

EVALUATION AND VALIDATION OF METHODS TO DETERMINE PARASITEMIA IN MALARIA CELL CULTURES

CHRIZAAN SLABBERT
(B. PHARM.)

Dissertation submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE (PHARMACEUTICS)

at the

POTCHEFSTROOM CAMPUS OF THE NORTH-WEST UNIVERSITY

Supervisor: Dr. L.H. du Plessis

Co-supervisor: Prof. A.F. Kotzé

DEDICATED TO MY PARENTS

JAN AND MAGDA SLABBERT

&

MY GRANDFATHER, ATTIE VAN NIEKERK

THERE ARE MOMENTS IN OUR LIVES WHEN WE
FIND OURSELVES AT CROSSROADS.
AFRAID, CONFUSED, WITHOUT A ROAD MAP.
THE CHOICES WE MAKE IN THOSE MOMENTS
CAN DEFINE THE REST OF OUR DAYS.
OF COURSE, WHEN FACED WITH THE UNKNOWN,
MOST OF US PREFER TO TURN AROUND
AND GO BACK. BUT ONCE IN A WHILE, PEOPLE PUSH ON
TO SOMETHING BETTER. SOMETHING FOUND
JUST BEYOND THE PAIN OF GOING IT ALONE,
AND JUST BEYOND THE BRAVERY AND COURAGE
IT TAKES TO LET SOMEONE IN, OR TO GIVE SOMEONE
A SECOND CHANCE. SOMETHING BEYOND THE QUIET
PERSISTENCE OF A DREAM.

BECAUSE IT'S ONLY WHEN YOU'RE TESTED THAT
YOU DISCOVER WHO YOU CAN BE.
THE PERSON YOU WANT TO BE DOES EXIST,
SOMEWHERE ON THE OTHER SIDE OF
HARD WORK AND FAITH AND BELIEF.
AND BEYOND HEARTACHE AND FEAR OF
WHAT LIES AHEAD.

~ CHAD MICHAEL MURRAY - ONE TREE HILL ~

ACKNOWLEDGEMENTS

This research would not appear in its present form without the kind assistance and support of the following individuals and organizations to whom I feel compelled to say thank you.

This dissertation would not have been possible without the kind support, the positive critiques, remarkable patients and friendship of my supervisor, **Dr. Lissinda du Plessis**, I cannot thank you enough.

I would like to express my gratitude to my co-supervisor, **Prof. Awie Kotzé**, for always having an open door and the support during these two years.

Thanks must go to the **Innovation Fund** for the financial support.

I would like to thank **Prof. Braam Louw** and **Mr. Jaco de Ridder** for the strains they supplied and the training I received. Without it this would not have been possible.

Further thanks should go to the Library staff at the North-West University, in particular Mrs. **Anriëtte Pretorius**, for all their help and their time.

In recognition to all the blood, sweat and tears I would like to give a special thank **Mr. RW Odendaal** and **Mr. Dewald Steyn**.

For the manufacturing of the Pheroid™, I would like to thank **Ms. Liezl-Marié Nieuwoudt**, **Ms. Silverani Padayachee** and **Mr. RW Odendaal**.

I am indebted to my friends, especially **Hester Delpont, Helanie van der Merwe and Jaco van Heerden**, for all their love and support and ability to endure without complaint. And a special thanks to **Kevin Helena** for the support and help in particularly with the illustrations.

To my brothers, sister and brother in law, **Riaan, Hannes, Suzette and Danie**, thank you for always being there for me during the highs and lows of life.

I am forever grateful to my parents, **Jan and Magda Slabbert**, whose foresight and values paved the way for a privileged education and the gentle counsel and unconditional support at each turn of the road.

To my grandfather, **Ds. Attie van Niekerk**, to whom I dedicate this work, thanks for the love and interest you always showed and given.

And lastly, I would like to praise God for His unfailing love and strength. Without whom I would not be able.

TABLE OF CONTENTS

TABLE OF CONTENTS	i
LIST OF FIGURES	viii
LIST OF TABLES	xi
LIST OF ABBREVIATIONS	xiii
LIST OF EQUATIONS	xvii
ABSTRACT	xix
UITTREKSEL	xxi
INTRODUCTION AND AIM OF STUDY	1

CHAPTER 1 ~ MALARIA

1.1 INTRODUCTION	3
1.2 EPIDEMIOLOGY	4
1.3 PARASITE BIOLOGY	4
1.4 CLINICAL DISEASE	5
1.5 DRUG RESISTANCE	8
1.6 ANTIMALARIAL DRUGS	9

1.6.1 Quinoline antimalarial drugs	9
1.6.1.1 Chloroquine	11
1.6.1.2 Quinine	11
1.6.1.3 Mefloquine	12
1.6.1.4 Halofantrine and lumefantrine	12
1.6.1.5 Primaquine	12
1.6.2 Artemisinin and its derivatives	13
1.6.3 Antibiotics: Doxycycline	14
1.6.4 Other	14
1.6.4.1 Atovaquone-proguanil	15
1.6.4.2 Sulfadoxine-pyrimethamine	15
1.6.5 Pharmacokinetics	15
1.7 COMBINATION THERAPY	17
1.8 MALARIA IN SOUTH AFRICA	18
1.9 CONCLUSION	20

CHAPTER 2 ~ PHEROID™ TECHNOLOGY AS DRUG DELIVERY SYSTEM

2.1 INTRODUCTION	21
2.2 CLASSIFICATION OF LIPID-BASED COLLOIDAL DELIVERY SYSTEMS	22
2.3 CHARACTERISTICS OF PHEROID™ TECHNOLOGY	23

2.3.1	Structure and classification of the different types of Pheroid™ formulations	24
2.3.2	Toxicity	26
2.3.3	Mechanism of uptake.....	26
2.3.4	Pharmacokinetic properties of Pheroid™ formulations	27
2.3.4.1	Drug entrapment	27
2.3.4.2	Protection of the drug	27
2.3.4.3	Absorption	28
2.3.4.4	Volume of distribution	28
2.4	CLINICAL APPLICATIONS OF PHEROID™	28
2.4.1	Transdermal and cosmetic therapies.....	29
2.4.2	Preparations for the prevention and treatment of infectious diseases	30
2.4.2.1	The treatment of tuberculosis	30
2.4.2.2	The treatment of malaria	31
2.4.2.3	The treatment of HIV/AIDS.....	31
2.4.3	Vaccines as preventative therapy	32
2.4.3.1	Virus-based vaccines	32
2.4.3.2	Peptide-based vaccines	32
2.4.4	Peptide drug delivery	33
2.4.4.1	Calcitonin: Polypeptide hormone.....	33
2.4.4.2	Insulin: Polypeptide hormone	34
2.4.4.3	Human growth hormone: Polypeptide hormone	34
2.4.4.4	Vasopressin: Nanopeptide hormone	34
2.5	CONCLUSION.....	35

CHAPTER 3 ~ DRUG EFFICACY TESTS

3.1	INTRODUCTION	36
3.2	CULTIVATION OF <i>PLASMODIUM FALCIPARUM</i> IN CONTINUOUS CULTURES	38
3.3	POLYMERASE CHAIN REACTION (PCR)	39
3.4	MICROSCOPE EVALUATION	40
3.5	MICRO-TECHNIQUE AND WHO TEST	40
3.6	RADIO-ISOTOPE METHOD.....	41
3.6.1	[³ H]hypoxanthine-based assay	42
3.7	NON RADIO-ISOTOPE METHODS	42
3.7.1	Fluorometric assays.....	43
3.7.2	Enzyme-Linked ImmunoSorbent Assay (ELISA based assays).....	43
3.7.2.1	Histidine-Rich Protein 2 (HRP2)	44
3.7.2.2	Double-site enzyme-linked lactate dehydrogenase immunodetection (DELI)	44
3.7.3	Non-ELISA based colorimetric assays – <i>Plasmodium</i> Lactate Dehydrogenase (pLDH).....	45
3.7.4	Flow cytometry.....	47
3.8	CONCLUSION.....	49

CHAPTER 4 ~ METHOD DEVELOPMENT: METHODS, RESULTS & DISCUSSION

4.1 INTRODUCTION	50
4.2 CULTIVATION OF <i>PLASMODIUM FALCIPARUM</i> CULTURES	51
4.2.1 Materials	51
4.2.2 Cultivation	51
4.2.3 Synchronization	52
4.3 MICROSCOPE EVALUATION	53
4.3.1 Materials	53
4.3.2 Method	53
4.4 METHOD VALIDATION.....	55
4.5 NON-ELISA BASED COLORIMETRIC ANALYSIS – pLDH	56
4.5.1 Materials	56
4.5.2 Experimental design	56
4.5.3 Results and discussion	59
4.5.3.1 Influence of haematocrit and blood type on the pLDH method.....	59
4.5.3.2 Sensitivity and repeatability	61
4.5.4 Conclusion	67
4.6 FLOW CYTOMETRIC EVALUATION – FLUORESCENCE ACTIVATED CELL SORTER (FACS).....	67
4.6.1 Materials	68
4.6.2 Experimental design	68

4.6.3 Results and discussion	71
4.6.4 Conclusion	80
4.7 CORRELATIONS BETWEEN THE DIFFERENT METHODS	81
4.8 CONCLUSION.....	84

<i>CHAPTER 5 ~ DRUG EFFICACY & FORMULATION: METHODS, RESULTS & DISCUSSION</i>
--

5.1 INTRODUCTION	86
5.2 MATERIALS	87
5.3 GENERAL EXPERIMENTAL DESIGN.....	87
5.4 DETERMINING THE OPTIMAL PHEROID™ RATIO.....	88
5.4.1 Experimental design	89
5.4.2 Results and discussion	89
5.5 INFLUENCE OF PHEROID™ MICROSPONGES ON THE METHODS TO EVALUATE PARASITE DENSITY	92
5.6 DRUG EFFICACY TESTS: MEFLOROQUINE IN DIFFERENT PHEROID™ FORMULATIONS	94
5.6.1 Experimental design	95
5.6.2 Results and discussion	95
5.7 CONCLUSION.....	102

SUMMARY, ANNEXURES & REFERENCES
--

SUMMARY AND FUTURE PROSPECTS	103
ANNEXURE A	106
ANNEXURE B	108
ANNEXURE C	111
ANNEXURE D	116
REFERENCES	121

LIST OF FIGURES

Figure 1.1:	Schematic representation of the lifecycle of <i>P. falciparum</i> . The figure indicates the two different parts of the lifecycle. The asexual cycle is shown in green and the sexual cycle is shown in blue	6
Figure 1.2:	Malaria distribution in South Africa	19
Figure 2.1:	Schematic representation of a liposome vesicle (a). The lipid bilayer entraps lipid soluble drug (b) and the aqueous compartment water soluble drugs (c)	23
Figure 2.2:	Confocal laser scanning of the Pheroid™ formulations. (a) A mixture of vesicles and microsponges. (b) An elastic vesicle containing rifampicin. (c) A small pro-Pheroid™ for oral administration. (d) A depot with a hydrophobic core with pro-Pheroid™ and surrounded by hydrophilic zone	25
Figure 2.3:	Comparison between the antibody production of the Pheroid™ formulation and existing vaccines over time	33
Figure 3.1:	Different approaches to assess the sensitivity of antimalarial drugs	37
Figure 3.2:	The final step of the anaerobic glycolysis metabolic pathway. LDH reduces the lactate to pyruvate with the production of NADH.....	45
Figure 3.3:	The basic concept of the colorimetric assay utilizing pLDH	46
Figure 3.4:	The basic principle of flow cytometry. Cells pass in single file through a laser. Light and fluorescence scattering takes place and detected by a series of mirrors, filters, detectors and amps and digitally converted to quantitative data	47
Figure 4.1:	The procedure to follow when making a thin blood smear.	54
Figure 4.2:	Graphical representation of the pLDH method showing the enzymatic reaction and how it relates to the assay.	57

Figure 4.3:	Average of the percentage parasitemia of a serial dilution with the NBT solution containing diaphorase as co-enzyme.	61
Figure 4.4:	Average of the percentage parasitemia of a serial dilution with the NBT solution containing PES as co-enzyme (Experiment 1).....	62
Figure 4.5:	Correlation graph between the experimental percentage survival and the theoretical percentage survival of experiment one.	66
Figure 4.6:	Schematic representation of the flow cytometric evaluation of infected and uninfected erythrocyte samples using acridine orange as fluorochrome.....	69
Figure 4.7:	Presentation of the FL-1/FL-2 graph with the uninfected erythrocytes (green) in the lower left (LL) quadrant and the infected erythrocytes (blue) in the upper right (UR) quadrant. The table represents the statistical analysis done on the graph. (UL – Upper left; LR – Lower right).	70
Figure 4.8:	A FSC/SCC dot plot of infected and uninfected erythrocytes.....	71
Figure 4.9:	SSC/FL1 dot plots of infected and uninfected erythrocytes. The infected erythrocytes are gated.....	72
Figure 4.10:	FL1/FL2 dot plots of infected and uninfected erythrocytes. The lower left quadrant represents the uninfected erythrocytes and the upper right quadrant represents the infected erythrocytes.	73
Figure 4.11:	A histogram showing the difference in the fluorescence intensity between the infected and uninfected erythrocytes.....	73
Figure 4.12:	Percentage parasitemia of the serial dilution of experiment one.....	75
Figure 4.13:	Correlation between the theoretical and experimental percentage parasitemia of experiment one.....	77
Figure 4.14:	(a) Dot plot of the uninfected erythrocytes (green) and infected erythrocytes in the ring phase (red); (b) Dot plot of the uninfected erythrocytes (blue) and infected erythrocytes in different phase, ring (red) and schizont (green); (c) A histogram with uninfected erythrocytes (blue) and infected erythrocytes in different phase, ring (red) and schizont (green) (FlowJo version 7.2.5 was used draw the graphs).....	79
Figure 4.15:	Correlation between the theoretical percentage parasitemia and the percentages parasitemia as determined by (a) the slides (red) and pLDH (blue) and (b) the slides (red) and FACS (green).....	81

Figure 4.16:	Correlation between the percentage parasitemia as determined by slides and FACS.	82
Figure 4.17:	Correlation between the percentage parasitemia as determined by slides and pLDH.	83
Figure 5.1:	Percentage parasitemia as determined by microscope evaluation of the 1:1750 ratio of Pheroid™ microsponges to nitrous oxide water compared to the control. The percentages indicated on the bar represent the relative percentage parasitemia.	91
Figure 5.2:	Difference between the average percentage survival of samples with and without Pheroid™ microsphere as determined by colorimetric evaluation of pLDH.	92
Figure 5.3:	Uninfected erythrocytes without Pheroid and only Pheroid as read on the FACS with AO. (a) Dot plot of FL-2 against FL-1 and (b) overlay histogram of the uninfected erythrocytes and Pheroid.	93
Figure 5.4:	Micrographs of the Pheroid with mefloquine entrapped in the membrane. The auto fluorescent property of mefloquine gives the green colour to the mefloquine crystals and Pheroid is red in colour.	98
Figure 5.5:	The percentage parasitemia of the control compared to the Pheroid formulation three (MQ was incorporated into the Pheroid). (The average of the triplicate was used in the graph).	99
Figure 5.6:	The percentage survival of the three Pheroid™ microsphere formulations at different mefloquine concentrations. (The average of the triplicate was used in the graph).	101
Figure A.1:	Percentage parasitemia of the two Pheroid formulations, with and without an organic solvent.	107
Figure C.1:	Micrographs of Pheroid™ microsponges (Batch number: S08001)	112
Figure C.2:	Micrographs of Pheroid™ microsponges (Batch number: S08010)	112

LIST OF TABLES

Table 1.1:	Dates of first reported resistance to antimalarial drugs	8
Table 1.2:	A list of drugs used for treatment and prophylaxis of malaria	10
Table 1.3:	Pharmacokinetics of the malaria drugs	17
Table 1.4:	Different artemisinin based combinations and non-artemisinin based combination therapies	19
Table 2.1:	Active compounds used in combination with Pheroid™ for different therapeutic applications	29
Table 4.1:	The average of the absorbance value (optical density) of uninfected erythrocytes at three different haematocrit percentages and three different blood types.....	60
Table 4.2:	Sensitivity of the pLDH method - average percentage survival of the different dilutions on five occasions.	63
Table 4.3:	The precision and accuracy of the colorimetric evaluation using PES as co-enzyme at different parasite densities.....	64
Table 4.4:	Spearman's correlation (r) and the equation of the linear function of each experiment.	65
Table 4.5:	The average percentage parasitemia and standard error of mean of different serial dilutions on three different occasions.	74
Table 4.6:	The precision and accuracy of the flow cytometric evaluation using acridine orange as fluorochrome at different parasite densities.	76
Table 4.7:	Spearman's correlation (r) and the equation of the linear function of each experiment.	77
Table 4.8:	The average and standard error of mean of the same culture as read on the FACSCalibur at two different times, at two different phases	78

Table 5.1:	Percentage parasitemias as obtained from microscope evaluation to determine the optimal Pheroid ratio.	90
Table 5.2:	The relative percentage parasitemia of the different Pheroid ratios compared with the control. The highlighted values indicate the highest relative percentage decrease of parasitemia.	91
Table 5.3:	The percentage parasitemia of the different mefloquine concentration of Pheroid formulation one (no entrapment) and the control. The results are presented as the average $\pm$ SEM and the t-test values are also given.	96
Table 5.4:	The percentage parasitemia of the different mefloquine concentration of Pheroid formulation two (24 hour entrapment) and the control. The results are presented as the average $\pm$ SEM and the t-test values are also given.	97
Table 5.5:	The percentage parasitemia of the different mefloquine concentration of Pheroid formulation 3 (incorporated in) and the control. The results are presented as the average $\pm$ SEM and the t-test values are also given. The highlighted values indicate statistical significant results ($p \leq 0.05$).	100
Table A.1:	Percentage parasitemia of three mefloquine concentration in different formulation with and without an organic solvent, ethanol.	107

LIST OF ABBREVIATIONS

µl	Micro litre
18S ss rRNA	Small sub-unit ribosomal ribonucleic acid
3TC	Lamivudine
ACT	Artemisinin-based combination therapy
ADH	Anti-diuretic hormone
AIDS	Acquired immune deficiency syndrome
AO	Acridine orange
APAD	3-Acetylpyridine-adenine dinucleotide
APADH	Reduced 3-Acetylpyridine-Adenine dinucleotide
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate enzyme
Ave	Average
C.V	Coefficient of variation
CDC	Centre for Disease Control
CNS	Central Nervous System
CO ₂	Carbon dioxide

CQ	Chloroquine
DELI	The double-site enzyme-linked lactate dehydrogenase immunodetection
DHA	Dihydroartemisinin
DNA	Deoxyribonucleic acid
DOH	Department of Health
EDL	Essential Drug List
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtOH	Ethanol
FACS	Flourescence activated cell sorter
FCP	Flow cytometry principles
FDA	Food and drug administration
GI	Gastro Intestinal
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
HRP2	Histidine-Rich Protein 2
IC ₅₀	50% Inhibitory Concentration
IM	Intramuscular
IV	Intravenous
LDH	Lactate dehydrogenase
MIC	Minimum inhibitory concentration
ml	Millilitre

MQ	Mefloquine hydrochloride
mRNA	Messenger ribonucleic acid
N ₂	nitrous gas
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
nm	Nanometre
nM	Nano molar
NW	Nitrous oxide water
O ₂	Oxygen
OD	Optical densities
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PES	Phenazine ethosulphate
pLDH	<i>Plasmodium</i> Lactate Dehydrogenase
pRBC	<i>Infected erythrocytes</i>
r	Correlation
RBC	Erythrocytes
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute

RPP	Relative percentage parasitemia
SAMCP	South African Malaria Control Program
SERCA	plasmodia sarcoplasmic / endoplasmic calcium ATPase
TB	Tuberculosis
tRNA	Transfer ribonucleic acid
UV	Ultra violet
WHO	World Health Organization

LIST OF EQUATIONS

EQUATION 1 ~ PERCENTAGE PARASITEMIA CALCULATED FOR MICROSCOPE EVALUATION

$$\% \text{ Parasitemia} = \frac{\text{Total amount of infected erythrocytes}}{\text{Total amount of erythrocytes}} \times 100$$

EQUATION 2 ~ PERCENTAGE SURVIVAL CALCULATED FOR pLDH

$$\% \text{ Survival} = \frac{\text{OD of the sample}}{\text{OD}_{\text{AVE normal pRBC}}} \times 100$$

Were OD = Optical density as obtained after the background absorbance was subtracted

OD_{AVE} = The average of the optical density repeats

pRBC = Infected erythrocytes (normal growth) (X_{100%})

EQUATION 3 ~ PERCENTAGE PARASITEMIA CALCULATED FOR pLDH

$$\% \text{ Parasitemia} = \frac{\% \text{ survival of sample}}{\% \text{ parasitemia of the initial parasitemia}} \times 100$$

EQUATION 4 ~ PERCENTAGE COEFFICIENT OF VARIATION

$$\% \text{ C.V.} = \frac{\text{Standard error of mean}}{\text{Average}} \times 100$$

EQUATION 5 ~ PERCENTAGE SURVIVAL CALCULATED FOR FACS

$$\% \text{ Survival} = \frac{\% \text{ parasitemia of the sample}}{\% \text{ parasitemia of the initial parasitemia}} \times 100$$

EQUATION 6 ~ RELATIVE PERCENTAGE PARASITEMIA

$$\text{Relative \% Parasitemia} = \frac{\% \text{ Sample zero} - \% \text{ Sample}}{\% \text{ Sample zero}} \times 100$$

ABSTRACT

Malaria is a leading cause of death in Africa and other subtropical regions in the world. There is an urgent need for new drugs and vaccine development. The effect of these drugs is often investigated *in vitro*. Microscopic analysis of blood smears is the gold standard to determine parasitemia levels in experiments of drug efficacy studies but is subjective, time consuming and labour intensive. Alternative methods for the determination of parasitemia levels include methods utilizing flow cytometry and colorimetry. Apart from new drug development, drug delivery systems can also play a role in malaria treatment. Pheroid™ is a patented colloidal delivery system consisting of plant and essential fatty acids. This emulsion type formulation consists of lipid-based submicron sized stable structures dispersed in liquid and nitrous oxide gas phase. The morphology, structure, size and function of these dispersed structures can be manipulated and entrap pharmaceutical active compounds that can possibly enhance the therapeutic effect. This could probably enhance the efficacy of current antimalarial drugs.

The aim of this study was twofold. Firstly, to develop and validate a faster and more effective analysis method to determine parasitemia levels in drug efficacy studies. Parasitemia levels were determined with flow cytometry (FACS) and colorimetrically (pLDH-method). These methods were compared with microscope evaluation. The second aim was to entrap existing antimalarial drugs in Pheroid™ microsponges and evaluate the effect on *Plasmodium falciparum* growth *in vitro*.

The results showed that both methods are accurate at high parasite densities with a sensitivity of only 1% for the pLDH-method and 0.2% for FACS. The FACS also complied with the FDA regulations. Different Pheroid™ ratios in combination with a serial dilution chloroquine were incubated for 48 hours to determine the optimal Pheroid™ ratio needed in the drug efficacy tests and evaluated microscopically. The chloroquine in the Pheroid™ microsponges was more

effective compared to water. The best ratio was 1:1750 with a 23%, 55% and 80% reduction in growth for the different concentrations without a negative influence on the cells. Three different Pheroid™ microsphere-mefloquine formulations were evaluated with FACS. The most effective formulation was when the mefloquine was incorporated into the Pheroid™ during manufacturing. The results showed a 46% reduction in growth at the highest concentration. There was a significant difference between Pheroid™ and control at the higher concentrations. It can be concluded that mefloquine and chloroquine in combination with Pheroid™ microsphere delivery systems can enhance the antimalarial effect of the drugs.

KEY WORDS: Malaria, mefloquine, chloroquine, Pheroid™ technology, *P. falciparum*, flow cytometry, colorimetric evaluation, pLDH-method.

