.E., Prichard, R.K., 1993. Clinical pharmacokinetics and metabolism of benzimidazole anthelmintics in ruminants. Drug. Metab. Rev. 25, 235–279.
Li, Q., Cantilena, L.R., Leary, K.J., Saviolakis, G.A., Miller, R.S., Melendez, V., Weina, P.J., 2009. Pharmacokinetic profiles of artesunate after single intravenous doses at 0.5, 1, 2, 4, and 8 mg/kg in healthy volunteers: a phase I study. Am. J. Trop. Med. Hyg. 81, 615–621.
Li, Q., Xie, L., Zhang, J., Weina, P.J., 2008. The distribution pattern of intravenous [(14)C] artesunate in rat tissues by quantitative whole-body autoradiography and tissue dissection techniques. J. Pharm. Biomed. Anal. 48, 876–884.
Li, Q.G., Peggins, J.O., Fleckenstein, L.L., Masonic, K., Heiffer, M.H., Brewer, T.G., 1998. The pharmacokinetics and bioavailability of dihydroartemisinin, arteether, artemether, artesunic acid and artelinic acid in rats. J. Pharm. Pharmacol. 50, 173–182.
Maggs, J.L., Madden, S., Bishop, L.P., O'Neill, P.M., Park, B.K., 1997. The rat biliary metabolites of dihydroartemisinin, an antimalarial endoperoxide. Drug Metab. Dispos. 25, 1200–1204.
Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant-borne trematode zoonoses. Int. J. Parasitol. 35, 1255–1278.
McManus, D.P., Dalton, J.P., 2006. Vaccines against the zoonotic trematodes *Schistosoma japonicum*, *Fasciola hepatica* and *Fasciola gigantica*. Parasitology 133, S43–S61.
Medhi, B., Patyar, S., Rao, R.S., Byrav, D.S.P., Prakash, A., 2009. Pharmacokinetic and toxicological profile of artemisinin compounds: an update. Pharmacology 84, 323–332.
Musella, V., Catelan, D., Rinaldi, L., Lagazio, C., Cringoli, G., Biggeri, A., 2011. Covariate selection in multivariate spatial analysis of ovine parasitic infection. Prev. Vet. Med. 99, 69–77.
Newton, P., Suputtamongkol, Y., Teja-Isavadharm, P., Pukrittayakamee, S., Navaratnam, V., Bates, I., White, N., 2000. Antimalarial bioavailability and disposition of artesunate in acute falciparum malaria. Antimicrob. Agents Chemother. 44, 972–977.
O'Neill, J.F., Johnston, R.C., Halferty, L., Brennan, G.P., Keiser, J., Fairweather, I., 2009. Adult triclabendazole-resistant *Fasciola hepatica*: morphological changes in the tegument and gut following *in vivo* treatment with artemether in the rat model. J. Helminthol. 83, 151–163.
Prichard, R.K., Hennessy, D.R., 1981. Effect of oesophageal groove closure on the pharmacokinetic behaviour and efficacy of oxfendazole in sheep. Res. Vet. Sci. 30, 22–27.
Porter, C.J., Pouton, C.W., Cuine, J.F., Charman, W.N., 2008. Enhancing intestinal drug solubilisation using lipid-based delivery systems. Adv. Drug Deliv. Rev. 60, 673–691.
Robinson, M.W., Dalton, J.P., 2009. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos. Trans. R. Soc. Lond. B: Biol. Sci. 364, 2763–2776.
Silamut, K., Newton, P.N., Teja-Isavadharm, P., Suputtamongkol, Y., Siriyanonda, D., Rasameesoraj, M., Pukrittayakamee, S., White, N.J., 2003. Artemether bioavailability after oral or intramuscular administration in uncomplicated falciparum malaria. Antimicrob. Agents Chemother. 47, 3795–3798.
Sinou, V., Taudon, N., Mosnier, J., Aglioni, C., Bressolle, F.M., Parzy, D., 2008. Pharmacokinetics of artesunate in the domestic pig. J. Antimicrob. Chemother. 62, 566–574.
Spithill, T.W., Smooker, P.M., Copeman, D.B., 1999. *Fasciola gigantica*: epidemiology, control, immunology and molecular biology. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, Oxford (UK), pp. 465–525.
Utzinger, J., Xiao, S.H., Tanner, M., Keiser, J., 2007. Artemisinins for schistosomiasis and beyond. Curr. Opin. Investig. Drugs 8, 105–116.
Vandamme, T.F., Ellis, K.J., 2004. Issues and challenges in developing ruminal drug delivery systems. Adv. Drug Deliv. Rev. 56, 1415–1436.
White, N.J., 2008. Qinghaosu (artemisinin): the price of success. Science 320, 330–334.

Please cite this article in press as: Duthaler, U., et al., Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.076

# *Chapter 7*

# Update on the Diagnosis and Treatment of Food-borne Trematode Infections

Jennifer Keiser [a], Urs Duthaler [a], and Jürg Utzinger [b]

[a] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

[b] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

**Published in Current Opinion in Infectious Disease 23 (2010): p. 513-520**

# Update on the diagnosis and treatment of food-borne trematode infections

Jennifer Keiser[a,b], Urs Duthaler[a,b] and Jürg Utzinger[b,c]

[a]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, [b]University of Basel and [c]Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland

Correspondence to Jennifer Keiser, Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, P.O. Box, CH–4002 Basel, Switzerland
Tel: +41 61 284 8218; fax: +41 61 284 8105; e-mail: jennifer.keiser@unibas.ch

**Current Opinion in Infectious Diseases** 2010, 23:513–520

**Purpose of review**
More than 40 million people are affected by food-borne trematode infections. Diagnosis is unsatisfactory and there are only two drugs available for treatment and control: praziquantel and triclabendazole. This review provides an update on recent developments in the diagnosis and treatment of food-borne trematodiases.
**Recent findings**
The trematocidal properties of tribendimidine and peroxidic drugs (e.g. artemisinins and synthetic trioxolanes) have been characterized, including in-vitro and in-vivo studies, elucidating structure-activity relationships and pharmacokinetics and their efficacies have been evaluated in large animal models. Tribendimidine achieved high worm burden reductions against *Opisthorchis viverrini* and *Clonorchis sinensis* harboured in rodents and the antimalarial drug mefloquine showed promising opisthorchicidal activity *in vivo*. Advances have been made with immunological and molecular diagnostics. Metabolic profiling investigations in rodents experimentally infected with *Fasciola hepatica* and *Echinostoma caproni* yielded parasite-specific candidate biomarkers, which might give rise to novel diagnostic targets. The FLOTAC technique showed a higher sensitivity and efficiency for detecting *F. hepatica* eggs in rat faecal samples than the sedimentation method.
**Summary**
Progress has been registered with trematocidal drug candidates that need to be studied in greater detail preclinically, with the most promising ones progressing into proof-of-concept trials. Drug development research should go hand-in-hand with innovation and application into new and improved diagnostic tools.

**Keywords**
chemotherapy, control, diagnosis, food-borne trematodiasis, in-vitro studies, in-vivo studies, treatment

Curr Opin Infect Dis 23:513–520

0951-7375

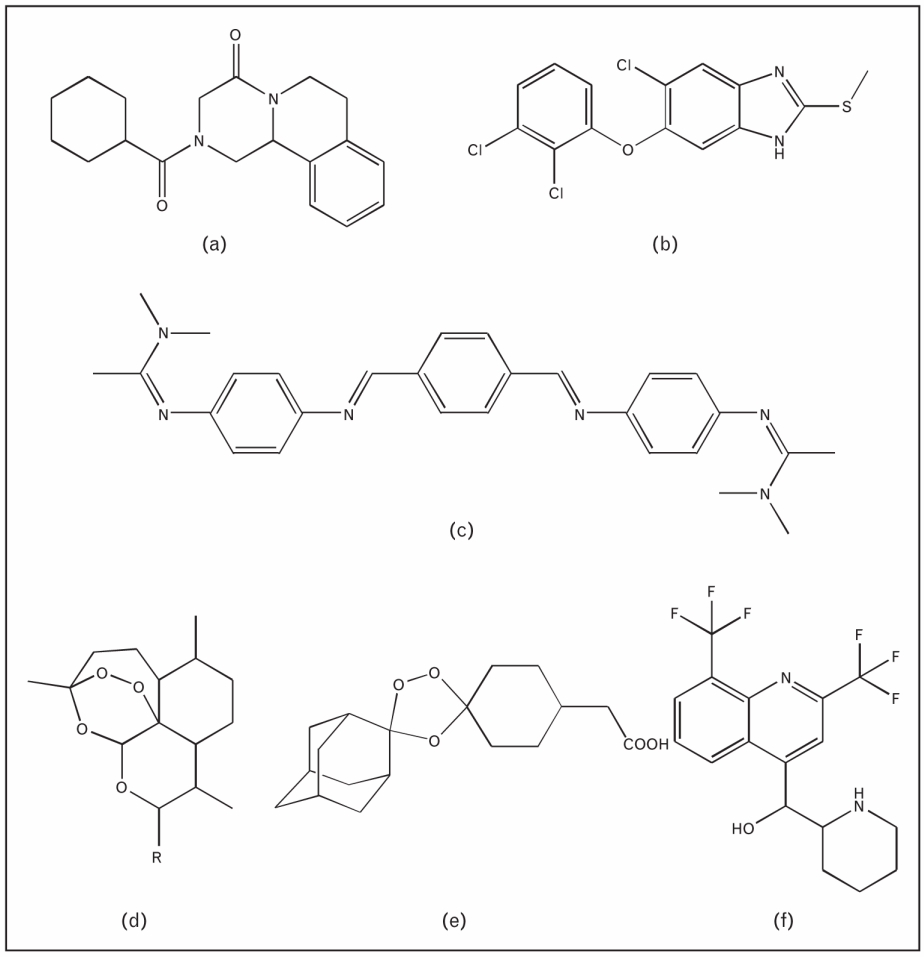
## Introduction

At least 750 million people are at risk of food-borne trematodiases and over 40 million individuals are infected with particularly high infection rates recorded in Southeast Asia, the People's Republic of China (P.R. China) and the Altiplano in the Andes of South America [1,2••]. Yet, food-borne trematodiases are some of the most neglected tropical diseases and their global burdens have yet to be determined [3,4••]. Thus far, more than 80 food-borne trematode species have been found to parasitize humans. However, only about two handfuls are of public health importance, including the liver flukes (*Clonorchis sinensis*, *Fasciola gigantica*, *Fasciola hepatica*, *Opisthorchis felineus* and *Opisthorchis viverrini*), the lung flukes (*Paragonimus* spp.) and the intestinal flukes (e.g. *Fasciolopsis buski* and the heterophyids) [2••,4••].

Praziquantel (Fig. 1a) and triclabendazole (Fig. 1b) are the only drugs used for the treatment of food-borne trematode infections. Discussions are under way as to how these two drugs could be utilized at a larger scale within the framework of 'preventive chemotherapy' in national control programmes. Praziquantel is recommended against clonorchiasis, opisthorchiasis, paragonimiasis and intestinal fluke infections, but it achieves only moderate cure rates in the treatment of fascioliasis [5], for which triclabendazole is the drug of choice [6,7•]. Although triclabendazole resistance is common and poses a huge problem in veterinary public health, no clinically relevant resistance has been documented against either drug in humans. The need to develop new trematocidal drugs – now that praziquantel and triclabendazole are still efficacious – cannot be overemphasized, should resistance emerge against praziquantel and triclabendazole [8].

There is growing awareness of the importance of linking the development and validation of accurate, affordable and robust diagnostic tools to the drug development process. Indeed, the diagnosis of trematode infections

**Figure 1 Diagrammatic representation of current (a and b) and potential future drugs (c–f) against food-borne trematodiases**




Chemical structures of praziquantel (a), triclabendazole (b), tribendimidine (c), artemisinin core (d), the synthetic trioxolane OZ78 (e), and mefloquine (f).

plays an important role in supporting surveillance of control programmes. Features of future diagnostic assays might include the ability of an early diagnosis in the prepatent phase of an infection, accurate monitoring of disease progression and rigorous appraisal of drug efficacy trials. The most widely used method to detect a food-borne trematode infection is direct parasitological diagnosis (e.g. Kato-Katz technique for detection of parasite eggs in stool samples). Immunodiagnosis and molecular tests are also employed widely, particularly in specialized laboratories [4••,9]. Ultrasound, computer tomography, MRI and tissue harmonic imaging complement the main diagnostic approaches [2••]. Challenges and dilemmas remain, for example, misdiagnosis of trematode eggs in direct diagnostic approaches, which have been discussed elsewhere [2••,4••,9,10•].

The purpose of this review is to provide an update on the diagnosis and treatment of food-borne trematodiases. Results obtained in recent in-vitro, in-vivo and pharmacokinetic studies with experimental trematocidal drugs, and drug development candidates that progressed into sheep (target animals), are summarized. Emphasis is placed on research undertaken with tribendimidine (Fig. 1c), different peroxide compounds, such as the artemisinins (Fig. 1d), the synthetic trioxolanes (ozonides; Fig. 1e), and the aminoalcohol mefloquine (Fig. 1f). With regard to diagnostics, new insight gained with FLOTAC, a series of new copromicroscopic techniques [11], polymerase chain reaction (PCR) immunological tests and metabolic profiling approaches are highlighted.

## Progress in drug discovery against food-borne trematodiases

Below we summarize studies undertaken *in vitro*, progress made with peroxidic drugs, and recent results obtained with myrrh, tribendimidine and mefloquine.

**In-vitro studies**

While reviewing the literature, we could not identify any recent studies investigating the in-vitro efficacy of novel trematocidal drugs against *O. viverrini*, *C. sinensis*, *Paragonimus* spp. or intestinal flukes. However, the fasciocidal effects of three natural products were studied *in vitro*, namely extracts from the stem and leaves of *Meryta denhamii* Seem. (family: Araliaceae) [12]; crude extracts of *Artocarpus lakoocha* (family: Moraceae) [13]; and extracts from fruits of *Balanites aegyptiaca* (family: Balantiaceae) [14]. Extracts derived from these natural products have shown promising in-vitro activities against *F. gigantica*. For example, flukes exposed to a medium containing 750–1000 μg/ml *A. lakoocha* died within 12–24 h [13]. Whether these natural extracts also exhibit fasciocidal properties *in vivo* remains to be investigated.

