ANNEXURES

ANNEXURE A

Annexure A contains the following poster:

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).

The effect of Pheroid® technology on the bioavailability of artemisone in primates

Lizette Grobler[a], Anne Grobler[b], Richard Haynes[b], Faans Steyn[c] and Lubbe Wiesner [d]

[a] Department of Pharmaceutics, Unit for Drug Research and Development, School of Pharmacy, North-West University, Potchefstroom, South Africa, [b] DST/NWU Preclinical Drug Development Platform , Faculty of Health Sciences, North-West University, Potchefstroom, South Africa, [c] Statistical Consultation Service, North-West University, Potchefstroom, South Africa, [d] Division of Clinical Pharmacology, University of Cape Town, Medical School, Observatory, 7925, Cape Town, South Africa

Introduction

Artemisinin-based combination treatments is recommended by the World Health Organization as first line treatment of uncomplicated malaria[1]. Artemisinins have a low aqueous solubility that causes poor, inconsistent absorption upon oral administration, which in turn results in low bioavailability. Their short elimination half-life is also a limitation[2].

The new artemisinin derivative, artemisone, shows enhanced antimalarial activity against chloroquine-sensitive and -resistant *P. falciparum* cell lines *in vitro* and against chloroquine sensitive *P. berghei* and chloroquine resistant *P. yoelii* rodent models and in *Aotus* monkey-*P. falciparum* models *in vivo* when compared to the most widely used artemisinin derivative, artesunate[3].

Pheroid® technology is an emulsion type formulation that can be manipulated in terms of structure, size, morphology and function in order to adapt to the chemical class and size of the drug molecules that need to be delivered. It can entrap, transport and deliver pharmacologically active compounds and other molecules[4]. In terms of artemisone, bioavailability studies have been performed for reference and Pheroid®-entrapped artemisone in a C57 BL/6 mouse model[5]. Significant differences where found between these two formulations with a greatly increased C_{max} (1550.0 ng/ml vs. 809.5 ng/ml) for artemisone-Pheroid® vesicle formulation compared to the artemisone reference formulation). The AUC_{0-last} and AUC_{0-inf} also increased (2219.0 ng h/ml and 3094.0 ng h/ml vs. 458.7 ng h/ml and 604.6 ng h/ml) with the Pheroid® vesicle formulation .

Aim

The purpose of this study was to progress the investigation into the enhancement of the pharmacokinetic profile of artemisone in the Pheroid® delivery system to a primate model.

Methods

Artemisone was prepared at the Hong Kong University of Science and Technology. Pro-Pheroid® was prepared by gassing an oil phase consisting of the active pharmaceutical ingredient (API), PEG 400, vitamin F ethyl ester, Cremaphor EL, d *D*-α-tocopherol, BHA and BHT with N_2O

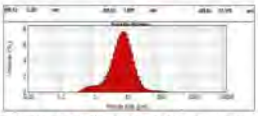

Figure 1: Particle size distribution of artemisone-entrapped artemisone incorporated into pro-Pheroid®.

In vivo PK

Reference (artemisone) and test formulations (artemisone entrapped into 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.

Blood samples were drawn in heparin through percutaneous venipuncture of the femoral vein pre-dose and 7 times post dose up to 10 hours. Plasma samples were prepared by centrifugation (4 °C) and immediately frozen at -196 °C and then stored at -80 °C. Parent drug concentrations and metabolite M1 in the plasma samples were determined using an LC/MS/MS assay. Calibrations standards and quality control standards were prepared in blank primate plasma. Artemisinin was used as internal standard. An Agilent 1200 series autosampler injected 2 µl onto an HPLC column with detection by API 3200 mass spectrometer.

In vitro metabolic stability

Artemisone-reference and pro-Pheroid® formulations were incubated with enzyme (0.5 mg/mL) and 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADPH. An aliquot was taken at various time points up to 45 min. 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 on a UPLC-QTOF MS/MS with a Waters Sample Manager (autosampler) and Waters Synapt HDMS G1 (spectrometer).