UITTREKSEL

Malaria is een van die grootste oorsake van sterftes in Afrika en ander subtropiese dele van die wêreld. Daar is 'n groot behoefte vir die ontwikkeling van nuwe malaria middels en vaksienes. Die effek van hierdie middels kan *in vitro* bestudeer word. Die algemeenste metode om parasitemie te bepaal is deur gebruik te maak van mikroskoop evaluering van verdunde en volbloedsmere. Hierdie goue standaard is subjektief en arbeids intensief. Alternatiewe metodes sluit in vloeisitometrie en kolorimetriese analise. Geneesmiddel afleweringssysteme kan ook 'n belangrike rol speel in die stryd teen malaria. Pheroid™ is 'n gepatenteerde kolloïedale afleweringstelsel, wat bestaan uit plant- en ander essensiële vetsure. Die formulering is 'n emulsie waarin klein stabiele submikron deeltjies gesuspenseer is in 'n water en stikstof gasfase. Die struktuur en grootte van die deeltjies kan gemanipuleer word om aktiewe bestandele vas te vang om sodoende die terapeutiese effek te verhoog.

Die doel van hierdie studie was tweeledig. Die eerste doelstelling was die optimalisering en validering van verskillende analise metodes om die parasitemie vlakke te bepaal. Die metodes sluit in die kolorimetriese evaluering van *Plasmodium* laktaat dehydrogenase (pLDH) en deur vloeisitometrie (FACS). Hierdie metodes is vergelyk met die mikroskoop evaluering metode. Die tweede doelstelling was om malaria geneesmiddels in die Pheroid™ mikrosponsie vas te vang en die effektiwiteit daarvan op *Plasmodium falciparum* kulture te ondersoek.

Die resultate het getoon dat beide die pLDH-metode en FACS die parasietvlakke akkuraat kan bepaal by hoë vlakke van parasitemie. Die pLDH-metode kan die parasitemie bepaal tot by 'n minimum van 1%, terwyl die FACS dit tot by 0.2% kan bepaal. Die FACS het ook aan die Food and Drug Administration (FDA) se regulasie voldoen. Verskillende Pheroid™ verhoudings met 'n verdunningreeks van chloroquine is getoets om die Pheroid™ se effek te

optimaliseer. Die resultate soos verkry deur mikroskoop evaluering het getoon dat die Pheroid™ by al die verhoudings hoër effektiwiteit het as die kontrole. Die mees optimale verhouding was 1:1750 waar dit 'n 23%, 55% en 80% verlaging in die groei van die parasiete by die verskillende geneesmiddel konsentrasies gegee het sonder enige negatiewe effekte op die selle. Drie verskillende Pheroid™ formulerings van mefloquine is ook by hierdie verhouding getoets met FACS. Resultate het getoon dat die formule waar mefloquine in die Pheroid™ geïnkorporeer was, die effektiwiefste was. Daar was 'n 46% afname in die parasitemie vlakke by die hoogste geneesmiddel konsentrasie. Daar was ook 'n noemenswaardige verskil tussen die Pheroid™ formule en die kontrole. Die gevolgtrekking wat gemaak kan word met die studie is dat die Pheroid™ mikrosponsie afleweringstelsel die terapeutiese effek van die malaria geneesmiddels verhoog.

SLEUTEL WOORDE: Malaria, mefloquine, chloroquine, Pheroid™ tegnologie, *P. falciparum*, vloeisitometrie, kolorimetriesse evaluering, pLDH-metode

INTRODUCTION & AIM OF STUDY

Malaria is a parasitic disease caused by *Plasmodium falciparum*. Malaria is still a major cause of death in the epidemic areas (CDC, 2008; Daily, 2006; WHO, 2007a) killing more than a million people annually (CDC, 2008; WHO, 2007a). The problem surrounding malaria is partly due to inadequate health infrastructures and poor social-economic conditions especially in sub-Saharan Africa (Lewison & Srivastava, 2008). Malaria research accounts for only 2.3% of the research done on all the diseases at present. Between 1980 and 2004 the research done for malaria reflects the needs of that country. Even though the burden of malaria is the greatest in Africa, less than 10% of research is done in Africa. The high mortality rate of malaria will not be eradicated by research, but will lay the necessary foundation for the policies needed to fight this disease (Lewison & Srivastava, 2008).

Malaria is a curable disease (CDC, 2008; Daily, 2006; Shapiro & Goldberg, 2007; WHO, 2007a). The success in treatment is the prompt diagnosis and correct treatment regime (CDC, 2008). It is therefore not only necessary for new treatment options but also faster and more sensitive methods for drug efficacy test and diagnosis of malaria (Stratton *et al.*, 2008). Resistance to almost all of the antimalarial drugs has increased the malaria burden (White, 2004; WHO, 2006). The resistance is worse now than 20 years ago and is due to the incorrect use of antimalarial drugs (Hyde, 2007; Walliker *et al.*, 2005).

The efficacy of a drug to eradicate the parasite can be measured by different approaches. One of these approaches is to measure the sensitivity of malaria parasites quantitatively *in vitro*. An important reason why drug efficacy studies are done is to measure the resistance of the parasite to the drugs (Noedl *et al.*, 2003). Microscope evaluation is seen as the gold standard (Guy *et al.*, 2007). Microscopy is however subjective and labour intensive (Basco, 2007; Piper *et al.*, 1999; Sio *et al.*, 2006). Several other methods can be used to determine drug efficacy *in vitro* (Noedl *et al.*, 2003). The colorimetric pLDH detection and flow

cytometric evaluation is two methods that can be more sensitive to determine parasite densities.

Pheroid™ is a patented colloidal delivery system consisting of plant and essential fatty acids (Grobler, 2004). This emulsion type formulation consists of lipid-based micron sized stable structures dispersed in liquid and nitrous oxide gas phase can be manipulated in morphology, structure, size and function. Pheroid™ can entrap molecules that are water and lipid soluble with high efficacy (85 – 90%) (Grobler, 2004; Grobler *et al.*, 2007). The *in vitro* drug efficacy studies of antimalarial drugs showed better efficacy in the Pheroid™ formulation enhancing the therapeutic effect (Langley, 2007).

The broad objective of this study was to develop and validate methods that can be used in *in vitro* drug efficacy tests and to evaluate the possible drug enhancement of antimalarial drugs entrapped in Pheroid™.

The aims of this study were:

- ◆ Determining the sensitivity of the colorimetric detection of pLDH in *P. falciparum* cultures.
- ◆ Determining the sensitivity of flow cytometric evaluation of parasite densities using a fluorochrome.
- ◆ Comparing the colorimetric evaluation and flow cytometric evaluation with the golden standard of microscope evaluation.
- ◆ Optimising the Pheroid™ microsphere ratio for drug efficacy test.
- ◆ Evaluating the efficacy of mefloquine hydrochloride against a chloroquine resistant *P. falciparum* strain in three different Pheroid™ formulations and comparing it to a control formulation consisting of mefloquine hydrochloride in water.

Chapter 1 to 3 are a literature study comprising of a basic overview of malaria, Pheroid™ technology and drug efficacy tests respectively. Chapter 4 focuses on the colorimetric and flow cytometric method development. Chapter 5 focuses on drug efficacy and formulation of mefloquine in combination with Pheroid™ microspheres. Chapter 4 and 5 describes the experimental design, results and discussion.

CHAPTER 1

MALARIA

1.1 INTRODUCTION

Malaria is one of the oldest diseases known to mankind with symptoms described more than 4000 years ago. It wasn't until the 19th century that the causative agent was identified (CDC, 2008). Alphonse Laveran discovered the presence of malaria parasites in blood of patients in 1880 (CDC, 2008; Lewison and Srivatava, 2008). The different forms of malaria were only discovered in 1885 by Camillo Golgi (CDC, 2008). Ronald Ross demonstrated the transmission of the human malaria parasites by mosquitoes in 1897 (CDC, 2008; Lewison and Srivatava, 2008). Giovanni Batista Grassi described transmission of malaria to humans by *Anopheles* mosquitoes between 1898 and 1899 (CDC, 2008). Laveran and Ross both received the Nobel Prize for their discoveries (CDC, 2008; Lewison and Srivatava, 2008). Through the years to come, a big effort was made to eradicate malaria. In 1955 the World Health Organization (WHO) introduced a world wide eradication campaign which included a combination of antimalarial drugs and insecticides. Malaria was eradicated in certain countries whereas other countries had a significant reduction in number of malaria cases. Countries in sub-Saharan Africa were not included in this campaign. The campaign was abandoned due to resistance to drugs and insecticides, population movements and lack of funding. The focus was shifted to the control of malaria (CDC, 2008). In the following section the disease will be discussed in detail in terms of the epidemiology, biology, etiology and treatment of the disease.

1.2 EPIDEMIOLOGY

According to the WHO, malaria is a leading cause of death. The parasitic disease kills more than 1 million people and more than 300 million is infected annually (WHO, 2007a; Worrall *et al.*, 2007). Malaria accounts for 2.3% of the disease burden in the world with 90% in sub-Saharan Africa (Daily, 2006; Lewison and Srivatava, 2008) and it kills more people today than 40 years ago (Stratton *et al.*, 2008). The disease is mostly found in the tropical and sub-tropical regions of the world where children and pregnant women are most at risk (WHO, 2007a; Worrall *et al.*, 2007). Malaria accounts for 8% of deaths in children under the age of five years (CDC, 2008). Malaria in pregnant women can lead to the death of the mother and have other adverse pregnancy effects. The low birth weight of the infants has a fatality rate of 37,5% (Worrall *et al.*, 2007).

Malaria has a high financial burden on individuals and governments (CDC, 2008). The greatest risk factor of malaria is poverty (Gallup and Sachs, 2001; Stratton *et al.*, 2008). More than 60% of malaria infections occur in the poorest countries in the world (Stratton *et al.*, 2008). The high cost for the treatment, maintenance of clinics, vector control through insecticides, the patients that are unable to work and high death toll are all factors that lead to impeded economic growth (CDC, 2008). Poverty is an important factor of malaria, but malaria contributes to poverty (Stratton *et al.*, 2008).

1.3 PARASITE BIOLOGY

Malaria is an infection caused by any of the four different species of *Plasmodium* (Beers and Berkow, 1999). *Plasmodium falciparum* and *Plasmodium vivax* is the most prevalent species worldwide whereas *Plasmodium ovale* and *Plasmodium malariae* are less prevalent (Daily, 2006). *P. falciparum* is the most dangerous of these four species (Foley and Tilley, 1998). A fifth human malaria parasite, *Plasmodium knowlesi*, a parasite that was thought to be confined to monkeys, can also be transmitted to humans by the mosquito *Anopheles abaoenais*. There are other plasmodium species that infect non-human mammals like *Plasmodium berghii* and *Plasmodium yeolii* (Tripathi *et al.*, 2005).

As seen in Figure 1.1 when an infected *Anopheles* mosquito takes a blood meal, it injects *P. falciparum* sporozoites into the bloodstream from the salivary glands. Circulating sporozoites invade the liver cells where they multiply and develop into tissue schizonts. This is known as the exoerythrocytic cycle. In *P. falciparum* and *P. malariae* infections there are only one liver stage but in *P. vivax* and *P. ovale* infections the parasite persists in the liver leading to relapse infections months to years after primary infections. The tissue schizonts rupture and release merozoites into circulation 5 to 15 days after infection. During the erythrocytic cycle, the merozoites infect the erythrocytes, using haemoglobin as nutrient. In erythrocytes the parasites undergo asexual development from young ring forms to trophozoites and finally mature schizonts. The erythrocytes containing schizonts rupture and release merozoites allowing the cycle to start again (Rosenthal, 2004; Shapiro and Goldberg, 2007; Daily, 2006). The erythrocytic cycle for *P. falciparum*, *P. vivax* and *P. ovale* is 48 hours. The microscopic evaluation of the younger stages of *P. knowlesi* and *P. malariae* are much the same. But where the *P. malariae* has a three day lifecycle and never reaches dangerous levels in the blood, *P. knowlesi* only has a 24 hour lifecycle and is extremely dangerous (White, 2008). It is the erythrocytic stages of the parasite that causes the disease pathology and is most susceptible to antimalarial drugs (Foley and Tilley, 1998). During sexual development in the bloodstream the merozoites develop into the male and female gametocytes. When the mosquito takes a blood meal, the gametocytes develop into sporozoites in the gut of the mosquito. The sporozoites are taken up in the salivary gland. The mosquito infects another person when it takes a blood meal and injects the sporozoites into the circulation (Rosenthal, 2004; Shapiro and Goldberg, 2007; Daily, 2006; Hyde, 2007).

1.4 CLINICAL DISEASE

Malaria symptoms appear 9-14 days after an infectious mosquito bite (WHO, 2007a). This varies with the species and the pattern and intensity of malaria transmission in the area. A natural immunity can be acquired by the local people in a malaria area (WHO, 2006). There are two distinctive categories the disease can be grouped into. The first is uncomplicated malaria (CDC, 2008; Daily, 2006). The symptoms include flu-like symptoms characterized by headache, fever, body and joint pains, fatigue and vomiting (WHO, 2007a; WHO, 2006; Shapiro and Goldberg, 2007; Daily, 2006). The symptoms are related to the release of the

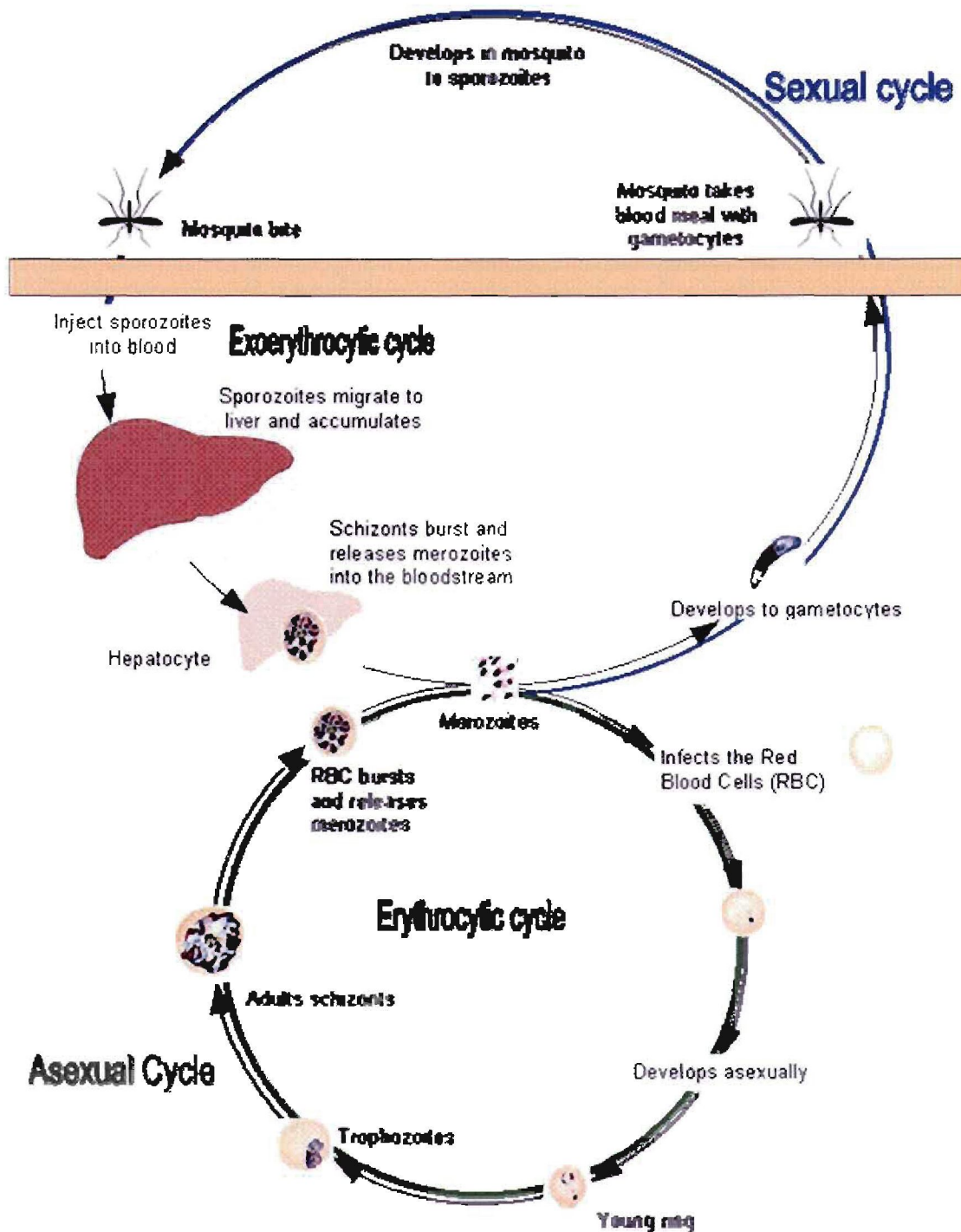


Figure 1.1 Schematic representation of the lifecycle of *P. falciparum*. The figure indicates the two different parts of the lifecycle. The asexual cycle is shown in green (----) and the sexual cycle is shown in blue (---) (Adapted from Rosenthal, 2004; Shapiro and Goldberg, 2007; Daily, 2006)

merozoites into the bloodstream (Daily, 2006). It invades erythrocytes of any age and produces endotoxin-like products, which in turn, induces release of cytokines resulting in fever and chills. It causes an overwhelming parasitemia that when left untreated, is life-threatening. When treated inadequately due to resistance against the drug or incomplete or inadequate dosage regimes, re-infection can occur. *P. ovale* and *P. vivax* have a lower mortality rate if not treated in adults. Both are characterized by relapses caused by the reactivation of the latent tissue forms (Shapiro and Goldberg, 2007).

The second category is severe or complicated malaria that is characterized by serious organ failures (CDC, 2008; Daily, 2006). As the lifecycle progress and the merozoites are released the erythrocytes are destroyed, causing anaemia and clogging of the capillaries that carry blood and other essential products to the vital organs (WHO, 2007a). The symptoms include:

- ◆ Cerebral malaria characterized by impaired consciousness, seizures, coma and other neurological abnormalities.
- ◆ Anaemia caused by erythrocyte loss.
- ◆ Respiratory distress.
- ◆ Renal failure.
- ◆ Hypoglycemia and metabolic acidosis.
- ◆ Cardiovascular collapse (CDC, 2008; Daily, 2006).

Children and pregnant women are at high risk of dying if not treated quickly and effectively. Pregnant women are the main adult risk for malaria (WHO, 2007a). The physiological changes in women during pregnancy as well as the immuno suppression and loss of acquired immunity to malaria contribute to this fact. Symptoms include anaemia, fever, hypoglycemia, cerebral malaria, pulmonary oedema and puerperal sepsis. The effect on the unborn baby is low birth weight, prematurely, malaria illness and death. The treatment of malaria in pregnant women is limited due to most drugs being contraindicated. This makes the treatment difficult and increases the risk to mother and child (Gibbon, 2005).

1.5 DRUG RESISTANCE

Drug resistance is the reduction in effectiveness of a drug in curing a disease. Antimalarial drug resistance is the ability of the parasite to survive despite administration of drug given in doses higher than those normally given (Bloland, 2001). The global malaria burden has increased due to resistance against almost all of the antimalarial drugs (White, 2004; WHO, 2006) except the artemisinins (White, 2004). The malaria burden is worse now than 22 years ago because of drug resistance (Hyde, 2005). Reasons for drug resistance are the inappropriate use of drugs in treatment and prophylaxis (Hyde, 2007; Walliker *et al.*, 2005), the ability of the parasite to change their genetic code and the ability of the rapid multiplication in certain species (Hyde, 2007). The change or mutation on genetic levels is related to the antimalarial target site and the influx/efflux pump (Hyde, 2007; White, 2004). The altered gene expression and the mutation of protein structure reduce the efficacy of drug-binding. The mutated parasite can then resist the effect of the drug (Hastings and Donnelly, 2005). Resistance occurs about 10-15 years after it has been introduced into a specific area (Wongsrichanalai *et al.*, 2002). Table 1.1 shows the year when resistance to the antimalarial drugs was first observed. When resistance was observed to a specific drug and that drug was not longer used in that area, a decrease in resistance can be observed (Hyde, 2005).

Table 1.1 Dates of first reported resistance to antimalarial drugs (Adapted from Wongsrichanalai *et al.*, 2002).

Antimalarial drug	First reported resistance to the drug
Quinine	1910
Proguanil	1949
Chloroquine	1957
Sulfadoxine-pyrimethamine	1967
Mefloquine	1982
Atovaquone	1996

Drug resistance has been documented for both *P. falciparum* and *P. vivax* but not for *P. ovale* or *P. malariae* (Wongsrichanalai *et al.*, 2002). Resistance can be minimized by combination therapy and correct drug regimes (WHO, 2006). When two drugs are combined with different mechanism of action the emerging of resistance is reduced (White, 2004). Multidrug resistance does occur especially in South Asia. The occurrence of multidrug resistance is limited but must be monitored (Dua *et al.*, 2003).

1.6 ANTIMALARIAL DRUGS

Antimalarial drugs can be grouped in one of two ways. Firstly by their intended use of the drugs as either treatment or prophylaxis. Table 1.2 gives a list of the drugs that can be given during treatment and prophylaxis. The second is by what part of the parasites' lifecycle they affect (Shapiro and Goldberg, 2007). The parasites' lifecycle consist of three distinctive parts, the exoerythrocytic cycle, erythrocytic cycle or asexual development and the sexual development namely the gametocytes (Shapiro and Goldberg, 2007; Rosenthal, 2004). When drugs are active against the exoerythrocytic cycle they are classified as tissue schizonticides. These drugs include primaquine and proguanil (Rosenthal, 2004). These drugs are mostly used in *P. ovale* and *P. vivax* infections to eradicate all liver merozoites (Foley and Tilley, 1998). Most drugs are active against the erythrocytic cycle and called blood schizonticides. These drugs include mefloquine, chloroquine, quinine, pyrimethamine and artemisinins. The last drug group, the gametocides, kills the gametocytes and prevents transmission to the mosquito. The drug mostly used is primaquine (Rosenthal, 2004).

1.6.1 Quinine antimalarial drugs

Since 1630 the bark of the cinchona tree was used to treat malaria (Foley and Tilley, 1997; Foley and Tilley, 1998; Raynes, 1999). In 1820 Pelletier and Caventou isolated quinine from the bark (Foley and Tilley, 1998; Raynes, 1999). Early drug research resulted in only few

Table 1.2 A list of drugs used for treatment and prophylaxis of malaria (Adapted from Rosenthal, 2004).

Treatment	Prophylaxis
Chloroquine	Chloroquine
Quinine	Mefloquine
Mefloquine	Proguanil
Primaquine	Doxycycline
Sulfadoxine-pyrimethamine	Atovaquone-proguanil
Doxycycline	
Halofantrine	
Lumefantrine	
Artemisinins	
Atovaquone-proguanil	

compounds that were used in humans (Foley and Tilley, 1998). In 1920 two 8-aminoquinolines were synthesized. The toxic pamaquine was used as radical cure that lead to primaquine that is less toxic. The biggest discovery was in 1940 when the synthetic 4-aminoquinoline chloroquine was developed. The misuse of the drug leads to widespread resistance. Other 4-aminoquinolines include amodiaquine that has been use as prophylaxis for over 40 years. Mefloquine, a quinolinemethanol like quinine was develop for the treatment of chloroquine resistance malaria. An analogue and subclass of the quinolinemethanol is halofantrine a 9-phenanthrenemethanol (Foley and Tilley, 1998).

1.6.1.1 Chloroquine

This 4-aminoquinoline has been the drug of choice for the treatment and prevention of malaria (Rosenthal, 2004; Shapiro and Goldberg, 2007). There is still some controversy about the exact mechanism of action of chloroquine, however two main mechanisms have emerged (Foley and Tilley, 1998). The parasite produces a toxic haem molecule that is polymerizes to form a non toxic molecule haemozoin. Haemozoin collects in the food vacuole as crystals. Chloroquine is proposed to inhibit the polymerizing of the haem to haemozoin. The haem concentration increases to toxic levels, which disrupts normal cell membrane function (Foley and Tilley, 1998; Rosenthal, 2004; Shapiro and Goldberg, 2007). A second proposed mechanism of action is that chloroquine interferes with the feeding process in parasites. Haemoglobin taken up by the parasite by endocytosis is transported to the food vacuole. The haemoglobin is then digested by protease. Chloroquine causes swelling of the food vacuole and indigested haemoglobin accumulation (Foley and Tilley, 1998). During treatment adverse effects have been observed including retinal toxicity, blurred vision, blindness, gastro intestinal problems, headache, pruritus, mood changes, depression and anxiety (Gibbon, 2005; Rosenthal, 2004; Shapiro and Goldberg, 2007). An overdose can be fatal (Foley and Tilley, 1998; Shapiro and Goldberg, 2007). It is not safe during pregnancy. It is a category C drug meaning it has adverse effects on the fetus (Gibbon, 2005). It can be used in children, but the bitter taste makes oral administration difficult (Gibbon, 2005; Foley and Tilley, 1998).