**Artemisinins and ozonides**

We have recently reported that artesunate and artemether (two semisynthetic derivatives of artemisinin) and the 1,2,4-trioxolane OZ78 possess trematocidal properties *in vitro* and in rodent hosts [15]. Indeed, artesunate, artemether and OZ78 cured chronic *F. hepatica* and *C. sinensis* infections in the rat, and achieved high *O. viverrini* worm burden reductions in a hamster model [15]. Moreover, artemether showed considerable in-vitro activity against *F. gigantica* [16,17].

The fasciocidal properties of artesunate have also been studied in Vietnamese patients suffering from acute fascioliasis. Patients subjected to a 10-day treatment course with artesunate, administered daily at a dose of 4 mg/kg, were less likely to report abdominal pain at hospital discharge than patients treated with a standard oral dose of 10 mg/kg triclabendazole. However, 3 months post-treatment, clinical and serological response rates were lower among artesunate recipients compared to triclabendazole-treated patients [18]. A proof-of-concept trial assessing the efficacy and safety of different artemether regimens against patent *Fasciola* infections among Egyptian patients has been completed recently and data analysis is under way.

Over the past 18 months, the trematocidal properties of the artemisinins and ozonide compounds have been further elucidated, including structure-activity relationships (SAR) and studies in large animal hosts. With the aim to identify an ozonide lead candidate, 26 OZ78 derivatives were studied in the *F. hepatica*-rat model, with the key results summarized here. Interestingly, OZ78 revealed a higher activity than any of the derivatives tested. Seemingly small structural changes commonly reduced the fasciocidal activity significantly. A spiroadamantane substructure and an acidic functional group were required for fasciocidal activity. Furthermore, the activity of OZ78 was peroxide bond-dependent, suggesting that haemoglobin digestion plays a role in the fasciocidal efficacy of peroxidic drugs [19]. The later hypothesis is supported by the fact that tegumental changes on *F. hepatica* are exacerbated in the presence of haemin [20,21]. Additional results from scanning electron microscopic (SEM) and transmission electron microscopic (TEM) studies with the artemisinins and OZ78 revealed progressive and time-dependent alterations of the tegument and gut of *F. hepatica* [22,23] and *C. sinensis* [24]. Disruption of the gut was even more pronounced than that of the tegument, suggesting an oral uptake of artemether by adult *F. hepatica* worms [23].

Since liver infections often strongly influence disposition kinetics and metabolism, the pharmacokinetic parameters of artesunate and its main metabolite dihydroartemisinin were studied in *F. hepatica*-infected rats following oral and intravenous administration. Pharmacokinetic parameters such as elimination half life ($t_{1/2}$), maximum plasma concentration ($C_{max}$) and area under the curve (AUC) were considerably altered in infected rats. For example, following oral administration, AUC and $C_{max}$ of artesunate and dihydroartemisinin were 1.7–4.4 fold higher in infected rats. Although the clinical significance of these findings is not yet clear, it is recommended that more detailed pharmacokinetic studies be pursued in patients infected with *Fasciola* or *Schistosoma*, as well as patients suffering from liver diseases, following artemisinin administration [25].

Additional supportive evidence of the trematocidal properties of the artemisinins and ozonides have recently been generated in large animal models. In brief, artesunate and artemether, administered at single 120 mg/kg oral doses, achieved statistically significant worm burden reductions of 88.8 and 67.2%, respectively, in *C. sinensis*-infected rabbits, a model characterized by close proximity to human disease [26]. On the other hand, *Paragonimus westermani*, harboured in dogs, were not affected by artemether [27]. The efficacy of artesunate, artemether and OZ78 was also studied in target animals, i.e. sheep naturally infected with *F. hepatica*. Artesunate given intramuscularly at a single dose of 40 mg/kg reduced faecal egg counts (FECs) and worm burdens by 97.8 and 87.1%, respectively [28]. A higher dose of intramuscular artemether (160 mg/kg) was necessary to achieve a significant FEC reduction (64.9%) and worm burden reduction (91.3%) [29]. In both studies, plasma samples have been taken from sheep and pharmacokinetic parameters, according to different drug administration routes, are currently being analysed.

Interestingly, a single 50 mg/kg dose of OZ78, given orally or subcutaneously to lambs harbouring an experimental *F. hepatica* infection, showed no effect on FECs and worm burden [30]. Additional preclinical studies with

OZ78 are warranted to determine whether the promising activity observed in rats can be translated into efficacy in sheep or cattle. Additionally, artesunate should be studied in sheep, experimentally-infected with *F. hepatica*.

Mathematical modelling suggests that the likelihood of anthelminthic drug resistance development is significantly delayed when combination therapies are used [31]. In a combination chemotherapy study against *C. sinensis* in experimentally-infected rats, the peroxidic drugs were combined with praziquantel or tribendimidine. Synergistic interactions were observed when artesunate and tribendimidine (12.5–50 mg/kg) were combined with 150 mg/kg praziquantel. Co-administration of either artemether or OZ78 (each at a dose of 12.5–50 mg/kg) with praziquantel (150 mg/kg) showed independently additive effects, compared with single drug regimens. However, at lower doses of the two peroxides (3.1 and 6.25 mg/kg) even antagonistic effects were seen [32]. SEM studies confirmed differences in the onset of action and the extent of tegumental alterations of the three peroxide-praziquantel combinations in *C. sinensis*-infected rats [33].

#### Myrrh

It has been claimed that myrrh (also known under the market name of Mirazid), an oleo gum resin derived from the stem of *Commiphora molmol* (family: Burseraceae), has antischistosomal [34] and fasciocidal properties [35]. However, laboratory investigations and clinical trials produced conflicting results [36]. Recent data suggest that myrrh (administered at 10 mg/kg/day for six consecutive days) lack activity against ovine fascioliasis [37], and hence it seems unlikely that myrrh will ever play a role for the treatment and control of fascioliasis.

#### Tribendimidine

Tribendimidine, an aminophenyldimidine derivative of the Bayer drug amidantel has been developed and approved in P.R. China in 2004 for the treatment of soil-transmitted helminth infections [38]. Interestingly, tribendimidine has not only shown nematocidal but also trematocidal properties: a single 150 mg/kg oral dose of tribendimidine achieved a worm burden reduction of 99.1% in *C. sinensis*-infected rats. Tribendimidine, administered at a single 400 mg/kg oral dose to hamsters infected with *O. viverrini*, resulted in a worm burden reduction of 95.7% [39]. More recently, it was shown that tribendimidine and its primary metabolite, deacylated amidantel, possess similar clonorchicidal activity *in vitro* and *in vivo*, whereas the secondary metabolite, acetylated deacylated amidantel, was slightly less active [40]. SEM observations revealed extensive tegumental alterations on *C. sinensis* recovered from rats after administration of a single 300 mg/kg oral dose of tribendimidine [24].

#### Mefloquine

Mefloquine, an aminoalcohol discovered at the Walter Reed Army Research Institute is widely used for the prevention and treatment of malaria. New research has shown that mefloquine also possesses antischistosomal properties [41,42,43•,44•]. Laboratory studies indicated that mefloquine has neither an effect against *F. hepatica* nor against *C. sinensis*. However, a single 300 mg/kg oral dose of mefloquine resulted in worm burden reductions of 88.5% (juvenile infection) and 96.0% (adult infections), respectively, in hamsters infected with *O. viverrini* [45].

## Progress in the diagnosis of food-borne trematode infections

Below we summarize recent developments with direct parasitological diagnostic tests, immunological and molecular assays as well as metabolic profiling.

#### FLOTAC techniques

The FLOTAC techniques consist of a series of novel multivalent FEC techniques for qualitative and quantitative diagnosis of helminth infections in animals and humans [46•]. The basic principle is that the FLOTAC apparatus separates parasitic elements from faecal debris based on centrifugal flotation and subsequent translation of the apical portion of the floating suspension. Among a panel of nine flotation solutions with specific gravities ranging between 1.20 and 1.45, the most suitable ones are selected in a preceding calibration step, according to the parasitic elements of interest [46•]. The FLOTAC techniques allow processing up to 1 g of stool per analysis (0.2 g/ml) in contrast to traditional FEC techniques such as Kato-Katz or McMaster, where only small amounts of faeces are examined (e.g. 41.7 mg of stool for a single Kato-Katz thick smear) [47]. Results obtained thus far suggest that the FLOTAC techniques have higher sensitivities than traditional FEC techniques, including Kato-Katz, McMaster and ether-based concentration techniques in the diagnosis of human and veterinary parasitic infections [48,49].

With regard to the diagnosis of trematodes, the FLOTAC techniques have been utilized for the detection of *Dicrocoelium dendriticum* and *F. hepatica* in sheep and water buffaloes [50,51]. We recently compared the FLOTAC and sedimentation techniques for the detection and quantification of FECs of *F. hepatica* eggs in faecal samples obtained from experimentally infected rats before and after treatment [52]. A single FLOTAC showed a higher sensitivity (92.6%) than multiple sedimentation readings (63.0–85.2%) for detecting *F. hepatica* eggs in rat faecal samples characterized by low-intensity infections after experimental chemotherapy. Significantly higher FECs could be determined using the

sedimentation technique [mean of 10 051 and 176 eggs per gram of stool (EPG) before and after treatment, respectively] compared with the FLOTAC technique (6756 and 108 EPG, respectively). Additionally, operational and practical issues were studied. We found, for example that the time to prepare and examine a single FLOTAC was 21 min, whereas preparation and reading of eight sedimentation slides took 114 min.

#### Polymerase chain reaction

Several PCR-based methods have been developed and validated for the detection of trematode egg DNA in faecal samples [53], parasite DNA in biliary stones [54] and metacercariae in fish and snail intermediate hosts [55,56]. In contrast to copromicroscopic techniques, PCR-based methods offer high diagnostic sensitivities and specificities, and enable the discrimination between infections caused by different trematode species [2••].

The detection of eggs in faecal sample is the standard method to diagnose *O. viverrini* infections in humans. However, differential diagnosis poses problems since the size and shapes of *C. sinensis*, *O. viverrini*, *Haplorchis taichui* and *Haplorchis pumilio* eggs are similar [9,57]. Recently, highly sensitive PCR-based methods were developed for discrimination between *O. viverrini* and *H. taichui* [53,58], and between *O. viverrini*, *C. sinensis*, *H. pumilio* and *H. taichui* infections [59,60]. PCR amplicons of internal-transcribed-spacer-1/2 (ITS-1/2) regions in ribosomal DNA [59,61•,62•] and mitochondrial DNA sequences [53] were used for species differentiation. Importantly, one of the studies showed a significant correlation (correlation coefficient = 0.68, $P < 0.001$) of PCR cycle-threshold values (DNA loads) and egg counts, and hence not only detection but also quantification of *C. sinensis* infections might be feasible [61•]. The PCR tests developed were applied to estimate the prevalence of *O. viverrini* and *H. taichui* infections in Lao People's Democratic Republic [53] and enabled the first report of *C. sinensis* infections in central Thailand [62•]. In addition, a novel 256 bp random amplified polymorphic DNA (HAT-RAPD) marker for the identification of *H. taichui* was designed recently and tested for specificity on 13 parasite species (nine trematodes, two nematodes and two acanthocephalans) [63,64]. HAT-RAPD PCR showed a higher sensitivity than a formalin-ether sedimentation technique in the diagnosis of *H. taichui* infections in faecal samples from 51 Thai villagers [65].

A novel, real-time PCR-method for the diagnosis of *O. felineus* in human faeces, targeting 5.8S rRNA gen region, was developed and compared with traditional coproovoscopy and enzyme immunoassay. The sensitivity of the PCR method in detecting *O. felineus* is about $10^3$ copies of target DNA per ml. In a study with *O. felineus*-infected and uninfected individuals, this PCR approach revealed a high specificity (98%) and detected 13% infected persons whereas coproovoscopy and two different immunodiagnostic tests detected only 6.7, 5.5 and 1.6% *O. felineus* infections, respectively [66].

Finally, progress has been registered with regard to PCR methods for the differential diagnosis of infections with *F. hepatica* and *F. gigantica* targeting ITS-1 DNA [67,68], ITS-2 [69] and the nuclear small subunit ribosomal RNA gene [70].

#### Immunological assays

Immunological tests, such as the enzyme-linked immunosorbent assay (ELISA), have been studied thoroughly in the recent past with the ultimate goal to increase stability, sensitivity, specificity and reduce costs of these diagnostic assays [71••]. Immunological approaches offer the advantage that an infection can already be diagnosed in the acute phase of the disease. Crude worm extracts are frequently used as antigens, however cross-reactions against other trematode infections and false-positive results owing to past infections often occur. Today excretory-secretory antigen (ESA) tests are often used to increase the sensitivity and specificity of the immunodiagnostic assay, although it is labour intense to produce sufficient amounts of ESA. ESA proved to be a highly sensitive and specific diagnostic approach in human and veterinary fascioliasis. For example, indirect ELISA using crude worm, ESA and glutathione *S*-transferase antigens were recently compared for the diagnosis of *F. gigantica* in cattle, sheep and donkeys. The sensitivity and specificity of the ELISA method using ESA (>90 and >95%, respectively) were superior to the crude antigen (>90 and >85%, respectively) and the glutathione *S*-transferase antigen (>60 and >65%, respectively) in the three animal hosts investigated [72]. Furthermore, the development of a faecal antigen (ESA) diagnostic sandwich ELISA for the detection of *F. gigantica* in cattle showed a superior sensitivity and specificity than an anti-*F. gigantica* antibody ELISA in serum and higher sensitivity compared with FECs [73]. Additionally, a 27-kDa excretory/secretory protein of *F. gigantica*, purified using high-performance liquid chromatograpy (HPLC), demonstrated high sensitivity and specificity for the diagnosis of human infections [74]. On the contrary, a recent investigation found that an indirect ELISA test based on a secretion product of *O. viverrini*, 28-kDa glutathione *S*-transferase, was not applicable as a diagnostic tool in human infections owing to low specific antibody titre and an abundance of circulating antigen [75].

