

CHAPTER 3

This chapter consists of a full length text article that has been submitted to *Expert Opinion on Drug Metabolism and Toxicology*. In this chapter the author guidelines are given, followed by the article prepared according these guidelines. The aims discussed in this chapter were:

- 1) to investigate the bioavailability of artemisone entrapped in Pheroid® (Pheroid® test formulation) and artemisone only (reference formulation) in a non-human primate model.
- 2) to determine the *in vitro* metabolism of artemisone (reference and Pheroid® test formulations) by measuring the hepatic and intestinal metabolism of artemisone by using human and monkey liver and intestinal microsomes, as well as recombinant CYP3A4 enzymes. Results of this study were also presented at 3 conferences and are added as annexure A-C

EXPERT OPINION

Expert Opinion on Drug Metabolism and Toxicology: Guidelines for Original Research

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Date Submitted: 18-Nov-2013

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The effect of the Pheroid delivery system on the *in vitro* metabolism and *in vivo* pharmacokinetics of artemisone

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¹ Details concerning poster presentations of this work:

1. Grobler, L. Grobler, A., Haynes, R. Steyn, F. & Wiesner, L. The effect of Pheroid® technology on the bioavailability of artemisone in primates. 1st Pan-African summer School in Nanomedicine, CSIR, Pretoria, 2012. (Poster).;
2. Grobler, L. Grobler, A., Haynes, R. & Steyn, F. The effect of Pheroid® technology on the bioavailability of artemisone in primates. 33rd Annual Conference of the Academy of Pharmaceutical Sciences of South Africa, Rhodes University, Grahamstown, 2012. (Poster).;
3. Grobler, A.F, Steyn, J.D., Wiesner, L., Haynes, R., Meeding, L., Gibhard, L. & Kotze, A. Comparative pharmacokinetic analysis of novel antimalarial artemisinin derivatives after oral administration using PheroidT technology. 3rd Pharmaceutical Sciences Fair and Exhibition, Prague, Czech Republic. 2011 (Poster).

Abstract

The pharmacokinetics (PK) of artemisone and artemisone entrapped in the Pheroid drug delivery system were determined in healthy primates. The *in vitro* metabolism of the formulations was determined in human and monkey liver and intestinal microsomes. For the PK study, a single oral dose of 60 mg/kg artemisone was administered to Vervet monkeys using a crossover design. Plasma samples were analysed for artemisone and the principal metabolite M1 by means of LC-MS/MS. For the *in vitro* metabolism study, clearance was determined using microsomes and recombinant CYP3A4 enzymes and samples were analysed by means of UPLC-QTOF-MS.

Artemisone demonstrated rapid absorption with $T_{\max}$ of 5.11 and 3.44 hours for the reference and test formulations respectively. The metabolite, M1, appeared in the plasma after 30 minutes and had a $T_{\max}$ similar to the parent compound. Since artemisone and M1 plasma levels were unexpectedly low in view of that described for man and rodent, first-pass metabolism was evaluated. The *in vitro* intrinsic clearance (CL_{int}) of the reference formulation with monkey liver microsomes (MLM) is much higher (1359.33 ± 103.24 vs. 178.86 ± 23.42) than that of human liver microsomes (HLM). The *in vitro* results also suggest that microsomal metabolism of artemisone is inhibited by the Pheroid delivery system.

Keywords: artemisone, clearance, microsomes, monkey, pharmacokinetics, Pheroid

1. Introduction

A major limitation of the artemisinins is their low aqueous solubility that causes poor and inconsistent absorption upon oral administration, resulting in low bioavailability¹. Another limitation is their short half-life, which requires frequent administration, leading to non-compliance, recrudescence, and possibly the development of resistance^{2,3}. As a result, the World Health Organisation (WHO) recommends artemisinin-based combination (ACT) treatments as first line treatment of uncomplicated malaria⁴. Combinations are effective because the artemisinin component eradicates the majority of parasites at the start of the treatment, while the long half-life partner drug eliminates the remaining parasites⁵.

Artemisinins have a peroxide bridge incorporated within the trioxane unit, which is crucial for their antimalarial activity⁶. The new artemisinin derivative artemisone shows enhanced antimalarial activity, superior bioavailability to other derivatives, as well as the benefit of no detectable neurotoxic potential^{7,8}.

CYP3A4 is regarded as the fundamental enzyme for phase I biotransformation of artemisone in humans. The phase I reactions include the dehydrogenation in the thiomorpholine-*S,S*-dioxide moiety leading to metabolite M1, monohydroxylation in the methyloxepane and methylcyclohexyl moieties, leading to the metabolites M2 and M3 respectively and dehydrogenation of M2 and M3 leading to M4 and M5.

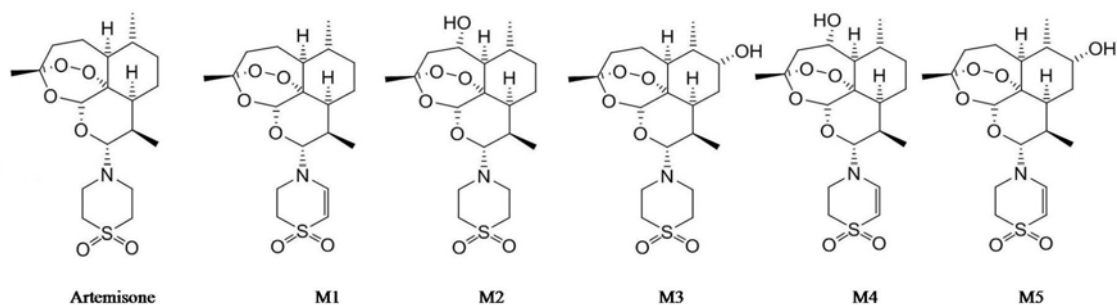


Figure 1: Artemisone and the metabolites M1-5 formed during metabolism

The main restriction to oral administration of a drug is the consequence of poor systemic bioavailability arising as a result of rapid clearance or poor absorption of the drug^{9,10}. Due to the first pass effect, drugs with a high metabolic clearance have a low bioavailability¹¹. Drug absorption in the gastrointestinal tract may be limited by poor physicochemical properties of the drug, insufficient contact time in transit through the gastrointestinal tract or poor permeability across the gastrointestinal mucosa. Also, because most drugs are recognized as foreign substances by the body, they are actively pumped back from enterocytes into the gastrointestinal lumen by intestinal efflux proteins^{12,13}.

Recently, artemisinin-loaded polyethylene glycol (PEGylated) liposomes were shown to increase the half-life of artemisinin by more than 5-fold compared to free artemisinin¹⁴. Antimalarial activity was also enhanced when artemisinin-loaded conventional liposomes, artemisinin-curcumin-loaded conventional liposomes, artemisinin-loaded PEGylated liposomes and artemisinin-curcumin-loaded PEGylated liposomes were assessed in *Plasmodium berghei* NK-65 infected mice¹⁵. It was therefore suggested that liposomes loaded with artemisinin may represent efficacious therapeutic nanocarriers for the treatment of tumors and parasitic diseases^{14,15}.