Results

In vivo bioavailability

Table1: Pharmacokinetic parameters of the oral artemisone reference and Pheroid® formulations

Dosage	Drug 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 parent drug	184.41 ± 134.40	0.07	47.39 ± 44.53	0.16	5.11 ± 2.52	0.14	3.17 ± 1.68	0.61
Artemisone-Pheroid®		421.83 ± 521.42		103.21 ± 51.57		3.3 ± 2.44		4.45 ± 3.47	
Artemisone	Metabolite M1	303.14 ± 320.23	0.45	75.73 ± 80.26	0.31	3 ± 1.25	0.5	3.52 ± 1.03	0.47
Artemisone-Pheroid®		635.48 ± 891.44		140.78 ± 43.42		2.8 ± 1.48		6.35 ± 4.45	

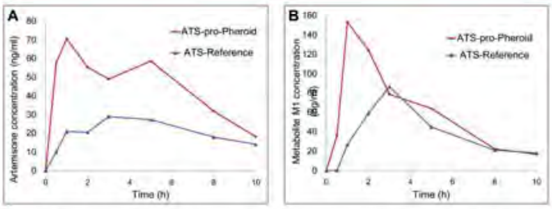

Figure 2: Mean concentration vs. time graph of the oral artemisone reference and pro-Pheroid® formulations. A) artemisone concentration in ng/ml and B) Metabolite M1 concentration in ng/ml.

In vitro metabolic stability

Table 2: *In vitro* intrinsic clearance (CL_{int}) of the artemisone reference and artemisone-pro-Pheroid® formulations in HLM, HIM, MLM and MIM and estimated *in vivo* hepatic clearance (CL_H) for HLM and MLM.

	In vitro CL_{int} (µl/min/mg)				Estimated in vivo CL_H (ml/min/kg)	
	HLM	MLM	HIM	MIM	HLM	MLM
ATS reference	111.00	1533.33	11.00	0.00 *	18.25	34.28
ATS-pro-Pheroid®	0.06	0.00 *	0.00 *	0.00 *	0.08	0.00

* No microsomal metabolism occurred

Discussion

After log-transformation of the pharmacokinetic parameters C_{max}, AUC and V_d (and a Box-Cox transformation of $t_{1/2}$) the two formulations did not differ significantly (p>0.05).

A: The mean artemisone C_{max}, AUC and $t_{1/2}$ are higher with the test formulation compared with the reference formulation and the T_{max} and V_d (6.24 ± 6.65 vs. 13.22 ± 20.81 litre, p = 0.17) were lower.

B: The mean M1 C_{max}, AUC and $t_{1/2}$ were higher with the test formulation and the T_{max} were similar for both formulations.

The artemisone plasma levels are much lower than expected for the dosages administered. Previous studies established that a lower bioavailability for certain drugs occurs in monkeys, compared to humans, due to incomplete gastrointestinal absorption, greater first-pass metabolism, or a combination of these factors[6].

The *in vitro* metabolism studies made it clear that very slow metabolism of the artemisone reference occurred in HIM with no metabolism in the MIM. The pro-Pheroid® formulation inhibited artemisone catabolism in all microsome preparations. The *in vitro* intrinsic clearance of the MLM is much higher than that of HLM. If extrapolated to the estimated hepatic clearance *in vivo*, the monkey CL_H is almost twice the CL_H in humans.

Also, if azamulin, a CYP3A4 inhibitor, is added to the reaction mixture, metabolism of artemisone is inhibited in the human liver microsomes (% drug remaining = 99.1%) but not in the monkey liver microsomes (% activity remaining = 2.81%). Therefore, the monkey CYP3A4 in the monkey might not be the enzyme responsible for the catabolism of artemisone or alternatively the monkey CYP3A4 is not inhibited by azamulin.

Conclusion

Entrapment of artemisone in the Pheroid® delivery system improves the pharmacokinetic profile of artemisone, although statistical significance could not be shown as a result of the low plasma levels in the limited number of animals used. The vervet monkey model may not be the most suitable model for evaluation of artemisone bioavailability.

The *in vitro* results suggest that artemisone is protected by the Pheroid® delivery system against microsomal metabolism.