Recombinant protein approaches might overcome the shortcomings of ESA-ELISA, but these tests are still under development. For example, the serodiagnostic applicability of four recombinant proteins (7-kDa

protein, 28-kDa cystein protease, and 26-kDa and 28-kDa glutathione *S*-transferase) from *C. sinensis* worms was evaluated recently. A low sensitivity (<47.3%) was observed compared with crude antigen ELISA, however, a high specificity (94.5–100%) was achieved [76]. In addition, a recent study evaluated the use of a recombinant-based ELISA (2.9-kDa recombinant protein; FhrAPS), as a test of cure following fasciocidal treatment. Promisingly, a significant reduction of the IgG response to FhrAPS was measured post-treatment. However, difficulties to discriminate between long persisting *Fasciola*-specific serum IgG, new parasitic infections and infections refractory to treatment need to be addressed [77].

An alternative approach is to coat wells with antibodies with the aim to captivate antigens in patient's biological samples (e.g. blood). A high sensitivity (94.5%) and specificity (100%) was recently observed using a monoclonal antibody (MoAb)-based sandwich ELISA for the detection of circulating 28.5-kDa tegumental antigen in the sera of *F. gigantica*-infected mice. Two additional advantages of this assay are the ability for an early diagnosis (day 1 postinfection) and the lack of any cross-reactions (e.g. between *O. viverrini* and *S. mansoni*) [78]. Finally, the MM3-COPRO method, a commercial monoclonal antibody (MM3) kit, showed 100% sensitivity and 100% specificity for diagnosing *Fasciola* coproantigens in human stool samples [79]. MM3-COPRO and MM3-SERO were recently also evaluated in sheep. Detectable amounts of coproantigens were observed 4–7 and 3–6 weeks before patency (egg shedding) in *F. hepatica* and *F. gigantica* infections in sheep [80].

#### Metabolic profiling

Metabolic profiling of biological samples (e.g. blood, stool and urine) employing spectroscopic methods [e.g. nuclear magnetic resonance (NMR) spectroscopy], coupled with multivariate data analysis (e.g. principal component analysis), is a widely used approach for studying drug toxicity and gene function, and for enhancing drug development and disease diagnosis [81,82]. Recently, $^{1}$H NMR spectroscopy has been applied for global and temporal profiling of host metabolism, altered by parasite infections, including *F. hepatica*-rat and *Echinostoma caproni*-mouse models. The comparison of biological samples obtained from mice infected with *E. caproni* [83,84] and rats infected with *F. hepatica* [85•] to samples recovered from corresponding noninfected control animals revealed a number of candidate biomarkers. Importantly, infection-specific metabolic fingerprints were identified in the majority of the assessed compartments. For example, in the case of *E. caproni*, 10 biomarkers, many of which were stable over the course of infection, were identified in mouse urine, including phenylacetylglycine, 2-oxoisocaproate and trimethylamine, whereby many of these urinary metabolites were reflective of changed gut-microbial activity and/or composition. In different tissue samples of *E. caproni*-infected mice, changes in concentrations of different osmotically active substances were observed, such as betaine, which increased in the liver, *scyllo*-inositol (increased), and glycerophosphocholine in the kidney (decreased), and *myo*-inositol in jejunum (decreased). A striking finding was the increased faecal excretion of the branched chain amino acids (leucine, isoleucine and valine) and the subsequent depletion in plasma, liver and ileum, which is indicative of an intestinal malabsorption induced by the fluke.

Plasma samples obtained from *F. hepatica*-infected rats reflected the destruction of liver tissue and the resulting disruption of glycogen in elevated levels of glucose. Furthermore, *O*-acetylated glycoprotein signals, which result from acute phase proteins, including α1 acid glycoprotein [86], were found to be significantly higher in the plasma of *F. hepatica*-infected rats and might reflect hepatic inflammation induced by the migrating larvae. Finally, significant perturbations of the nucleotide balance in the brain were observed together with an increase of the anti-inflammatory cytokine IL-13, which suggests a shift towards modulation of immune reactions to minimize inflammatory damage, which possibly favours presence of the trematode in the host [87].

## Conclusion

Treatment of food-borne trematodiases has remained unchanged over the last 3 decades: only two drugs are available, praziquantel and triclabendazole. Data summarized in this review suggest that marketed drugs currently used against malaria (e.g. the artemisinins and mefloquine) or common soil-transmitted helminths (e.g. tribendimidine) possess interesting trematocidal properties, and hence might be further developed into proper trematocidal drugs with relative speed and limited economic costs. Hence, there is a need to further study these drugs preclinically and, sequentially, in proof-of-concept clinical trials. Immunological and molecular diagnostic tests have improved significantly. Metabolic profiling studies might uncover candidate biomarkers, which, in the long-term, might provide novel diagnostic targets. The diagnostic accuracy of the FLOTAC technique for human *Fasciola* spp. infections should be validated with utmost speed.

## Acknowledgements

We thank Dr Robert Bergquist for carefully revising our manuscript. We are grateful to the Swiss National Science Foundation for financial support (project no. PPOOA-114941 to J. Keiser and U. Duthaler) and project no. PPOOB-102883 and PPOOB-119129 to J. Utzinger).

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 538–539).


**1** WHO. Control of foodborne trematode infections. Report of a WHO study group. WHO Tech Rep Ser No. 849. Geneva: World Health Organization; 1995.

**2** •• Keiser J, Utzinger J. Food-borne trematodiases. Clin Microbiol Rev 2009; 22:466–483.
This is a detailed review of food-borne trematodiases, including taxonomy, geographical distribution, historical account, clinical manifestations, epidemiology, diagnosis, prevention, treatment and control.

**3** Hotez PJ, Molyneux DH, Fenwick A, *et al.* Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. PLoS Med 2006; 3:e102.

**4** •• Sripa B, Kaewkes S, Intapan PM, *et al.* Food-borne trematodiases in Southeast Asia: epidemiology, pathology, clinical manifestation and control. Adv Parasitol 2010; 72:305–350.
The authors provide a comprehensive review pertaining to the epidemiology, clinical manifestations, pathogenesis and pathology, diagnosis, prevention and control of the clinically relevant food-borne trematode infections in Southeast Asia.

**5** Keiser J, Utzinger J. Chemotherapy for major food-borne trematodes: a review. Expert Opin Pharmacother 2004; 5:1711–1726.

**6** Keiser J, Engels D, Büscher G, Utzinger J. Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Invest Drugs 2005; 14:1513–1526.

**7** • Fairweather I. Triclabendazole progress report, 2005–2009: an advancement of learning? J Helminthol 2009; 83:139–150.
In this expert review, the main aspects of triclabendazole activity, including drug pharmacokinetics and pharmacodynamics and an understanding of the mechanism(s) of resistance are summarized.

**8** Keiser J, Utzinger J. Advances in the discovery and development of novel trematocidal drugs. Expert Opin Drug Discov 2007; 2:S9–S23.

**9** Johansen MV, Sithithaworn P, Bergquist R, Utzinger J. Towards improved diagnosis of zoonotic trematode infections in Southeast Asia. Adv Parasitol 2010; 73:171–195.

**10** • Bergquist R, Johansen MV, Utzinger J. Diagnostic dilemmas in helminthology: what tools to use and when? Trends Parasitol 2009; 25:151–156.
An opinion piece, discussing current challenges and dilemmas in the diagnosis of major helminthiases and emphasizing that according to the stage of a helminth control programme specific diagnostics are needed.

**11** Cringoli G. FLOTAC, a novel apparatus for a multivalent faecal egg count technique. Parassitologia 2006; 48:381–384.

**12** Shehab NG, Monem AR, Hassan SE, Toaleb NI. Botanical study of *Meryta denhamii* Seem. and its antihelminthic activity against *Fasciola gigantica*. J Egypt Soc Parasitol 2009; 39:269–288.

**13** Saowakon N, Tansatit T, Wanichanon C, *et al. Fasciola gigantica*: anthelmintic effect of the aqueous extract of *Artocarpus lakoocha*. Exp Parasitol 2009; 122:289–298.

**14** Al Ashaal HA, Farghaly AA, Abd El Aziz MM, Ali MA. Phytochemical investigation and medicinal evaluation of fixed oil of *Balanites aegyptiaca* fruits (Balantiaceae). J Ethnopharmacol 2010; 127:495–501.

**15** Keiser J, Utzinger J. Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol 2007; 23:555–562.

**16** Shalaby HA, El Namaky AH, Kamel RO. *In vitro* effect of artemether and triclabendazole on adult *Fasciola gigantica*. Vet Parasitol 2009; 160:76–82.

**17** Diab TM, Mansour HH, Mahmoud SS. *Fasciola gigantica*: parasitological and scanning electron microscopy study of the *in vitro* effects of ivermectin and/or artemether. Exp Parasitol 2010; 124:279–284.

**18** Hien TT, Truong NT, Minh NH, *et al.* A randomized controlled pilot study of artesunate versus triclabendazole for human fascioliasis in central Vietnam. Am J Trop Med Hyg 2008; 78:388–392.

**19** Zhao Q, Vargas M, Dong Y, *et al.* Structure-activity relationship of an ozonide carboxylic acid (OZ78) against *Fasciola hepatica*. J Med Chem 2010; 53:4223–4233.

**20** Keiser J, Morson G. *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. Exp Parasitol 2008; 118:228–237.

**21** Keiser J, Morson G. *Fasciola hepatica*: surface tegumental responses to *in vitro* and *in vivo* treatment with the experimental fasciolicide OZ78. Exp Parasitol 2008; 119:87–93.

**22** Halferty L, O'Neill JF, Brennan GP, *et al.* Electron microscopical study to assess the *in vitro* effects of the synthetic trioxolane OZ78 against the liver fluke, *Fasciola hepatica*. Parasitology 2009; 136:1325–1337.

**23** O'Neill JF, Johnston RC, Halferty L, *et al.* Adult triclabendazole-resistant *Fasciola hepatica*: morphological changes in the tegument and gut following *in vivo* treatment with artemether in the rat model. J Helminthol 2009; 83:151–163.

**24** Xiao SH, Keiser J, Xue J, *et al.* Effect of single-dose oral artemether and tribendimidine on the tegument of adult *Clonorchis sinensis* in rats. Parasitol Res 2009; 104:533–541.

**25** Keiser J, Gruyer MS, Perrottet N, *et al.* Pharmacokinetic parameters of artesunate and dihydroartemisinin in rats infected with *Fasciola hepatica*. J Antimicrob Chemother 2009; 63:543–549.

**26** Kim TI, Yoo WG, Li S, *et al.* Efficacy of artesunate and artemether against *Clonorchis sinensis* in rabbits. Parasitol Res 2009; 106:153–156.

**27** Xue J, Utzinger J, Zhang YN, *et al.* Artemether and tribendimidine lack activity in experimental treatment of *Paragonimus westermani* in the dog. Parasitol Res 2008; 102:537–540.

**28** Keiser J, Veneziano V, Rinaldi L, *et al.* Anthelmintic activity of artesunate against *Fasciola hepatica* in naturally infected sheep. Res Vet Sci 2010; 88:107–110.

**29** Keiser J, Rinaldi L, Veneziano V, *et al.* Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol Res 2008; 103:517–522.

**30** Keiser J, Kirchhofer C, Haschke M *et al.* Efficacy, safety and pharmacokinetics of 1,2,4 trioxolane OZ78 against an experimental infection with *Fasciola hepatica* in sheep. Vet Parasitol 2010. doi:10.1016/j.vetpar.2010.06.031.

**31** Barnes EH, Dobson RJ, Barger IA. Worm control and anthelmintic resistance: adventures with a model. Parasitol Today 1995; 11:56–63.

**32** Keiser J, Xiao SH, Smith TA, Utzinger J. Combination chemotherapy against *Clonorchis sinensis*: experiments with artemether, artesunate, OZ78, praziquantel, and tribendimidine in a rat model. Antimicrob Agents Chemother 2009; 53:3770–3776.

**33** Keiser J, Vargas M. Effect of artemether, artesunate, OZ78, praziquantel, and tribendimidine alone or in combination chemotherapy on the tegument of *Clonorchis sinensis*. Parasitol Int 2010. doi:10.1016/j.parint.2010.04.003.

**34** Sheir Z, Nasr AA, Massoud A, *et al.* A safe, effective, herbal antischistosomal therapy derived from myrrh. Am J Trop Med Hyg 2001; 65:700–704.

**35** Massoud A, El Sisi S, Salama O. Preliminary study of therapeutic efficacy of a new fasciolicidal drug derived from *Commiphora molmol* (myrrh). Am J Trop Med Hyg 2001; 65:96–99.

**36** Abdul-Ghani RA, Loutfy N, Hassan A. Myrrh and trematodoses in Egypt: an overview of safety, efficacy and effectiveness profiles. Parasitol Int 2009; 58:210–214.

**37** Botros SS, El-Lakkany NM, Badawy AA, *et al.* Mirazid shows insignificant activity against ovine fascioliasis. Ann Trop Med Parasitol 2009; 103:605–616.

**38** Xiao SH, Wu HM, Tanner M, *et al.* Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 2005; 94:1–14.

**39** Keiser J, Xiao SH, Chollet J, *et al.* Evaluation of the *in vivo* activity of tribendimidine against *Schistosoma mansoni*, *Fasciola hepatica*, *Clonorchis sinensis*, and *Opisthorchis viverrini*. Antimicrob Agents Chemother 2007; 51:1096–1098.

**40** Xiao SH, Xue J, Xu LL, *et al.* The *in vitro* and *in vivo* effect of tribendimidine and its metabolites against *Clonorchis sinensis*. Parasitol Res 2009; 105:1497–1507.

**41** Van Nassauw L, Toovey S, Van Op den Bosch J, *et al.* Schistosomicidal activity of the antimalarial drug, mefloquine, in *Schistosoma mansoni*-infected mice. Trav Med Infect Dis 2008; 6:253–258.

**42** Keiser J, Chollet J, Xiao SH, *et al.* Mefloquine: an aminoalcohol with promising antischistosomal properties in mice. PLoS Negl Trop Dis 2009; 3:e350.

**43** • Keiser J, N'Guessan NA, Adoubryn KD, *et al.* Efficacy and safety of mefloquine, artesunate, mefloquine-artesunate, and praziquantel against *Schistosoma haematobium*: randomized, exploratory open-label trial. Clin Infect Dis 2010; 50:1205–1213.
This is the first proof-of-concept study documenting antischistosomal activity of mefloquine and an artesunate-mefloquine combination in school-aged children in Côte d'Ivoire.