The aim of this study was to investigate the effect of the Pheroid[®] delivery system on the *in vitro* metabolism and *in vivo* pharmacokinetics (PK) of artemisone. The Pheroid technology is a stable colloidal formulation that is able to entrap, transport and deliver pharmacologically active compounds and other molecules^{16,17}. The multifunctional properties of the Pheroid technology led to a number of different applications^{18,19,20,21}. It primarily consists of an oil phase and a nitrous oxide saturated water phase (N₂O)-water phase^{18,21,17}. The Pheroid vesicle oil phase consists mostly of vitamin F ethyl ester, pegylated ricinoleic acid (Kolliphor) and α -tocopherol^{18,19,20}. The size and structure of the Pheroid formulations can be manipulated to form lipid-bilayer vesicles, microsponges or pro-Pheroid, by altering the ratios of components and/or the process of preparation²². The pro-Pheroid formulations do not contain a water phase and therefore only consist of the oil phase saturated with nitrous oxide gas. Pheroid micro- and nano-particles form spontaneously upon addition of a water phase to the pro-Pheroid. During this reaction, the active pharmaceutical ingredient (API) present in the pro-Pheroid is packaged into the particles²².

In a C57 BL/6 mouse model, the Pheroid delivery system was shown to enhance the bioavailability of artemisone¹⁷. Significant differences were found between these two formulations with a greatly increased C_{max} (1550.0 vs. 809.5 ng/mL) for the artemisone-Pheroid vesicle formulation compared to the artemisone reference formulation. The AUC_{0-last} and AUC_{0-inf} also increased (2219.0 and 3094.0 ng h/mL vs. 458.7 and 604.6 ng h/mL) with the Pheroid vesicle formulation. It was proposed that by entrapping artemisone in the Pheroid delivery system, the bioavailability of artemisone may be increased. Here we examine this aspect by using a Vervet monkey model.

2. Methods

2.1 Materials

Artemisone and metabolite M1 were prepared by Ho-Ning Wong and Wing-Chi Chan in the Department of Chemistry at the Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong. Vitamin F ethyl ester was obtained from Chemimpo (South Africa) and Kolliphor[®] EL was obtained from BASF (South Africa). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), polyethylene glycol (PEG 400) and dl- α -tocopherol were obtained from Chempure (South Africa). Purified water was obtained from SABAX (Johannesburg, South Africa). Recombinant CYPs 3A4 was obtained from CYPEX (Dundee, UK). Human liver microsomes (HLM), human intestinal microsomes (HIM), Cynomolgus monkey liver microsomes (MLM) and Cynomolgus monkey intestinal microsomes (MIM) were obtained from Xenotech (Kansas, USA). NADPH and azamulin were purchased from Sigma Chemical Co. (St. Louis, MO). Capsules were purchased from CapsuGel (Durban, South Africa). All other chemicals and reagents used in this study were of analytical reagent grade.

2.2 In vivo pharmacokinetic studies

2.2.1 Preparation of artemisone reference and test formulations

To prepare the reference formulation, capsules were filled with artemisone in accordance to the weight of each monkey to obtain a dosage of 60 mg/kg. To prepare the Pheroid formulation, artemisone (0.18 g) was dissolved in PEG 400 (20 g), heated to 70 °C and sonicated. Vitamin F ethyl ester (55.72 g), Kolliphor EL (23.22 g), BHA (0.01 g) and BHT (0.01 g) were added and heated to 70 °C and sonicated. DL- α -tocopherol (1.0 g) was then added and the mixture gassed with nitrous oxide (N₂O) under pressure (200 kPa). The

capsules were filled with the artemisone-pro-Pheroid for a comparative dose of 60 mg/kg in accordance with the weight of each monkey.

2.2.2 Drug administration and sample analysis

Vervet monkeys (*Chlorocebus Aetiops*) were maintained at the North-West University, Potchefstroom, South Africa. The NWU Ethics Committee for animal experimentation approved all experiments (approval number of NWU-00027-10-A5). Reference (artemisone) and test formulations (artemisone entrapped in pro-Pheroid) were administered to Vervet monkeys (n = 10) as a single dose of 60 mg/kg, using capsules as dosage form. A crossover design with a one week washout period was used. The monkeys were fasted overnight and were given access to food approximately 5 h post dose. The monkeys were anaesthetized with ketamine hydrochloride (10 mg/kg) to enable handling and blood sampling. Blood samples were drawn in heparin through percutaneous venipuncture of the femoral vein pre-dose and at 0.5, 1, 2, 3, 5, 8 and 10 hours post-dose. Plasma samples were prepared by centrifugation at 4 °C and obtained plasma samples were immediately frozen at -196 °C and then stored at -80 °C. The samples were transported overnight on dry ice to the University of Cape Town for analysis.

Parent drug concentrations and metabolite M1 in the plasma samples were determined using a validated quantitative LC-MS/MS assay. The liquid-liquid extraction procedure was performed on ice. Calibrations standards and quality control standards were prepared in drug free (blank) primate plasma. Twenty microlitres of monkey plasma sample were extracted using 200 µL of a universal Britton Robinson buffer (pH 10), artemisinin (internal standard) and 1 mL of the organic solvent 1-chlorobutane. Each sample was thoroughly mixed by vortexing for 2 minutes and centrifuged at 2000 g for 5 minutes. The organic phase (800 µL)

was transferred to clean tubes and evaporated under vacuum for 30 min at room temperature. The dried samples were reconstituted with 100 μ L mobile phase consisting acetonitrile and 0.1 % formic acid, mixed by vortexing and transferred to 96 well polypropylene plates. Gradient chromatography was performed on a Phenomenex Luna 5 μ m PFP(2), 100 A, 50 mm $\times$ 2 mm analytical column. The mobile phase was delivered at a flow rate of 500 μ L/min. The organic phase was increased from 50% to 90% over 3 minutes, and the organic phase returned to 50% between 3.1 and 7 minutes to equilibrate the column. An Agilent 1200 series autosampler injected 2 μ l onto the HPLC column. An AB Sciex API 4000 mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 402.1 to the product ions at m/z 267.0 for artemisone, and the protonated molecular ions at m/z 400.1 to the product ions m/z 267.1 for M1, and the protonated molecular ions at m/z 283.2 to the product ions m/z 209.1 for artemisinin.

2.2.3 Statistical analysis

Descriptive statistics are reported for each characteristic in both reference and pro-Pheroid groups as mean $\pm$ SD. Log-transformation of the pharmacokinetic parameters $C_{\max}$, $AUC_{0-\text{last}}$, $T_{\max}$ and $t_{1/2}$ was used to compare differences in pharmacokinetic parameters of artemisone when given alone and when artemisone was entrapped in the Pheroid. Statistical comparison of the data was done by analysis of variance (ANOVA) at a significance level of $p < 0.05$, using the GLM-procedure of SAS²³. Since a two-way crossover design was used, the product effect was tested for by controlling for the phase, the sequence and the subjects by sequence effects.