1.6.1.2 Quinine

This quinolinemethanol is a natural alkaloid that has antipyretic, antimalarial, analgesic and anti-inflammatory properties (Daily, 2006). The mechanism of action relies on the toxic effect on the parasite because of haem build-up. The drug inhibits the parasites ability to break the haem down (Daily, 2006; Rosenthal, 2004). The adverse effect of quinine is characterized by cinchonism. Cinchonism symptoms include sweaty skin, ringing in the ears, blurred vision, impaired hearing, confusion, reversible hearing loss, headache, abdominal pain, rashes,

vertigo, dizziness, dysphoria, nausea, vomiting and diarrhoea (Gibbon, 2005; Rosenthal, 2004). It is recommended during pregnancy and with extreme caution in paediatrics (Gibbon, 2005).

1.6.1.3 Mefloquine

It is a quinolinemethanol structurally related to quinine (Shapiro and Goldberg, 2007). It is active against blood schizonts and used for the treatment and prevention of malaria infections caused by *P. falciparum* and *P. vivax*. The mechanism of mefloquine is unknown (Rosenthal, 2004; Shapiro and Goldberg, 2007). The adverse effects include GI disturbances, headache, chest pains, oedema, sinus bradycardia, severe anxiety, paranoia, nightmares, insomnia, seizures, peripheral motor sensory neuropathy, vestibular damage and CNS damage (Gibbon, 2005; Rosenthal, 2004; Shapiro and Goldberg, 2007). It is not recommended for children or pregnant women because of the adverse effects (Gibbon, 2005; Shapiro and Goldberg, 2007).

1.6.1.4 Halofantrine and lumefantrine

Halofantrine is a phenanthrene methanol and effective against the blood stage of all four *Plasmodium* species (Rosenthal, 2004). Side effects include cardio toxicity, abdominal pain, diarrhoea, vomiting, cough, rash, headache and pruritus (Gibbon, 2005; Rosenthal, 2004). Use in pregnancy is not safe, but it can be used in children (Gibbon, 2005). Lumefantrine is an aryl alcohol and is used in combination. It is safer than halofantrine because it is not cardio toxic (Rosenthal, 2004). No data is available for the use during pregnancy, but it can be used in children (Gibbon, 2005).

1.6.1.5 Primaquine

It is an 8-aminoquinoline used in the treatment of the latent phase of *P. vivax* and *P. ovale*. The mechanism of action is unknown, but it is effective against the *P. falciparum* gametocytes and the hypnozoite stages of *P. vivax* and *P. ovale* (Rosenthal, 2004; Shapiro and Goldberg, 2007). It is given as radical cure to prevent the relapse of *P. vivax* and *P. ovale* infections

(Rosenthal, 2004). Adverse effects include nausea, vomiting, stomach cramps, anaemia, headache, visual disturbance and intense itching (Gibbon, 2005; Rosenthal, 2004; Shapiro and Goldberg, 2007). It should not be given during pregnancy or for children under the age of 1 year (Gibbon, 2005).

1.6.2 Artemisinin and its derivatives

Artemisinin is a natural product used for centuries for the treatment of fever and it was later discovered that it can be used for the treatment of malaria (Rosenthal, 2004; Shapiro and Goldberg, 2007). It is an extract of sweet wormwood that has been used for more than 2000 years in China (Haynes and Krishna, 2004; Eckstein-Ludwig *et al.*, 2003). It is extremely valuable in the treatment of malaria and is very effective against multidrug resistance strains (Eckstein-Ludwig *et al.*, 2003; Golenser *et al.*, 2006; Haynes and Krishna, 2004). It is rapid acting with few side effects (Haynes and Krishna, 2004). There are different derivatives including artesunate and artemether (Golenser *et al.*, 2006). Artesunate and artemether are prodrugs for dihydroartemisinin (DHA) that is the most effective against the malaria parasite, but is neurotoxic (Golenser *et al.*, 2006; Newton *et al.*, 2000). Artesunate is more water-soluble than artemether but is still highly lipid-soluble (Golenser *et al.*, 2006; Rosenthal, 2004). Adverse effects include nausea, vomiting and diarrhoea after oral administration (Golenser *et al.*, 2006; Rosenthal, 2004). The safety during pregnancy and in children has not yet been established (Shapiro and Goldberg, 2007). The artemisinin derivatives have the following advantages above quinine:

- ◆ No stimulation of insulin release.
- ◆ No local toxicity during IV or IM administration.
- ◆ Simpler dosage regimes.
- ◆ Safer therapeutic margins.
- ◆ Decreased mortality especially in children.
- ◆ Can be administered in a rectal form (Haynes and Krishna, 2004).

Artemisinin affects the asexual stages and the early development of sexual development (Haynes and Krishna, 2004; Eckstein-Ludwig *et al.*, 2003). The mechanism of action is not resolved but possible mechanisms have been established (Golenser *et al.*, 2006). It is now widely accepted theory that artemisinins inhibits important pathophysiological processes (Haynes and Krishna, 2004). It interferes with plasmodia sarcoplasmic / endoplasmic calcium ATPase (SERCA), which causes excess free radicals mediated by iron. The free radicals kill the parasite by inactivating crucial enzymes (Golenser *et al.*, 2006)

1.6.3 Antibiotics: Doxycycline

It is a tetracycline antibiotic used to treat malaria, chronic prostatitis, sinusitis, syphilis, chlamydia, pelvic inflammatory disease, acne and rosacea. Doxycycline is lipophilic and can enter the cell through the lipid membrane. It binds to the 20S ribosomal subunit. This inhibits the binding of the aminoacyl tRNA to the mRNA. Because tRNA can't bind to the mRNA, it inhibits protein synthesis (Daily, 2006). The adverse reactions of doxycycline include photosensitivity reactions, anorexia, nausea, diarrhoea, glossitis, dysphagia, enterocolitis and inflammatory lesions (Daily, 2006; Gibbon, 2005). It is contraindicated for children under the age of 8 years and should not be used in pregnancy (Gibbon, 2005; Shapiro and Goldberg, 2007). It is mostly used in combination with rapid acting schizonticide such as quinine and for prophylaxis (Daily, 2006).

1.6.4 Other

The following synergistic drugs are used in the treatment and prophylaxis of malaria:

- ◆ Atovaquone-proguanil
- ◆ Sulfadoxine-pyrimethamine

1.6.4.1 Atovaquone-proguanil

It is a combination used for the prevention of malaria. Proguanil is a biguanide derivate and atovaquone belongs to the class naphthalenes. Proguanil acts by inhibiting the dihydrofolate reductase enzyme that is involved in the reproduction of the parasite. Atovaquone inhibits the electron transport that in turn inhibits the metabolic enzymes linked to the mitochondria. Giving only one of the drugs causes a rapid development to resistance (Daily, 2006). Adverse effects include fever, rash, nausea, vomiting, diarrhoea, headache, insomnia, mouth ulcers and alopecia. The safety of the combination in pregnancy is not established. It is considered safe in children over 10kg (Gibbon, 2005).

1.6.4.2 Sulfadoxine-pyrimethamine

It is antifolate inhibitor consisting of two drugs that work synergistic and therefore considered as monotherapy (WHO, 2006). The mechanism of action is that it targets the parasite's folate enzymes that cause decreased production of nuclei acids, serine and methionine (Daily, 2006). Side effects include nausea, vomiting, abdominal discomfort, headaches, dizziness and dermatological reactions (Gibbon, 2005; Rosenthal, 2004). It should not be used during pregnancy or in neonates (Gibbon, 2005).

1.6.5 Pharmacokinetics

Table 1.3 gives a summary of the pharmacokinetic properties of the antimalarial drugs. The oral absorption of most of the drugs is good except artesunate that has an oral absorption of less than 30% (Daily, 2006; Gibbon, 2005; Rosenthal, 2004; Shapiro and Goldberg, 2007; Drugbank, 2008). Alternatively artesunate can be given as an IV or IM preparation as well as rectally (Golenser *et al.*, 2006). All the antimalarial drugs are metabolised in the liver and excreted either through the faeces or urine. The half life of the drugs differs from hours to days to months. This

Table 1.3 Pharmacokinetics of the malaria drugs (Adapted from Daily, 2006; Drugbank, 2008; Gibbon, 2005; Rosenthal, 2004; Shapiro and Goldberg, 2007)

Drug	Absorption	Metabolism	Half life	Excretion	Protein binding	Solubility
Chloroquine	Completely from GI tract	Hepatic	1-2 months	Urine	55%	Slightly in water
Quinine	Well absorbed from GI tract	Hepatic	18 hours	Urine	70%	Water
Mefloquine	Well from the GI tract, food lower absorption rate	Hepatic	1-2 weeks	Bile and Faeces	98%	Slightly in water
Halofantrine Lumefantrine	Well absorbed, food enhances absorption	Hepatic	6-10 days	Faeces	60-70%	-
Primaquine	Well absorbed orally (only)	Hepatic	6 hours	Urine	-	-
Artesunate	Less than 30% orally	Hepatic	1-2 hours	-	59%	Lipid but more water soluble
Artemether	-	Hepatic	-	-	-	Lipid
Doxycycline	Well absorbed orally	Hepatic	18-22 hours	Urine and faeces	>90%	Lipid
Atovaquone-proguanil	Rapidly absorbed orally. Fatty food enhances absorption	Hepatic	A - 2-3 days P - 20 hours	Faeces	A- 99% P - 75%	A - Lipid P - Water
Sulfadoxine- pyrimethamine	Well absorbed	Hepatic	4-9 days	Urine	87%	Lipid

is an important consideration when choosing a drug for prophylactic use. Drugs with a short half life, like artesunate, are not as suitable for prophylaxis (Haynes and Krishna, 2004). Whereas drugs with longer half life like mefloquine and chloroquine are better suited for prophylaxis. The protein binding of all the drugs are high (Daily, 2006; Drugbank, 2008; Gibbon, 2005; Rosenthal, 2004; Shapiro and Goldberg, 2007). This means that the more the drug is bound to the protein the less efficiently it can traverse cell membranes (Shargel, 1995). The solubility as well as the protein binding properties of the drugs has an effect on the plasma level concentration of each drug (Daily, 2006).

1.7 COMBINATION THERAPY

The WHO defines combination therapy as the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and thus unrelated biochemical targets in the parasite. Drugs that works in synergy with each other and are not used alone, is considered monotherapy and includes sulfadoxine-pyrimethamine. Combination therapy should improve therapeutic efficacy and delay the onset of resistance. These combinations can be divided into two groups. The first is the artemisinin-based combination therapy (ACT) (Geyer, 2001; WHO, 2006). This is the preferred first-line drug treatment (Ekland and Fiddock, 2008). ACT's is unique because of the rapid clearance of parasites given by the artemisinin derivatives. It is also effective against multidrug resistance parasites and has few side effects. However the safety in young children and pregnant women has not yet been established (Geyer, 2001). The second group is the non-artemisinin based combination therapy. Because there is already drug resistance against some of these drugs, it is less effective. There is still the advantage that the combination will be more effective than monotherapy (WHO, 2006). Table 1.4 shows the different combinations that can be used in combination therapy.

Table 1.4 Different artemisinin based combinations and non-artemisinin based combination therapies (Geyer, 2001; WHO; 2006).

Artemisinin based combination therapy	Non-artemisinin based combination therapy
Artesunate and chloroquine	Chloroquine and sulfadoxine-pyrimethamine
Artesunate and amodiaquine	Amodiaquine and sulfadoxine-pyrimethamine
Artesunate and sulfadoxine-pyrimethamine	Atovaquone and proguanil
Artesunate and mefloquine	Mefloquine and sulfadoxine-pyrimethamine
Artemether and lumefantrine	Quinine and tetracycline or doxycycline

1.8 MALARIA IN SOUTH AFRICA

Malaria caused by *P. falciparum* is the most prevalent in South Africa. The areas most affected are Limpopo, Mpumalanga and KwaZulu Natal as seen in Figure 1.2. Only 10% of South Africans live in these areas (Gerritsen *et al.*, 2008; Tren and Bate, 2004). Malaria is seasonal with an increase in the number of cases during an increase in rainfall (Tren and Bate, 2004). In the early 19th century about 20000 deaths were reported annually with cases as far inland as Pretoria. In 1945 the South Africa Malaria Control Program (SAMCP) was established (Gerritsen *et al.*, 2008; Blumberg and Frean, 2007). The following are in the plan of the SAMCP to eradicate malaria:

- ◆ Vector control through indoor residual house spraying.
- ◆ Case management.
- ◆ Treatment of uncomplicated malaria with ACT.

- ◆ Disease surveillance.
- ◆ Epidemic preparedness and response.
- ◆ Health Promotion (Gerritsen *et al.*, 2008; Blumberg and Frean, 2007).

With the residual house spraying in the 1940s, malaria was eradicated in most of South Africa except the north eastern part (Blumberg and Frean, 2007). In 2006 the case fatality rate was 0.7% and 12098 cases of malaria were reported. Limpopo has the highest infection rate followed by Mpumalanga and KwaZulu Natal (Gerritsen *et al.*, 2008).

Treatment for uncomplicated malaria is oral dose of ACT consisting of lumafantrine and artemether, sulfadoxine and pyrimethamine or quinine with either doxycycline or clindamycin. The treatment regime for complicated malaria includes IV quinine. Prophylactic measures when entering a malaria area is a weekly dose of mefloquine or a daily dose of doxycycline (EDL, 2003).

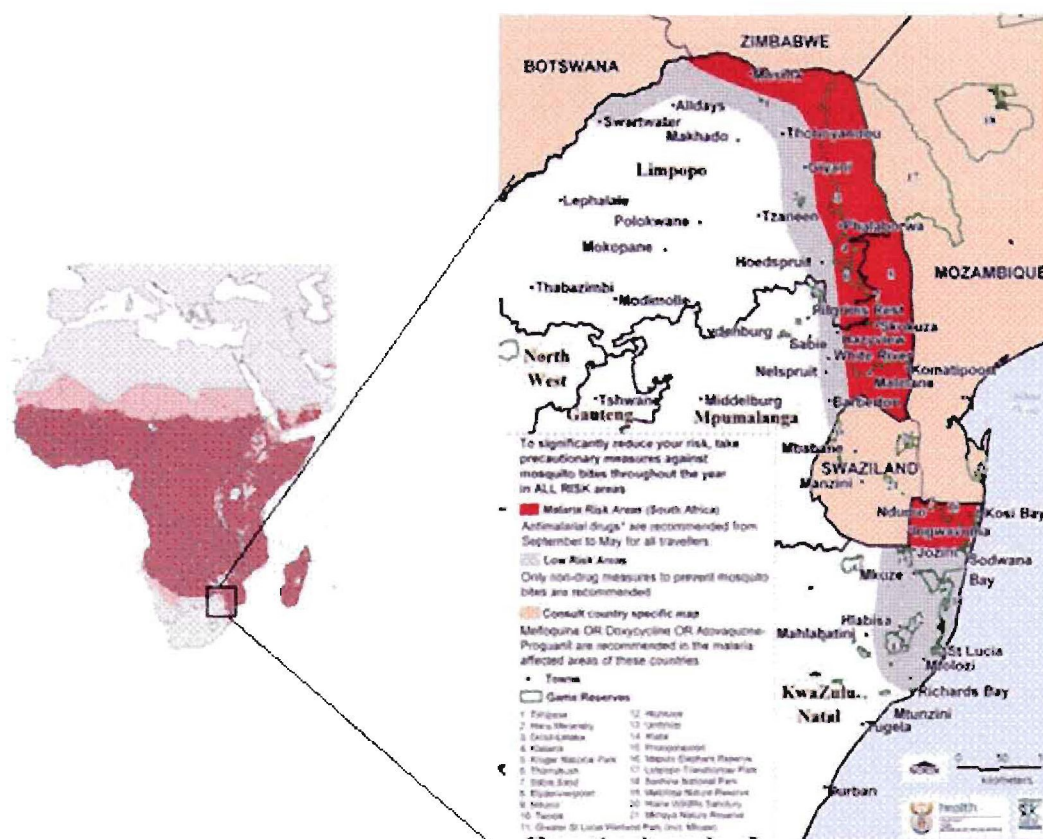


Figure 1.2 Malaria distribution in South Africa (Adapted from Geurra *et al.*, 2008; DOH, 2002)

1.9 CONCLUSION

Malaria kills more than 1 million people annually, especially in sub-Saharan Africa. Children accounts for 8% of these deaths. The economic burden of malaria is high on the households and governments. Research done on malaria only accounts for 2.3% of research done on diseases. The lifecycle of the parasite is complex and has three distinctive phases. These phases are the exoerythrocytic cycle, the erythrocytic cycle or asexual development and the sexual development. The erythrocytic cycle is responsible for the clinical diseases. The symptoms include headache, fever, chills, body pain, fatigue and vomiting. When malaria is left untreated it is life threatening. The symptoms of severe malaria include impaired consciousness, seizures, coma, anaemia, respiratory distress, renal failure and hypoglycemia. Most of the drugs used in malaria works on the erythrocytic cycle. Antimalarial drugs can be grouped into treatment and prophylaxis. Because of incorrect dosage regimes and misuse of drugs, resistance especially to the quinolines is now widespread. Because of resistance to most of the antimalarial drugs and to avoid drug resistance to the artemisinins, it is important that combination therapy is used to treat malaria.

CHAPTER 2

PHEROID™ TECHNOLOGY AS DRUG DELIVERY SYSTEM

2.1 INTRODUCTION

Drug delivery systems are primarily used to control the rate and period of drug delivery and have target specific delivery to decrease adverse effects (Vogelson, 2001). A colloidal system consists of two separate phases: the dispersed phase and continuous phase (Martin, 1993; Attwood, 2003). Pheroid™ is a patented colloidal delivery system consisting of plant and essential fatty acids (Grobler, 2004). This emulsion type formulation consists of lipid-based submicron- and micron sized stable structures dispersed in liquid and nitrous oxide gas phase. The morphology, structure, size and function of these dispersed structures can be manipulated (Grobler, 2004; Grobler *et al.*, 2007). The particles have a diameter of between 200 nm and 2 μ m (Grobler *et al.*, 2007) and can entrap pharmaceutical active compounds that can possibly enhance the therapeutic effect (Grobler, 2004).

Drug delivery systems in parasitic diseases like malaria are necessary to improve the efficacy, specificity, tolerability and therapeutic index of the existing drugs. The drug delivery systems should allow oral administration of the drugs with specific intracellular targeting to maximise effect and reduce toxicity. The duration of the treatment should be reduced and

more cost effective. It should also be versatile to allow simultaneous administration of combination drugs. Colloidal drug carriers have great advantages to deliver these drugs. Antimalarial drugs encapsulated in liposomes colloids protected the drug against degradation. It also has the ability of sustained release with reduction in adverse effects. Drug delivery systems can play an important role in the management of the global burden of malaria (Date *et al.*, 2007).

2.2 CLASSIFICATION OF LIPID-BASED COLLOIDAL DELIVERY SYSTEMS

Colloidal delivery systems consist of two distinctive phases, the disperse phase in a continuous phase (Attwood, 2002). Colloidal systems can be grouped into three groups. The first group is lyophilic colloids, the second is lyophobic and the last is association colloids (Martin, 1993). Examples of colloidal dosage forms include liposomes, emulsions and micro-emulsions, polymeric micro-spheres and macromolecular micro-spheres (Grobler *et al.*, 2007). Liposomes are vesicles consisting of lipid bilayer spheres separated by an aqueous phase (Date *et al.*, 2007). The molecules in the lipid bilayer consist of a hydrophilic head and a hydrophobic tail (Honeywell-Nguyen & Bouwstra, 2007). The vesicle is 80 nm to 100 µm in size and as seen in Figure 2.1 can entrap lipid and water soluble drugs. One of the first application of liposomes as a delivery system was the targeting of macrophages in leishmanial infections (Date *et al.*, 2007). An emulsion consists of two immiscible liquid phases. The dispersed phase is small droplets in the disperse medium usually stabilized by an emulsifying agent (Attwood, 2003; Martin, 1993). Polymeric nanoparticles consist of biocompatible polymeric matrices between 1 nm and 1000 nm in size able to entrap, adsorb or covalently attach to drugs (Date *et al.*, 2007).

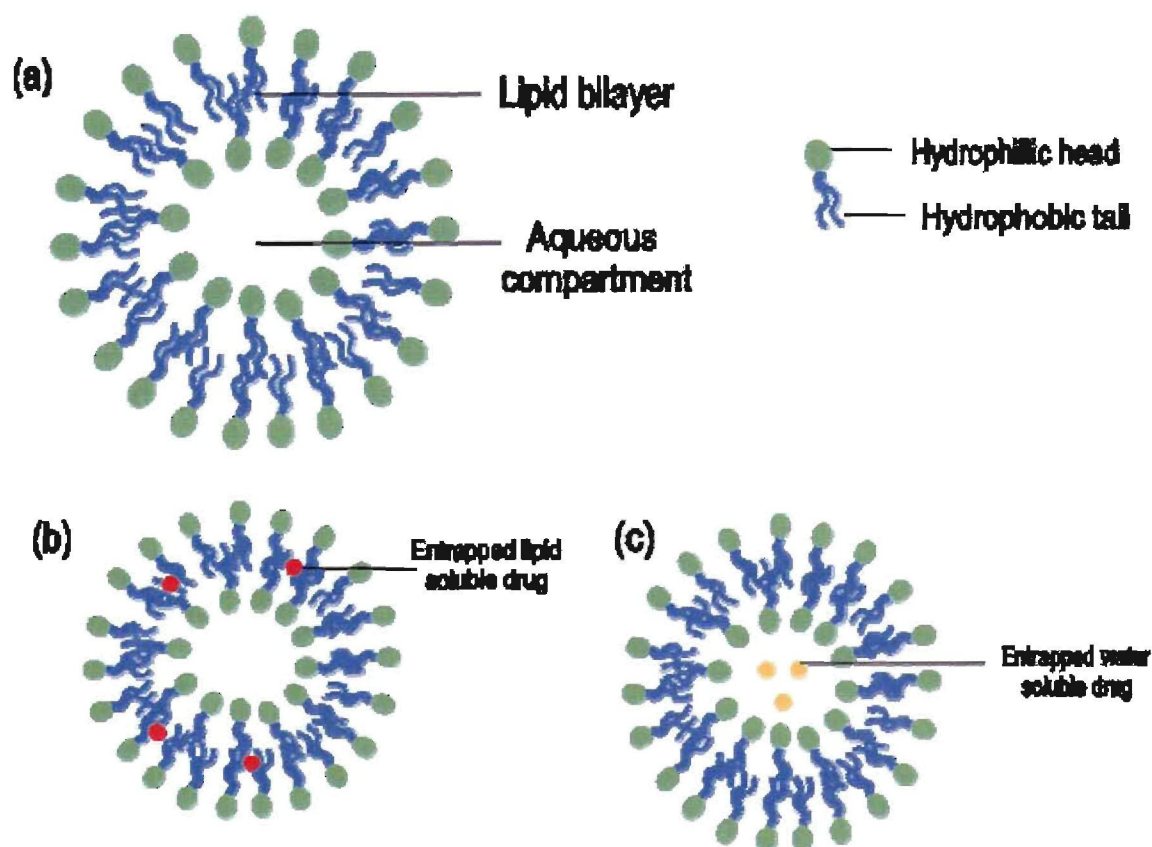


Figure 2.1 Schematic representation of a liposome vesicle (a). The lipid bilayer entraps lipid soluble drug (b) and the aqueous compartment water soluble drugs (c). (Adapted from Honeywell-Nguyen & Bouwstra, 2007).