**44** • Xiao SH, Keiser J, Chen MG, *et al.* Research and development of antischistosomal drugs in the People's Republic of China: a 60-year review. Adv Parasitol 2010; 73:231–295.
This is the definitive review on antischistosomal drug discovery, development and use in P.R. China between 1949 and today.

**45** Keiser J, Odermatt P, Tesana S. Dose-response relationships and tegumental surface alterations in *Opisthorchis viverrini* following treatment with mefloquine *in vivo* and *in vitro*. Parasitol Res 2009; 105:261–266.

**46** • Cringoli G, Rinaldi L, Maurelli MP, Utzinger J. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat Protoc 2010; 5:503–515.
A comprehensive review discussing the development of the FLOTAC techniques, presenting protocols for the FLOTAC basic, dual, double and pellet techniques and summarizing experiences made thus far in the diagnosis of animal and human parasites.

**47** Knopp S, Glinz D, Rinaldi L, *et al.* FLOTAC: a promising technique for detecting helminth eggs in human faeces. Trans R Soc Trop Med Hyg 2009; 103:1190–1194.

**48** Utzinger J, Rinaldi L, Lohourignon LK, *et al.* FLOTAC: a new sensitive technique for the diagnosis of hookworm infections in humans. Trans R Soc Trop Med Hyg 2008; 102:84–90.

**49** Levecke B, De Wilde N, Vandenhoute E, Vercruysse J. Field validity and feasibility of four techniques for the detection of *Trichuris* in simians: a model for monitoring drug efficacy in public health? PLoS Negl Trop Dis 2009; 3:e366.

**50** Cringoli G. Coprological diagnosis: what's new? Parassitologia 2004; 46:137–139.

**51** Rinaldi L, Musella V, Veneziano V, *et al.* Helmintic infections in water buffaloes on Italian farms: a spatial analysis. Geospat Health 2009; 3:233–239.

**52** Duthaler U, Rinaldi L, Maurelli MP *et al. Fasciola hepatica*: comparison of the sedimentation and FLOTAC techniques for the detection and quantification of faecal egg counts in rats. Exp Parasitol 2010. doi:10.1016/j.exppara.2010.04.020.

**53** Lovis L, Mak TK, Phongluxa K, *et al.* PCR diagnosis of *Opisthorchis viverrini* and *Haplorchis taichui* infections in an endemic Lao community: a comparison of diagnostic methods for parasitological field surveys. J Clin Microbiol 2009; 47:1517–1523.

**54** Jang JS, Kim KH, Yu JR, Lee SU. Identification of parasite DNA in common bile duct stones by PCR and DNA sequencing. Korean J Parasitol 2007; 45:301–306.

**55** Intapan PM, Thanchomnang T, Lulitanond V, *et al.* Detection of *Opisthorchis viverrini* in infected bithynid snails by real-time fluorescence resonance energy transfer PCR-based method and melting curve analysis. Parasitol Res 2008; 103:649–655.

**56** Intapan PM, Thanchomnang T, Lulitanond V, *et al.* Real-time fluorescence resonance energy transfer PCR with melting curve analysis for the detection of *Opisthorchis viverrini* in fish intermediate hosts. Vet Parasitol 2008; 157:65–71.

**57** Sayasone S, Vonghajack Y, Vanmany M, *et al.* Diversity of human intestinal helminthiasis in Lao PDR. Trans R Soc Trop Med Hyg 2009; 103:247–254.

**58** Sato M, Pongvongsa T, Sanguankiat S, *et al.* Copro-DNA diagnosis of *Opisthorchis viverrini* and *Haplorchis tachui* infection in an endemic area of Lao PDR. Southeast Asian J Trop Med Public Health 2010; 41:28–35.

**59** Sato M, Thaenkham U, Dekumyoy P, Waikagul J. Discrimination of *O. viverrini*, *C. sinensis*, *H. pumilio* and *H. taichui* using nuclear DNA-based PCR targeting ribosomal DNA ITS regions. Acta Trop 2009; 109:81–83.

**60** Van Van K, Dalsgaard A, Blair D, Le TH. *Haplorchis pumilio* and *H. taichui* in Vietnam discriminated using ITS-2 DNA sequence data from adults and larvae. Exp Parasitol 2009; 123:146–151.

**61** • Kim EM, Verweij JJ, Jalili A, *et al.* Detection of *Clonorchis sinensis* in stool samples using real-time PCR. Ann Trop Med Parasitol 2009; 103:513–518.
This is a promising PCR method for the detection and quantification of *C. sinensis* in stool samples.

**62** • Traub RJ, Macaranas J, Mungthin M, *et al.* A new PCR-based approach indicates the range of *Clonorchis sinensis* now extends to central Thailand. PLoS Negl Trop Dis 2009; 3:e367.
Using a novel PCR-based method, the authors describe the first report of a *C. sinensis*-endemic community in central Thailand.

**63** Wongsawad P, Wongsawad C. Development of PCR-based diagnosis of minute intestinal fluke, *Haplorchis taichui*. Southeast Asian J Trop Med Public Health 2009; 40:919–923.

**64** Wongsawad C, Wongsawad P, Chai JY, Anuntalabhochai S. *Haplorchis taichui*, Witenberg, 1930: development of a HAT-RAPD marker for the detection of minute intestinal fluke infection. Exp Parasitol 2009; 123:158–161.

**65** Wongsawad C, Wongsawad P, Chuboon S, Anuntalabhochai S. Copro-diagnosis of *Haplorchis taichui* infection using sedimentation and PCR-based methods. Southeast Asian J Trop Med Public Health 2009; 40:924–928.

**66** Ogorodova LM, Petrova IV, Sazonov AE, *et al.* Genetic diagnostic technique for opisthorchiasis [in Russian]. Klin Lab Diagn 2009; 7:37–39.

**67** Ichikawa M, Itagaki T. Discrimination of the ITS1 types of *Fasciola* spp. based on a PCR-RFLP method. Parasitol Res 2010; 106:757–761.

**68** Rokni MB, Mirhendi H, Mizani A, *et al.* Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method. Exp Parasitol 2010; 124:209–213.

**69** Ai L, Dong SJ, Zhang WY, *et al.* Specific PCR-based assays for the identification of *Fasciola* species: their development, evaluation and potential usefulness in prevalence surveys. Ann Trop Med Parasitol 2010; 104:65–72.

**70** El-Gozamy BR, Shoukry NM. Identification of Egyptian *Fasciola* species by PCR and restriction endonucleases digestion of the nuclear small subunit ribosomal RNA gene. J Egypt Soc Parasitol 2009; 39:429–438.

**71** •• Blair D, Agatsuma T, Wang W. Paragonimiasis. In: Murrell KD, Fried B, editors. Food-borne parasitic zoonoses. New York: Springer; 2007. pp. 117–150.
This is an excellent summary of recent developments in the epidemiology, treatment, diagnosis and clinical manifestations of paragonimiasis.

**72** Awad WS, Ibrahim AK, Salib FA. Using indirect ELISA to assess different antigens for the serodiagnosis of *Fasciola gigantica* infection in cattle, sheep and donkeys. Res Vet Sci 2009; 86:466–471.

**73** Estuningsih E, Spithill T, Raadsma H, *et al.* Development and application of a fecal antigen diagnostic sandwich ELISA for estimating prevalence of *Fasciola gigantica* in cattle in central Java, Indonesia. J Parasitol 2009; 95:450–455.

**74** Nguyen TG, Le TH, De NV, *et al.* Assessment of a 27-kDa antigen in enzyme-linked immunosorbent assay for the diagnosis of fasciolosis in Vietnamese patients. Trop Med Int Health 2010; 15:462–467.

**75** Eursitthichai V, Viyanant V, Tesana S, *et al. Opisthorchis viverrini*: evaluation of 28 kDa glutathione *S*-transferase as diagnostic tool in human opisthorchiasis. Acta Trop 2010; 114:76–80.

**76** Shen C, Lee JA, Allam SR, *et al.* Serodiagnostic applicability of recombinant antigens of *Clonorchis sinensis* expressed by wheat germ cell-free protein synthesis system. Diagn Microbiol Infect Dis 2009; 64:334–339.

**77** Arias MS, Suarez JL, Hillyer GV, *et al.* A recombinant-based ELISA evaluating the efficacy of netobimin and albendazole in ruminants with naturally acquired fascioliasis. Vet J 2009; 182:73–78.

**78** Anuracpreeda P, Wanichanon C, Chawengkirtikul R, *et al. Fasciola gigantica*: immunodiagnosis of fasciolosis by detection of circulating 28.5 kDa tegumental antigen. Exp Parasitol 2009; 123:334–340.

**79** Ubeira FM, Muino L, Valero MA, *et al.* MM3-ELISA detection of *Fasciola hepatica* coproantigens in preserved human stool samples. Am J Trop Med Hyg 2009; 81:156–162.

**80** Valero MA, Ubeira FM, Khoubbane M, *et al.* MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep experimentally infected with *Fasciola hepatica* and *F. gigantica*. Vet Parasitol 2009; 159:77–81.

**81** Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999; 29:1181–1189.

**82** Lindon JC, Holmes E, Nicholson JK. Metabonomics and its role in drug development and disease diagnosis. Expert Rev Mol Diagn 2004; 4:189–199.

**83** Saric J, Li JV, Wang Y, *et al.* Metabolic profiling of an *Echinostoma caproni* infection in the mouse for biomarker discovery. PLoS Negl Trop Dis 2008; 2:e254.

**84** Saric J, Li JV, Wang Y, *et al.* Panorganismal metabolic response modeling of an experimental *Echinostoma caproni* infection in the mouse. J Proteome Res 2009; 8:3899–3911.

**85** • Wang Y, Li JV, Saric J, *et al.* Advances in metabolic profiling of experimental nematode and trematode infections. Adv Parasitol 2010; 73:373–404.
The authors provide a detailed background of metabolic profiling strategies and then summarize applications and key findings made with this strategy for biomarker discovery in different host models experimentally infected with trematodes and nematodes.

**86** Bell JD, Brown JC, Nicholson JK, Sadler PJ. Assignment of resonances for 'acute-phase' glycoproteins in high resolution proton NMR spectra of human blood plasma. FEBS Lett 1987; 215:311–315.

**87** Saric J, Li JV, Utzinger J, *et al.* Systems biology: effects of *Fasciola hepatica* on the neurochemical profile in the rat brain. Mol Syst Biol 2010. [Epub ahead of print]

# *Chapter 8*

# General Discussion and Conclusion

## Artemisinins for the Treatment of Fascioliasis: Progress in Preclinical and Diagnostic Research

# General Discussion

## Rationale, Objectives and Aim of the Present Thesis

Fascioliasis has a cosmopolitan distribution, a huge veterinary impact, and since the 1990s, it has been recognized as an increasing global public health problem [1, 2]. Nonetheless, not many people are aware of this neglected tropical disease and only a few institutions and companies are interested in research of this parasitic disease [3]. Consequently, no novel fasciocidals have been registered on the market since the 1980s and as a result, the drug arsenal is small with only one drug, triclabendazole, recommended for human use, which is licensed in only four countries worldwide [4-6]. To make matters worse triclabendazole resistance is a spreading problem in livestock and there are no vaccines available for disease control [7, 8]. To counteract this deficiency, novel drugs or drug combinations against fascioliasis need to be discovered and developed urgently.

In my PhD thesis, I tackled preclinical investigations in order to treat *Fasciola hepatica* infections with semisynthetic artemisinins, and furthermore evaluated the FLOTAC techniques, an innovative copromicroscopic tool for diagnosis of fascioliasis and quantification of treatment outcome. The finding that peroxidic drugs, including the semisynthetic artemisinin derivatives and the artemisinin-like synthetic 1,2,4-trioxolane OZ78, exhibit promising fasciocidal activity preceded this project and was the starting point of this thesis. In brief, it was described that artemether, artesunate, and OZ78 kill *F. hepatica* flukes *in vitro* and possess *in vivo* activity against adult and juvenile *F. hepatica* in experimentally infected rats [9, 10]. In addition, it was shown that artemether and OZ78 are effective against a triclabendazole resistant *F. hepatica* strain in the rat model [11]. Finally, the efficacy of artemether was assessed in sheep naturally infected with *F. hepatica*: intramuscular treatments were effective while oral applications lacked activity [12].

The aim of the present thesis was to advance our knowledge of the fasciocidal properties of the artemisinins by means of preclinical investigations including pharmacokinetics, further activity studies in ruminants, and combination therapies. The work conducted can be divided in two main parts and shall be summarized briefly (*chapter 2-6*).

First, since a sensitive, accurate, and user-friendly tool was needed for the detection and quantification of *F. hepatica* eggs in our chemotherapy studies (*chapter 3/4*), we compared the sedimentation technique, which is considered as the reference coprodiagnostic method of *F. hepatica* infections, with the recently developed and promising FLOTAC techniques (*chapter 2*). Copromicroscopy was also used to identify naturally infected sheep, to allocate the sheep in treatment groups according to their *F. hepatica* egg count, to confirm the experimental infections in rats and finally to estimate the treatment effect on egg excretion (*chapter 3/4*).
Second, we observed large differences in treatment outcomes in sheep naturally infected with *F. hepatica*. While orally applied artemether lacked activity and high doses of intramuscular artemether were needed to obtain good activity [12], intramuscular artesunate revealed a high treatment efficacy with a fourth of the used effective artemether dose. This result is different from the observations in rats (*chapter 3/4*). For this reason, we conducted pharmacokinetic studies of the artemisinin derivatives in sheep in order to obtain explanations for the diverse treatment outcomes following different routes of application and artemisinin derivatives (*chapter 6*). In a first step, an appropriate analytical method, in our case a liquid chromatography tandem mass spectrometry method (LC-MS/MS), had to be developed and validated, since drug and metabolite concentrations have to be quantified simultaneously, accurately, precisely, and selectively in sheep plasma for the later pharmacokinetic application (*chapter 5*).

The interrelation of the covered thesis objectives in the drug development process of fasciocidal compounds is depicted in Figure 1.