2.3 *In vitro* metabolism studies

2.3.1 *Preparation of artemisone reference and test formulations*

The 1 mM reference formulation for the *in vitro* metabolism studies was prepared with artemisone (0.0401 g) in acetonitrile (100 mL). Deionised water was used for dilutions. Artemisone pro-Pheroid formulations were prepared as described (section 2.2.1.2) but with artemisone concentrations of 0.0400 g artemisone per 1 g pro-Pheroid. A drug-free pro-Pheroid formulation, containing no entrapped drug, was also prepared according to the above described method and used for dilution of the study formulations.

2.3.2 *Metabolic stability and inhibitor phenotype assays*

Artemisone-reference and pro-Pheroid formulations were incubated with microsomes (0.5 mg/mL) and 0.1 M phosphate buffer (pH 7.4). After pre-incubation for 10 minutes, the reaction was initiated by the addition of NADPH. Aliquots were collected at various time points up to 45 minutes. The reaction was stopped by the addition of ice cold acetonitrile. Samples were stored at 4 °C to allow protein precipitation and centrifuged at 10 000 g for 15 minutes at 4 °C. Supernatants were stored at -20 °C until analysis. For inhibitor phenotype assays, similar reaction mixtures were used as described above but azamulin (5µM) was added as a CYP3A4 inhibitor. In addition, artemisone-reference and pro-Pheroid formulations were incubated with CYP3A4 recombinant enzyme (10 pmol/mL). These reactions were initiated by the addition of NADPH, and stopped at zero (baseline) and 30 minutes with the addition of ice cold acetonitrile. The samples were transported on ice to the Council for Scientific and Industrial Research (CSIR) for analysis. The qualitative analysis of artemisone and its metabolites were performed on a Waters ultra performance HPLC system coupled to a G1 hybrid quadrupole Time-of-Flight mass spectrometer (UPLC-QTOF-

MS, (Waters, Milford, MA, USA). Chromatographic separation was achieved on a Waters Acquity UPLC using Acquity HSS C18 150 x 2.1 mm (1.7 μ m) column maintained at 60 °C. The initial mobile phase consisted of 80% water containing 0.1% (v/v) formic acid and 20% acetonitrile at a flow rate of 400 μ L/min. The composition was changed to 10% water and 90% acetonitrile at 10 minutes. These conditions were maintained for 2 minutes whereafter the column was re-equilibrated at the initial conditions. The run time was 15 minutes and the injection volume was 10 μ L. Metabolynx, a Masslynx application software program, was used for metabolite identification. The reaction samples were scanned for specific m/z values corresponding to reported metabolites, which in turn confirms microsomal metabolism. The M1 metabolite standard was analysed on the UPLC-QTOF-MS and displayed a $2x [M+H]^+$ product of m/z 799.3509 suggesting a dimer-type structure formed in gas phase ($C_{38}H_{58}N_2O_{12}S_2$). Cationisation was also observed resulting in a mass ion at m/z 422.1613 corresponding to $C_{19}H_{28}NO_6SNa$.

2.3.3 Determination on intrinsic clearance

The log percentage remaining was plotted against incubation time and the gradient of the line was used to calculate the elimination rate constant (k , equation 1) and the half life ($t_{1/2}$, equation 2)²⁴. The *in vitro* intrinsic clearance was calculated according to equation 3.

$$\text{Elimination rate constant (k)} = (-\text{gradient}) \quad \text{equation 1}$$

$$\text{Half life (t}_{1/2}\text{) (min)} = \ln 2/k \quad \text{equation 2}$$

$$\text{Intrinsic clearance (CL}_{\text{int}}\text{) (mL/min/mg)} = \frac{\ln 2 \times \text{volume of incubation (mL)}}{t_{1/2} \text{ (min)} \times \text{protein incubation (mg)}} \quad \text{equation 3}$$

In the case of the liver microsomal study, the units of CL_{int} were converted to the apparent clearance for the whole liver (whole liver CL_{int}) by using equation 4. Because there is no

widely used physiological conversion model from mL/min/mg protein to mL/min/kg applicable to the intestine, only CL_{int} was calculated.

$$\text{Whole liver } CL_{int} \text{ (mL/min/kg)} = \text{In vitro } CL_{int} \times \text{MPPGL} \times \text{liver weight} \quad \text{equation 4}$$

MPPGL is the microsomal protein per gram of liver. Assuming that MPPGL is the same for Cynomolgus monkeys and humans, 48.8 mg/g liver was used for both species²⁵. For humans and Cynomolgus monkeys, 5.7 and 30.0 liver weight per kilogram of body weight were used respectively²⁶. The *in vivo* hepatic clearance (CL_H) was estimated according to the ‘well-stirred liver’ model²⁷ by using equation 5.

$$\text{In vivo } CL_H \text{ (mL/min/kg)} = \frac{Q_H \times \text{whole liver } CL_{int}}{Q_H + \text{whole liver } CL_{int}} \quad \text{equation 5}$$

Q_H is the hepatic blood flow. Q_H is given a value of 21 mL/min/kg and 43.6 mL/min/kg for human and monkeys respectively²⁶. The monkey microsomes used in this study were from Cynomolgus monkeys because Vervet monkey microsomes were not commercially available. Therefore it was assumed that all Cynomolgus monkey parameters used to calculate clearance is the same as in the Vervet monkey.

2.3.4 Statistical analysis

One-way ANOVA's were used to determine the significance product and system effects. This was followed by Tukey multiple comparison tests to compare group means mutually or by Dunnett's test to compare the means of groups with that of a control group. When comparing only two group means, the Student t-test was used. Statistical testing of the data was done at a significance level of $p < 0.05$, using the Statistica computer package²⁸.

2.4 Pro-Pheroid characterization

The particle size and zeta potential of the artemisone entrapped Pheroid formulation (P11015 and P11022) for *in vivo* PK studies, artemisone entrapped Pheroid formulation (P11018) and blank Pheroid formulation (P11020) for *in vitro* metabolism studies were measured.

The pro-Pheroid was mixed with 0.1 N hydrochloric acid (1:100 v/v) and the size and surface charge (zeta potential) of the resulting Pheroid vesicles was measured using a Hydro Malvern Mastersizer 2000MU and a Malvern 2000 zetasizer (Malvern Instruments Ltd, Malvern, Worcestershire, UK). For particle size measurement, samples were measured in duplicate by diluting samples with water before injection. Span value was calculated by means of the equation $(d_{0.9} - d_{0.1}) / d_{0.5}$ where $d_{0.9}$, $d_{0.5}$ and $d_{0.1}$ are the particle diameters determined respectively at the 90th, 50th and 10th percentile of the particles. For surface charge measurements, the analyses were performed in de-ionized water and all measurements were performed in triplicate. The morphology of the particles was characterized by confocal laser scanning electron microscopy (CLSM, Nikon D-eclipse C1 confocal laser scanning microscope, the Netherlands) using the method described by Slabbert and colleagues²⁹ by staining the Pheroid formulations with Nile Red.

3. Results and discussion

3.1 Pro-Pheroid characterization

Table 1 shows the particle size distribution of the Pheroid formulations. Span value, as an index of polydispersity, of the formulations was calculated from the particle diameters from the 90th ($d_{0.9}$), 50th ($d_{0.5}$) and 10th ($d_{0.1}$) percentile of the particles.