2.3 CHARACTERISTICS OF PHEROID™ TECHNOLOGY

Pheroid™ formulations are a colloidal system. The Pheroid™ formulations incorporate different features of the different colloidal dosage forms (Grobler *et al.*, 2007). The following characteristics make the Pheroid™ technology unique:

- ◆ Structure and classification of the different types of Pheroid™ formulations.
- ◆ Toxicity profile of Pheroid™.
- ◆ Mechanism of uptake.
- ◆ Pharmacokinetic properties - drug entrapment, protection of drugs, absorption and volume of distribution.

2.3.1 Structure and classification of the different types of Pheroid™ formulations

Pheroid™ consists of essential fatty acids that are a natural ingredient of the body dispersed in a liquid and nitrous oxide gas phase (Grobler, 2004). It has no cholesterol or phospholipids but it is the main ingredient of liposomes (Grobler, 2004; Grobler *et al.*, 2007). Other lipid based delivery systems consist of artificial polymers that are foreign to the body (Grobler, 2004). Pheroid™ Technology consists of different types of Pheroid™ formulations that depends on the composition and method of manufacturing (Uys, 2006). The three main formulations are:

- ◆ Pheroid™ vesicles.
- ◆ Pheroid™ microsponges.
- ◆ Pro-Pheroid™ in depots or reservoirs.

Every Pheroid™ formulation has a unique composition (Uys, 2006). The size of the Pheroid™ particle can be manipulated for the different formulations and intended use (Grobler, 2004; Uys, 2006). The vesicle is a lipid-bilayer molecule. The size of each vesicle is in the range of 0.5 μm to 1.5 μm (Grobler, 2004). The microsphere formulation has prolonged release characteristics and is between 1.5 μm and 5 μm in size (Steyn, 2006). The microspheres consist of a central hydrophilic aqueous space with a thick sponge-like membrane. This structure gives the microsphere the ability to entrap hydrophilic compounds in the centre and hydrophobic compounds in the membrane (Grobler *et al.*, 2007). The amount of pro-Pheroid™ in the reservoirs or depots determines the size. This formulation gives prolonged release that is dependent on the concentration gradient (Uys, 2006). Figure 2.2 shows the different types of Pheroid™ formulations.

Pheroid™ formulations are sterically stabilized by the composition of the formula. There is no increase in particle size or decrease in elasticity. The elasticity and fluidity enables the Pheroid™ to be extravasated from the vascular system. Other lipid based delivery systems needs to be sterically stabilized that leads to an increase in size and rigidity. Because liposomes contain cholesterol the system loses fluidity and elasticity that makes

extravasating difficult. Repeatability of the manufacturing of Pheroid™ formulations have been achieved where as other lipid systems vary in size that make batch repeatability difficult (Grobler, 2004).

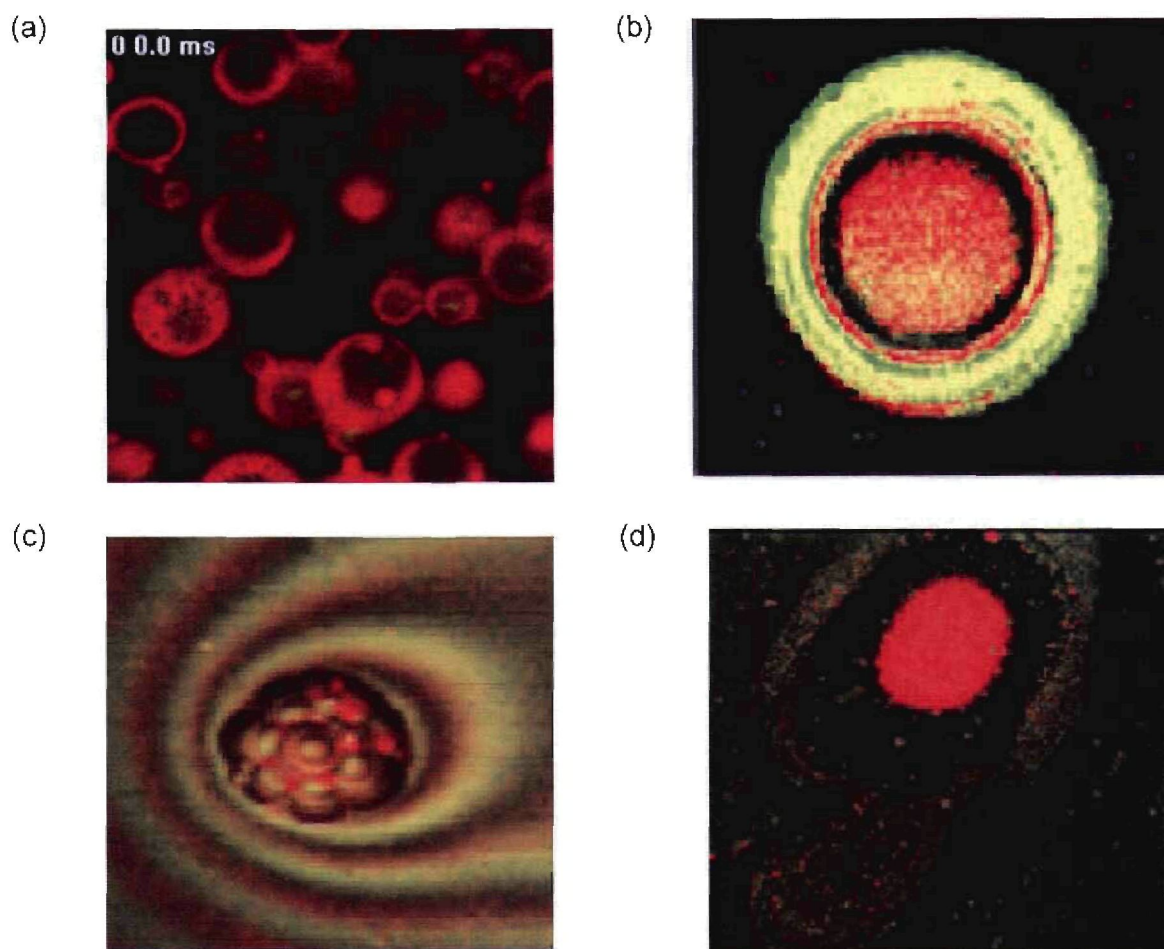


Figure 2.2 Confocal laser scanning of the Pheroid™ formulations. (a) A mixture of vesicles and microsponges. (b) An elastic vesicle containing rifampicin. (c) A small pro-Pheroid™ for oral administration. (d) A depot with a hydrophobic core with pro-Pheroid™ and surrounded by hydrophilic zone (Adapted from Grobler, 2004; Grobler *et al.*, 2007).

2.3.2 Toxicity

During an extensive *in vivo* toxicity test on Sprague Dawley rats, no signs of toxicity was observed with the administration of oral pro-Pheroid™ formulation at a concentration of 50 mg/kg. The toxicity test included the following:

- ◆ Blood samples to determine the possible effect of Pheroid™.
- ◆ Urinalysis.
- ◆ Necropsy, organ weight and histopathology.
- ◆ Bodyweight and feed consumption (Elgar, 2008).

No cytotoxicity was observed. Pheroid™ assists in membrane maintenance. Because Pheroid™ consists of fatty acids that are a natural ingredient, no immune response is elicited. In other lipid based delivery systems cytotoxicity and impaired cell integrity is common. Especially in liposome formulations, an immune response can be elicited (Grobler, 2004).

2.3.3 Mechanism of uptake

The proposed mechanism of uptake of the Pheroid™ into the cell is the high affinity of the fatty acids for the cell membranes. It interacts with the cell membrane and penetrates the cell through the endosome sorting mechanism resulting in effective and fast delivery. It is able to target the sub cellular level to some extent depending on the formulation. Mammalian cell mechanism of other lipid based delivery systems have not yet been described (Grobler, 2004).

2.3.4 Pharmacokinetic properties of Pheroid™ formulations

The kinetic properties of the delivery system include the drug absorption, distribution and elimination including metabolism and excretion. The study of pharmacokinetics is experimental and theoretic (Shargel and Yu, 1999). Entrapment of the drug and the protection given by the delivery system has a direct influence on the bioavailability and absorption (Grobler, 2004). The following aspects will be discussed:

- ◆ Drug entrapment in the delivery system.
- ◆ Protection of the drug by the delivery system.
- ◆ Absorption.
- ◆ Volume of distribution.

2.3.4.1 Drug entrapment

Pheroid™ is a polyphillic system. Pheroid™ can entrap molecules that are water and lipid soluble with high efficacy (85 – 90%). Pheroid™ has the ability to entrap one drug in the interior space and another in the membrane. This characteristic decreases drug interaction making combination therapy possible with a single preparation. The Pheroid™ can be manipulated in size, charge, lipid composition and membrane packing to optimise formulation to ensure that the maximum effect is achieved. Other lipid based delivery systems can either entrap water soluble or lipid soluble drugs (Grobler, 2004).

2.3.4.2 Protection of the drug

A current problem is that many drugs have reduced therapeutic effects because of the partial degradation of the drug before it reaches the specific target (Vogelson, 2001). Pheroid™ protects the entrapped drug against degradation before it reaches the target site. No leakage of the drug out of the delivery system is observed. Other lipid based delivery systems can protect the drug after IV administration but leakage is present (Grobler, 2004).

2.3.4.3 Absorption

Shargel and Yu defines bioavailability as the measurement of the rate and extent of active drug that reaches the systemic circulation (1999). Pheroid™ increases the absorption and bioavailability in oral, topical, nasal and buccal preparations. An increase in the bioavailability leads to a reduction in the minimal inhibitory concentration with increased therapeutic efficacy. Other lipid based delivery systems can also improve the bioavailability whereas some can decrease it (Grobler, 2004).

2.3.4.4 Volume of distribution

The volume of distribution of a drug represents the volume that must be taken into consideration when calculating the drug concentration needed. A drug with a large volume of distribution means that there is more drug concentrated in the extravascular tissue than intravascularly. Drugs that have high protein binding have lower volume of distribution (Shargel and Yu, 1999). Drugs with high volume of distribution can be entrapped in the Pheroid™ to reduce the volume of distribution. This leads to an increase of drug concentration at the target site. The narrow therapeutic index is enhanced with less toxicity (Grobler, 2004).

2.4 CLINICAL APPLICATIONS OF PHEROID™

The different Pheroid™ formulations make it possible to have a wide range of possible clinical applications (Grobler, 2004). The applications include:

- ◆ Transdermal and cosmetic therapies.
- ◆ Preparations for the prevention and treatment of infectious diseases.
- ◆ Vaccines as preventative therapy.
- ◆ Peptide drug delivery (Grobler, 2004; Grobler *et al.*, 2007).

2.4.1 Transdermal and cosmetic therapies

When designing a topical product, it is important to maintain the natural homeostasis of the skin. The product should therefore be designed according to the requirements of the skin. The delivery system of active compounds should not disturb the homeostasis of the skin. Fatty acids, like in the Pheroid™ help maintain the homeostasis of the skin. Pheroid™ enhances the cellular uptake of the entrapped active compound. It also shows the ability to decrease the immune response and adverse reactions. The dosing frequency and cytotoxicity are decreased and therapeutic effect increased (Grobler *et al.*, 2007). Some of the active compounds that can be used in combination with Pheroid™ are listed in table 2.2.

Table 2.1 Active compounds used in combination with Pheroid™ for different therapeutic applications (Adapted from Grobler *et al.*, 2007).

Skin proliferation	Anti-infective agents	Moisturising creams and lotions
Retinoids	Gentamycin	Alpha and beta –hydroxy acids like fruit extract and salicylic acid
Vitamin D	Sulphamethoxazole	Various types of cologne
Hydrocortisone	Trimethoprim	Hydrolyzed elastin
Epinephrine	Cloxacillin	Vitamin C
Extracellular adhesion and signalling molecules	Acyclovir	Anti-oxidants and preservatives
Calcium	Ciprofloxacin	Essential oils
	Miconazole nitrate	
	Neomycin	

Acyclovir is an antiviral agent effective against the herpes simplex virus 1 and 2. When formulated in a Pheroid™ micro-formulation it was compared *in vitro* with phosphate buffer solution (PBS) and had a better drug delivery across the skin (Van der Walt, 2007).

Transdermal delivery of antituberculosis drugs, isoniazid and rifampicin in a Pheroid™ micro-formulation showed an increase of drug delivery. The drugs were delivered in the *in vitro* test above the minimum inhibitory concentration (MIC) that is important to minimize drug resistance (Botes, 2007)

2.4.2 Preparations for the prevention and treatment of infectious diseases

Infectious diseases are a major problem in the world especially in Africa. The prevention and treatment of infectious diseases are important. The following clinical applications have been investigated:

- ◆ The treatment of tuberculosis.
- ◆ The treatment of malaria.
- ◆ The treatment of AIDS.

2.4.2.1 The treatment of tuberculosis

Tuberculosis (TB) and multidrug resistant TB is a big health concern in South Africa (Hoek *et al.*, 2008). TB-drugs combined with Pheroid™ showed to be very effective in *in vitro* and *in vivo* studies (Grobler, 2004; Matthee, 2007). For the pre-clinical trial, a Pheroid™ with 60% of the prescribed dosage regime was compared to the normal prescribed regime of TB-drugs. The oral preparation of pro-Pheroid™ and TB-drugs combination lead to an increase and faster absorption rate. This increase in drug plasma levels lead to an enhanced therapeutic effect over a longer time period. The target specific delivery of the drug decreases the MIC. Less of the drugs are necessary to give the same effect with less side effects and developing of drug resistance (Grobler, 2004). The *in vivo* efficacy of the drug regimes in a 60% pro-Pheroid™ formulation compared with the drugs in water showed an increase in plasma concentration. Rifampicin in pro-Pheroid™ showed a 305% increase in the plasma concentration where isoniazid and pyrazinamide showed a 20% and 19% increase

respectively. This 60% pro-Pheroid™ formulation showed better absorption and bioavailability (Matthee, 2007). The *in vitro* transdermal delivery of isoniazid and rifampicin lead to an increase when formulated in a Pheroid™ micro-formulation. The drug quantities as determined over 12 hours showed levels above the MIC (Botes, 2007). Accelerated stability tests of isoniazid, pyrazinamide and ethambutol remained within the 90-110% of the original concentration (Matthee, 2007).

2.4.2.2 The treatment of malaria

Malaria kills more than one million people annually with a big economic burden especially on the poor countries in sub-Saharan Africa. Resistance to antimalarial drugs make it necessary for developing new drugs or better delivery of the current drugs (WHO, 2007a). The *in vitro* drug efficacy studies showed that chloroquine, mefloquine, artesunate and artemether had better efficacy in the Pheroid™ formulation than in water. Chloroquine showed an efficacy enhancement of 1544% against a chloroquine resistant strain. Where as mefloquine, artesunate and artemether had an efficacy enhancement of 314%, 238% and 254% respectively (Langley, 2007). The ability of Pheroid™ to reduce or eliminate drug resistance can be due to the drug that is delivered beyond the efflux pump (Grobler, 2004). The *in vivo* study using the *P. berghei* mouse model showed better efficacy of chloroquine in Pheroid™ during the first 11 days after infection compared to chloroquine in water (Langley, 2007).

2.4.2.3 The treatment of HIV/AIDS

Pheroid™ possibly enhances the uptake of drugs into cells during an *in vitro* efficacy test of lamivudine (3TC) in Pheroid™ (Botha, 2007). In the *in vitro* efficacy study of HIV drugs the M7-Luc cells (T-cell line) when incubated for a short period with Pheroid™ show enhanced viability (Van der Merwe *et al.*, 2008). Accelerated stability testing of HIV drugs in Pheroid™ formulations showed a decrease in drug contents over three months (Cassim, 2007). A nevirapine in pro-Pheroid™ formulation were stable in an accelerated stability test done over three months (Kühn, 2008). Lamivudine derivates were tested *in vitro* in a transdermal preparation and compared with PBS. These derivates did not show better transdermal flux but the derivates that had a higher water solubility had better transdermal flux in Pheroid™ (Gerber, 2007). Stavudine derivatives synthesised had better transdermal flux than stavudine

in Pheroid™. When compared to the control of PBS, Pheroid™ showed an increase in transdermal flux (Holmes, 2006)

2.4.3 Vaccines as preventative therapy

Vaccination of smallpox led to the elimination of smallpox. Vaccination strategy is therefore important but synthetic and recombinant peptide vaccines elicit a human immune response. It is necessary for effective vaccine adjuvants to enhance the immunogenicity and immunostimulatory properties. The vaccine adjuvants can be grouped in 2 classes, the immunostimulatory or –modulatory adjuvants and vaccine delivery systems. Pheroid™ can be used as a vaccine delivery system (Grobler, 2004).

2.4.3.1 Virus-based vaccines

Rabies is a virus-based vaccine. Carnivores and certain bat species host the rabies virus. Infections of humans from rabid animal bites are fatal. Post-exposure vaccination prevents the death of millions of people each year. The inactivated virus is used in the vaccine formulation. Animal studies of the virus in combination with Pheroid™ were compared with other formulations. The Pheroid™ formulations showed a significant increase in antibody response. This study was repeated in other animal studies with similar results (Grobler, 2004).

2.4.3.2 Peptide-based vaccines

The non-recombinant hepatitis B vaccines are a peptide-based vaccine. A surface molecule of the virus is used as antigen. An antibody response needs to be elicited to ensure the effectiveness of the vaccine. In combination with Pheroid™ there was an increase in the antibody production. This animal study was executed by the SA state vaccine Institute and the department of Immunology at the University of Cape Town (Grobler, 2004). Figure 2.3 show the comparison between the Pheroid™ formulation and that of existing vaccines.

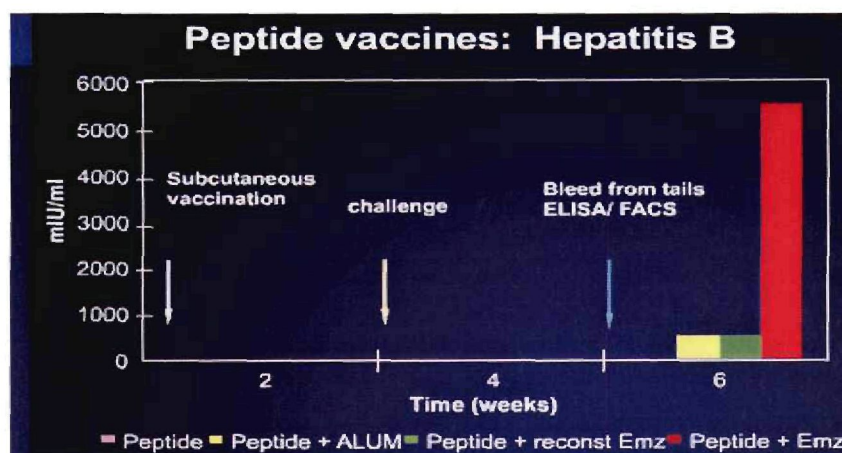


Figure 2.3 Comparison between the antibody production of the Pheroid™ formulation and existing vaccines over time (Grobler, 2004).

2.4.4 Peptide drug delivery

Protein drugs are used in neurological, endocrinological and haematological diseases and disorders (Edwards *et al.*, 1999). Peptide and other protein drugs are poorly absorbed after oral delivery (Johnson and Tracey, 1999). The possible enhancement of the absorption of these peptide drugs using Pheroid™ technology have been investigated (Coetzee, 2007; De Bruyn, 2006; Kotzé, 2005; Steyn, 2006; Strauss, 2005). These drugs include:

- ◆ Calcitonin for the treatment of osteoporosis.
- ◆ Insulin for the treatment of insulin dependent diabetes mellitus.
- ◆ Human growth hormone important for the maintenance of optimal cellular performance.
- ◆ Vasopressin needed to regulate blood pressure.

2.4.4.1 Calcitonin: Polypeptide hormone

Calcitonin is necessary for regulating the calcium concentration in plasma levels. The nasal administration of calcitonin in combination with Pheroid™ microsponges and vesicles lead to an increase in nasal absorption saline *in vivo* (Kotzé, 2005; Lubbe, 2007). When a smaller

dose was given in a comparative study, no significant increase in nasal absorption was observed (Maritz, 2008). The *in vivo* study of oral administration of calcitonin formulated in Pheroid™ had a better intestinal absorption (Strauss, 2005).

2.4.4.2 Insulin: Polypeptide hormone

Insulin dependant diabetes mellitus is a fatal disorder when left untreated. This chronic disorder is treated by injection of recombinant human insulin subcutaneously. The *in vivo* effect of the nasal administration of insulin was determined using Sprague Dawley rats. The insulin formulated in either Pheroid™ microsponges or Pheroid™ vesicles showed a decrease in glucose levels after nasal administration (De Bruyn, 2006).

2.4.4.3 Human growth hormone: Polypeptide hormone

Human growth hormone is used in the treatment of short stature in children. This polypeptide hormone in combination with Pheroid™ showed an increase in plasma concentration after nasal administration. In this *in vivo* study male Sprague Dawley rats were used. Pheroid™ technology shows great potential to enhance absorption (Steyn, 2006).

2.4.4.4 Vasopressin: Nanopeptide hormone

Vasopressin, also known as anti-diuretic hormone (ADH) increases the water permeability of the cells for long term blood pressure control (Reid, 2004). Pheroid™ increases the flux of the vasopressin compared to the control in a transdermal preparation. Pheroid™ formulation increased the peptide drug delivery (Coetzee, 2007).

2.5 CONCLUSION

Drug delivery systems like Pheroid™ are primarily used to control drug delivery with target specific delivery. Pheroid™ is a patented drug delivery system. It has a unique submicron emulsion type formulation of fatty acids capable of encapsulating various drugs. The ability to entrap both water and lipid soluble drugs makes combination therapy possible. The structure, morphology, size and function can be manipulated according to the intended use. It has a diversity of possible clinical applications including transdermal, preparation for infectious diseases, vaccines and peptide drug delivery. Pheroid formulations include oral, topical, nasal and buccal. Pheroid™ has the ability to increase delivery of the active compound at lower minimum inhibitory concentration with higher therapeutic efficacy. It has reduced cytotoxicity with little toxicity at the prescribed dose. Drug delivery can play an important role in the management of the global burden of malaria.

CHAPTER 3

DRUG EFFICACY TESTS

3.1 INTRODUCTION

The efficacy of antimalarial drugs is measured by the ability of the drugs to eradicate the parasite from the host. One of the most important functions of a drug efficacy test is to ensure treatment of resistant *Plasmodium* infections. To assess the sensitivity of antimalarial drugs, one of several different approaches can be followed. As seen in Figure 3.1, drug efficacy can be determined by *in vitro* studies, *in vivo* studies and in clinical trials. Parasites in the blood of an infected patient are detected by polymerase chain reaction (PCR) or the golden standard of microscope evaluation (Basco, 2007; Noedl *et al.*, 2003). *In vivo* drug efficacy studies are a quantitative evaluation of drug efficacy carried out with accepted drug dosages to ensure tolerability. Additional bioassays to determine the pharmacokinetic factors like absorption, metabolism and excretion should be done (Noedl *et al.*, 2003). *In vivo* studies to assess drug efficacy were first conducted in 1965 after chloroquine resistance was observed. Clinical trials on infected patients are done using a standardised protocol that was developed by the Centres of Diseases Control and Prevention and the WHO to assess therapeutic efficacy (WHO, 2002).