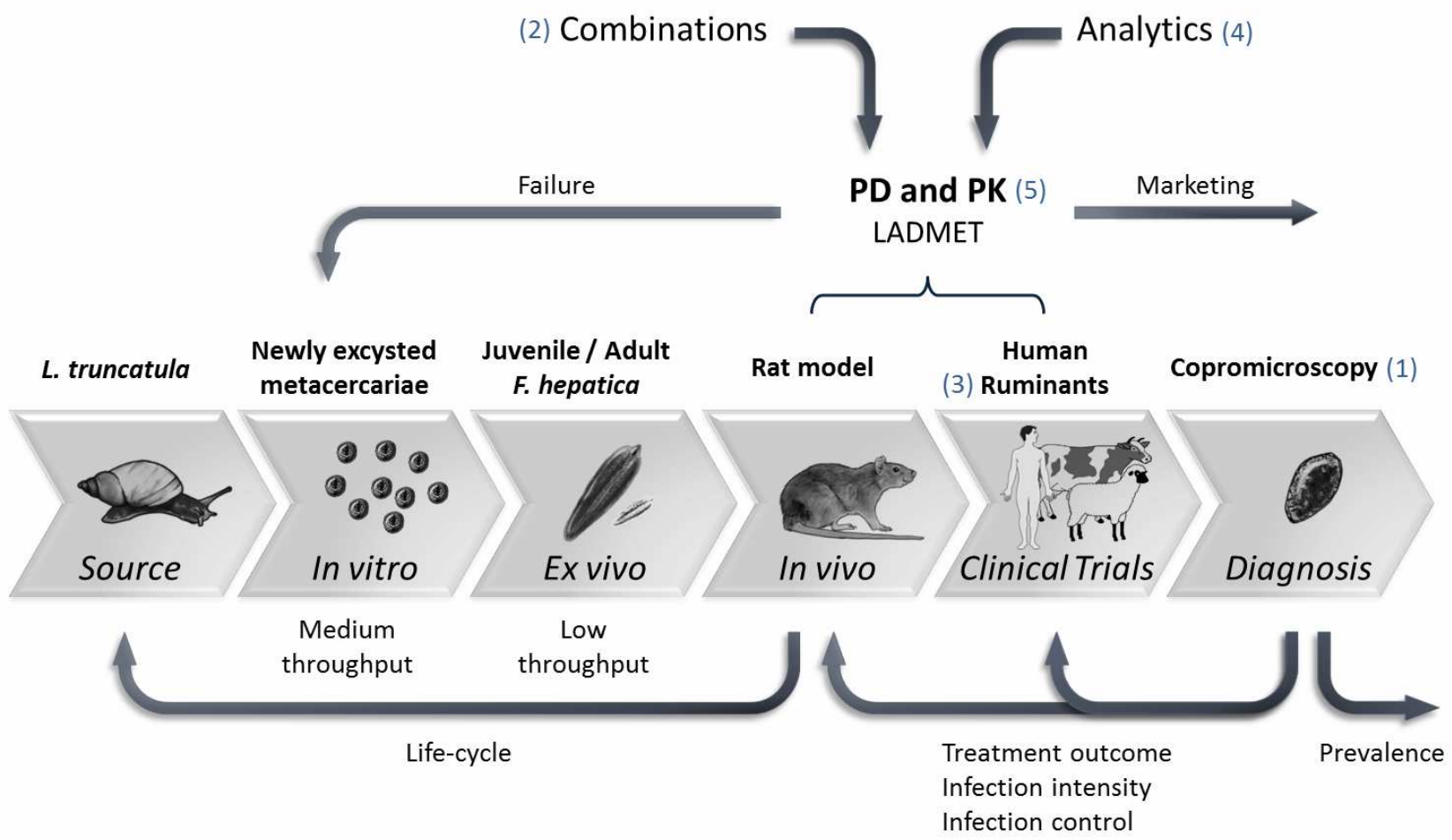



**Figure 1. Drug development process of fasciocidal compounds.** Adapted from [3, 13, 14]

PD: Pharmacodynamic, PK: Pharmacokinetic, LADMET: Drug Liberation, Absorption, Distribution, Metabolism, Excretion, Toxicity

(№): Thesis objectives 1-5 (*chapter 1, section 8*)

The results generated in these five objectives present my thesis, which will be discussed in a general manner and put into a wider context in the following pages. “Objective specific” issues have already been discussed in the last section of each *chapter* and therefore they will be taken into account in only a few instances to avoid unnecessary repetitions. Based on the obtained results and my own experience, novel ideas, open questions, different approaches, future perspectives, and my personal opinion will be considered in this general discussion. The following four topics have been chosen for a more detailed discussion.

(1) Further thoughts in copromicroscopic techniques with particular emphasis on the FLOTAC techniques.

(2) The way forward to evaluate drug activity against *F. hepatica*. Ethical and methodological considerations.

(3) Improvement of the LC-MS/MS method for artemisinins quantification

(4) Potential and limitations of the artemisinins for treatment of fascioliasis

# 1 Further Thoughts about the FLOTAC Techniques

An appropriate copromicroscopic technique was required to verify the experimental *F. hepatica* infections in rats and to estimate the egg burden reduction after treatment in the combination chemotherapy study. Therefore, we compared the sedimentation and FLOTAC techniques in terms of sensitivity, faecal egg count (FEC), and feasibility. In brief, the sedimentation technique needed a rigorous reading effort with 8 slides examined to achieve a comparable sensitivity to a single FLOTAC in rats characterised by low infection intensities (85.2% vs. 92.6%). Sensitivity was not an issue for both techniques analysing high infection intensities. Interestingly, significantly higher mean FECs were determined with the sedimentation technique. Although the FLOTAC preparation is laborious, the reading effort needed to obtain a good sensitivity is lower than for the sedimentation technique and therefore FLOTAC is more time-efficient. So that the reader can get an idea of what time-efficient means; without exaggeration, it was possible to perform 32 FLOTAC analyses a day, whereas 8 analyses with the sedimentation technique with 8 slides examined each, filled a working day. For this reason FLOTAC, and not the sedimentation technique, was chosen for the future copromicroscopic measurements carried out in our laboratories.

As described in *chapter* 2, several studies in humans and animals showed that FLOTAC possesses an advantageous sensitivity compared to various other coprodiagnostic techniques including Kato-Katz thick smear, ether-based concentration technique, McMaster, and parasep solvent free technique [15-18]. However, the advantages observed with FLOTAC depend on the parasite species of investigation and also on the individual host organism. For example, new studies showed that the Koga agar plate method and the repeated adhesive tape test are not replaceable by FLOTAC, and remain the method of choice for the diagnosis of *Strongyloides stercoralis* and *Enterobius vermicularis* in humans, respectively [19, 20]. In addition, a recent study observed that FLOTAC was less sensitive for diagnosing hookworm infections than the Kato-Katz method [21], which is contradictory to observations from three other studies [15, 16, 19]. A further controversial point is that many studies, my own included, detected lower mean FEC for FLOTAC in parallel to a higher sensitivity than the comparative method [15, 16, 19].

One explanation for this finding is that the FEC might be overestimated in the case of the applied sedimentation technique or Kato-Katz, where the analytical sensitivity is high and a large multiplication factor is used for estimating FECs [22, 23][personal communication Prof G. Cringoli]. For example, while the FLOTAC technique allow for analysing of up to 1 gram of faeces due to its amazing ability to separate faecal debris and parasitic eggs, in a single Kato-Katz analysis only 41.7 mg of faeces are used [15, 24]. However, another explanation might be that the observed differences in FEC arises, because not all the eggs float to the upper layer or additionally they might be cleared by preservatives (formaldehyde), ether or the flotation solution itself [21, 25]. Hence, it would be interesting to count the eggs in the flotation chamber after translating the reading disk in order to estimate the percentage of eggs which did not float or were not extracted by translation. Stool samples of experimentally infected laboratory animals are, in particular, suitable for these experiments, since these samples are characterised by especially high FECs. This experiment is not that easy to perform, because the FLOTAC apparatus has to be destroyed in a way, without loosing the faecal sample or re-combining the translated egg layer with the flotation chamber compartment. To avoid this problem, the centrifugal flotation of the eggs could be carried out in a conic tube instead of the FLOTAC apparatus and the sediment analysed for remaining eggs.
To sum up, the egg recovery of FLOTAC might not be as good as that of other copromicroscopic techniques resulting in lower FECs, but its ability to analyse a large amount of faeces makes it frequently more sensitive than the other techniques.

After analysing more than 500 hundred faecal samples using FLOTAC in my thesis, I noticed one important shortcoming, which will be emphasized here. Sometimes (interestingly, predominantly observed in rats, which had undergone treatment) the reading grid was overfilled with faecal debris, which complicated an accurate counting of *F. hepatica* eggs. Importantly, this phenomenon was never observed with the sedimentation techniques. Similar findings have been described for human faecal samples [21]. It is not a severe drawback for the detection of *F. hepatica* eggs, since these eggs are large and yellow in colour, but the detection sensitivity of the smaller and transparent hookworm eggs and the small-sized intestinal protozoa might be negatively impacted. It has been suggested that more organic debris floats and hence worsens the microscopic picture, if the lipid content of faecal samples is increased [25]. One might speculate that chemotherapy applied to rats might have influenced the faecal

composition, possibly triggered by adverse drug reactions or healing process, which would explain that contaminated reading slides were mainly observed in treated rats. The suggested theory could be verified by analysing faecal samples of people who are medicated with orlistat (Xenical®), a lipase inhibitor, and therefore have a higher fat level in faeces. The use of an ether or ethyl acetate cleaning step has been proposed to overcome this problem [25]. However, it has been shown that ether might negatively impact the diagnosis of *Ascaris lumbricoides* and hookworms [25]. Therefore further investigations regarding an improvement of flotation solutions or the use of surface-active agents (tensides) are needed so that an accurate and robust application is possible with the FLOTAC techniques for all the diverse parasites and faecal samples.

Furthermore, it is worthwhile mentioning that FLOTAC is applicable in field conditions in developing countries, where poly-parasitism is a norm [26]. Recently, it has been shown in several studies, that the FLOTAC techniques can be executed also in rural field conditions [16, 20, 27]. This is not that obvious as for example centrifugation is essential for faecal sample preparation. Therefore, a special adaptor was produced to connect the FLOTAC apparatus with hand centrifuges, which enabled the use of FLOTAC also without electricity [20]. FLOTAC holds promise to detect simultaneously the three common soil-transmitted helminths (*A. lumbricoides*, *T. trichiura*, and hookworms), *Schistosoma mansoni*, and intestinal protozoa infections using only two flotation solutions (FS 4 and 7) and might therefore compete with the Kato-Katz method for the detection of poly-parasitism [21]. Of note, two different faecal samples can be examined simultaneously using a single FLOTAC apparatus. It should be highlighted however, that some flotation solutions contain hazardous components such as zinc sulphate, which is toxic to water organisms and harmful to users, or mercury iodide, which is additionally very poisonous [28]. A proper disposal of the flotation solutions must thus be ensured, which is not an issue under laboratory conditions, but might be a limitation of FLOTAC under field conditions. Furthermore, it has recently been demonstrated that FLOTAC is more expensive than the Kato-Katz method, where the estimated costs to perform a duplicate Kato-Katz thick smear and a FLOTAC dual technique were US$ 2.06 and US$ 2.83, respectively [29]. All these results should be taken into account before deciding whether FLOTAC is an applicable diagnostic tool for developing countries.

My opinion is that FLOTAC is an excellent invention, which has been shown to be more sensitive than comparable copromicroscopic methods. However, its performance is difficult, because multiple operation steps including centrifugation, FLOTAC assembly, choice of the right flotation solution, and possible additional sample cleaning steps are needed to ensure an accurate measurement. Therefore, further investigations are needed to improve the robustness of FLOTAC and hence make it simple to perform under various conditions.

## 2 Evaluating Drug Activity against *F. hepatica*

The *in vivo* and *in vitro* drug activity of single and combination chemotherapy against *F. hepatica* have been presented in *chapter 3* and *4* of the thesis. In brief, intramuscular applied artesunate and artemether showed good effects against *F. hepatica* in naturally infected sheep, whereas artesunate was more potent than artemether. This observation was different from the one in the rat model, where artemether showed a higher activity. Specifically, 50% effective dosages ($ED_{50}$) of 113, 78, 23, and 2.7 mg/kg were estimated for artesunate, artemether, OZ78, and triclabendazole, respectively against adult *F. hepatica* in experimentally infected rats. The juvenile *F. hepatica* appeared to be less susceptible to treatment than adult flukes (for example 5 mg/kg triclabendazole showed only marginal effects against juvenile worms, whereas 2.7 mg/kg triclabendazole killed approximately 50% of adult *F. hepatica in vivo*). The sensitivity of adult and juvenile *F. hepatica* to combinations of triclabendazole plus one of the peroxidic drugs, artesunate, artemether, and OZ78 was evaluated. The observed drug-drug interactions are difficult to interpret and it is therefore not easy to decide how to continue with the investigated drug combinations. For example, we observed great variations in combinations of artesunate or artemether plus triclabendazole in the dose response following slight titrations in the triclabendazole doses. I would therefore like to discuss here, how to continue to evaluate drug activity and combination effects against *F. hepatica*, including ethical and methodological considerations. Alternative approaches will be suggested here, which might help to improve drug screening and to find effective drug combinations.

To date, it is not possible to cultivate *F. hepatica in vitro* throughout the entire life cycle [3, 30], and consequently laboratory animals are indispensable for drug screening against fascioliasis. However, ethical considerations for how to reduce, refine, and replace live animals (3Rs) have to be implemented in the laboratory work [31]. Wherever applicable, drug candidates should be screened first *in vitro* and the most promising ones should be selected for *in vivo* models. Therefore reproducible, accurate, and rapid performing *in vitro* assays are needed. In the case of *F. hepatica*, drugs can be screened *in vitro* against newly excysted metacercariae/flukes or *ex vivo* against juvenile and adult *F. hepatica* worms isolated from infected animals [3].