References

1. WORLD HEALTH ORGANIZATION (WHO). 2010. World Malaria Report 2010. [illegible] Date of access: 4 January 2011.
2. STEPNIEWSKA K. & WHITE N.J. 2008. Pharmacokinetic determinants of the window of selection for antimalarial drug resistance. *Antimicrob Agents Chemother* [illegible]
3. HAYNES R.K., FUGMANN, B., STETTER J., HIECKMANN, K., EILMANN, H., CHAN, H., CHEUNG, M., LAM, W., WONG, H., CROFT, S.L., VIVAS, L., RATTRAY, L., STEWART, L., PETERS, W., ROBINSON, B.L., EDSTEIN, M.D., KOTECKA, B., KYLE, D.E., BECKERMANN, B., GERISCH, M., RADTKE, M., SCHMUCK, G., STEINKE, W., WOLLBORN, U., SCHMEER, K. & ROMER, A. 2006. Artemisone [illegible] *Angewandte Chemie* [illegible]
4. GROBLER, A., KOTZE, A., DU PLESSIS, J. The design of a skin-friendly carrier for cosmetic compounds using Pheroid® technology. (*In* Wiechers, J., ed. Science and Applications of Skin Delivery Systems. Wheaton, IL: Allured Publishing Corporation, 2008).
5. STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W., HAYNES, R.K. & KOTZE, A.F. 2011. [illegible] artemisone and artemiside: Potential application of Pheroid® technology. *International journal of pharmaceutics*, [illegible]
6. AKABANE, T., TABATA, K., KADONO, K., SAKUDA, S., TERASHITA, S. & TERAMURA, T. 2010. A comparison of pharmacokinetics between humans and monkeys. *Drug metabolism and disposition: The biological fate of chemicals*, [illegible]

Acknowledgements

We wish to acknowledge:
1. The South African Technology Innovation Agency (TIA), the Swiss South African Joint Research Project Initiative and the NWU for funding.
2. The participation of the Swiss Tropical and Public Health Institute for their advise and support.

science & technology — Department: Science and Technology, REPUBLIC OF SOUTH AFRICA | PCDDP | NORTH-WEST UNIVERSITY YUNIBESITI YA BOKONE-BOPHIRIMA NOORDWES-UNIVERSITEIT POTCHEFSTROOM CAMPUS

ANNEXURE B

Annexure B contains the following poster:

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).

The effect of Pheroid® technology on the bioavailability of artemisone in primates

Lizette Grobler [a], Anne Grobler [b], Richard Haynes [b], Faans Steyn [c]

[a] Department of Pharmaceutics, Unit for Drug Research and Development, School of Pharmacy, North-West University, Potchefstroom, South Africa, [b] DST/NWU Preclinical Drug Development Platform , Faculty of Health Sciences, North-West University, Potchefstroom, South Africa, [c] Statistical Consultation Service, North-West University, Potchefstroom, South Africa

Introduction

Artemisinin-based combination treatments have been recommended by the World Health Organization as the first line treatment of uncomplicated malaria[1]. Artemisinins have low aqueous solubility that causes poor and inconsistent absorption upon oral administration which in turn results in low bioavailability. Another limitation is their short elimination half-life[2].

The new artemisinin derivative, artemisone, shows enhanced antimalarial activity against chloroquine-sensitive and -resistant *P. falciparum* cell lines *in vitro* and against chloroquine sensitive *P. berghei* and chloroquine resistant *P.yoelii* rodent models and in *Aotus* monkey-*P. falciparum* models *in vivo* when compared to the most widely used artemisinin derivative, artesunate[3].

Pheroid® technology is an emulsion type formulation that can be manipulated in terms of structure, size, morphology and function depending on the type and size of the drug molecules that needs to be delivered. It can entrap, transport and deliver pharmacologically active compounds and other molecules[4]. In terms of artemisone, bioavailability studies have been performed for reference and Pheroid®-entrapped artemisone in a C57 BL/6 mouse model[5]. Significant differences where found between these two formulations with a greatly increased Cmax of 1550.0 ng/ml for artemisone-Pheroid® vesicle formulation compared to a Cmax of 809.5 ng/ml for the artemisone reference formulation. The $AUC_{0\text{-last}}$ and $AUC_{0\text{-inf}}$ also increased from 458.7 ng h/ml and 604.6 ng h/ml for the artemisone reference formulation and 2219.0 ng h/ml and 3094.0 ng h/ml for the artemisone-Pheroid® vesicle formulation .

Aim

The purpose of this research was to investigate the enhancement of the pharmacokinetic profile of artemisone when incorporated in the Pheroid® delivery system by using a primate model.

Methods

Artemisone was prepared at the Hong Kong University of Science and Technology. Pro-Pheroid® were prepared by gassing an oil phase consisting of the active pharmaceutical ingredient (API), PEG 400, vitamin F ethyl ester, Cremaphor EL, d *D*-α-tocopherol, BHA and BHT with N_2O under high pressure.