Drug efficacy tests done in *in vitro* studies have a different approach. It measures the sensitivity of malaria parasites quantitatively (Noedl *et al.*, 2003). The first antimalarial *in vitro* drug sensitivity test was preformed by Bass in 1922. Forty years later the macro-test, later adopted by the WHO, was developed by Karl Rieckmann. The main reason for this assay

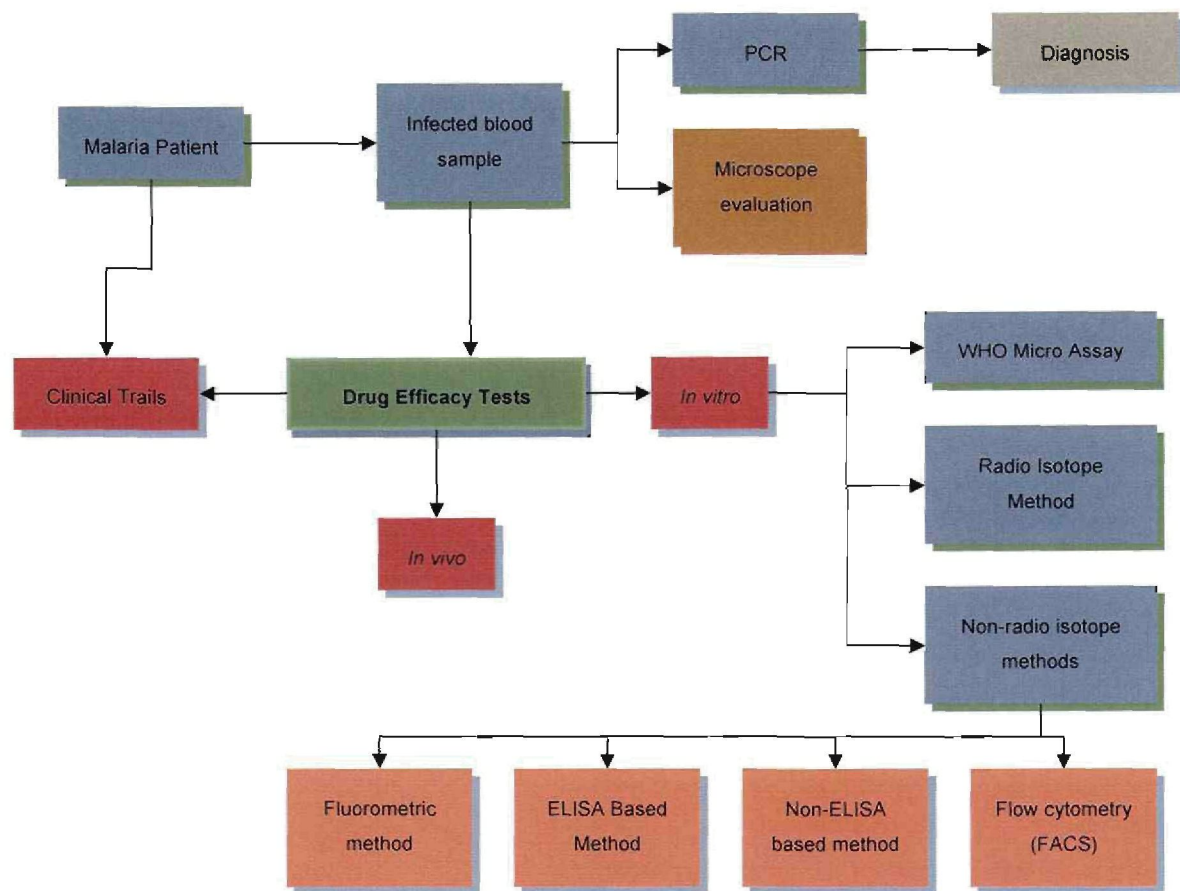


Figure 3.1 Different approaches to assess the sensitivity of antimalarial drugs
(Adapted from Noedl *et al.*, 2003; Basco, 2007).

developments was to monitor the antimalarial drug resistance development. The WHO brought out a standard test kit and procedure based on the macro-test of Rieckmann. This test kit was used during 1976 and 1987. The advantages of this kit were that it was simple and no sophisticated equipment was necessary. The disadvantages were that the volume of blood needed was very high and it had a low success rate in short term cultures. *In vitro* drug efficacy tests are a research tool for drug development and can be used for:

- ◆ Screening of new drugs.
- ◆ Evaluating drug interaction in combination therapy.
- ◆ Evaluating resistance and cross resistance (Basco, 2007).

In vitro assays can be grouped according to method used to quantify the parasite density. The WHO micro-assay is the basic procedure to follow during a drug efficacy test and is usually evaluated microscopically. The radio isotope method utilizes radio active precursors to determine the parasite density. Non-radio isotope methods can be grouped into four different sub groups. The sub groups include fluorometric method, ELISA based and non-ELISA based methods and flow cytometry (Noedl *et al.*, 2003; Basco, 2007).

3.2 CULTIVATION OF *PLASMODIUM FALCIPARUM* IN CONTINUOUS CULTURES

Malaria parasites were first successfully cultivated by Trager and Jensen during the 1970 (Basco, 2007; Schuster, 2002; Trager & Jensen, 1976). The cultivation of these parasites leads to drug efficacy tests and method development (Basco, 2007). *In vitro* cultivation of *Plasmodium* requires the imitating of the conditions during the erythrocytic cycle (Schuster, 2002). Culture medium is supplemented with RPMI 1640 medium, HEPES, human serum, erythrocytes and sodium bicarbonate. The cultures are incubated at 37 °C and an atmosphere of between 3-10% carbon dioxide (CO₂), 2-17% oxygen (O₂) and the balance nitrous gas (N₂) (Basco, 2007; Schuster, 2002). During the drug assays the incubation period can vary from 24 to 96 hours depending on the assay and drug used. The CO₂ alters the pH of the culture and can alter the 50% inhibitory concentration (IC₅₀) of drugs that is pH dependant like chloroquine. The presence of leukocytes in the cultures has no effect because of the rapid degradation it undergoes. The fraction erythrocytes and parasite density used in drug efficacy tests can also have an influence on the IC₅₀ value. Higher initial parasitemia levels and erythrocyte fractions increases the IC₅₀ value. The best starting parasitemia should be between 0.3 and 0.8% with the haematocrit at 2% (Basco, 2007). Synchronized cultures used in efficacy studies were first reported by Lambros and Vanderberg (1979). The sorbitol treatment to synchronize the cultures in a specific stage, can lead to quantitative studies on the immunological, biochemical and physiological differences during stage development (Lambros & Vanderberg, 1979).

3.3 POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) was developed by Kary Mullis during the 1980's and received the Nobel Prize in Chemistry in 1993 for his work. Mullis summarized his work as beginning with a single molecule of genetic material and the PCR generates 100 billion similar molecules in an afternoon. This reaction is easy to execute and only requires no more than a test tube, a few simple reagents and a source of heat (Mullis, 1990). PCR is a molecular method (Mens *et al.*, 2006) that uses DNA polymerase enzymes to amplify a specific sequence in the DNA by replicating it several million-fold (Bloom, 2008). It is a specific and sensitive method (Hang *et al.*, 1995; Mens *et al.*, 2008) that can be used in the diagnosis of a wide range of diseases including malaria (Mens *et al.*, 2008). In the diagnosis of malaria the blood sample is thawed and the lysed erythrocytes are treated with proteinase K and phenol-chloroform mixture. The parasite's DNA is extracted and processed by PCR to amplify the species-specific sequence of the small sub-unit ribosomal ribonucleic acid (18S ss rRNA). The amplified product is stained with ethidium bromide and visually detected by UV transillumination (Zakeri *et al.*, 2002). PCR has the ability to single out the different *Plasmodium* species because the band pairs lie at different distances (Hang *et al.*, 1995; Zakeri *et al.*, 2002). Advantages of the PCR method are the high sensitivity, ability to complete the diagnosis in an afternoon, are not affected by the subjectivity of the observer and can differentiate between the different *Plasmodium* species (Rougemont *et al.*, 2004; Swan *et al.*, 2005; Zakeri *et al.*, 2002). Problems associated with the PCR method are the complex methodologies and need of specially trained personnel (Rougemont *et al.*, 2004; Swan *et al.*, 2005). Real-time PCR uses fluorescent labels for continuous monitoring with the advantages of fast results (Rougemont *et al.*, 2004) and there is no need for trained technologists (Swan *et al.*, 2005). PCR can be implemented in the diagnosis of the different species of *Plasmodium* with high efficacy and sensitivity (Mens *et al.*, 2006; Rougemont *et al.*, 2004; Swan *et al.*, 2005.)



3.4 MICROSCOPE EVALUATION

Microscope evaluation of drug efficacy tests was first used in the “48-h *in vitro* reinvasion test” by Trager (Basco, 2007). Microscope evaluation is based on the manual visual evaluation of stained thin and thick blood smears by trained personnel (Rougemont *et al.*, 2004). Slides are usually stained with giemsa solution and evaluated under a microscope. It is based on the principle that erythrocytes do not contain any DNA while the parasites do (Cohen, 1982). When using a stain like giemsa that is DNA specific only the parasite is stained making it possible to manually count the amount of parasites present (Bio Medicine, 2008). To determine the level of parasitemia, the total amount of infected erythrocytes is divided by the total amount of erythrocytes and multiplied by 100. The erythrocytes of different microscope fields are counted (Basco, 2007). Microscope evaluation is seen as the golden standard (Guy *et al.*, 2007) and is a relative inexpensive, rapid and simple method compared to other assays (Rougemont *et al.*, 2006). Microscope evaluation does not use expensive or dangerous reagents (Russell *et al.*, 2003). Skilled personnel can differentiate between the different *Plasmodium* species (Rougemont *et al.*, 2006), but to diagnose mixed infections is difficult (Ohrt *et al.*, 2002). Microscopy is labour intensive and needs skilled and trained personnel to avoid false negative results at very low parasitemias (Basco, 2007; Piper *et al.*, 1999; Sio *et al.*, 2006). These problems can be overcome by making use of automated image analysis software like MalariaCount (Sio *et al.*, 2006). Variations on microscope evaluation include the use of different stains to evaluate the slide. Fluorescent dyes can potentially improve the accuracy of microscopy (Guy *et al.*, 2007). Even though Giemsa staining of thin and thick blood smears is the golden standard for drug efficacy tests, it leaves a lot to be desired (Basco, 2007; Guy *et al.*, 2007; Piper *et al.*, 1999).

3.5 MICRO-TECHNIQUE AND WHO TEST

The micro-technique was developed by a group under guidance of Karl Rieckmann in 1970 (Basco, 2007; WHO, 2007b). The micro-test was based on the same principle as the macro-test that was developed by Rieckmann in 1968. The major difference between these two

methods is the volume of infected blood needed is much less in the micro-technique (WHO, 2007b; Noedl *et al.*, 2003). The principle of these methods is the collection of *Plasmodium falciparum* infected capillary blood and adding it to pre-coated microtitre plates. The plate is coated with a series of different drug concentrations (Basco, 2007; WHO, 2007b). The plates are placed in a candle jar and incubated for 24 hours at 38 - 39°C. A candle jar is an airtight container you light a candle in to minimize the presence of oxygen in the container. After the 24 hour incubation period, Giemsa stained thick blood smears are made and visually analyzed with a microscope (Basco, 2007; WHO, 2007b). The schizont maturation is determined by counting the total amount of schizont present compared to the total amount of asexual parasites on the slide (Noedl *et al.*, 2003; WHO, 2007b). An advantage of determining schizont maturation is that the parasites don't burst to reinfect other cells (Noedl *et al.*, 2003). The WHO adapted this method for field application and it was first used during the late 1970. During 1981 to 2000 the WHO micro-technique assay kit was available as Mark I to Mark III (WHO, 2007b). These days this method is mostly used to determine the epidemiology of drug resistance and to assess drug sensitivity (Noedl *et al.*, 2003; WHO, 2007b). Problems that arise from this method are that it is labour intensive and needs skilled personnel for the microscope evaluation (Noedl *et al.*, 2003).

3.6 RADIO-ISOTOPE METHOD

Radio-isotope methods are indirect methods to determine the metabolic activity of parasites. Labelled precursors like nucleic acids, proteins or phospholipids are added to the culture and incorporated into different metabolic activity of the parasite. Purines and pyrimidines are nucleic acids that are used in RNA and DNA synthesis. The widely used [²H]hypoxanthine method makes use of purine precursors. Other purines include [2,8-³H]adenosine and [G-³H]hypoxanthine. Proteins or amino acids like [³⁵S]methionine, [³H]leucine and [¹⁴C]isoleucine can be used to analyse the amount of protein produced by the parasite. [³H]ethanolamine, [³H]choline, [¹⁴C]serine and [³H]inositol is radio labelled phospholipids that can be used to quantitatively determine the parasite density (Basco, 2007). The disadvantages of these methods are the radio-active compounds that is used and the need of a well equipped laboratory (Basco, 2007; Kaddouri *et al.*, 2006).

3.6.1 [³H]hypoxanthine-based assay

The purine incorporated [³H]hypoxanthine isotopic assay is the most widely used *in vitro* method to determine the IC₅₀ value of drugs (Bacon *et al.*, 2007; Basco, 2007; Noedl *et al.*, 2002). Hypoxanthine is converted through a series of reaction to adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (Tulane, 2008) and incorporated in RNA and DNA (Kicska *et al.*, 2001; Tulane, 2008). The method measures the specific metabolic pathways (Noedl *et al.*, 2003) and provide quantitative measurements (Desjardins *et al.*, 1979). The amount of [³H]hypoxanthine is directly proportional to the number of infected erythrocytes (Basco, 2007). Beside the use of radio-active compounds (Bacon *et al.*, 2007; Basco, 2007; Noedl *et al.*, 2003), this method is also time consuming (Bacon *et al.*, 2007) and has technical limitation (Kaddouri *et al.*, 2006).

3.7 NON RADIO-ISOTOPE METHODS

Microscopic evaluation and radio isotope methods offered some advantages and were effective in determining the parasitemia. These methods also had a great number of disadvantages including the use of radio active reagents and being labour intensive. This made the need for alternative methods necessary (Bacon *et al.*, 2007; Noedl *et al.*, 2002). The non radio-isotope methods include the following:

- ◆ Fluorometric assays.
- ◆ ELISA based assays.
- ◆ Non-ELISA based colorimetric assays.
- ◆ Flow cytometry (Basco, 2007).

3.7.1 Fluorometric assays

The basic principle of fluorometric assays is that erythrocytes don't contain any RNA or DNA, but infected erythrocytes do (Basco, 2007). DNA specific fluorochromes label DNA of the parasite and is quantitatively measured (Corbett *et al.*, 2004). Fluorochromes include ethidium bromide, Hoechst 33358, Pico green and SYBR® green (Basco, 2007), acridine orange (Bosch *et al.*, 1996) and YOYO-1 (Jiménez-Díaz *et al.*, 2005). The fluorescence intensity emitted is proportional to the amount of DNA or RNA present (Basco, 2007). Fluorochromes have successfully been utilized in the microscopic diagnosis of malaria (Bosch *et al.*, 1996; Guy *et al.*, 2007). The advantages of this method are that it is less expensive, rapid and simple to perform. The technician needs to be skilled to be able to extract the DNA and further studies needs to be done to establish the sensitivity of the method (Basco, 2007).

3.7.2 Enzyme-Linked ImmunoSorbent Assay (ELISA based assays)

ELISA based assays are based on the detection of parasite antigens (Tomar *et al.*, 2006). This *in vitro* test measures the bio-molecules produced by the *Plasmodium* parasite (Noedl *et al.*, 2005) through the optical density on a spectrophotometer (Appawu *et al.*, 2003). This method was developed as an alternative method for microscopy in drug efficacy tests (Tomar *et al.*, 2006). It can detect low parasitemias levels accurately (Noedl *et al.*, 2006). It is easy and rapid to perform, doesn't need specialised equipment, has no radio-active reagents and is less labour intensive than other methods. Disadvantages of ELISA based assays are the large amount of washing steps (Basco, 2007; Noedl *et al.*, 2003; Noedl *et al.*, 2004; Noedl *et al.*, 2006; Tomar *et al.*, 2006). Two types of ELISA mostly used are:

- ◆ Histidine-Rich Protein 2 (HRP2).
- ◆ Double-site enzyme-linked lactate dehydrogenase immunodetection (DELI) (Noedl *et al.*, 2005).

3.7.2.1 Histidine-Rich Protein 2 (HRP2)

The HRP2 assay measures the histidine- and analine-rich protein produced by *P. falciparum* (Noedl *et al.*, 2003) in the late ring and trophozoites phase (Basco, 2007). The HRP2 is localized in pockets in the cytoplasm of *P. falciparum* and the cytoplasm and infected erythrocyte membranes of the host (Noedl *et al.*, 2002). HRP2 is an indicator of the multiplication and development of the parasite (Basco, 2007; Noedl *et al.*, 2002). HRP2 is specific to *P. falciparum* and can not be used to detect *P. vivax*, *P. malariae* or *P. ovale* (Iqbal *et al.*, 2001). Another version of the assay, a rapid diagnostic test (RDT) is used to diagnose malaria in the field. A RDT based on the detection of *P. falciparum* HRP2 shows good sensitivity but can detect the antigen several weeks after parasites have been eliminated from the patient's blood (Hopkins *et al.*, 2007). The high sensitivity and relative simple method makes the detection of HRP2 ideal for the diagnosis in the field (RDT) and drug efficacy tests in the laboratory (Basco, 2007).

3.7.2.2 Double-site enzyme-linked lactate dehydrogenase immunodetection (DELI)

Lactate dehydrogenase (LDH) is an enzyme used in the glycolysis pathway necessary for energy production in humans and *Plasmodium* species (Kaddouri *et al.*, 2006; Tulane, 2008). This *Plasmodium* lactate dehydrogenase (pLDH) structurally differs from human LDH and can be targeted in the diagnosis of malaria and assessment of drug efficacy tests. The double-site enzyme-linked lactate dehydrogenase immunodetection (DELI) ELISA is based on the pLDH presence in infected blood. The amount of pLDH present is directly proportional to the parasite density, and as the parasite dies the level of the enzyme decreases (Kaddouri *et al.*, 2006; Noedl *et al.*, 2003). This method is sensitive and can detect low parasitemia densities (Basco, 2007; Brockman *et al.*, 2004).

3.7.3 Non-ELISA based colorimetric assays – *Plasmodium* Lactate Dehydrogenase (pLDH)

Lactate dehydrogenase (LDH) is an oligomeric enzyme (Palmer, 2001) used in the reduction reaction of lactate to pyruvate during the anaerobic glycolytic metabolic pathway (Basco, 2007; Noedl *et al.*, 2003; Makler *et al.*, 1998). As seen in Figure 3.2 glucose is metabolised for energy production with lactate as byproduct (Palmer, 2001). LDH catalysis the conversion of lactate to pyruvate with the production of reduced nicotinamide adenine dinucleotide (NADH) necessary for the continuous production of ATP (Basco, 2007; Palmer, 2001).

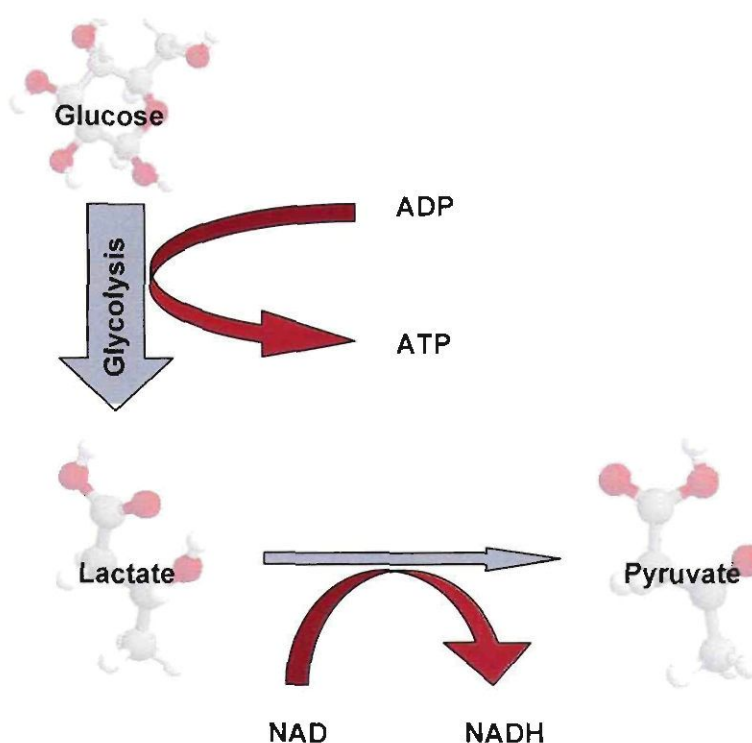


Figure 3.2 The final step of the anaerobic glycolysis metabolic pathway. LDH reduces the lactate to pyruvate with the production of NADH (Adapted from Palmer, 2001; Noedl *et al.*, 2003; Voet and Voet, 1995).

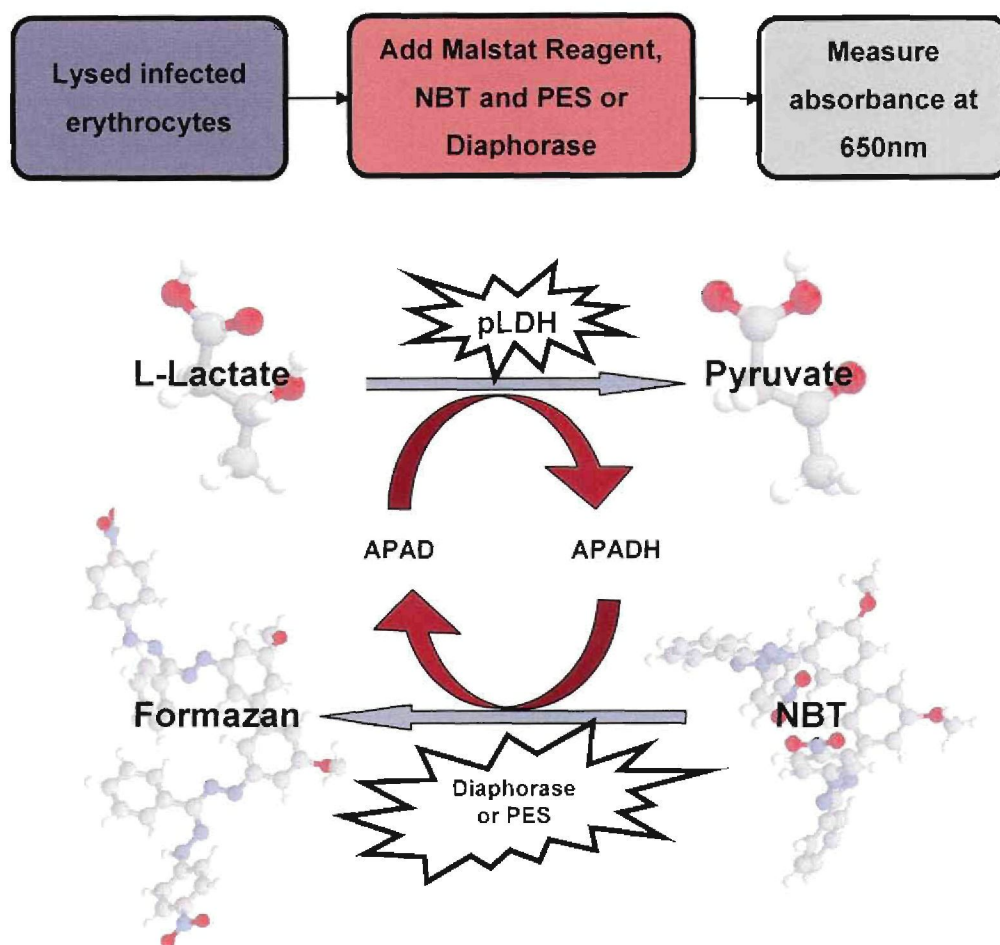


Figure 3.3 The basic concept of the colorimetric assay utilizing pLDH (Adapted from Basco, 2007; Makler *et al.*, 1998; Noedl *et al.*, 2003; Palmer, 2001; Voet and Voet, 1995).

The colorimetric assays make use of the difference in the LDH between the *Plasmodium* species and the host (Basco, 2007; Makler *et al.*, 1998; Noedl *et al.*, 2003). pLDH has distinctive differences in the amino acid and can thus be electrophoretically separated from the human LDH present in the erythrocytes (Basco, 2007). Makler and his co-workers developed a drug sensitivity assay based on this difference (Basco, 2007; Makler *et al.*, 1998; Noedl *et al.*, 2003). Figure 3.3 explains the basic concept of the colorimetric assay utilizing pLDH. Erythrocytes are thawed and lysed before adding Malstat® reagent, nitro blue tetrazolium (NBT) and phenazine ethosulphate (PES) or the enzyme diaphorase. pLDH has the ability to convert the NAD analogue, 3-acetylpyridine-adenine dinucleotide (APAD) very rapidly to the reduced form, APADH and simultaneously produce pyruvate. The reduction

reaction of NBT to formazan is catalyzed by diaphorase or PES in the presence of APADH. Formazan is blue in colour and the absorbance can be measured at 650nm. pLDH convert the APAD to APADH at a much faster rate than the host LDH (Basco, 2007; Makler *et al.*, 1998; Noedl *et al.*, 2003).