First, to screen drugs against newly excysted flukes might be the most ethical approach because only a few animals are needed to maintain the life-cycle. A further advantage might be that drugs can be screened in a medium throughput manner, since numerous metacercariae can be harvested from snails, and thus parasite material is not a limiting factor [32]. However, metacercariae are expensive to buy particularly due to shipment and customs charges. In addition, the entire life cycle would be necessary to generate metacercariae in the laboratory, which is difficult because the maintenance of the snails is very sensitive. So far we were not successful in maintaining uninfected snails longer than a month in our laboratories.

One could speculate that colorimetric *in vitro* assays such as Alamar blue or MTT formazan could be established for medium throughput screening to assess drug effects on newly excysted flukes and replace the so far applied work-intensive phenotype-based drug assay [33, 34]. This approach allows the estimation of 50% and 90% effective concentrations ($EC_{50/90}$), and hence enables the comparison of drug candidates and the selecting of the most promising candidates for *in vivo* investigations. Furthermore, if drug combinations are evaluated, the precise determination of $EC_{50/90}$ allows for calculating the combination index or the construction of isobolograms, which is required to estimate synergism or antagonism between two drugs [35, 36]. One could hypothesise, that with such a method it would have been possible to evaluate the activity of artesunate, artemether, OZ78, and triclabendazole combinations at several concentrations and not only at one fixed concentration as applied in our combination chemotherapy study (*chapter 3*). This may have allowed for a better understanding of the interaction between triclabendazole and peroxidic drugs. Nevertheless a disadvantage of this screening method might be that novel drug candidates or

synergistic drug combinations are missed, since newly excysted flukes were not always affected by known fasciocidal drugs [3, 37].

Secondly, drugs can be screened *ex vivo* against juvenile and adult flukes isolated from laboratory animals. Of note, in our combination chemotherapy study, juvenile and adult *F. hepatica* worms were used, which were recovered from rats after ineffective treatments, and hence our experimental design was limited (a median of 6 to 7 *F. hepatica* flukes were recovered from untreated control rats). More ethically, as now also done in our laboratories, adult worms can be obtained from naturally infected animals, mainly sheep and cattle, from the local slaughterhouse. However, it is difficult to plan such experiments, because it is not known when a *F. hepatica* infected liver can be obtained from the slaughterhouse and how many worms can be isolated from it. Furthermore, to my knowledge, only phenotype-based assays are currently used for drug screening against adult *F. hepatica*, which are time and work intensive and consequently only a few compounds can be tested in a short period. It was not possible so far to evaluate drug effect on adults colorimetrically [unpublished observation], though microcalorimetry might play a role [38].

Another possibility for an *F. hepatica* assay is to infect rats with approximately 80 metacercariae and euthanize the rats within 4 weeks post-infection. Hence, the number of juvenile flukes recovered from rat livers might be significantly increased. This approach allows for a low-medium throughput screening of drugs. In my opinion, it would be particularly interesting to evaluate drug activity in a first run against juvenile flukes, since many commercially available fasciocidals lack activity against immature worms and furthermore a potential drug candidate should be effective against all parasite stages (Introduction, Section 7.1). In addition, similarly to *in vitro* assays of newly excysted flukes, the determination of $EC_{50/90}$ and combination indices would be absolutely feasible, since the assays might be performed in 96-well plates using better performing screening methods than phenotype-based assays. For example, promising results were achieved in our laboratory using a microcalorimetric viability assay to evaluate drug efficacy against juvenile *F. hepatica* [unpublished observation]. However, this approach would only be proposed if it can be demonstrated that the *ex vivo* assay against juvenile flukes models the *in vivo* effect of drugs better than the *in vitro* assay of newly excysted flukes. Moreover, it should be deliberated from an ethical point of view,

whether rats suffer pain at these high infection intensities, for the larger amount of worms recovered.

Promising *in vitro* or *ex vivo* activities of lead compounds frequently can not be translated to an *in vivo* efficacy, because biopharmaceutical properties such as low drug solubility, poor bioavailability, extensive metabolism, and fast drug elimination might negate a potential drug effect [37, 39]. Moreover, with regard to *F. hepatica* infections, it has been shown that the pharmacokinetics of drugs including the artemisinins can be altered due to liver damage [40, 41]. In addition, adult flukes lodge in the bile, whereas juvenile flukes migrate through the liver and therefore a biphasic activity might only be achieved if compounds reach both host organs. It is tempting to speculate that this is a reason for many fasciocidals lacking activity against one of the infection stages. Additionally, this might be a reason for why the drug combinations showed a lower activity against juvenile than against adult worms (*chapter 3*).
Given that novel fasciocidal drugs should be active against both stages, in my opinion, *in vivo* drug activity should be assessed first against juvenile and not adult worms, because of the following reasons. First, a juvenile infection takes only about 3-4 weeks and is therefore less expensive. In addition, rats suffer for a shorter time from the *F. hepatica* infection. Second, one can speculate that more drug candidates fail to be active against juvenile worms as opposed to against adult worms and thus fewer animals are used. And third, higher infection intensities can be generated in juvenile infections, which might improve the interpretation of treatment outcomes. For example, high infection intensities of ~200 *F. hepatica* flukes were observed in experimentally infected sheep [42], and hence one could speculate that results obtained in rats with higher infection intensities are more representative for the situation in the natural end host sheep.

Our combination chemotherapy study highlighted a substantial loss of precision if egg instead of worm counts were used to estimate dose-response relationships (*chapter 3*). Hence, I would suggest using egg analysis only for infection control and for allocating rats in treatment groups according to their *F. hepatica* egg count to equally balance the groups. Nevertheless, the faecal egg count reduction test (FECRT) is an important tool to evaluate treatment outcome in humans or after drenching farm animals, and is widely used in preclinical and clinical studies [43, 44].

In conclusion, in a next combination chemotherapy study, it would be advisable to incorporate the afore-mentioned approaches and start with evaluating the *ex vivo* or *in vitro* drug-drug interactions and if promising results arise, *in vivo* studies in rats can be added. In addition, in upcoming studies, it would be wise to focus on a single drug combination and only on a single metabolite, which would simplify the whole study design and potentially result in a more conclusive outcome.

## 3 Improvement of the LC-MS/MS Method

We developed and validated a LC-MS/MS method for the simultaneous determination of artesunate and artemether and their metabolites DHA and DHA-glucuronide in sheep plasma. The validation results demonstrated that the LC-MS/MS method is precise, accurate, sensitive, and selective and can be applied for the quantification of the artemisinins in sheep plasma in pharmacokinetic studies. However, the method has its limitations, and therefore feasible improvements of the method should be discussed here.

The developed LC-MS/MS method achieved a lower limit of quantification (LLOQ) between 10-100 ng/ml depending on the investigated analyte, which is relatively high compared to other published methods where LLOQs of about 1 ng/ml were demonstrated for this compound class [41, 45-47]. In addition, the performance of the method is somewhat outdated; this means long run times of 23 minutes and laborious sample work-up. Hence, these variables might be improvable, which would be especially important if a high throughput of many samples is needed.

The method sensitivity may depend amongst others, on the used instrumentation, the molecular structure of the analytes, the sample work-up, the mode of analyte detection, the selected fragmentation, and the used chromatographic elution. The instrumentation and the molecules are more or less fixed parameters, whereas particularly the sample work-up, the mode of detection, and the chromatographic settings could be improved.

In the framework of the method development, the sensitivity and selectivity of the method had been rigorously increased by replacing the mode of artemisinins detection using selected reaction monitoring (SRM: *m/z* 267.4→163.0) instead of detecting protonated fragments in single MS mode (*m/z* 221.5, 267.4, and 284.4). This change in

detection increased the sensitivity of the artemisinins of about 10-100 times. However, at this point of method development we were not able to further enhance the sensitivity notably with only small adaptations. The following suggestions might improve the method sensitivity and performance significantly.

An advanced analyte recovery and the reduction of the matrix effect might boost sensitivity. It is attractive to speculate, that for instance, the sensitivity of artemether could be increased about 3-4 times, in the case suppressing matrix effects could be eliminated and a complete recovery could be realized. In retrospect, increased method sensitivity would have been valuable, since in our artemether PK study, the concentrations of artemether and DHA were low and not quantifiable for some animal at each time point (*chapter 6*). Different operational strategies have been suggested such as laborious cleanup procedures, including solid phase or liquid-liquid extraction to minimize the interferences of coeluting matrix compounds and consequently to eliminate matrix effects [48]. The use of an online solid-phase extraction method using column switching systems might be the most promising possibility to minimize matrix effects, increase sensitivity, improve analytical separation, and concurrently simplify sample work-up [49]. In brief, plasma samples are precipitated and the supernatants are injected in the LC-MS/MS system. The analytes are concentrated and cleaned with a trapping column and subsequently separated using an analytical column. This technique might be less laborious and allow for a much larger sample throughput compared to the method we used, since all steps can be accomplished in 96-well plates and there is no need for time consuming evaporation of the organic phase [personal communication M. Donzelli]. Establishment of an online column switch technique might be of particular interest, if the method has to be adapted to analyse bile fluid, since biliary matrix components (e.g. biliary salts, biliary acid) might interfere strongly with the analytics [50][personal observation]. In addition, this technique would also be useful and transferable to the development of analytical methods of upcoming experimental fasciocidal analytes.

A further benefit regarding sample turnover would be to shorten the chromatographic elution program of the method. All analytes had to be baseline separated, since they were all detected by selected reaction monitoring (SRM) with a similar transition of *m/z* 267.4→163.0. The used run time was long, primarily because a slow mobile phase gradient had to be applied to separate β-DHA from artesunate. In addition the column

had to be washed for several minutes to avoid carry-over effects. However, it might be possible to shorten the 23-minute run time using chromatographic columns with smaller particles such as pellicular silica columns (Phenomenex Kinetex), as it was proposed by one reviewer. Afore-mentioned column-switching techniques might additionally assist in shortening the analytical run time. A major upgrade of the method would be to detect the artemisinins by multiple reaction monitoring (MRM), given that the artemisinins must not be baseline separated and thus the run times can be shortened [51][personal communication M. Donzelli]. However, this implies that a specific reaction of the parent mass of each artemisinin is monitored and not of an identical fragment of the parent mass. Therefore, the parent mass must be ionised in the interface of the mass spectrometer but without fragmenting it extensively. The use of fragmentation the ammonium ion adducts from the parent mass has been demonstrated in other studies, but we were so far not successful with our instrumentation [52, 53]. It is tempting to speculate that higher ammonium concentrations added to mobile phase A, the use of another interface, or different MS settings might lead to better results. Finally, MRM of the parent masses would allow for the usage of isotope-labelled internal standards such as artesunate-D4 or artemether-C13-D3, which might positively influence the accuracy, since suppressing or enhancing matrix effects can be better adjusted [48].

In conclusion, our developed method might be improvable in regard to method sensitivity, accuracy, sample throughput, and practicality with moderate adaptations without exchanging the whole instrumentation.

## 4 Potential of the Artemisinins for the Treatment of Fascioliasis

Label extension of marketed drugs is one key strategy for drug discovery for tropical diseases, because market launch can be achieved with relative speed and limited economic cost [54]. The artemisinins may exhibit promise for label extension, since they are currently registered for treatment of malaria and promising trematocidal properties including *Fasciola* spp. have been demonstrated [55, 56]. For this reason, it was certainly important to investigate the fasciocidal characteristics of the artemisinins, artesunate and artemether, during the last years in more detail. Nonetheless, after having taken my

personal observations and data from different publications into account, I feel that the artemisinins are rather promising lead structures for the development of more active next generation analogues than a true alternative to triclabendazole in treatment of human and veterinary fascioliasis.

To begin with, reasons are summarized why the sesquiterpene lactone scaffold of the artemisinins holds promise to be a lead structure for the development of novel fasciocidal drugs. First, artesunate and artemether showed fasciocidal activity *in vitro* and *in vivo* in the rat and the sheep model [9, 12]. Second, molecules derived from the artemisinin core such as the 1,2,4-trioxolanes OZ78 or 1,2,4,5-tetraoxane MT04 showed higher activity than the artemisinins itself [10, 38]. Third, the observed activity was not stage specific, with both juvenile and adult worms being affected [9, 10, 38]. Fourth, artemether and OZ78 were active against a triclabendazole resistant *F. hepatica* strain, and thus it might be speculated that these peroxidic compounds exhibit a different mode of action than triclabendazole [11]. In addition, this might be important for the development of effective combination treatments. Fifth, to my knowledge neither the semisynthetic nor the synthetic artemisinins molecular structures are related to any marketed fasciocidals, which underlines the originality of this compound class. Finally, the artemisinins showed additional activity against other trematodes including *Schistosoma*, *Clonorchis*, and *Opisthorchis* spp., and artemisinin-like structures may therefore hold the promise to become a broad spectrum trematocidal drug [56].

Nevertheless, in my opinion the semisynthetic artemisinins might not be an alternative to triclabendazole while the synthetic artemisinins, such as OZ78 and MT04, might be more promising, based on the following reasons.
First, triclabendazole exhibit a higher potency than the artemisinins, since we estimated an $ED_{50}$ of 2.7 mg/kg for triclabendazole, which is approximately 30-40 times lower than the estimated $ED_{50}$s of artemether (78 mg/kg) and artesunate (113 mg/kg) following a single oral dose in rats. In addition, we could show that OZ78 achieved an improved estimated $ED_{50}$ of 23 mg/kg than the artemisinins. Contradictory results were achieved in sheep. While OZ78 failed to cure sheep experimentally infected with *F. hepatica*, the artemisinins were active against *F. hepatica* in naturally infected sheep [12, 42] (*chapter 4*). However, this comparison is incomplete, since the activity of the artemisinins was evaluated in sheep with lower infection intensities (natural infections) compared to the

study with OZ78. For this reason, I am not so convinced that artemether or artesunate could cure high infection intensities in a single dose regimen. Further investigations including metabolic, bile and urine excretion studies of OZ78 in sheep and rat are needed to understand the discrepancy in activity between the two hosts better (A further issue where the column switching technique might become necessary). Moreover, two independent clinical studies revealed that artemether lacks activity for treatment of chronic human fascioliasis and the response rate in patients treated with artesunate was lower than for triclabendazole for the treatment of acute human fascioliasis [44, 57]. Still, artesunate may play a role in treatment of acute fascioliasis, as patients treated with artesunate were significantly more likely to be free of abdominal pain at hospital discharge when compared to triclabendazole-treated patients. Of note, 4 mg/kg artesunate was applied once daily for 10 days, whereas only two doses of 10 mg/kg triclabendazole were administered [57].