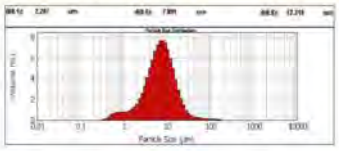

Figure 1: Particle size distribution of artemisone-entrapped artemisone incorporated into pro-Pheroid®

Reference (artemisone) and test formulations (artemisone incorporated into pro-Pheroid®) were administered to vervet monkeys (n = 10) as a single dose of 60 mg/kg, using capsules as dosage form. A crossover model was used with a one week washout period in-between.

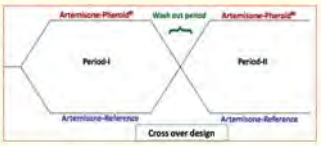

Figure 2: Primate study design

Blood samples were drawn in heparin through percutaneous venipuncture of the femoral vein at pre-dose and 0.5, 1, 2, 3, 5, 8 and 10 hr post dose time intervals. Plasma samples were prepared by centrifugation (4 °C) and immediately frozen at -196 °C and then stored at -80 °C. Parent drug concentrations and metabolite M1 in the plasma samples were determined using an LC/MS/MS method.

Calibrations standards and quality control standards were prepared in blank primate plasma. Artemisinin was used as the internal standard. An Agilent 1200 series autosampler injected 2 µl onto HPLC column with detection by API 3200 mass spectrometer.

Results

Table1: Pharmacokinetic parameters of the oral artemisone reference and Pheroid® formulations

Dosage	Drug measured	$AUC_{0\text{-last}}$ (ng·h/ml)	C_{max} (ng/ml)	T_{max} (hours)	$t_{1/2}$ (hours)
Artemisone	Artemisone parent drug	184.41 ± 134.40	47.39 ± 44.53	5.11 ± 2.52	3.17 ± 1.68
Artemisone-Pheroid®		421.83 ± 521.42	103.21 ± 51.57	3.3 ± 2.44	4.45 ± 3.47
Artemisone	Metabolite M1	303.14 ± 320.23	75.73 ± 80.26	3 ± 1.25	3.52 ± 1.03
Artemisone-Pheroid®		635.48 ± 891.44	140.78 ± 43.42	2.8 ± 1.48	6.35 ± 4.45

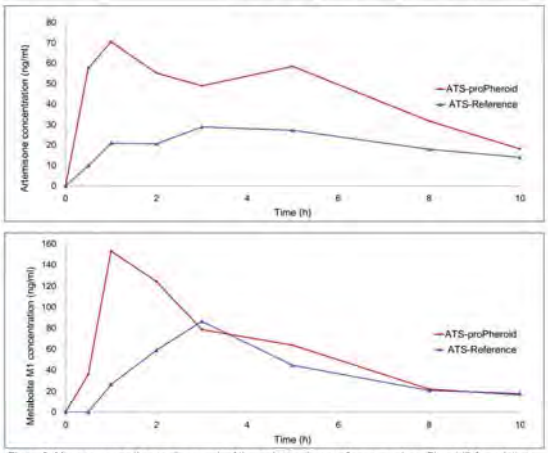

Figure 3: Mean concentration vs. time graph of the oral artemisone reference and pro-Pheroid® formulations.
A) artemisone concentration in ng/ml and B) Metabolite M1 concentration in ng/ml

Discussion

After log-transformation of the pharmacokinetic parameters C_{max}, AUC and V_d (and a Box-Cox transformation of $t_{1/2}$) the two formulations did not differ significantly (p>0.05).

A: The mean artemisone C_{max} (103.21 ± 151.57 vs. 47.39 ± 44.53 ng/ml, p = 0.16). AUC (421.83 ± 521.42 vs 184.41 ± 134.40 ng h/ml, p = 0.07) and $t_{1/2}$ (4.45 ± 3.47 vs. 3.17 ± 1.68 hours, p = 0.61) seems to be higher with the test formulation compared with the reference formulation and the T_{max} (3.3 ± 2.44 vs 5.11 ± 2.52 hours, p = 0.14) and V_d (6.24 ± 6.65 vs. 13.22 ± 20.81 litre, p = 0.17) were lower.

B: The mean M1 C_{max} (140.78 ± 243.42 vs. 75.73 ± 80.26 ng/ml, p = 0.31), AUC (635.48 ± 891.44 vs 303.14 ± 320.23 ng h/ml, p = 0.45) and t1/2 (6.35 ± 4.45 vs. 3.52 ± 1.03 hours, p = 0.47) seems to be higher with the test formulation and the T_{max} (2.8 ± 1.48 vs 3 ± 1.25 hours p = 0.50) were similar for both formulations.