3.7.4 Flow cytometry

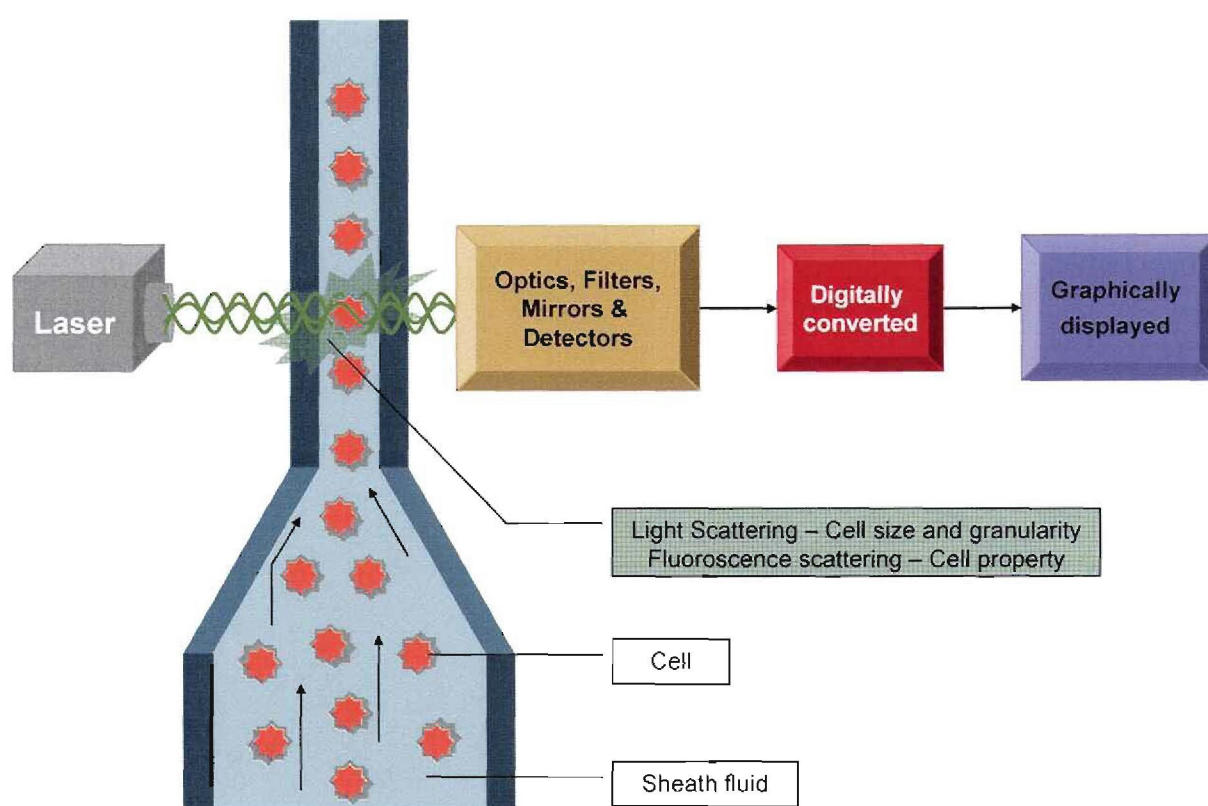


Figure 3.4 The basic principle of flow cytometry. Cells pass in single file through a laser. Light and fluorescence scattering takes place and detected by a series of mirrors, filters, detectors and amps and digitally converted to quantitative data (Adapted from Basco, 2007; FCP, 2008; Nolla, 2008; Riley and Idowu, 2008).

Flow cytometry is a technique developed in the 1970's (Riley and Idowu, 2008) to measure different cell properties as they flow through a column in single file (Basco, 2007; Riley and Idowu, 2008). A fluorescence activated cell sorter (FACS) combines techniques in fluidics,

laser optics, electronics and computer software to determine the properties of cells (Basco, 2007; Nolla, 2008). The basic principle of flow cytometry is illustrated in Figure 3.4. The cells in the sample are injected and pass through a beam of laser light one by one. When a cell passes through the laser two events occur. The first is light scattering analyzing the size, shape and granularity of the cell. The second only occurs if the cell is tagged with antibodies conjugated with fluorochromes for cell surface markers or DNA and RNA specific dyes. The fluorescent dye absorbs the laser light, is excited and emits at a longer wavelengths. The characteristics are detected by a series of mirrors, detectors and filters. The electrical impulse is analysed and processed into quantitative data (Basco, 2007; Riley and Idowu, 2008). This fast and effective method of analysis has a wide range of clinical application. The clinical applications include:

- ◆ Diagnosis of haematological malignancies.
- ◆ Detection of minimal residual disease (acute lymphoblastic leukaemia).
- ◆ Analysis of DNA ploidy and cell cycle.
- ◆ Cell kinetics.
- ◆ Parasitology.
- ◆ Bioterrorism (Nolla, 2008; Riley and Idowu, 2008).

The application in malaria diagnosis and drug efficacy tests has been investigated with great success (Basco, 2007). The absence of DNA in erythrocytes makes the specific DNA fluorochromes like acridine orange and YOYO-1 ideal to bind to the parasite. Because the intensity of the emitting fluorescene is directly proportional to the amount of DNA present, it is possible to distinguish between the different stages in the life cycle of the parasite (Basco, 2007; Riley and Idowu, 2008, Whaun *et al.*, 1983). Flow cytometry is a promising method for assessing drug efficacy (Saito-Ito *et al.*, 2001). A distinctive feature of flow cytometry is that it measures the characteristic of single cells whereas spectrophotometry measures the properties of the bulk volume (FCP, 2008). Flow cytometry is a rapid, automated, accurate and highly sensitive method that can be successfully utilized in drug efficacy tests, but the high cost and difficulty in operating the instrument limits its use in field applications (Basco, 2007)

3.8 CONCLUSION

The global burden of malaria and the persistent emergence of resistance to antimalarial drugs make it necessary for new faster and more sensitive ways to determine parasitemia densities. To effectively determine the parasitemia levels in drug efficacy tests is a big step in the search of new treatments and vaccines. It is important to monitor the epidemiology of resistance for the continuous monitoring and adjustment of dosage regimes for effective treatment. The advantages of some of the new methods for drug sensitivity and efficacy studies can help in the burden of malaria.

CHAPTER 4

METHOD DEVELOPMENT: METHODS, RESULTS & DISCUSSION

4.1 INTRODUCTION

In vitro drug efficacy tests are a research tool for drug development and can be used for screening of new drugs or current drugs in a new delivery system (Basco, 2007). Drug efficacy tests done in *in vitro* studies measures the sensitivity of malaria parasites to the various drugs quantitatively (Noedl *et al.*, 2003). It is also used to monitor the development of antimalarial drug resistance (Basco, 2007). The advent of continuous culture of *P. falciparum* in 1976 made it possible to evaluate the effect of new or current drugs *in vitro* (Trager and Jensen, 1976). In order to accurately describe the effect of drugs, the amount of parasites needs to be counted. Manual microscopic counting of thin blood smears of parasitized samples are labour intensive and subjective. This lead to the search for other methods to determine the parasite density much faster. Several methods have been described since and two of the more popular methods are the colorimetric detection of pLDH and flow cytometric evaluation using a fluorochromes like acridine orange.

4.2 CULTIVATION OF *PLASMODIUM FALCIPARUM* CULTURES

The continuous cultivation of *P. falciparum* was first described by Trager and Jensen as the candle jar method (Basco, 2007; Schuster, 2002; Trager and Jensen, 1976). *In vitro* growth of *Plasmodium* requires imitating of the conditions during the erythrocytic cycle (Schuster, 2002). The original technique requires *P. falciparum* infected human red blood cells, buffered RPMI 1640 medium and human serum (Basco, 2007; Schuster, 2002). Later cultivation techniques used human serum substitutes like Albumax II (Ringwalde *et al.*, 1999). Synchronized cultures used in efficacy studies were first reported by Lambros and Vanderberg (1979). The sorbitol treatment of cultures to synchronize the cultures in a specific stage, can lead to quantitative studies on the immunological, biochemical and physiological differences during stage development (Lambros and Vanderberg, 1979).

4.2.1 Materials

D-(+)-glucose powder, gentamicin solution, HEPES, hypoxanthine, RPMI 1640, sodium bicarbonate, sodium chloride, and sorbitol were purchased from Sigma Aldrich® (South Africa). Albumax II was purchased from Scientific Group and the three component gas mix, 5% oxygen (O₂), 5% carbon dioxide (CO₂) and balance nitrogen gas (N₂) from Afrox (Germiston, South Africa).

4.2.2 Cultivation

P. falciparum was cultivated according to a modified method of Trager and Jensen (1976) and De Ridder (2007). The continuous cultivation of *P. falciparum in vitro* uses a culture medium consisting of the following reagents:

- ◆ RPMI 1640 medium ~ 1.04 g/100 ml.
- ◆ D-(+)-glucose ~ 0.4 g/100 ml.
- ◆ HEPES ~ 0.6 g/100 ml.
- ◆ Hypoxanthine ~ 0.0044 g/100 ml.
- ◆ Gentamicin (40 mg/ml) ~ 0.12 ml/100 ml.
- ◆ Sodium bicarbonate ~ 4.2 ml/100 ml of a 5% solution.
- ◆ Albumax II ~ 0.5 g/100 ml.

These reagents were dissolved in water and filtered through a 0.22 μ m filter under vacuum. The culture medium (10 ml) was added to the RSA11 chloroquine resistant strain and the 3D7 chloroquine sensitive strain on a biweekly basis. The cultures were kept in a culture flask with an air tight cap purchased from Scientific Group (South Africa). The cultures were maintained at an atmosphere of 5% O₂, 5% CO₂ and the balance N₂. Fresh erythrocytes were added on a weekly basis to maintain a haematocrit and parasitemia of approximately 5%. Erythrocytes were obtained from human A+ whole blood. Wash medium, which consisted of the same reagents as the culture medium but contained no Albumax II, was used to remove the leukocytes. This was done by repeatable steps of centrifuging of the blood and wash medium and the removal of the Buffy coat or supernatant by aspiration. After the washing the erythrocytes were stored at 4 °C (De Ridder, 2007). Ethics approval was obtained from the Ethics Panel of the North West University (NWU-0008-08-S5).

4.2.3 Synchronization

For the purpose of these studies, synchronized cultures were used. Cultures are synchronized to ensure that all the parasites in the cultures are in the same phase (De Ridder, 2007; Lambros and Vanderberg, 1979). Cultures are normally synchronized in the ring phase or early trophozoite phase (see Figure 1.1, Chapter 1). This is achieved by adding 4 ml of a sterile 15% (w/v) sorbitol solution to the packed infected erythrocytes. This is left to incubate for five minutes at 37°C after which 8 ml of a sterile 0.1% (w/v) glucose solution is added. It is incubated at 37°C for five minutes. The solution is then centrifuged at 2000 revolutions per minute (r.p.m) for eight minutes. The supernatant is removed and the infected erythrocytes are resuspended in culture medium. The cultivation as described in 4.2.2 is then followed.

4.3 MICROSCOPE EVALUATION

Microscope evaluation is based on the manual visual evaluation of stained thin and thick blood smears by trained personnel (Rougemont *et al.*, 2004). Slides are usually stained with giemsa solution and evaluated under a microscope. It is based on the principle that erythrocytes do not contain any DNA while the parasites do (Cohen, 1982). When using a stain like giemsa that is DNA specific only the parasite is stained making it possible to manually count the amount of parasites present (Bio Medicine, 2008). Microscope evaluation is seen as the golden standard (Guy *et al.*, 2007) and is a relative inexpensive, rapid and simple method compared to other assays (Rougemont *et al.*, 2006). Microscope evaluation does not use expensive or dangerous reagents (Russell *et al.*, 2003). Skilled personnel can differentiate between the different *Plasmodium* species (Rougemont *et al.*, 2006), but to diagnose mixed infections is difficult (Ohrt *et al.*, 2002). Microscopy is however labour intensive and needs skilled and trained personnel to avoid false negative results at very low parasitemias (Basco, 2007; Piper *et al.*, 1999; Sio *et al.*, 2006).

4.3.1 Materials

Giemsa stain, sodium phosphate and potassium phosphate were purchased from Sigma Aldrich® (South Africa). Methanol was purchased from MERCK (South Africa). First grade, frosted, 1.2 mm, ground edge microscope slides were purchased from Lasec (South Africa) or Separations (South Africa).

4.3.2 Method

Giemsa stained thin blood smear microscope slides were made described in Figure 4.1. A small amount of erythrocytes was placed on the slide near the edge. A second slide was placed on this slide and moved backwards into the drop of erythrocytes spreading it across

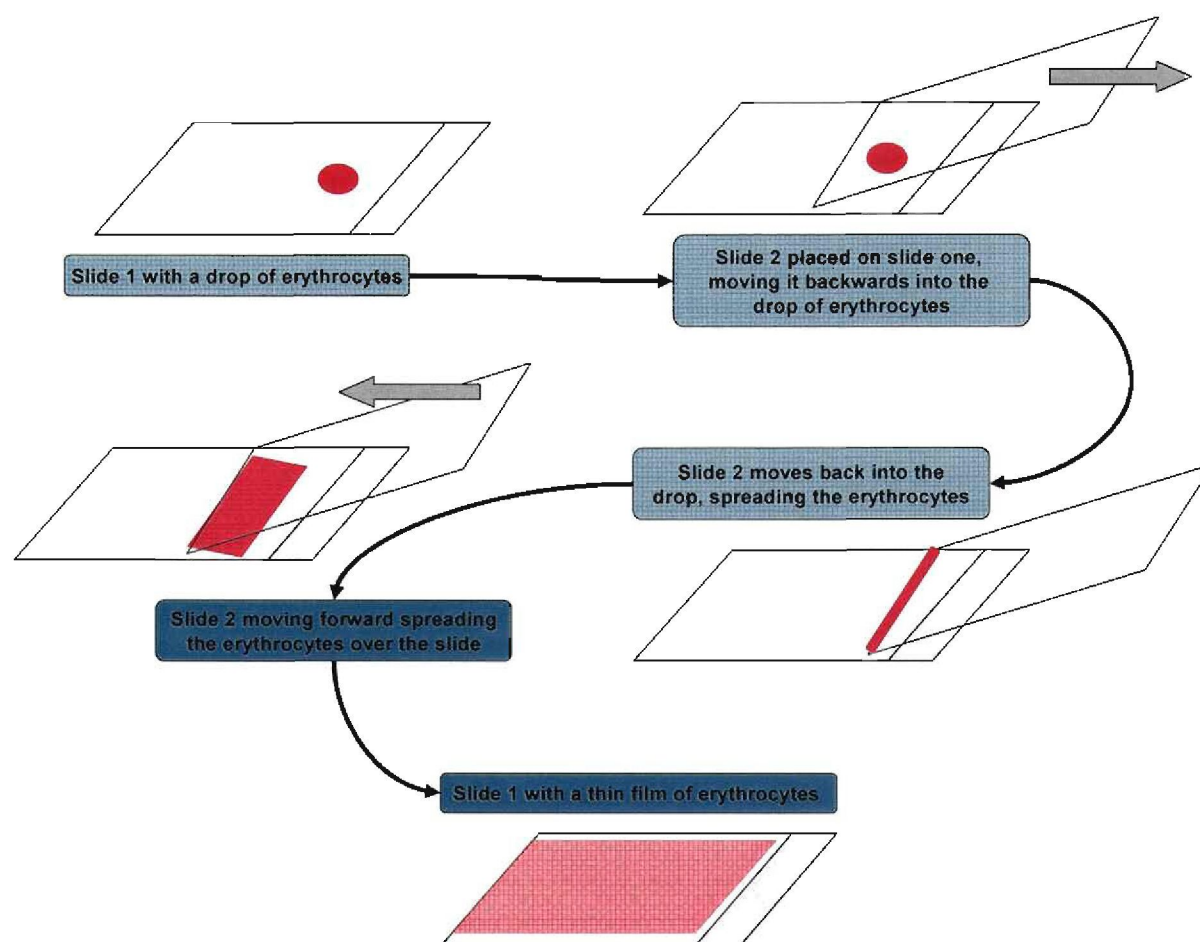


Figure 4.1 The procedure to follow when making a thin blood smear.

the width of the slide. The second slide was moved forward smearing the erythrocytes in a thin film over the first slide. The slide was left to air dry after which it was fixed with methanol. The slide was then covered with giemsa solution consisting of one part giemsa and four parts phosphate buffer (0.65 g sodium phosphate and 0.41 g potassium phosphate to one litre of water). The giemsa was filtered to remove any small crystals before it was used. After three to five minutes the excess stain was removed by running water. The slide was left to air dry and then evaluated under a light microscope with a 100x objective (De Ridder, 2007). To determine the level of parasitemia, the total amount of infected and uninfected erythrocytes was counted in 10 different microscope fields (Basco, 2007). The percentage parasitemia was calculated as follows:

$$\% \text{ Parasitemia} = \frac{\text{Total amount of infected erythrocytes}}{\text{Total amount of erythrocytes}} \times 100$$

(Equation 1)

Giemsa is DNA specific and stains the parasite. Microscope slides were used to determine the biweekly cultivation percentage parasitemia and the initial percentage parasitemia before an experiment. This method was also used to validate the colorimetric and flow cytometric evaluation.

4.4 METHOD VALIDATION

According to the Food and Drug Administration (FDA) it is necessary for drug efficacy tests to have a sensitive and selective analysis method. Method validation includes procedures to demonstrate that the method is repeatable and is a quantitative measure for the intended use. There are different types and levels of validation. Full validation is done when a new method is done for the first time. Partial validation is when a previously validated method is modified for use in a different laboratory. Cross-validation is when a method is compared to another validated and accepted method (FDA, 2001). In this study a combination of partial validation and cross-validation were done. The parameters included selectivity, accuracy, precision, sensitivity, reproducibility and stability. Selectivity is the ability of the method to selectively measure the determinant in the sample. Accuracy portrays the closeness between the average and the true or theoretical values. The average should be within 15% of the theoretical value and within 20% of the lowest level of quantification. The precision illustrates the closeness of individual measures on repeat basis. This value should also be within 15% of the value. Sensitivity is an indication of how accurate and precise the method is able to detect the lowest quantification value. Reproducibility indicates if the method can be repeated (FDA, 2001). Stability is an indication of product integrity and this was done in a separate study (Pretorius *et al.*, 2008).

4.5 NON-ELISA BASED COLORIMETRIC ANALYSIS – pLDH

Lactate dehydrogenase (LDH) is an oligomeric enzyme (Palmer, 2001) used in the reduction reaction of lactate to pyruvate during the anaerobic glycolytic metabolic pathway (Basco, 2007; Noedl *et al.*, 2003; Makler *et al.*, 1998). The colorimetric assay detects pLDH by making use of the distinctive differences in the amino acid sequence of the LDH between the *Plasmodium* species and the host (Basco, 2007; Makler *et al.*, 1998; Noedl *et al.*, 2003). This assay can be utilized for the detection of *P. falciparum* in culture for drug efficacy tests (Basco, 2007; Basco *et al.*, 1995; Makler *et al.*, 1998). This method has been standardized and validated previously (Makler *et al.*, 1998; Persson *et al.*, 2006), but needed to be optimized for use in our laboratory.

4.5.1 Materials

The materials purchased from Sigma-Aldrich® (South Africa) for this assay included 3-acetylpyridine adenine dinucleotide (APAD), diaphorase, lithium L-lactate, nitroblue tetrazolium (NBT), phenazine ethosulfate (PES), Triton® X-100 and Trizma® hydrochloride.

4.5.2 Experimental design

To determine the sensitivity of this assay one of two methods was used (Figure 4.2). The only difference between these methods is the use of either PES or diaphorase as co-enzyme. A reagent mixture was prepared and consisting of Malstat® reagent and NBT solution that contained either PES or diaphorase. Malstat® reagent was prepared by adding 0.2% (v/v)

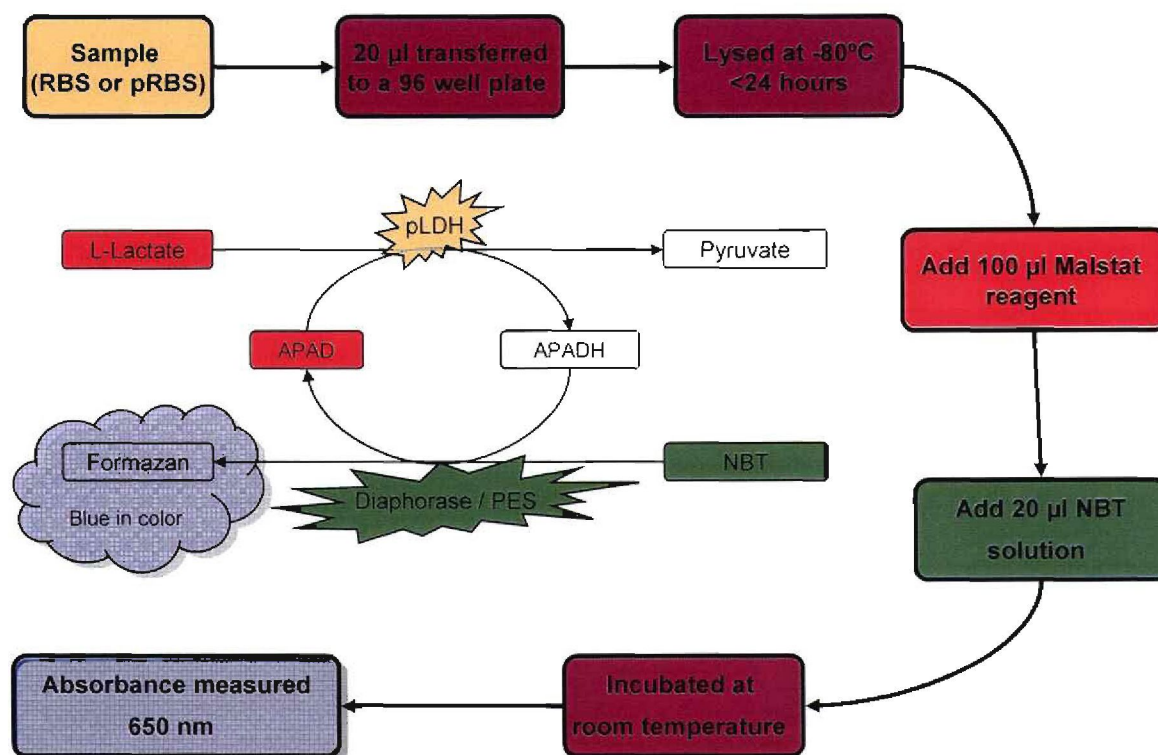


Figure 4.2 Graphical representation of the pLDH method showing the enzymatic reaction and how it relates to the assay.

Triton® X-100, 20 mg/ml lithium L-lactate, and 6.6 mg/ml Trizma® hydrochloride to distilled water. This solution was heated to ensure that all the reagents had dissolved properly. After the solution has cooled down to room temperature, 0.1 mg/ml APAD was added. NBT solution consisted of 1.6 mg/ml NBT and either 0.08 mg/ml PES or 0.1 mg/ml diaphorase as co-enzyme. The assay was performed in a 96 flat bottom well plate. The samples (20 µl) were added to the wells and lysed by freezing at -80°C for a minimum of 24 hours (see Figure 4.2). Malstat® reagent at a volume of 100 µl and 20 µl of NBT solution were added to individual wells. After the reagents have been added, the well plate was incubated at room temperature for 10 minutes or until a positive colour change has taken place. The well plate was gently shaken before the optical density was measured with a 650 nm filter using ELX800™ Absorbance Microplate Reader together with GEN5™ Data Analysis Software to generate the data (Makler *et al.*, 1993).