A second aspect concerns the safety of the artemisinins treatments. Artesunate and artemether exhibit a good safety profile and are well tolerated with only minor adverse drug events observable, if applied on a malaria treatment schedule [55]. However, a large single oral dose is needed to achieve a high activity against *F. hepatica* in rats and sheep and hence treatment safety was not guaranteed particularly in the case of artesunate. For instance, the $ED_{90}$ of single oral artesunate in rats was calculated at 489 mg/kg, whereas the 50% lethal dose in healthy rats ($LD_{50}$) was estimated to be 351 mg/kg [58]. For comparison, the safety profile of triclabendazole is exceptionally good, since we estimated an $ED_{90}$ of 11.7 mg/kg following a single oral dose in the *F. hepatica* rat model and the $LD_{50}$ in rats was described to be >8000 mg/kg [4]. In sheep, single intramuscular doses of artesunate (<60 mg/kg) were well tolerated and no physical clinical sign of toxicity were observed, while double doses of artesunate (40 mg/kg) caused death to three of six sheep (*chapter 3*). Artemether treatment regimens for fascioliasis were in general safe; however embryotoxicity in sheep is of concern [12]. Importantly, only minor adverse drug events and no severe toxicity was observed in OZ78 activity studies in sheep and rats [10, 42].

A third issue is that artemisinin is required as a precursor molecule for the synthesis of artesunate and artemether, which has to be extracted from the leaves of Artemisia annua [55]. The herb has to be planted each year and the artemisinin extraction yield depends considerably on temperature, humidity, and soil type [55]. For this reason, the

chemical purity is of issue as well as the high and volatile artemisinin prices, which is controlled by the extraction yields and number of companies extracting artemisinin ($350-$1700 per kg) [55, 59, 60]. On the other hand, these factors are not present for totally synthetic artemisinins such as OZ78, because they exhibit structural simplicity and an economically feasible and scalable synthesis [60].

Finally, the artemisinins are characterised by limiting pharmacokinetics such as poor bioavailability and fast elimination [56, 60]. Progress has been made by semisynthetic derivatization of the artemisinins, which improved the oral bioavailability and in addition enabled the use of different routes of drug administration such as intravenous and intramuscular applications [55]. However, the artemisinins were principally modified only at one position of the sesquiterpene lactone scaffold, and hence the possibility to improve the pharmacokinetic properties is limited. This is in line with the experiences we had in the PK study of artemether and artesunate in sheep, where for instance, artemether was poorly liberated out of the oil-based intramuscular drug formulation with consequently low maximal plasma concentrations. And furthermore, both artemisinins were extensively transformed to their glucuronide-metabolite, which showed no activity *in vitro*. Therefore, it is attractive to speculate that other artemisinin derivatives than artemether or artesunate, with higher metabolic stability or better physicochemical properties, might exhibit better fasciocidal activity. Totally synthetic drugs have the great advantage that physicochemical properties as well as structure activity relationships can be assessed in the development process of the lead structures, whereas these possibilities might be more limited for semi-synthetic drugs [61]. Hence, several synthetic artemisinins (such as OZ78) possess an enhanced oral bioavailability due to an appropriate balance between lipophilicity and aqueous solubility and additionally a longer elimination half-life, and therefore a superior *in vivo* drug disposition over the artemisinins [56, 60].

Taking all points into consideration, it might be reasonable to conclude that the artemisinins are promising lead structures for the development of novel fasciocidal entities and not yet an alternative to marketed drugs.

# Conclusion

In conclusion, in the framework of my PhD thesis, I have worked on different aspects of the drug development process ranging from diagnostics, analytics, and pharmacokinetics to activity studies *in vitro*, in rodents and in ruminants (Figure 1).

The FLOTAC techniques demonstrated a high efficiency and sensitivity for detecting *F. hepatica* eggs in faecal rat samples and have the potential to become a valuable tool for experimental work in the *F. hepatica* rat model. In addition, the FLOTAC techniques might be a useful diagnostic tool for other helminth models (e.g. hookworms and schistosomes) in our laboratories. Nonetheless, further work is required to achieve a better analytical robustness and ease of performance before the FLOTAC techniques can compete with established copromicroscopic methods such as the sedimentation techniques.

Artesunate and artemether exhibit interesting fasciocidal activity *in vitro* and in *F. hepatica* infected rats and sheep. The developed LC-MS/MS method was capable to determine PK parameters of the artemisinins and their major metabolites in sheep plasma. The main disposition parameters suitably reflected the observed activities of artesunate and artemether following different routes of administration against *F. hepatica* in naturally infected sheep. This LC-MS/MS method might be of value to determine the artemisinins in other biological fluids including bile and rumen fluid of sheep, which might provides further details why oral treatments lacked activity in sheep. Combinations with triclabendazole improved the efficacy of the peroxide treatments, but in the case of the artemisinins, the observed treatment effect dependent on the applied triclabendazole dose. Therefore, further experiments including studies using triclabendazole-resistant *F. hepatica* strains, pharmacokinetic studies and combination trials in ruminants, are necessary to thoroughly evaluate the potential of triclabendazole-peroxide drug combinations.

Overall, the novelty of the peroxidic core, the broad spectrum of activity against juvenile and adult flukes and the observed activity against a triclabendazole resistant *F. hepatica* strain classify the artemisinins as a lead structure for the development of novel peroxidic fasciocidal drugs.

# References


1. **Torgerson, P. and J. Claxton**, Epidemiology and Control, in *Fasciolosis*, J.P. Dalton, Editor. 1999, CABI Publishing: Oxford (UK). p. 113-149.
2. **Mas-Coma, S., M.A. Valero, and M.D. Bargues**, Chapter 2. Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol, 2009. **69**: p. 41-146.
3. **Keiser, J.**, *In vitro* and *in vivo* trematode models for chemotherapeutic studies. Parasitology, 2010. **137**(3): p. 589-603.
4. **Keiser, J., D. Engels, G. Buscher, and J. Utzinger**, Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Investig Drugs, 2005. **14**(12): p. 1513-26.
5. **Fairweather, I. and J.C. Boray**, Fasciolicides: efficacy, actions, resistance and its management. Vet J, 1999. **158**(2): p. 81-112.
6. **WHO**, Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control. 2006: Geneva, Switzerland. p. 31.
7. **Fairweather, I.**, Triclabendazole progress report, 2005-2009: an advancement of learning? J Helminthol, 2009. **83**(2): p. 139-50.
8. **McManus, D.P. and J.P. Dalton**, Vaccines against the zoonotic trematodes Schistosoma japonicum, *Fasciola hepatica* and *Fasciola gigantica.* Parasitology, 2006. **133 Suppl**: p. S43-61.
9. **Keiser, J., X. Shu-Hua, M. Tanner, and J. Utzinger**, Artesunate and artemether are effective fasciolicides in the rat model and *in vitro.* J Antimicrob Chemother, 2006. **57**(6): p. 1139-45.
10. **Keiser, J., J. Utzinger, M. Tanner, Y. Dong, and J.L. Vennerstrom**, The synthetic peroxide OZ78 is effective against *Echinostoma caproni* and *Fasciola hepatica.* J Antimicrob Chemother, 2006. **58**(6): p. 1193-7.
11. **Keiser, J., J. Utzinger, J.L. Vennerstrom, Y. Dong, G. Brennan, and I. Fairweather**, Activity of artemether and OZ78 against triclabendazole-resistant *Fasciola hepatica.* Trans R Soc Trop Med Hyg, 2007. **101**(12): p. 1219-22.
12. **Keiser, J., L. Rinaldi, V. Veneziano, L. Mezzino, M. Tanner, J. Utzinger, and G. Cringoli**, Efficacy and safety of artemether against a natural *Fasciola hepatica* infection in sheep. Parasitol Res, 2008. **103**(3): p. 517-22.
13. **Keiser, J. and J. Utzinger**, Food-borne trematodiases. Clin Microbiol Rev, 2009. **22**(3): p. 466-83.
14. **Stein, M.** Animal-Health-Online "Leberegel" Survey 2003 [cited 2011 15.05.2011]; Available from: http://leberegel.de/.
15. **Utzinger, J., L. Rinaldi, L.K. Lohourignon, F. Rohner, M.B. Zimmermann, A.B. Tschannen, K. N'Goran E, and G. Cringoli**, FLOTAC: a new sensitive technique for the diagnosis of hookworm infections in humans. Trans R Soc Trop Med Hyg, 2008. **102**(1): p. 84-90.
16. **Knopp, S., L. Rinaldi, I.S. Khamis, J.R. Stothard, D. Rollinson, M.P. Maurelli, P. Steinmann, H. Marti, G. Cringoli, and J. Utzinger**, A single FLOTAC is more sensitive than triplicate Kato-Katz for the diagnosis of low-intensity soil-transmitted helminth infections. Trans R Soc Trop Med Hyg, 2009. **103**(4): p. 347-54.
17. **Levecke, B., N. De Wilde, E. Vandenhoute, and J. Vercruysse**, Field validity and feasibility of four techniques for the detection of Trichuris in simians: a model for monitoring drug efficacy in public health? PLoS Negl Trop Dis, 2009. **3**(1): p. e366.

18. **Rinaldi, L., G. Calabria, S. Carbone, A. Carrella, and G. Cringoli**, Crenosoma vulpis in dog: first case report in Italy and use of the FLOTAC technique for copromicroscopic diagnosis. Parasitol Res, 2007. **101**(6): p. 1681-4.
19. **Glinz, D., K.D. Silue, S. Knopp, L.K. Lohourignon, K.P. Yao, P. Steinmann, L. Rinaldi, G. Cringoli, E.K. N'Goran, and J. Utzinger**, Comparing diagnostic accuracy of Kato-Katz, Koga agar plate, ether-concentration, and FLOTAC for Schistosoma mansoni and soil-transmitted helminths. PLoS Negl Trop Dis, 2010. **4**(7): p. e754.
20. **Jeandron, A., G. Abdyldaieva, J. Usubalieva, J.H. Ensink, J. Cox, B. Matthys, L. Rinaldi, G. Cringoli, and J. Utzinger**, Accuracy of the Kato-Katz, adhesive tape and FLOTAC techniques for helminth diagnosis among children in Kyrgyzstan. Acta Trop, 2010. **116**(3): p. 185-92.
21. **Knopp, S., B. Speich, J. Hattendorf, L. Rinaldi, K.A. Mohammed, I.S. Khamis, A.S. Mohammed, M. Albonico, D. Rollinson, H. Marti, G. Cringoli, and J. Utzinger**, Diagnostic Accuracy of Kato-Katz and FLOTAC for Assessing Anthelmintic Drug Efficacy. PLoS Negl Trop Dis, 2011. **5**(4): p. e1036.
22. **Levecke, B., L. Rinaldi, J. Charlier, M.P. Maurelli, M.E. Morgoglione, J. Vercruysse, and G. Cringoli**, Monitoring drug efficacy against gastrointestinal nematodes when faecal egg counts are low: do the analytic sensitivity and the formula matter? Parasitol Res, 2011.
23. **Cringoli, G., L. Rinaldi, V. Veneziano, G. Capelli, and A. Scala**, The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and Dicrocoelium dendriticum in sheep. Vet Parasitol, 2004. **123**(1-2): p. 121-131.
24. **Katz, N., A. Chaves, and J. Pellegrino**, A simple device for quantitative stool thick-smear technique in Schistosomiasis mansoni. Rev Inst Med Trop Sao Paulo, 1972. **14**(6): p. 397-400.
25. **Cringoli, G., L. Rinaldi, M.P. Maurelli, and J. Utzinger**, FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat Protoc, 2010. **5**(3): p. 503-15.
26. **Pullan, R. and S. Brooker**, The health impact of polyparasitism in humans: are we under-estimating the burden of parasitic diseases? Parasitology, 2008. **135**(7): p. 783-94.
27. **Becker, S.L., L.K. Lohourignon, B. Speich, L. Rinaldi, S. Knopp, K. N'Goran E, G. Cringoli, and J. Utzinger**, Comparison of the FLOTAC-400 Dual Technique and the Formalin-Ether Concentration Technique for the Diagnosis of Human Intestinal Protozoa Infection. J Clin Microbiol, 2011.
28. **Aldrich, S.** Category Safety. 2011 [cited 2011 19.05.2011]; Available from: http://www.sigmaaldrich.com/.
29. **Speich, B., S. Knopp, K.A. Mohammed, I.S. Khamis, L. Rinaldi, G. Cringoli, D. Rollinson, and J. Utzinger**, Comparative cost assessment of the Kato-Katz and FLOTAC techniques for soil-transmitted helminth diagnosis in epidemiological surveys. Parasit Vectors, 2010. **3**: p. 71.
30. **Smyth, J.D. and D.W. Halton**, The Physiology of Trematodes. 1983, Cambridge: Cambridge University Press.
31. **Autissier, C.**, [Ethical review in biomedical research]. Med Sci (Paris), 2008. **24**(4): p. 437-42.
32. **Krull, W.H.**, ed. The number of cercariae of *Fasciola hepatica* developing in snails infected with a single miracidium. *Proceedings of the Helminthological Society of Washington*, ed. J.R. Christie. 1941, The Helminthological Society of Washington: Washington. 55-58.