Table 1: The particle size of the different Pheroid formulations

Pheroid formulations	Mean particle size	d_{0.1} (μm)	d_{0.5} (μm)	d_{0.9} (μm)	Span
P11015	10.02 ± 0.77	2.07 ± 0.19	7.45 ± 0.51	18.09 ± 1.09	2.15 ± 0.03
P11022	13.33 ± 1.58	2.47 ± 0.68	12.01 ± 1.46	25.75 ± 3.93	1.93 ± 0.15
P11018	43.05 ± 1.20	6.05 ± 0.29	34.17 ± 0.85	92.80 ± 2.89	2.54 ± 0.03
P11020	34.45 ± 1.01	5.46 ± 0.25	27.35 ± 0.78	74.04 ± 2.47	2.51 ± 0.03

All formulations had a relatively low but positive (1-3 mV) zeta potential. This may be due to the formulations containing PEG. Pheroid vesicles that do not contain PEG display a negative zetapotential. Once a PEG layer coats nanoparticles, the zeta potential may change to positive³⁰. This low zeta potential is not regarded as unusual. According to the Gouy–Chapmann theory, the surface PEG chains’ hydrodynamic drag moves the hydrodynamic plane of shear away from the charge-bearing plane, and causes the electrophoretic mobilities to decrease³¹. Therefore, although PEG decreases the zeta potential, the amount of attraction between nanoparticles is also decreased by increasing the steric distance between them and, therefore, aggregation is inhibited³². The confocal laser scanning electron microscopy images indicates the successful formation of spherical particles.

3.2 *In vivo pharmacokinetics*

3.2.1 *LC-MS/MS assay*

The bioanalytical assay was validated for monkey plasma. The lower limit of quantification (LLOQ) for artemisone and metabolite M1 were 3.91 and 7.80 ng/mL, respectively (Figure 2). Calibration was performed in duplicate in the range from 3.91 ng/mL to 500 ng/mL for artemisone and 7.80 to 1000 ng/mL for M1.

The pharmacokinetic parameters of artemisone were determined after a single oral dose of 60 mg/kg artemisone to healthy monkeys. The pharmacokinetics of artemisone could not be determined in one monkey treated with the reference formulation, since this animal presented with an artemisone concentration of zero throughout the experiment (Table 2 and Figure 2).

Table 2: The plasma pharmacokinetics of artemisone (60 mg/kg orally) administered alone, or entrapped in the pro-Pheroid delivery system. Data are mean values $\pm$ SD in nine monkeys.

Dosage	Drug measured measured	AUC _{0-last} (ng·h/mL)	p-value	C _{max} (ng/mL)	p-value	T _{max} (hours)	p-value	t _{1/2} (hours)	p-value
Artemisone	Artemisone	184.4 $\pm$ 134.4	0.07	47.3 $\pm$ 44.5	0.16	5.1 $\pm$ 2.5	0.1	3.2 $\pm$ 1.7	0.51
Artemisone- Pheroid		466.0 $\pm$ 532.8		114.0 $\pm$ 156.7		3.4 $\pm$ 2.5		4.4 $\pm$ 3.7	

Log-transformation of C_{max}, AUC_{0-last}, T_{max} and t_{1/2} for artemisone and the metabolite M1 indicated that the pharmacokinetic parameters of the two formulations did not differ significantly (p>0.05). Taking into consideration the small groups of monkeys, indications of trends exist. The total drug exposure over 10 hours (AUC_{0-last}) was marginally (p=0.07) statistically significant and the test formulation appeared to be almost two fold higher than that of the reference formulation. The ratios between the geometric means for reference to test formulations (with 90% CI in brackets) were: C_{max}, 53% (28, 112) and AUC_{0-last} 46% (25, 93) and T_{max} 195% (101,378). Therefore the lower limits of the 90% CIs for the geometric mean ratios (reference to test formulations) of C_{max} and AUC_{0-last} and the upper limits of T_{max} were outside the 80–125% equivalence window, suggesting a clinically significant increase of C_{max} and AUC_{0-last} and a clinically significant decrease of T_{max} with the

test formulation. The Pheroid had no effect on artemisone $t_{1/2}$ values and the geometric mean ratios for reference to test formulations were not outside the 80–125% equivalence window.

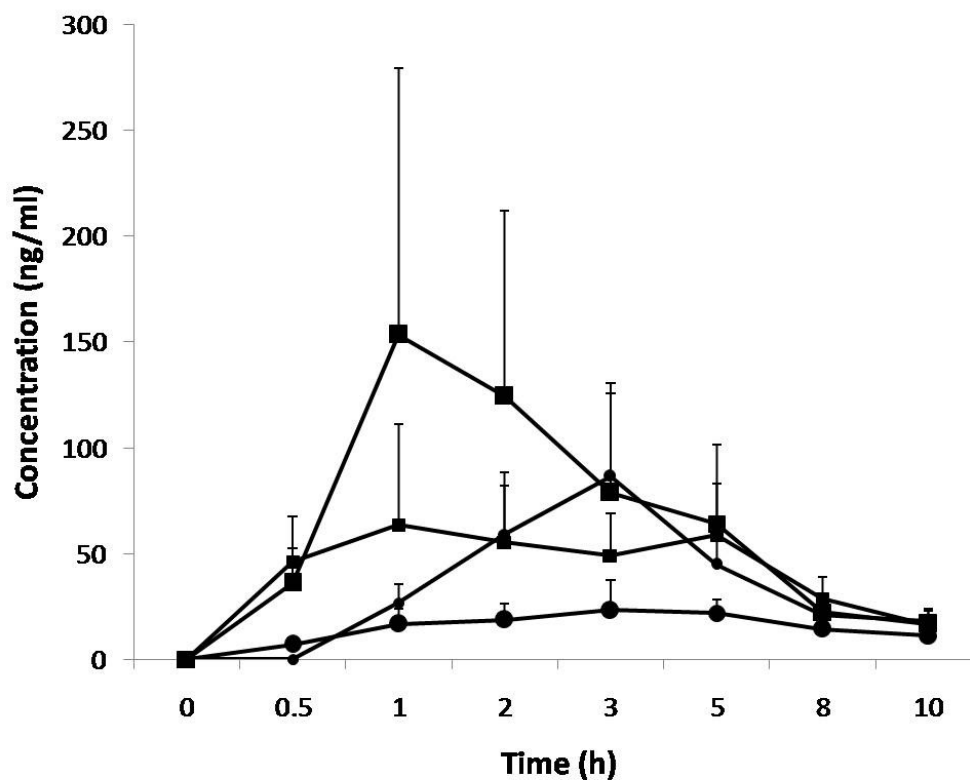


Figure 2: Effect of the Pheroid delivery system on artemisone and metabolite M1 (indicated with smaller circles or squares) plasma concentration in ng/mL; artemisone reference standard (circles) and pro-Pheroid (squares) formulation. Data depicted as the mean $\pm$ SE for each time interval.