Statistical analysis of this method included the calculation of the percentage survival, average, standard error of mean, coefficient of variation (C.V.), linear function equation and Spearman correlations using GraphPad® Prism 4. When calculating the percentage survival the normal growth of the infected erythrocytes is equal to 100%. When a dilution of the parasites is made or drugs are added, the amount of pLDH will decrease. This will lead to a percentage survival lower than 100%. The background absorbance, that is the uninfected erythrocytes (RBC), was subtracted from the sample optical density. This new optical density values were used to determine the percentage survival by expressing it as a percentage of the control sample (Persson *et al.*, 2006). The formula was as follows:

$$\% \text{ Survival} = \frac{\text{OD of the sample}}{\text{OD}_{\text{AVE normal pRBC}}} \times 100 \quad (\text{Equation 2})$$

Where OD = Optical density as obtained after the background absorbance was subtracted

OD_{AVE} = The average of the optical density repeats

pRBC = Infected erythrocytes (normal growth) (X_{100%})

To determine the percentage parasitemia the 100% survival was made equivalent to the starting parasitemia (X_{100%}) as calculated by a microscope evaluation. The formula was as followed:

$$\% \text{ Parasitemia} = \frac{\% \text{ survival of sample}}{\% \text{ parasitemia of the initial parasitemia}} \times 100 \quad (\text{Equation 3})$$

The intraday and interday repeatability was determined by calculating the precision and accuracy. The coefficient of variation (C.V.) was used to determine the precision and the ratio between the theoretical and experimental value to determine the accuracy. The percentage coefficient of variation (%C.V.) was calculated by the following formula:

$$\% \text{ C.V.} = \frac{\text{Standard error of mean}}{\text{Average}} \times 100$$

(Equation 4)

4.5.3 Results and discussion

Several different variations of the pLDH method have been described (El Bissati *et al.*, 2005; Makler *et al.*, 1993; Prudhomme and Sherman, 1999; Persson *et al.*, 2006). In this study it was important to adapt the method for the specific conditions in our laboratory. The influence of the percentage haematocrit and the blood type used in the experiment were determined to eliminate any external factors that may have an influence on the assay. The sensitivity of the method was determined using both the NBT solution with PES and diaphorase and verified by intra- and interday repeatability. Correlations between the theoretical and experimental values were made.

4.5.3.1 Influence of haematocrit and blood type on the pLDH method

To determine the influence of the different percentage haematocrit on the optical density aliquots of 20 µl of three different haematocrit percentages (2%, 5% and 10%) of O+ blood in triplicate was transferred to a 96 well plate. The plate was stored at -80 °C for a minimum of

24 hours. Malstat® reagent (100 µl) and 20 µl of NBT solution containing diaphorase was added to each sample. The absorbance was measured at 650 nm. Table 4.1 contains the average of the optical densities of the different percentage haematocrit. The same was done with O+, A+ and B+ blood types at a five percentage haematocrit to determine if different blood types may have an influence on the reading.

Table 4.1 The average of the absorbance value (optical density) of uninfected erythrocytes at three different haematocrit percentages and three different blood types.

Different haematocrit percentages (O+)		Different blood types (5%)	
2%	0.166	O+	0.205
5%	0.189	A+	0.209
10%	0.296	B+	0.224

The data shows a difference in the optical density of the different haematocrit percentages but not with the different blood types at a five percent haematocrit. An increase in the haematocrit leads to higher optical density values. The haematocrit has an influence on the optical density readings. The haematocrit is a direct relation to the amount of erythrocytes in a sample. The erythrocytes give red colour to the cell culture media. The colour of the media is also influenced by whether the haemoglobin within the erythrocytes is oxidised. All these factors can have an influence on the absorbance. A possible solution is to remove the haemoglobin after the cells are lysed by several washing steps with PBS, however this could lead to loss of pRBC and inaccurate results. Because the haematocrit have an influence on the absorbance readings, the haematocrit was kept between two and five percent for all concurrent experiments.

4.5.3.2 Sensitivity and repeatability

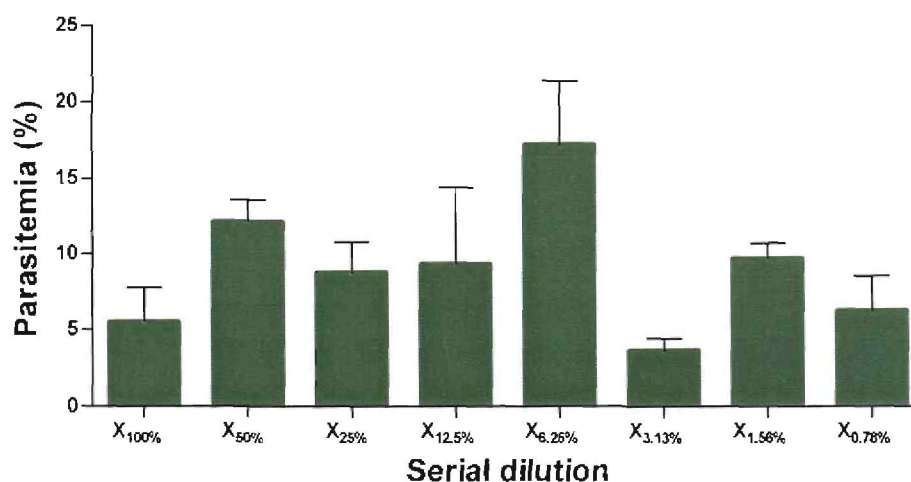


Figure 4.3 Average of the percentage parasitemia of a serial dilution with the NBT solution containing diaphorase as co-enzyme.

To ensure the method is sensitive, especially at a low parasitemia density, a serial dilution (X_{100%} - X_{0.78%}) of infected erythrocytes (20 μ l) was transferred to a 96 well plate in triplicate. The plate was stored at -80 °C until the assay could be performed. Malstat® reagent (100 μ l) and 20 μ l NBT solution consisting of NBT and diaphorase or PES was added to the lysed erythrocytes. After incubation at room temperature the absorbance was measured at 650 nm. Figure 4.3 display the percentage parasitemia of a serial dilution.

As seen in Figure 4.3, no visible decrease in parasitemia can be observed. The two fold serial dilution had to have a downward trend with X_{100%} as the highest percentage parasitemia. This method with diaphorase as co-enzyme can thus not be used to determine the parasite density with great sensitivity or accuracy. The second method that can be used is to determine the parasite density with the NBT solution containing PES as co-enzyme. Figure 4.4 displays the percentage parasitemia of the serial dilution where PES was used as co-enzyme.

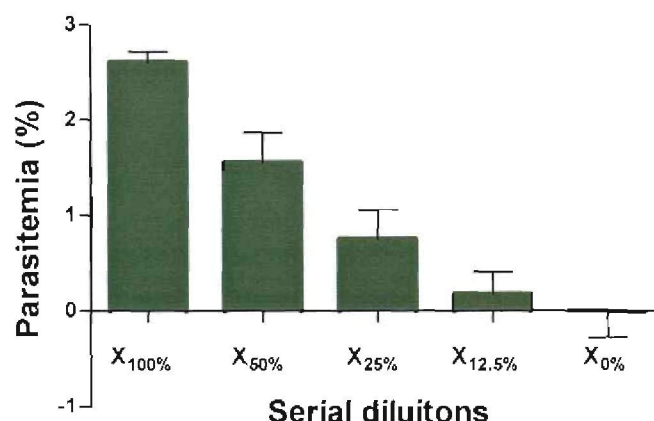


Figure 4.4 Average of the percentage parasitemia of a serial dilution with the NBT solution containing PES as co-enzyme (Experiment 1).

As seen in Figure 4.4, there is an accurate downward trend with X_{100%} having the highest percentage parasitemia. Thereafter the parasitemia (%) decreased twofold to 0%. This method with PES as co-enzyme can thus be used to determine the parasite density. The experiment with PES as co-enzyme was repeated five times to determine the precision and accuracy of this method. The precision and accuracy is some of the measures to determine the sensitivity of the method. The higher the precision and accuracy, especially at low parasite densities, the more sensitive the method is. The method is sensitive when it is able to quantify very low percentage parasitemias. This is very important during drug efficacy test to ensure accurate and precise results. Table 4.2 gives a summary of the average percent survival of the serial dilution of five different experiments. The results show that for all the experiments there was a downward trend. The higher parasitemia densities (X_{50%} to X_{12.5%}) showed better results compared to the low parasitemia densities (X_{6.25%} - X_{0.78%}). There was no trend visible in the dilution of X_{5.25%} to X_{0.78%}. This leads to the conclusion that the method using PES as co-enzyme is not sensitive at low parasitemia densities.

Table 4.2 Sensitivity of the pLDH method - average percentage survival of the different dilutions on five occasions.

		Experiment				
		1	2	3	4	5
Serial Dilution	X _{50%}	59.3 ± 12.2	113.6 ± 21.6	66.7 ± 27.3	55.2 ± 39.8	17.5 ± 4.3
	X _{25%}	28.8 ± 11.9	41.97 ± 9.6	35.56 ± 4.4	24.1 ± 13.8	25 ± 18.9
	X _{12.5%}	6.8 ± 9.0	62.96 ± 31	33.3 ± 17.8	41.4 ± 88.4	17.5 ± 13
	X _{6.25%}	-	50.6 ± 19.6	24.4 ± 17.6	10.3 ± 10.3	-5 ± 4.3
	X _{3.13%}	-	-	6.7 ± 4.4	103.5 ± 113	2.5 ± 8.7
	X _{1.56%}	-	-	53.3 ± 65.6	-31.03 ± 6	-25 ± 31.3
	X _{0.78%}	-	-	-	-	20 ± 6.6

Intraday repeatability is when the same sample is read multiple times on the same day. Interday repeatability is to determine if the method can give the same result on different days (Cheoymang *et al.*, 2007). The repeatability of the method was determined by means of the precision by calculating the coefficient of variation (C.V.). The precision of the intraday was determined by repeat analysis of the same sets of samples on the same day at different serial dilutions. This was done by reading the same sample on three occasions. The precision of the interday was determined by repeat analysis of different samples on different days at different dilutions. The different serial dilutions of the five experiments have different amount of samples (see Table 4.2). This lead to the precision and accuracy being calculated on the number of sample values available. The accuracy of the method is a relation between the theoretical values and the experimental values. Table 4.3 demonstrates the precision and accuracy of the method using PES as co-enzyme at the different dilutions (X_{50%} to X_{1.56%}).

Table 4.3 The precision and accuracy of the colorimetric evaluation using PES as co-enzyme at different parasite densities.

Two fold serial dilution	Precision (% C.V.)		Accuracy	
	Intra-assay*	Inter-assay**	Intra-assay*	Inter-assay**
X_{50%}	40.96	24.58786	75	80.07
X_{25%}	12.5	10.88234	70.31	80.39
X_{12.5%}	53.33	30.0435	37.5	38.59
X_{6.25%}	72.16	58.78019	25.57	31.09
X_{3.13%}	66.67	87.84871	48.86	8.32
X_{1.56%}	122.97	3017.91	2.93	173.54

* X_{50%} to X_{1.56%} n = 3; ** X_{50%} to X_{12.5%} n = 5, for X_{6.25%} n = 4, for X_{3.13%} and X_{1.56%} n = 2

Precision is given by the coefficient of variation (%C.V.) and should be close to zero. The intraday precision indicates that there is a variation on the readings of the sample on three different times, but can be due to the fact that the reduction of NBT to formazan has not yet been completed. By adding a reagent that has the ability to stop the reaction, this problem can be overcome. The lower the parasitemia density the higher the precision values. This means that the method is not accurate at low parasite densities because of the low precision for intra- and interday repeatability. The interday precision of the samples on different days showed lower values at the higher parasitemia densities and higher values at the lower parasitemia densities. The FDA guidelines states that the precision should be within 15% of zero. The precision do not meet the criteria of the FDA but this can be because of the different factors that has an influence on the method. The accuracy, which is the relation between the theoretical and experimental values, must be as close as possible to 100%. High accuracy was attained by the X_{50%} and X_{25%} dilutions for intra- and interday. At the lower parasite densities (X_{12.5%} to X_{1.56%}) the accuracy was very low especially with the intraday repeatability. The FDA criteria for accuracy states that it should be within 15% of the

theoretical values. The precision and accuracy was better at higher parasite densities than at low parasite densities. The method does not meet this criterion. This confirms that the colorimetric analysis is not sensitive at low parasitemia densities. The method can not be used according to the FDA for drug efficacy tests.

A correlation graphs shows the relationship between two set of values. The correlation between the theoretical and experimental values is another way to demonstrate the accuracy of the specific experiment. Table 4.4 shows the Spearman correlation values and the equation of a linear function of each experiment.

Table 4.4 Spearman's correlation (r) and the equation of the linear function of each experiment.

Experiment	Spearman's correlation (r)	Linear function equation
1	$r = 1.000$	$y = 1.034x + 0.2119$
2	$r = 0.7714$	$y = 0.8369x + 34.50$
3	$r = 0.7619$	$y = 0.8274x + 19.48$
4	$r = 0.5952$	$y = 0.7957x + 18.19$
5	$r = 0.5858$	$y = 0.9297x - 3.635$

To evaluate the correlation of two data sets it is necessary to look at both Spearman's correlation and the linear function equation. The closer the correlation value is to the number one, positive or negative, the better the data sets compares. For experiment one the correlation is exactly one. This means that the experimental percentage survival compares very well with the theoretical values (as seen in Figure 4.5). The correlation values for experiment two and three compare less but experiment four and five had the worst correlation values. The low correlation values of experiment four and five can be explained by the low sensitivity and accuracy of $X_{6.25\%}$ through $X_{0.78\%}$ (see Table 4.3 and Table 4.4). The linear function equation should be used in combination with the correlation value. The theoretical

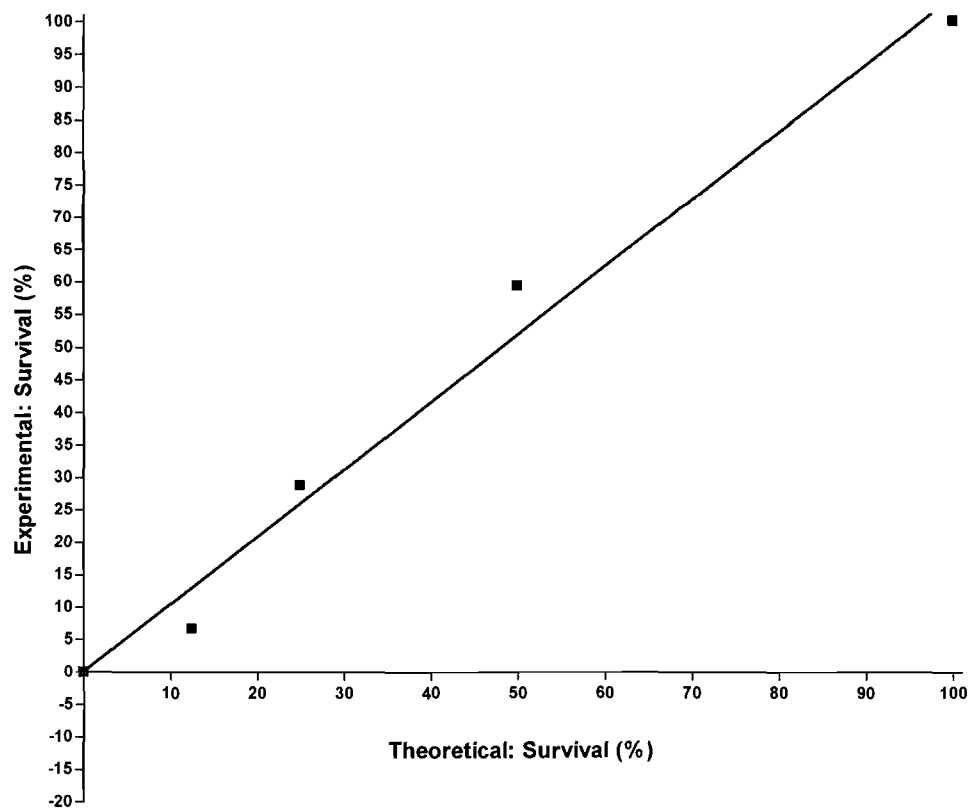


Figure 4.5 Correlation graph between the experimental percentage survival and the theoretical percentage survival of experiment one.

and experimental values should be equal. The equation for a linear function is $y = mx + c$ where m is the gradient and c is the y-intercept. If the value on the x-axis is equal to the value on the y-axis, $y = x$. The data that has the best correlation with the theoretical values has a y-intercept close to zero and the gradient close to one. The gradient of the linear function equation of all five experiments don't deviate too much from one with the best gradient in experiment one. The y-intercepts deviate a lot from zero with experiment one the closest to zero. When looking at the combination of Spearman's correlation and the linear function equation it is evident that better correlation could be made at higher parasite densities. Weak correlations between the experimental and theoretical values were found at low parasite densities. This method could be used to accurately quantify high parasite densities but has low sensitivity at low parasite densities.

4.5.4 Conclusion

This method is less labour intensive than microscope evaluation but erythrocytes need to be lysed before it can be analysed (Basco, 2007; Makler and Hinrichs, 1993). The analysis done with diaphorase as co-enzyme was less sensitive and accurate than when PES was used as co-enzyme. The method was comparatively accurate and sensitive at higher parasite densities with same results found by Makler and Hinrichs (1993). Low sensitivity and accuracy were found by low parasite densities and this was also documented by Basco (2007). Because the haematocrit and haemoglobin have an influence on the absorbance measurement, it can be possible that the haematocrit and haemoglobin of the different wells was not the same and thus having an influence on the absorbance measurement. This method can be used at higher parasite densities, but is not always feasible in drug efficacy tests (Makler, 1993). The method doesn't meet the standard of the FDA for use in drug efficacy tests.

4.6 FLOW CYTOMETRIC EVALUATION – FLUORESCENCE ACTIVATED CELL SORTER (FACS)

Flow cytometry was developed in the 1970's as a technique that measures different properties of cells as they flow through a column in single file (Basco, 2007; Riley and Idowu, 2008). A fluorescence activated cell sorter (FACS) combines techniques in fluidics, laser optics, electronics and computer software to determine the properties of cells (Basco, 2007; Nolla, 2008). As the cell passes through a laser one of two events occurs. The light scattering analyses the size, shape and granularity of the cell, while the fluorescence scattering analyses specific properties of the cell. The fluorescent dye absorbs the laser light, is excited and emits at a longer wavelength. The characteristics are detected by a series of mirrors, detectors and filters. The electrical impulse is analysed and processed into quantitative data

(Basco, 2007; Riley and Idowu, 2008). The application in malaria diagnostics and drug efficacy tests have been investigated with great success (Basco, 2007). Acridine orange (AO) is a metachromatic dye and bind differentially to DNA and RNA (Hare and Bahler, 1986). Because acridine orange is DNA specific it only binds to the infected erythrocytes. AO is excited at 488 nm and emits at 525 nm when bound to RNA and at 640 nm when bound to DNA (Bhakdi *et al.*, 2007). The intensity of the emitting fluorescence is directly proportional to the amount of DNA present making it possible to distinguish between the different stages in the life cycle of the parasite (Basco, 2007; Riley and Idowu, 2008, Whaun *et al.*, 1983).

4.6.1 Materials

Acridine orange, phosphate buffer solution (PBS), the FACSCalibur™ reagents and consumables were purchased from Scientific Group (South Africa). Gluturaldehyde solution and Triton® X-100 were purchased from Sigma Aldrich® (South Africa).

4.6.2 Experimental design

A two fold serial dilution of infected erythrocytes in triplicate was evaluated by flow cytometry using the fluorochrome AO. The procedure followed for staining the samples with AO is described in Figure 4.6. Aliquot samples (100 µl) was suspended in 1000 µl PBS and 2.5 µl of a 0.1 µg/ml AO prepared from a stock solution was added to each tube. AO is light sensitive and it was necessary to work in the dark. The tube was vortexed, incubated at room temperature for 15 minutes in the dark, vortexed and read on the FACSCalibur™. The prepared sample of infected erythrocytes and acridine orange (AO) passes through the flow cell of the FACSCalibur™ Flow cytometer (see Figure 4.6). As the cells pass through the

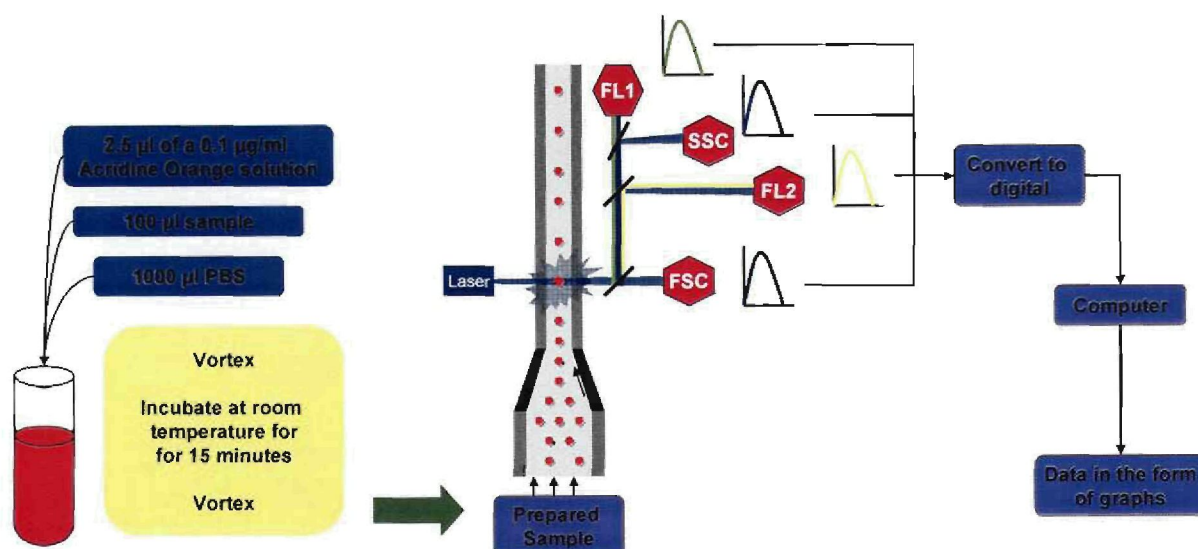


Figure 4.6 Schematic representation of the flow cytometric evaluation of infected and uninfected erythrocyte samples using acridine orange as fluorochrome.

488 nm argon ion laser in single file, the different properties of the cell is analysed. In each sample 50 000 to 100 000 cells were counted. These properties were analysed by a series of detectors. The properties that were first evaluated on all the cells were the size by the forward scatter (FSC) and the complexity by the side scatter (SSC). AO stained the DNA and RNA and will as a result only be present in infected erythrocytes. When infected erythrocytes tagged with the fluorochrome passes through the laser, the fluorochrome is excited and emits at longer wavelengths. This signal was detected by FL-1 or green channel with a 530/30 filter and by the FL-2 or yellow channel with a 585/42 filter. The signals from the FSC, SSC, FL-1 and FL-2 detectors were digitally converted to data that could be displayed in graphs. Data was collected on a MAC-OC computer and analysed with BD CellQuest™ Pro software. The data for each sample were displayed on a FL-1/FL-2 graph as seen in Figure 4.7. Statistical analysis from this graph was used to determine the percentage parasitemia. The negative population or uninfected erythrocytes is situated in the lower left (LL) quadrant while the positive population or infected erythrocytes predominantly located in the upper right (UR) quadrant. The percentage parasitemia was calculated by subtracting the percentage of the total number of cells in the lower left (LL) quadrant from 100.

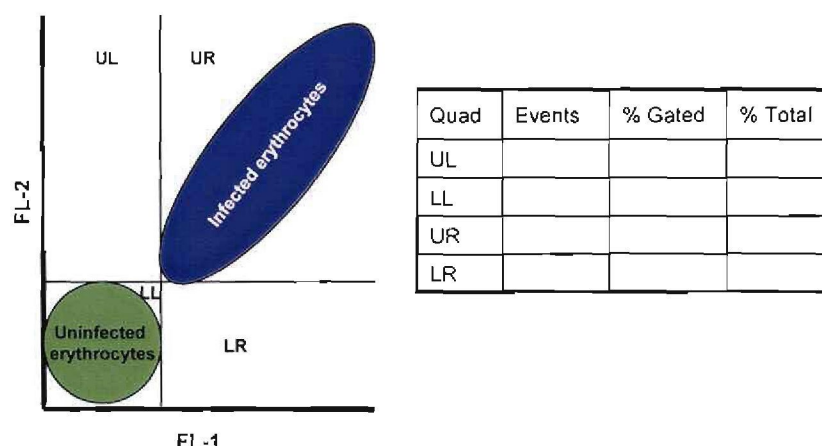


Figure 4.7 Presentation of the FL-1/FL-2 graph with the uninfected erythrocytes (green) in the lower left (LL) quadrant and the infected erythrocytes (blue) in the upper right (UR) quadrant. The table represents the statistical analysis done on the graph. (UL – Upper left; LR – Lower right).