33. **Mansour, N.R. and Q.D. Bickle**, Comparison of microscopy and Alamar blue reduction in a larval based assay for schistosome drug screening. PLoS Negl Trop Dis, 2010. **4**(8): p. e795.
34. **James, C.E. and M.W. Davey**, A rapid colorimetric assay for the quantitation of the viability of free-living larvae of nematodes *in vitro.* Parasitol Res, 2007. **101**(4): p. 975-80.
35. **Chou, T.C.**, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev, 2006. **58**(3): p. 621-81.
36. **Hu, Y., E.G. Platzer, A. Bellier, and R.V. Aroian**, Discovery of a highly synergistic anthelmintic combination that shows mutual hypersusceptibility. Proc Natl Acad Sci U S A, 2010. **107**(13): p. 5955-60.
37. **Ibarra, O.F. and D.C. Jenkins**, An *in vitro* screen for new fasciolicidal agents. Z Parasitenkd, 1984. **70**(5): p. 655-61.
38. **Kirchhofer, C., M. Vargas, O. Braissant, Y. Dong, X. Wang, J.L. Vennerstrom, and J. Keiser**, Activity of OZ78 analogues against *Fasciola hepatica* and *Echinostoma caproni.* Acta Trop, 2011. **118**(1): p. 56-62.
39. **Ruiz-Garcia, A., M. Bermejo, A. Moss, and V.G. Casabo**, Pharmacokinetics in drug discovery. J Pharm Sci, 2008. **97**(2): p. 654-90.
40. **Behm, C.A. and N.C. Sangster**, Pathology, Pathophysiology and Clinical Aspects, in *Fasciolosis* J.P. Dalton, Editor. 1999, CABI Publishing: Oxford (UK). p. 185-225.
41. **Keiser, J., M.S. Gruyer, N. Perrottet, B. Zanolari, T. Mercier, and L. Decosterd**, Pharmacokinetic parameters of artesunate and dihydroartemisinin in rats infected with *Fasciola hepatica.* J Antimicrob Chemother, 2009. **63**(3): p. 543-9.
42. **Keiser, J., C. Kirchhofer, M. Haschke, J. Huwyler, Y. Dong, J.L. Vennerstrom, K. Vanhoff, R. Kaminsky, and N. Malikides**, Efficacy, safety and pharmacokinetics of 1,2,4-trioxolane OZ78 against an experimental infection with *Fasciola hepatica* in sheep. Vet Parasitol, 2010. **173**(3-4): p. 228-35.
43. **Wood, I.B., N.K. Amaral, K. Bairden, J.L. Duncan, T. Kassai, J.B. Malone, J.A. Pankavich, R.K. Reinecke, O. Slocombe, S.M. Taylor, and J. Vercruysse**, World-Association-for-the-Advancement-of-Veterinary-Parasitology (Waavp) 2nd Edition of Guidelines for Evaluating the Efficacy of Anthelmintics in Ruminants (Bovine, Ovine, Caprine). Vet Parasitol, 1995. **58**(3): p. 181-213.
44. **Keiser, J., H. Sayed, M. El-Ghanam, H. Sabry, S. Anani, A.A. El-Wakeel, C. Hatz, J. Utzinger, S. Seif el-Din, W. El-Maadawy, and S. Botros**, Efficacy and Safety of Artemether in the Treatment of Chronic Fascioliasis in Egypt: Exploratory Phase-2 Trials. PLoS Negl Trop Dis, 2011 in press.
45. **Hodel, E.M., B. Zanolari, T. Mercier, J. Biollaz, J. Keiser, P. Olliaro, B. Genton, and L.A. Decosterd**, A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci, 2009. **877**(10): p. 867-86.
46. **Xing, J., H. Yan, S. Zhang, G. Ren, and Y. Gao**, A high-performance liquid chromatography/tandem mass spectrometry method for the determination of artemisinin in rat plasma. Rapid Commun Mass Spectrom, 2006. **20**(9): p. 1463-8.
47. **Lindegardh, N., J. Tarning, P.V. Toi, T.T. Hien, J. Farrar, P. Singhasivanon, N.J. White, M. Ashton, and N.P. Day**, Quantification of artemisinin in human plasma using liquid chromatography coupled to tandem mass spectrometry. J Pharm Biomed Anal, 2009. **49**(3): p. 768-73.
48. **Cappiello, A., G. Famiglini, P. Palma, E. Pierini, V. Termopoli, and H. Trufelli**, Overcoming matrix effects in liquid chromatography-mass spectrometry. Anal Chem, 2008. **80**(23): p. 9343-8.

49. **Campins-Falco, P., R. Herraez-Hernandez, and A. Sevillano-Cabeza**, Column-switching techniques for high-performance liquid chromatography of drugs in biological samples. J Chromatogr, 1993. **619**(2): p. 177-90.
50. **Srinivas, N.R. and R. Mullangi**, An overview of various validated HPLC and LC-MS/MS methods for quantitation of drugs in bile: challenges and considerations. Biomed Chromatogr, 2011. **25**(1-2): p. 65-81.
51. **Bhamidipati, R.K., J. Morizzi, F.C. Chiu, D.M. Shackleford, and S.A. Charman**, Simultaneous determination of OZ277, a synthetic 1,2,4-trioxolane antimalarial, and its polar metabolites in rat plasma using hydrophilic interaction chromatography. J Chromatogr B Analyt Technol Biomed Life Sci, 2009. **877**(27): p. 2989-95.
52. **Hanpithakpong, W., B. Kamanikom, A.M. Dondorp, P. Singhasivanon, N.J. White, N.P. Day, and N. Lindegardh**, A liquid chromatographic-tandem mass spectrometric method for determination of artesunate and its metabolite dihydroartemisinin in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci, 2008. **876**(1): p. 61-8.
53. **Cesar, I.C., J.A. Ribeiro, S. Teixeira Lde, K.B. Bellorio, F.C. de Abreu, J.M. Moreira, P.R. Chellini, and G.A. Pianetti**, Liquid chromatography-tandem mass spectrometry for the simultaneous quantitation of artemether and lumefantrine in human plasma: application for a pharmacokinetic study. J Pharm Biomed Anal, 2011. **54**(1): p. 114-20.
54. **Nwaka, S. and A. Hudson**, Innovative lead discovery strategies for tropical diseases. Nat Rev Drug Discov, 2006. **5**(11): p. 941-55.
55. **White, N.J.**, Qinghaosu (artemisinin): the price of success. Science, 2008. **320**(5874): p. 330-4.
56. **Keiser, J. and J. Utzinger**, Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. Trends Parasitol, 2007. **23**(11): p. 555-62.
57. **Hien, T.T., N.T. Truong, N.H. Minh, H.D. Dat, N.T. Dung, N.T. Hue, T.K. Dung, P.Q. Tuan, J.I. Campbell, J.J. Farrar, and J.N. Day**, A randomized controlled pilot study of artesunate versus triclabendazole for human fascioliasis in central Vietnam. Am J Trop Med Hyg, 2008. **78**(3): p. 388-392.
58. **Li, Q., L.H. Xie, T.O. Johnson, Y. Si, A.S. Haeberle, and P.J. Weina**, Toxicity evaluation of artesunate and artelinate in Plasmodium berghei-infected and uninfected rats. Trans R Soc Trop Med Hyg, 2007. **101**(2): p. 104-12.
59. **Stringham, R.W., K.G. Lynam, P. Mrozinski, G. Kilby, I. Pelczer, and C. Kraml**, High performance liquid chromatographic evaluation of artemisinin, raw material in the synthesis of artesunate and artemether. J Chromatogr A, 2009. **1216**(51): p. 8918-25.
60. **Vennerstrom, J.L., S. Arbe-Barnes, R. Brun, S.A. Charman, F.C. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. Santo Tomas, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin, and W.N. Charman**, Identification of an antimalarial synthetic trioxolane drug development candidate. Nature, 2004. **430**(7002): p. 900-4.
61. **Dong, Y., J. Chollet, H. Matile, S.A. Charman, F.C. Chiu, W.N. Charman, B. Scorneaux, H. Urwyler, J. Santo Tomas, C. Scheurer, C. Snyder, A. Dorn, X. Wang, J.M. Karle, Y. Tang, S. Wittlin, R. Brun, and J.L. Vennerstrom**, Spiro and dispiro-1,2,4-trioxolanes as antimalarial peroxides: charting a workable structure-activity relationship using simple prototypes. J Med Chem, 2005. **48**(15): p. 4953-61.

# Curriculum Vitae

Urs Philipp Duthaler

# Curriculum Vitae

## Urs Philipp Duthaler

Pharmacist


| | |
|---|---|
| **Name:** | Urs Philipp Duthaler |
| **Date of birth:** | November 22nd, 1983 |
| **Place of origin:** | Basel and Hittnau (ZH) |
| **Nationality:** | Swiss |


## PhD Study

| | |
|---|---|
| **02/2008 – 05/2011** | PhD in Pharmaceutical Sciences<br>Swiss Tropical and Public Health Institute (University of Basel) |
| **Thesis title** | Artemisinins for the Treatment of Fascioliasis:<br>Progress in Preclinical and Diagnostic Research |
| **Supervision** | Prof. Dr. Jennifer Keiser and Prof. Dr. Jörg Huwyler |
| **Collaborations** | University of Basel, Division of Pharmaceutical Technology<br>University of Applied Sciences Northwestern Switzerland |

**Presentations**

| | |
|---|---|
| March 2011 | Research Seminar, Swiss Tropical and Public Health Institute, Basel, Switzerland<br>"*Progress in the discovery of antischistosomal and fasciocidal drugs*" |
| November 2010 | The American Society of Tropical Medicine and Hygiene (ASTMH), 59th ASTMH Meeting, Atlanta, USA<br>"*The potential of combination chemotherapy in the treatment of* Fasciola hepatica *infections*" |
| January 2010 | Annual Research Meeting, Department of Pharmaceutical Sciences (University of Basel), Basel, Switzerland<br>"*Analysis of the artemisinins by high-performance liquid chromatography-tandem mass spectrometry*" |

**Lectures**

| | |
|---|---|
| Autumn semester 2010 | Master Program in Infection Biology and Epidemiology<br>Drug Discovery and Development for Parasitic Diseases (2 lectures) |

## Education

| | |
|---|---|
| **10/2002 – 10/2007** | Studies of Pharmaceutical Sciences at University of Basel, Switzerland |
| | *2007:* Federal diploma as pharmacist |
| | *2006:* Master in Pharmaceutical Sciences<br>Diploma thesis:<br>*"Determination of the carrier mediated and passive drug transport in Caco-2 cell cultures"* |
| **08/1997 – 06/2002** | High school diploma (Matura) at Gymnasium Bäumlihof Basel-Stadt (specialisation in music). |

## Work Experience

| | |
|---|---|
| **10/2007 – 06/2010** | Part-time work at Hard Apotheke as pharmacist, Birsfelden, Basel-Land |
| **10/2006 – 08/2007** | Practical year at Hard Apotheke, Birsfelden, Basel-Land (Assistenzjahr) |
| **02/2003 & 09/2003** | Six weeks Traineeship at Apotheke zum Wendelin, Riehen, Basel-Stadt |

## Additional Skills

| | | |
|---|---|---|
| **Languages** | German | (native speaker) |
| | English | (fluent) |
| | French | (basic) |
| **Trainings** | June 2009: | Seminar in Analytics - Applica 09 |
| | February 2008: | Laboratory animal course I (1 week) |
| | | LTK 1 - Labortierkunde Kurs 1 |
| **PC Literacy** | Proficient user | |
| **Applications** | Good command of Microsoft Office applications | |
| | Basic command of Adobe Photoshop & Illustrator | |
| **Laboratory skills** | Handling of laboratory animals (mouse, rat, hamster) | |
| | Development of analytical methods (HPLC-UV-MS/MS) | |
| | Coprodiagnostic techniques (helminthic diseases) | |
| | Cell culture technique (Caco-2 cells) | |

## List of publications

1. **Duthaler U**, Huwyler J, Rinaldi L, Cringoli G, Keiser J.
   *Evaluation of the pharmacokinetic profile of artesunate, artemether and their metabolites in sheep naturally infected with* Fasciola hepatica.

   Veterinary Parasitology, in press

2. **Duthaler U**, Keiser J, Huwyler J.
   *Development and validation of a liquid chromatography and ion spray tandem mass spectrometry method for the quantification of artesunate, artemether and their major metabolites dihydroartemisinin and dihydroartemisinin-glucuronide in sheep plasma.*

   Journal of Mass Spectrometry, 2011

3. **Duthaler U**, Smith TA, Keiser J.
   *In vivo and in vitro sensitivity of* Fasciola hepatica *to triclabendazole combined with artesunate, artemether, or OZ78.*

   Antimicrobial Agents Chemotherapy, 2010

4. **Duthaler U**, Rinaldi L, Maurelli MP, Vargas M, Utzinger J, Cringoli G, Keiser J.
   Fasciola hepatica*: comparison of the sedimentation and FLOTAC techniques for the detection and quantification of faecal egg counts in rats.*

   Experimental Parasitology, 2010

5. Keiser J, **Duthaler U**, Utzinger J.
   *Update on the diagnosis and treatment of food-borne trematode infections.*

   Current Opinion in Infectious Disease, 2010

6. Keiser J, Veneziano V, Rinaldi L, Mezzino L, **Duthaler U**, Cringoli G.
   *Anthelmintic activity of artesunate against* Fasciola hepatica *in naturally infected sheep.*

   Research in Veterinary Sciences, 2010

## References

Prof. Dr. Jennifer Keiser
Swiss Tropical and Public Health Institute
Socinstrasse 57
4002 Basel

E-mail: jennifer.keiser@unibas.ch

Prof. Dr. Jörg Huwyler
Department of Pharmaceutical sciences
Klingelbergstrasse 50
4056 Basel

E-mail: joerg.huwyler@unibas.ch

## Lectures

**As a student and PhD student I have attended lectures and courses given by**

B. Ernst, A. Zuberbühler, P. Hauser, M Lampert, Ch. Körner, H. Walser, P. Oelhafen, H.P. Hauri, W. Keller, W. Schaffner, W.D. Woggon, H. Wennemers, J. Seelig, U. Spornitz, K. Beier, D. Kunz, Ch. Dehio, U. Aebi, H. Im Hof, W. Gehring, U. Jenal, S. Grzesiek, T. Kiefhaber H. Reichert, T. Schirmer, M. Spiess, M. Affolter, Th. Boller, H. Müller, A. Vedani, T. Guenthert, H. Leuenberger, G. Imanidis, G. Betz, C. Rehmann-Sutter, K. Berger-Büter, M. Hamburger, St. Krähenbühl, B. Giese, J. Huwyler, J. Drewe, T.A. Bickle, O. Mayans, C. Zaugg, R. Schlienger, Ch. Meier, K. Hersberger, M. Kessler, St. Mühlebach, A. Scholer, C. André, E. Anderegg, M. Mütsch, A. Kropf, B. Wittwer, Ch. Surber, G. Folkers, U. Séquin, H.J. Güntherodt, G. Pluschke, P. Odermatt, P. Vounatsou, T. Smith, R. Brun, J. Keiser, M. Tanner.