The artemisone plasma levels are much lower ($C_{\max}$ of 47.39 and 113.97 ng/mL for reference and test formulations) than expected for the dosages administered. In a human Phase I study, where artemisone was administered at a dosage of 1 mg/kg, the $C_{\max}$ was 140.2 ng/mL⁸. Also in studies carried out by Steyn and colleagues¹⁷, artemisone reference or artemisone entrapped in the Pheroid vesicle formulations at a dose of 50.0 mg/kg to mice resulted in a

$C_{\max}$ of 809.5 ng/mL and 1550.0 ng/mL respectively. When these dosages are extrapolated to a human equivalent dose (HED) by using the body surface area normalization method³³, the 50 mg/kg bodyweight dosage given to the rodents are equivalent to a 4 mg/kg bodyweight human dose and, therefore, all of these results correlate well with human artemisone studies. Using this same extrapolation for the current Vervet monkey study, the dosage given is 20 times more than in the human clinical trials with a $C_{\max}$ that is in comparison, 3 fold lower and the AUC that is 1.5 fold lower for the reference formulation.

Although artemisone is highly crystalline with no polymorphs⁸, the likelihood of a possible polymorph being the cause of the lower plasma levels, were also investigated. The artemisone used in the studies was recrystallized at the Hong Kong University of Science and Technology (HKUST) using 50:50 hexane/ethylacetate (1:1) as solvent and the pharmacokinetics assessed in one Vervet monkey at the NWU. The AUC were again lower (140.8 vs 127.1 ng·h/mL) for the reference and higher (244.2 vs 267.8 ng·h/mL) for the Pheroid formulation.

3.4 In vitro metabolic stability and inhibition

The *in vitro* clearance of artemisone and the formation of metabolites were investigated in human liver and intestinal microsomes (HLM and HIM), and monkey liver and intestinal microsomes (MLM and MIM) to evaluate the effect of the Pheroid delivery system on the metabolic clearance of artemisone in humans and monkeys.

The percentage of artemisone remaining vs. time plots (Figures 3a-d) were subjected to linear regression analysis and the apparent terminal half-life ($t_{1/2}$) was calculated. The *in vitro* intrinsic clearance (CL_{int}) and estimated *in vivo* CL_H of artemisone were calculated as

indicated above. For the monkey liver microsomes, only time points zero to 10 h (time point where parent compound was depleted) were used to estimate the slope.

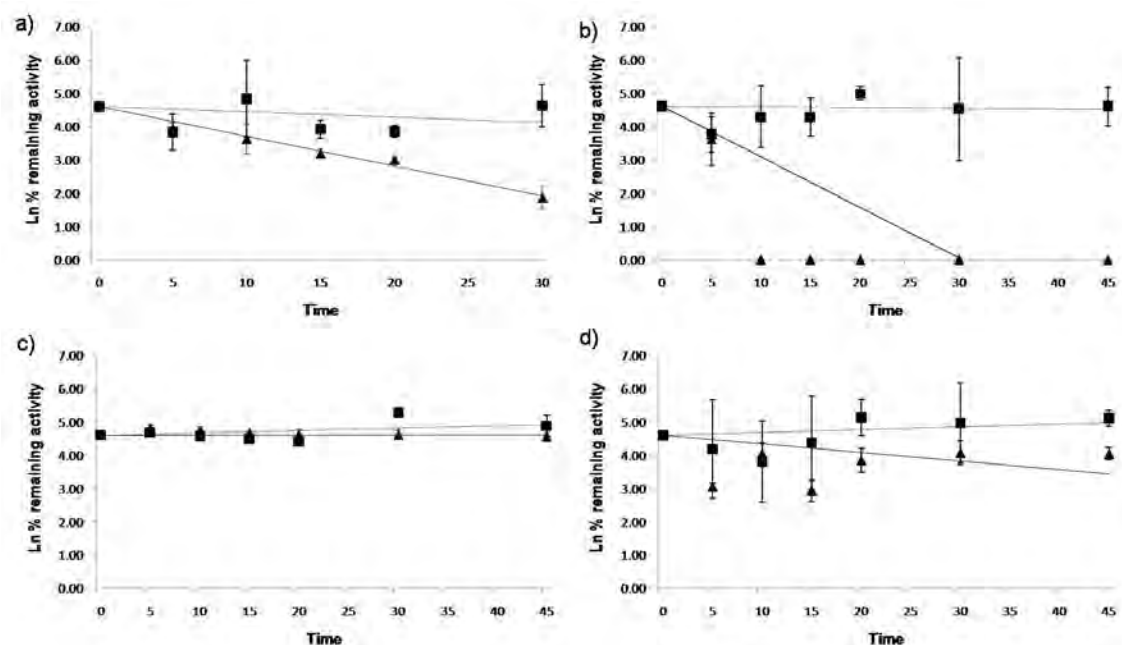


Figure 3: Metabolic stability of artemisone reference standard (triangle) and pro-Pheroid (squares) formulation in a) HLM, b) MLM, c) HIM and (d) MIM. Points are experimentally derived values (means of triplicate measurements at each time interval $\pm$ SD) while the solid (artemisone reference standard) and dashed lines (artemisone-pro-Pheroid) show computer-derived curves of best fit.

The results demonstrate that the Pheroid drug delivery system inhibits the metabolism of artemisone by the CYP450 system in both human and monkey model systems *in vitro* (Figure 3a-d). Also, as seen in Table 3, the *in vitro* intrinsic clearance (CL_{int}) of the reference formulation with MLM is much higher than that with HLM. If extrapolated to the estimated hepatic clearance *in vivo*, where the microsomal protein per gram of liver, the liver weight and hepatic blood flow are taken into account, the monkey CL_H is almost twice the CL_H in humans. It is clear that very slow metabolism of the artemisone reference occurred in HIM,

with faster metabolism occurring in the MIM (Figures 3b,d); thus, artemisone is metabolized in the liver as well as in the intestine of the monkey. These findings might be partly responsible for the species differences observed in the *in vivo* pharmacokinetics. Numerous drugs are subject to the first-pass effect and the organs responsible for this are mainly the intestine, liver and lung. These data suggest that artemisone undergoes extensive catabolism in both the liver and intestine of the monkey.

Table 3: The *in vitro* intrinsic clearance (CL_{int}) of the artemisone reference and artemisone-pro-Pheroid formulation in HLM, HIM, MLM and MIM and the estimated *in vivo* hepatic clearance (CL_H) for HLM and MLM for a 45 minute incubation period.

Parameter	<i>In vitro</i> CL_{int} ($\mu\text{L}/\text{min}/\text{mg}$)				Estimated <i>in vivo</i> CL_H ($\text{mL}/\text{min}/\text{kg}$)	
Species	HLM (a)	MLM (b)	HIM (c)	MIM (d)	HLM (e)	MLM (f)
Artemisone reference (A)	178.9 $\pm$ (Ba; Ab)	1359.3 $\pm$ (Bb; Aa;	4.0 $\pm$ 5.7 (Ab)	85.7 $\pm$ 3.0 (Ab)	19.2 $\pm$ 0.2 (Be; Af)	42.7 $\pm$ 0.1 (Ae)
Artemisone pro-Pheroid (B)	31.0 $\pm$ 3.3 (Aa)	0.0 $\pm$ 0.0 (Ab)	31.5 $\pm$	17.4 $\pm$	13.6 $\pm$ 0.5 (Ae)	14.8 $\pm$

*if $k > 0$, it was regarded as zero. Data depicted as mean $\pm$ SD of duplicate measurements.