The artemisone plasma levels are much lower than expected for the dosages administered. Previous studies established that a lower bioavailability for certain drugs occurs in monkeys, compared to humans, due to incomplete gastrointestinal absorption, greater first-pass metabolism, or a combination of these factors[5].

Conclusion

Entrapment of artemisone in the Pheroid® delivery system improves the pharmacokinetic profile of artemisone , although statistical significance could not be shown as a result of the low plasma levels in the limited number of animals used. The vervet monkey model may not be the most suitable model for evaluation of artemisone bioavailability.

References

1. WORLD HEALTH ORGANIZATION (WHO). 2010. *World Malaria Report 2010*. The http://whqlibdoc.who.int/publications/2010/9789241564403_eng.pdf) Date of access: 4 January 2011
2. STEPNIEWSKA K., & WHITE NJ. 2008. Pharmacokinetic determinants of the window of selection for antimalarial drug resistance. *Antimicrob Agents Chemother* 2:1589-96
3. HAYNES R.K., FUGMANN, B., STETTER,J. HEICKMANN, K., ELMANN, H., CHAN, H., CHEUNG, M., LAM, W., WONG, H., CROFT, S.L., VIVAS, L., RATTRAY, L., STEWART, L., PETERS, W., ROBINSON, B.L., EDSTEIN, M.D., KOTECKA, B. KYLE, D.E., BECKERMANN, B., GERISCH, M., RADTKE, M., SCHMUCK, G., STEINKE, W., WOLLBORN U., SCHMEER, K.& ROMER, A. 2006. Artemisone-a highly active antimalarial drug of the artemisinin class. *Angewandte Chemie*, 45: 2082-8.
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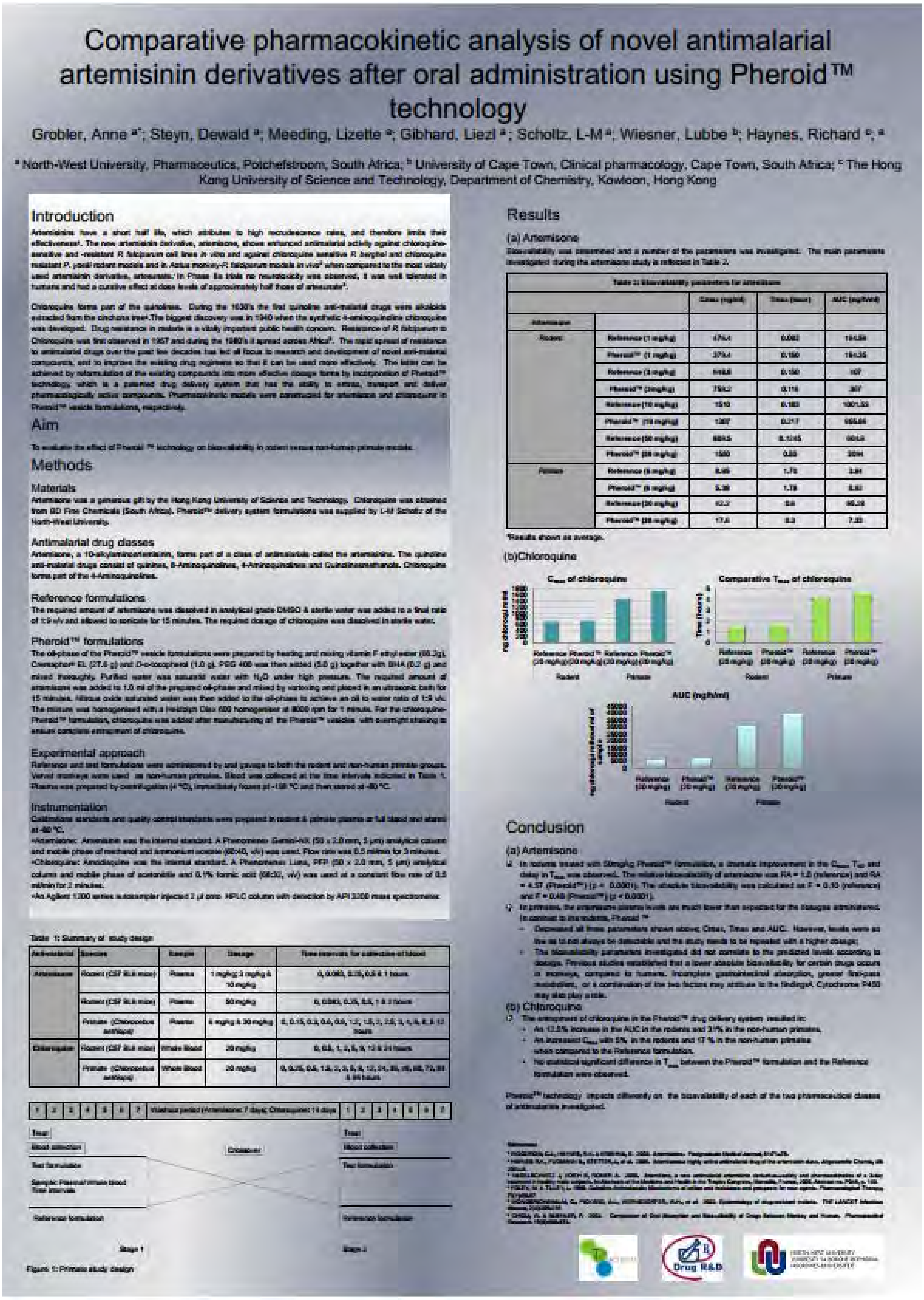
6. AKABANE, T., TABATA, K., KADONO, K., SAKUDA, S., TERASHITA, S. & TERAMURA, T. 2010. A comparison of pharmacokinetics between humans and monkeys. *Drug metabolism and disposition: The biological fate of chemicals*, 38(2):308-316.