Statistical differences ($p < 0.5$) are indicated in brackets by using the following keys:

Treatment A = artemisone; B = artemisone pro-Pheroid;

In vitro clearance of microsomal groups a = HLM; b = MLM; c = HIM and d = MIM

In vivo clearance of microsomal groups e = HLM; f = MLM

For example (Ba) states that there was a statistically significant difference between that parameter and the human liver microsomes treated with artemisone pro-Pheroid.

Figure 4 illustrates the chromatograms of the artemisone metabolites formed. The parent compound (formula: $C_{19}H_{31}NO_6S$, m/z : 402.1950, R_t 7.15 min), desaturation metabolite M1 dimer (formula: $C_{38}H_{59}N_2O_{12}S_2$, m/z : 799.3509, R_t 6.00 min), three other desaturation metabolites (formula: $C_{19}H_{30}NO_6S$, m/z : 400.1794, R_t 2.24, 4.79 and 6.04 min), hydroxylation-and-desaturation metabolite (formula: $C_{19}H_{30}NO_7S$, m/z : 416.1743, R_t 4.55, 4.79 and 6.04 min), mono-hydroxylation metabolite M2 (formula: $C_{19}H_{32}NO_7S$, m/z : 418.1899, R_t 4.25 min), mono-hydroxylation metabolite M3 (formula: $C_{19}H_{32}NO_7S$, m/z : 418.1899, R_t 3.59 min), three other mono-hydroxylation metabolites (formula: $C_{19}H_{32}NO_7S$, m/z : 418.1900, R_t 4.42, 3.82, and 4.79 min), di-hydroxylation metabolite (formula: $C_{19}H_{32}NO_8S$, m/z : 434.1849, R_t 4.22 min)

In total, 10 metabolites were produced by human liver microsomes. These consisted of M1, two other unsaturated metabolites, five mono-hydroxylated metabolites of which M2 and M3 are the major metabolites, one unsaturated mono-hydroxylated and one unsaturated di-hydroxylated metabolite were detected. In monkey liver microsomes, three monohydroxylated, metabolites of which M2 and M3 are the major metabolites, and one unsaturated monohydroxylated metabolite were observed. In the human and monkey intestinal microsomes two hydroxylated metabolites were observed. With the artemisone-Pheroid formulation, a unique unsaturated metabolite of retention time of 2.24 min was detected.

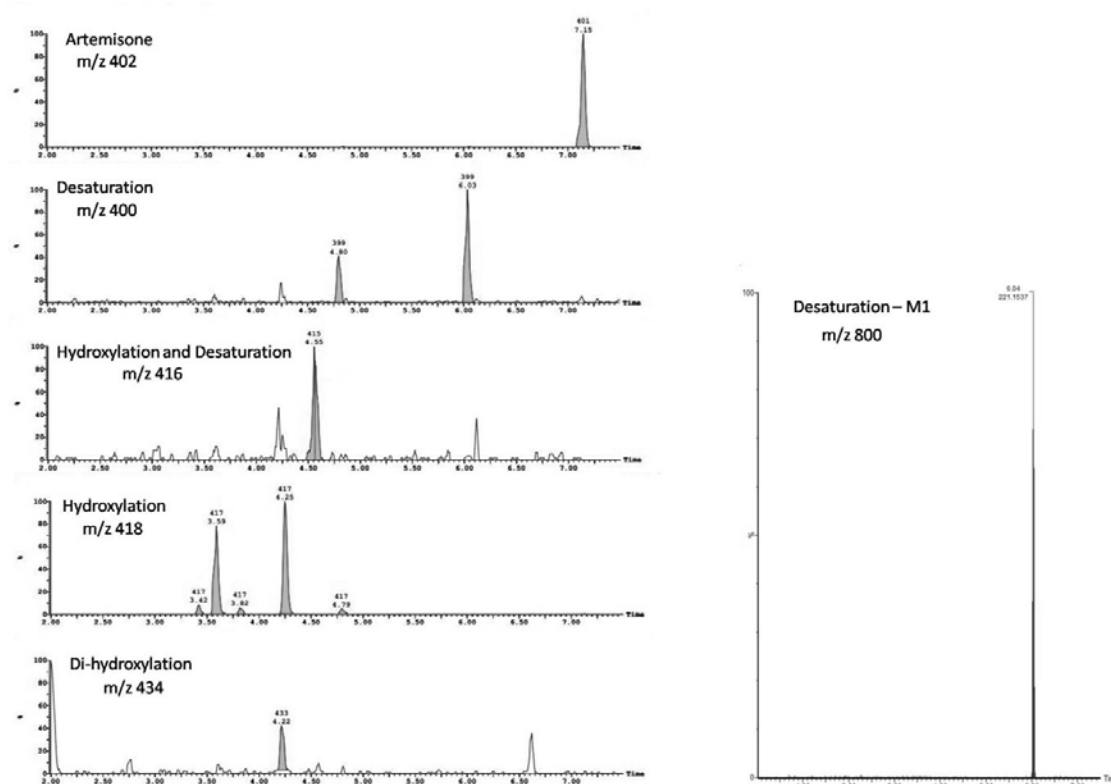


Figure 4: Selected ion chromatograms of artemisone and its metabolites obtained from the UPLC/MS scan (human liver microsomes at 45 min incubation).

The relative percentages of metabolites and parent compound were estimated by using the sum of peak areas of metabolites and parent compound at each time point (Figure 5). As illustrated by Figure 5, the formation of metabolites were inhibited with the Pheroid formulation. Only a small fraction of the artemisone was converted to M1 in the MLM, while a hydroxylation metabolite formed (RT 4.79) in all microsomes. The possibility exists that, in this particular *in vitro* system, artemisone is not released by the Pheroid and, therefore, cannot be metabolized by the enzymes. This might also explain the differences observed between the *in vitro* metabolism and *in vivo* PK studies where artemisone undergoes first pass metabolism once released in the body.

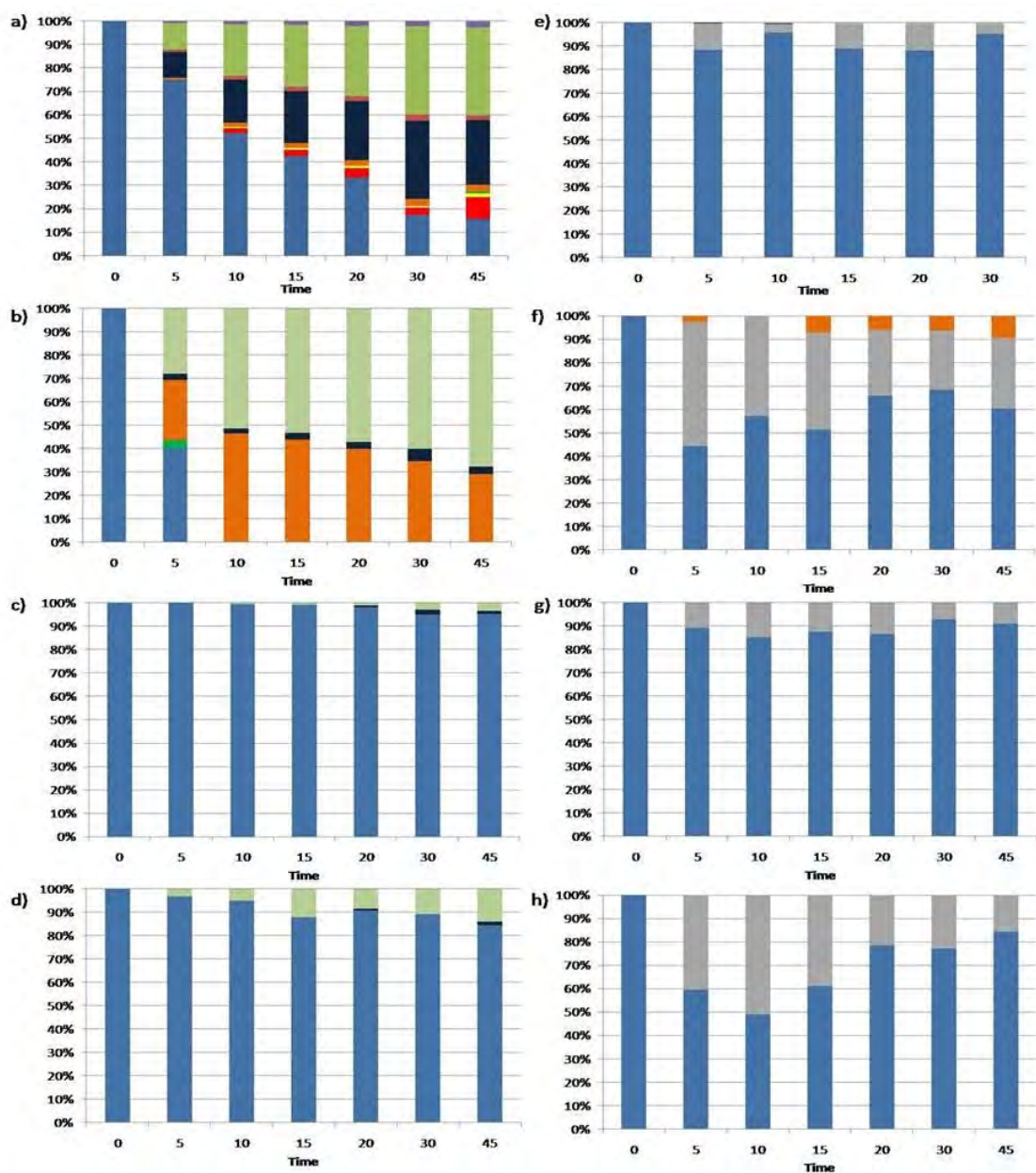


Figure 5: Percentage representation of the artemisone parent compound depletion and metabolite formation in a,e) HLM, c,g) HIM, b,f) MLM and d,h) MIM. Artemisone treatments depicted as a,b,c and d and artemisone entrapped in the Pheroid technology as e,f, g and h. The colours of artemisone and metabolites correspond with the colours indicated in Figure 7.

Figure 6 depicts the percentages of artemisone that remains following incubation in the different enzyme systems in the presence of azamulin. Previous studies have shown that CYP3A4 is the main hepatic CYP isoform responsible for the metabolism of artemisone in the liver⁷ and that azamulin is a specific inhibitor for CYP3A4³⁴. In the presence of azamulin, the percentage of artemisone remaining after the incubation time is close to 100% in HLM, HIM and MIM, indicating inhibition of artemisone metabolism by azamulin. For MLM, only $37.75 \pm 2.75\%$ of the parent compound was left, indicating that inhibition of artemisone was not achieved.

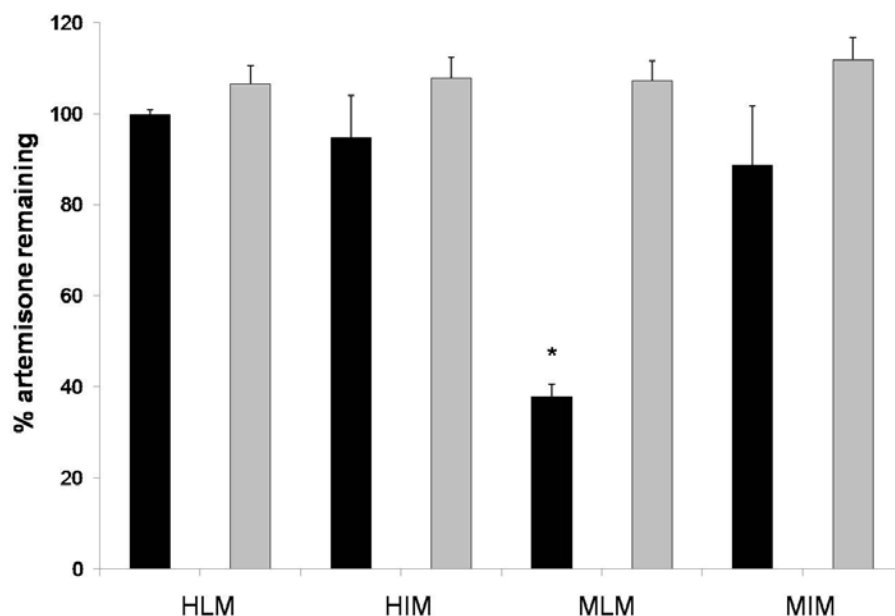


Figure 6: Effect of the Pheroid delivery system on artemisone clearance in the presence of the CYP3A4 inhibitor azamulin. Data of the artemisone reference standard (black) and pro-Pheroid (grey) formulation are depicted as mean $\pm$ standard deviation (SD) of triplicate measurements. Statistically significant differences are indicated by an asterisk (*) ($p < 0.05$).

It is apparent from Figure 7 that CYP3A4 is the responsible enzyme for artemisone metabolism. The percentage artemisone remaining after incubation with the recombinant CYP3A4 (rCYP3A4) compare well with the mixture with human liver microsomes containing other cytochromes in addition to CYP3A4 ($p > 0.05$). Also, having artemisone entrapped in the pro-Pheroid formulation during manufacturing of pro-Pheroid and adding drug-free pro-Pheroid to the final reaction mixtures of artemisone demonstrates similar inhibition of artemisone metabolism in the HLM and rCYP3A4 incubation mixtures ($p > 0.05$). When drug-free pro-Pheroid was added to the final reaction mixtures, the percentage of artemisone remaining for the rCYP3A4 was significantly lower than with the HLM incubation mixtures.