33rd Annual Conference of the Academy of Pharmaceutical Sciences of South Africa, 12-15 Sept 2012

ANNEXURE C

Annexure C contains the following poster:

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 Pheroid™ technology. 3rd Pharmaceutical Sciences Fair and Exhibition, Prague, Czech Republic. 2011(Poster).


Comparative pharmacokinetic analysis of novel antimalarial artemisinin derivatives after oral administration using Pheroid™ technology
Grobler, Anne a*; Steyn, Dewald a; Meeding, Lizette a; Gibhard, Liezl a; Scholtz, L-M a; Wiesner, Lubbe b; Haynes, Richard c; a
a North-West University, Pharmaceutics, Potchefstroom, South Africa; b University of Cape Town, Clinical pharmacology, Cape Town, South Africa; c The Hong Kong University of Science and Technology, Department of Chemistry, Kowloon, Hong Kong
Introduction
Aim
Methods
Materials
Antimalarial drug classes
Reference formulations
Pheroid™ formulations
Experimental approach
Instrumentation
Results
(a) Artemisone
(b)Chloroquine
Conclusion
(a) Artemisone
(b) Chloroquine

ANNEXURE D

Annexure D contains the Ethics application for cultivation of Plasmodium falciparum parasites in human blood (NWU-0008-08-S5) and for evaluation of bioavailability and efficacy of Pheroid®-entrapped anti-infective agents in non-human primates (NWU-00027-10-A5)

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Research Ethics Committee
North-West University
Potchefstroom Campus
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15 April 2008

Dear Ms Botha

FINAL RESPONSE: ETHICS APPLICATION: NWU-0008-08-S5

The abovementioned application has reference.

We have received satisfacory answers to all the questions posed by the Ethics panel and has therefore found the ethical aspects to be in order.

PROF. J. DU PLESSIS
DIRECTOR

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Ethics Committee
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2010-07-15

ETHICS APPROVAL OF PROJECT

This is to certify that the next project was approved by the NWU Ethics Committee:

Project title *Investigation into the comparative bioavailability and efficacy of Pheroid™-entrapped anti-infective agents in non-human primates*

Project leader: Dr. AF Grobler

Ethics number: **NWU-00027-10-A5**

Expiry date: 2015/07/11

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

The formal Ethics approval certificate will be sent to you as soon as possible.

Yours sincerely

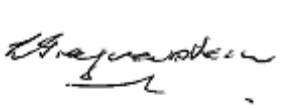
Me.Marietjie Halgryn
NWU Ethics Secretariate

ANNEXURE E

Annexure E contains the proof of language editing.

ENGLISH LANGUAGE EDITING CERTIFICATION

This is to certify that the English language editing of this thesis by Ms L Grobler was done by Prof. L.A. Greyvenstein.



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