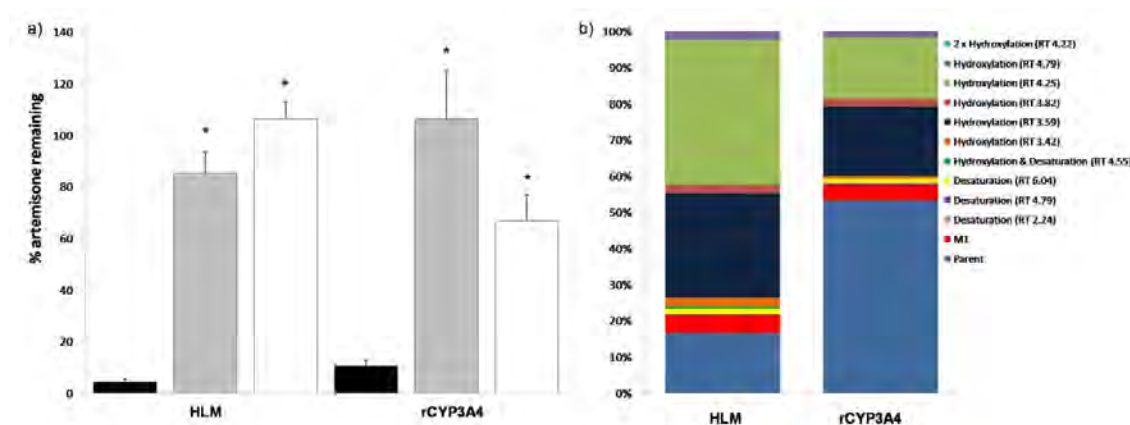


Figure 7: a) The effect of Pheroid technology on artemisone metabolism in HLM vs. rCYP3A4. Data depicted as mean $\pm$ SD of duplicate measurements; artemisone reference formulation (black), artemisone entrapped in pro-Pheroid during manufacturing (grey) and artemisone with drug free Pheroid added to the final reaction mixture (white). b) Percentage representation of the artemisone parent compound depletion and metabolite formation in HLM and rCYP3A4 treated with artemisone reference formulation. Statistically significant differences are indicated by an asterisk (*) ($p < 0.05$).

The metabolite pattern of the artemisone reference formulation observed in incubations with human CYP3A4 corresponds well with that found in incubations with human liver microsomes except that the percentage of parent compound, compared to the metabolites formed, are less in HLM than in rCYP3A4 (Figure 7b).

4. Discussion

Non-human primate models are utilized for predicting human pharmacokinetics of drugs^{35,36,37}, but poor correlations of bioavailability of various drugs have been observed between monkeys and humans^{38, 39, 40}. The extremely low plasma levels in monkeys may be due to incomplete gastrointestinal absorption, greater first-pass metabolism, or a combination of these factors^{39,40,41}. Akabane and colleagues³⁹ concluded that the lower bioavailabilities of drugs in monkeys were caused by intestinal metabolism rather than poor absorption or liver metabolism. Therefore, possible inter-species and possible inter-organ (intestine vs. liver) metabolism of the reference and test formulations were evaluated.

Also, CYP3A accounts for the largest portion of CYP450 in human liver and intestines. The average content of CYP3A in the liver and proximal small intestine represents 40% and 82% respectively of the total P450 enzyme based on total immunoquantified P450 content^{42,43}. Human CYP3A expression between individuals can vary up to 40-fold in liver and small intestine⁴⁴. Genetic and/or environmental factors may cause variation in CYP levels in Vervet monkeys³⁷. This might possibly explain the high degree of intersubject variability in artemisone plasma levels observed in this study.

In monkeys, CYP3A4 represents approximately 20% of the total P450 content of the liver⁴⁵. The total microsomal P450 content is approximately 3 fold higher in monkeys than in

man^{46,47}. Taking this into consideration, the levels of CYP3A4/unit of liver in the monkey are more than 4-fold higher compared to humans. In this study we used 48.8 mg/g microsomal protein per gram of liver for humans and monkeys according to the assumption of Naritomi and colleagues⁴⁸. Therefore the estimated *in vivo* clearance (CL_H) would actually be much higher than estimated here.

The CYP3A4 inhibitor, azamulin, exhibits species-dependent inhibition of artemisone metabolism in liver microsomes, possibly due to involvement of different cytochrome P-450 isozymes in the monkey or to structural differences at the active site of the CYP3A4 isozyme at which the azamulin interacts.

Nonhuman primates including the Cynomolgus monkey have been considered to be the most appropriate animal models for predicting drug metabolism in humans, and many publications have suggested that the monkey may provide more reliable predictions for human drug metabolism than rodents or dogs^{49,50,51,52,53}. This general belief is based on the sequence similarity of drug-metabolizing enzymes between man and within monkey species. For example the Rhesus, Cynomolgus and Vervet monkey CYP3A4 has a percentage homology of 93, 93 and 94% respectively with human CYP3A4⁵⁴. However, subtle changes in amino acid sequences can give rise to immensely different catalytic activities⁵⁵. Also, diverse cytochrome P450 enzymes can catalyze similar activity⁵⁶.

In a previous study, metabolites M1, M2 and M3 are the primary metabolites of artemisone in human liver microsomes⁷. Of the total radioactivity (TRA), M1, M2 and M3 were responsible for approximately 34%, 19%, 16% respectively while M4 and M5 were responsible for amounts of less than 6% each. In this study, M1 metabolite formation was low in HLM with

no M1 formation in MLM, HIM and MIM. The major metabolite, M2, formed in Rhesus monkey liver microsomes⁷ was also the major metabolite formed with Cynomolgus monkey liver microsomes in the current study.

The absence of M1 in MLM does not correlate well with *in vivo* PK results of this study where the M1 plasma levels were higher than the parent compounds in the plasma of Vervet monkeys. The monkey microsomes used in this study were from Cynomolgus monkeys because Vervet monkey microsomes were not commercially available. This, in part, could be responsible for the differences between *in vitro* PK and *in vitro* metabolism results. Tang and colleagues³⁷ showed that, in the regioselectivity of diclofenac hydroxylation, Rhesus and Cynomolgus monkeys are closer to humans than African green monkeys. They also conclude that there is a significant difference in the kinetic parameters and the reaction to chemical inhibitors between humans and all monkey species.

5. Conclusion

The *in vivo* results obtained in this study indicate that the Pheroid delivery system improves the pharmacokinetic profile of artemisone by increasing the maximum artemisone concentration and the area under the curve while decreasing the time to reach the maximum concentration in a clinically significant manner.

The *in vitro* results indicate that artemisone microsomal metabolism is inhibited by the Pheroid delivery system. Although inhibition of drug metabolism is typically regarded as potentially dangerous, or at least undesirable, the enhancement of plasma levels of artemisone in this case may be considered to be beneficial, given the very good safety profile of artemisone coupled with the relatively short half life of the unformulated drug.

6. Acknowledgements

Contract work (LC-MS/MS) conducted under supervision of Dr. Lubbe Wiesner at the University of Cape Town, South Africa. Financial support from the South African Technology Innovation Agency (TIA), the Swiss South African Joint Research Project Initiative and the North-West University.

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