Statistical analysis for this method included the calculation of the percentage parasitemia, average, standard error of mean, percentage survival, coefficient of variation (C.V.), linear function equation and Spearman correlations using GraphPad® Prism 4.

To determine the percentage survival the percentage parasitemia of the initial dilution, $X_{100\%}$ was made equivalent to 100%. The formula was as followed:

$$\% \text{ Survival} = \frac{\% \text{ parasitemia of the sample}}{\% \text{ parasitemia of the initial parasitemia}} \times 100 \quad (\text{Equation 5})$$

The intraday and interday repeatability was determined by calculation the precision and accuracy. The coefficient of variation (C.V.) was used to determine the precision (see equation 4) and the ratio between the theoretical and experimental value to determine the accuracy.

4.6.3 Results and discussion

As described in the experimental design of this method (4.6.2) there are various ways to determine the parasitemia. Figure 4.8 shows the difference between infected and uninfected erythrocytes on a FSC/SSC dot plot. This plot has a linear scale. The dot plot is a representation of two properties of the cells. Each dot represents a cell. The cell size increases to right on the x-axis and the complexity increases up on the y-axis. The difference between the infected and uninfected erythrocytes on this dot plot is not that distinctive but the complexity and size do increase with infected cells.

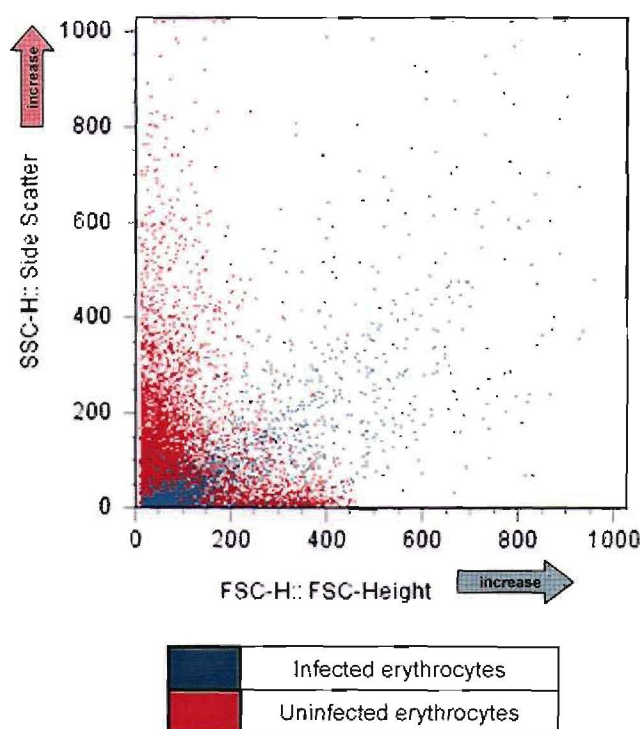


Figure 4.8 A FSC/SSC dot plot of infected and uninfected erythrocytes.

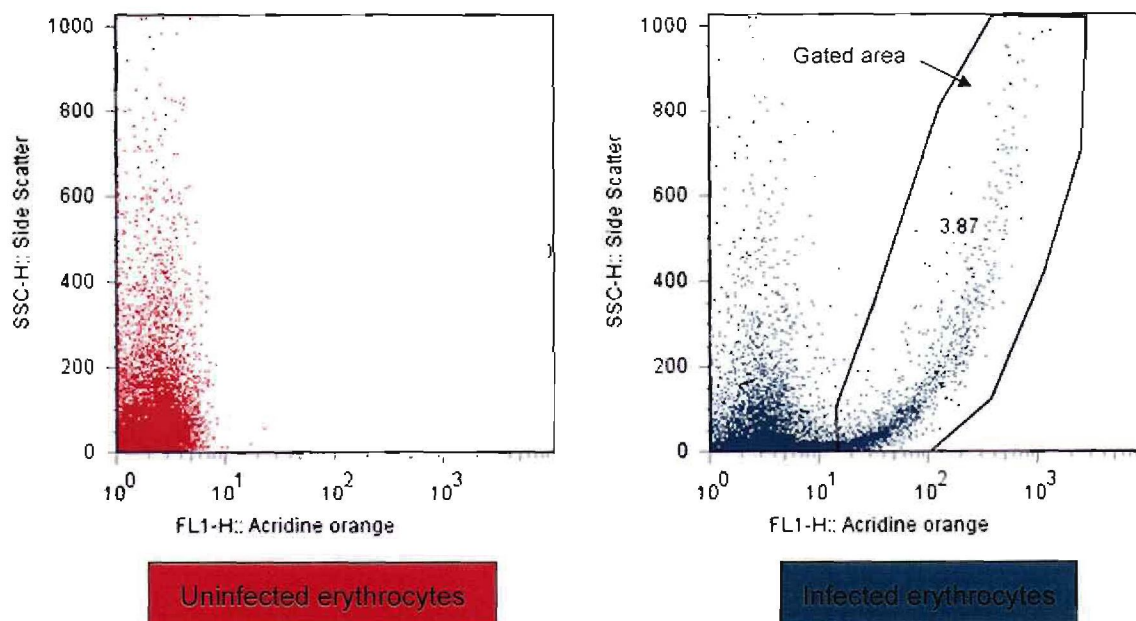


Figure 4.9 SSC/FL1 dot plots of infected and uninfected erythrocytes. The infected erythrocytes are gated.

Figure 4.9 shows the region of the uninfected erythrocytes and the differences of infected erythrocytes of a SSC/FL1 dot plot. This plot uses a log scale. Each dot represents a cell. In this plot a clearer difference can be seen between the infected and uninfected cells. The infected cells are marked with AO and the fluorescence intensity increases to right on the x-axis. Infected cells will be situated more to the right on the x-axis. The cells in the gated area are the infected erythrocytes. The percentage cells in that area is the percentage parasitemia. This plot can also be used to determine the percentage parasitemia. The FL2/FL1 dot plot, as seen in Figure 4.10 shows a distinct difference between the infected and uninfected erythrocytes. A log scale is used in this plot. The uninfected erythrocytes are gated in the lower left quadrant. The infected erythrocytes are the tail that forms in the upper right quadrant. The percentage cell in the lower left quadrant is subtracted from 100 to calculate the percentage parasitemia.

The histogram in Figure 4.11 measures the intensity of a cell property as a percentage of the total amount of cells. The x-axis shows the intensity of the signal. The intensity of the signal increases if more DNA is present. There is a difference in the pattern of the dot between infected and uninfected erythrocytes. This makes it possible to distinguish between different

cells present in the sample. The infected erythrocytes can be gated, like in Figure 4.11 and expressed as a percentage of the total number of cells.

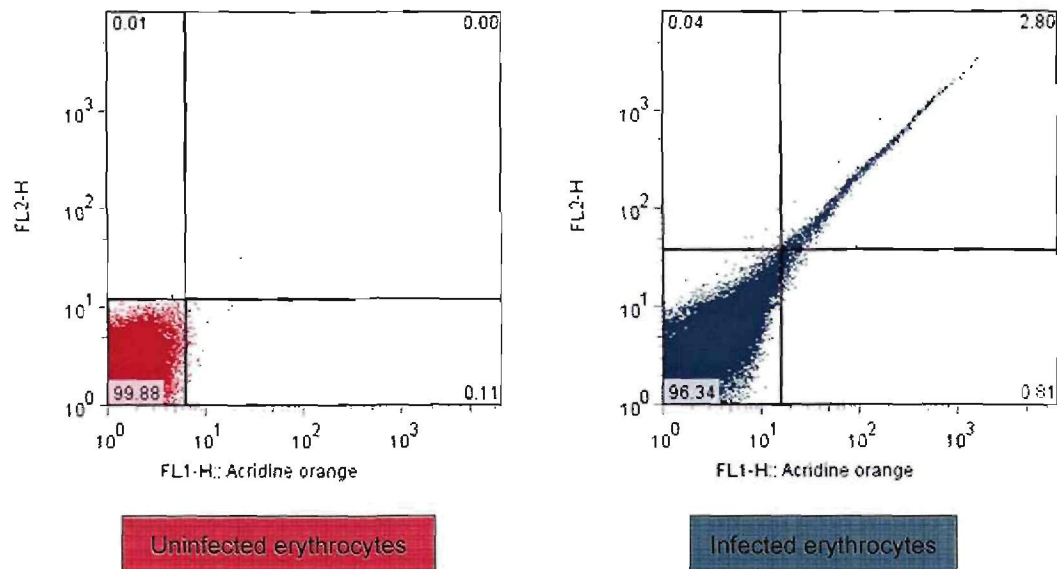


Figure 4.10 FL1/FL2 dot plots of infected and uninfected erythrocytes. The lower left quadrant represents the uninfected erythrocytes and the upper right quadrant represents the infected erythrocytes.

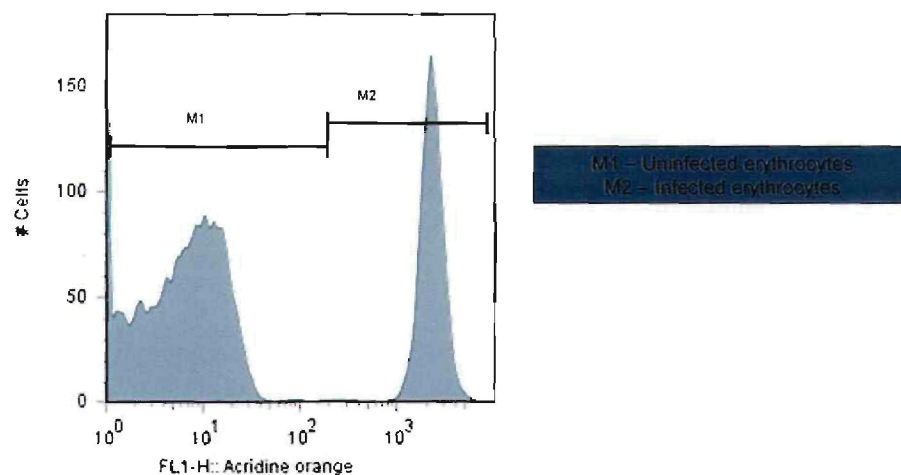


Figure 4.11 A histogram showing the difference in the fluorescence intensity between the infected and uninfected erythrocytes.

The different plots can be use to determine the percentage parasitemia. All the plots do give approximately the same percentage parasitemia. The plot that is mostly use is the FL1/FL2 dot plots.

The sensitivity of this method was determined by calculating the percentage parasitemia of a two fold serial dilution. The initial parasitemia ($X_{100\%}$) was diluted with uninfected erythrocytes to a parasitemia of $X_{12.5\%}$. The results of the experiments are displayed in Table 4.5. The first experiment had five dilutions in triplicate, the second seven in duplicate and the third experiment had eight dilutions in triplicate.

Table 4.5 The average percentage parasitemia and standard error of mean of different serial dilutions on three different occasions.

		Experiment		
		1	2	3
Serial Dilution	$X_{100\%}$	4.74 ± 0.04	1.56 ± 0.04	2.33 ± 0.07
	$X_{50\%}$	2.22 ± 0.10	0.53 ± 0.03	1.23 ± 0.06
	$X_{25\%}$	1.03 ± 0.04	0.30 ± 0.03	0.64 ± 0.04
	$X_{12.5\%}$	0.62 ± 0.04	0.22 ± 0.06	0.43 ± 0.01
	$X_{6.25\%}$	0.30 ± 0.02	0.23 ± 0.03	0.27 ± 0.03
	$X_{3.13\%}$	-	0.18 ± 0.06	0.19 ± 0.05
	$X_{1.56\%}$	-	0.16 ± 0.01	0.12 ± 0.02
	$X_{0.78\%}$	-	-	0.10 ± 0.02

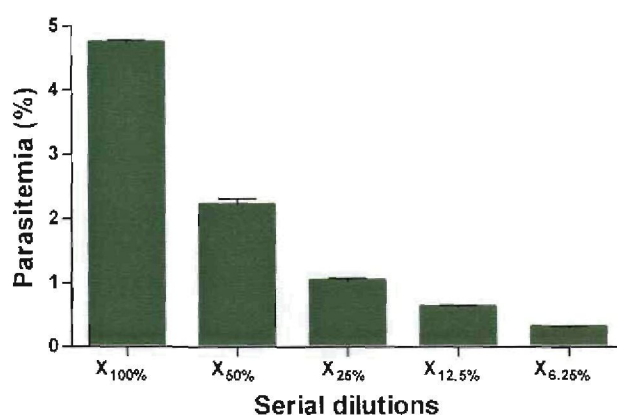


Figure 4.12 Percentage parasitemia of the serial dilution of experiment one.

As seen in Table 4.5, there is a definite linear decrease in parasitemia. At low parasite densities the flow cytometric evaluation showed better sensitivity even at a low parasitemia of 0.12%. The results of experiment one is shown in Figure 4.12.

The intraday and interday repeatability was determined in the same manner as the colorimetric evaluation (see 4.4.3.2). The percentage survival was first determined using equation 5. Intraday repeatability was measured on three repeat sample values for each dilution. For dilution X_{50%} to X_{6.25%} three sample values were used for interday repeatability but only two for X_{3.13%} and X_{1.56%}. The results are graphically depicted in Table 4.6.

The precision is better the closer the value is to zero. The intraday precision was very good with only one high value at X_{3.13%}. This can be because the sensitivity decreases a little at lower parasite densities. The repeatability from day to day (interday) is high because of the low coefficient of variation values. The lower parasite density dilutions have little higher values. Most of the precision values are within the 15% as stated by the FDA. The accuracy values should be close to 100%. Very high accuracy was found at dilution X_{50%} to X_{6.25%} for the intraday calculation but the percentage accuracy value was much higher at X_{3.13%} and X_{1.56%}. Because the accuracy is determined by the experimental value divided by the theoretical value expressed as a percentage, it means that the experimental value is much

Table 4.6 The precision and accuracy of the flow cytometric evaluation using acridine orange as fluorochrome at different parasite densities.

Two fold serial dilution	Precision (% C.V.)		Accuracy	
	Intra-assay*	Inter-assay**	Intra-assay*	Inter-assay*
$X_{50\%}$	4.34	12.57	106.02	89.19
$X_{25\%}$	4.23	10.94	109.46	90.75
$X_{12.5\%}$	2.77	11.40	105.13	121.46
$X_{6.25\%}$	7.21	21.74	102.32	173.68
$X_{3.13\%}$	26.3	16.61	256.73	307.85
$X_{1.56\%}$	6.25	31.85	339.26	497.83

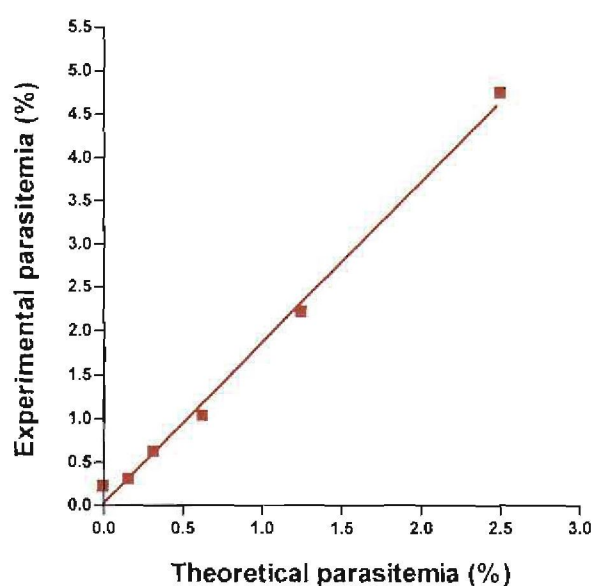
* $X_{50\%}$ to $X_{1.56\%}$ $n = 3$; ** $X_{50\%}$ to $X_{6.25\%}$ $n = 3$ and for $X_{3.13\%}$ and $X_{1.56\%}$ $n = 2$

higher than the theoretical value. This represents lower sensitivity at low parasite densities. For the interday repeatability the accuracy is below 100% for $X_{50\%}$ and $X_{25\%}$. Above 100% for $X_{12.5\%}$ to $X_{1.56\%}$, with lower sensitivity at $X_{3.13}$ and $X_{1.56\%}$ because of the very high values obtained. The accuracy values for the higher parasitemia densities are within the criteria of the FDA. The interday precision and accuracy is very good leading to the conclusion that even though there is a decrease in the sensitivity at very low percentage parasitemia, it can be used to determine the percentage parasitemia accurately. This method has good accuracy and precision at high parasitemia densities, with lower accuracy and precision at lower parasite densities.

Table 4.7 Spearman's correlation (r) and the equation of the linear function of each experiment.

Experiment	Spearman's correlation (r)	Linear function equation
1	$r = 1.0000$	$y = 1.847x + 0.028$
2	$r = 0.9762$	$y = 0.474x + 0.047$
3	$r = 0.9500$	$y = 0.851x + 0.116$

The correlation between theoretical and experimental values was determined by calculating Spearman's correlation and the linear function equation of the best fit line between the data points. Spearman's correlation values, as seen in Table 4.7, are very close to one. This shows very good correlation with high accuracy. The linear function, $y = x$ as ideal, illustrates weaker correlations between the theoretical and experimental values. Figure 4.13 show the correlation between the theoretical and experimental values of experiment one

**Figure 4.13 Correlation between the theoretical and experimental percentage parasitemia of experiment one.**

The theoretical values were determined by microscope evaluation. Because this is an imperfect gold standard, an error in determining the initial parasitemia could have been made. This will lead to lower correlation values and accuracy. The flow cytometric evaluation is more sensitive than the microscope evaluation, leading to higher percentage parasitemia. The amount of erythrocytes counted during flow cytometric evaluation is also much higher than during microscope evaluation.

Table 4.8 The average and standard error of mean of the same culture as read on the FACSCalibur at two different times, at two different phases

		Experiment 2	
		Ring	Schizont
Serial Dilutions	X _{100%}	1.56 ± 0.04	2.08 ± 0.07
	X _{50%}	0.53 ± 0.03	0.89 ± 0.01
	X _{25%}	0.30 ± 0.03	0.31 ± 0.01
	X _{12.5%}	0.22 ± 0.06	0.16 ± 0.00
	X _{6.25%}	0.23 ± 0.03	0.13 ± 0.01
	X _{3.13%}	0.18 ± 0.06	0.07 ± 0.01
	X _{1.56%}	0.16 ± 0.01	0.07 ± 0.01

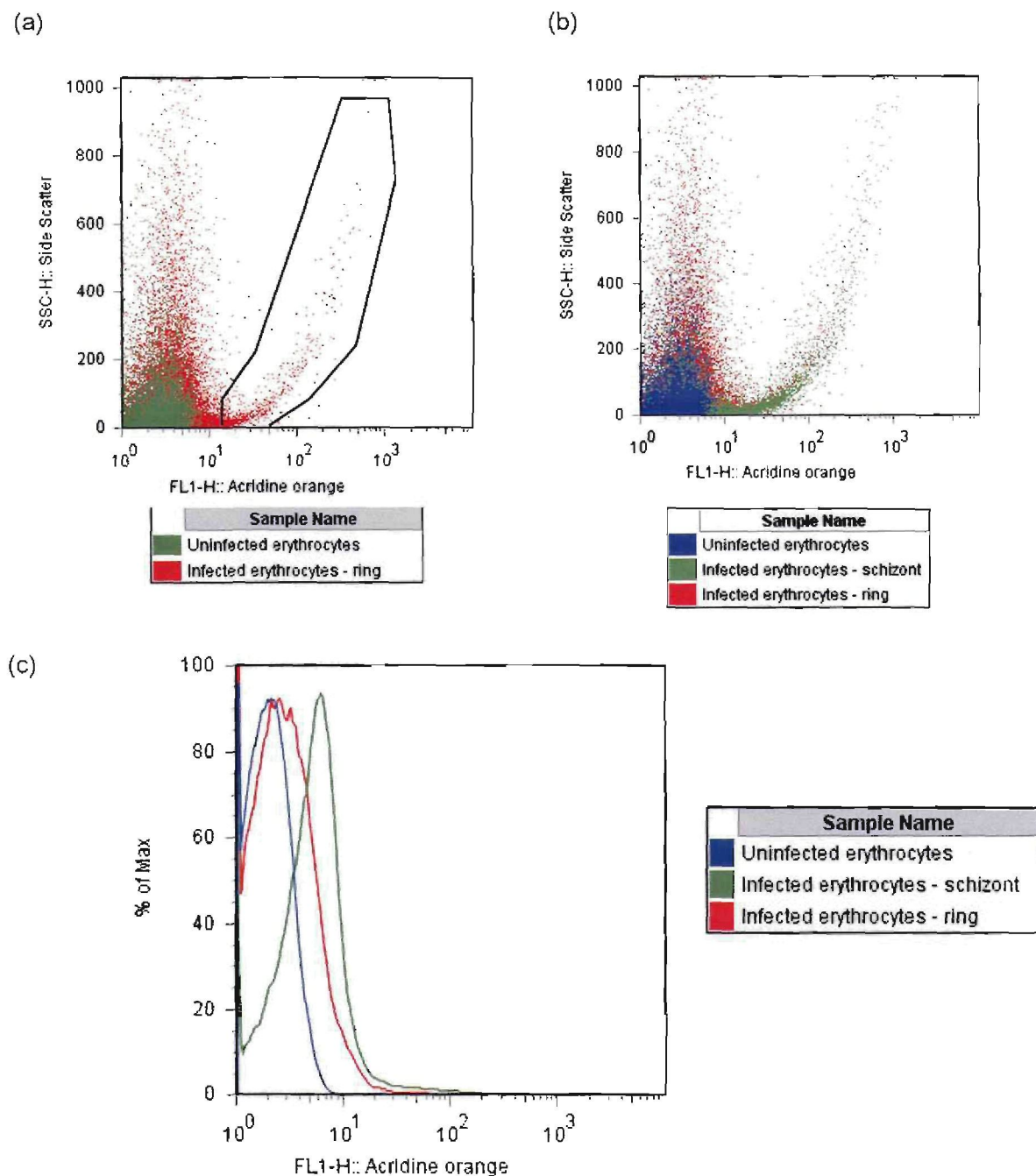


Figure 4.14 (a) Dot plot of the uninfected erythrocytes (green) and infected erythrocytes in the ring phase (red); (b) Dot plot of the uninfected erythrocytes (blue) and infected erythrocytes in different phase, ring (red) and schizont (green); (c) A histogram with uninfected erythrocytes (blue) and infected erythrocytes in different phase, ring (red) and schizont (green) (FlowJo version 7.2.5 was used draw the graphs)

The AO binds specifically to the DNA and RNA of the parasite and the intensity of the fluorescence is directly proportional to the amount of DNA and RNA present in the infected erythrocytes. Parasites that is in the schizont phase of the lifecycle, contains more DNA than parasites in the ring phase. Parasites in the schizont phase can be differentiated from the parasites in the ring phase. To determine if the flow cytometric evaluation using AO can distinguish between the phases, samples in duplicate of a serial dilution was run on time zero and time 24 hours. The synchronized cultures were in the ring phase at time zero and in the schizont phase at time 24 hours. This was confirmed by microscope evaluation. The data is showed in Table 4.8.

The graphs in Figure 4.14 show a clear difference in the uninfected and infected erythrocytes on the dot plot. This difference makes it possible to differentiate between the uninfected erythrocytes and the infected erythrocytes. More DNA is present in the schizont phase of the lifecycle than in the ring which increases the fluorescence signal making it possible to evaluate the different phases of the parasite's lifecycle (Figure 4.14(c)).

4.6.4 Conclusion

Flow cytometry is a promising method for assessing drug efficacy (Saito-Ito *et al.*, 2001). A distinctive feature of flow cytometry is that it measures the characteristic of single cells whereas spectrophotometry measures the properties of the bulk volume (FCP, 2008). The cell characteristics measured include the different phase of the parasite, different species and different properties of the erythrocytes. This can also be done by giemsa stained thin blood smears that is also DNA specific. However, microscope evaluation is manual and dependant on the ability of the operator. Flow cytometry is a rapid, automated, accurate and highly sensitive method able to analyse thousands of cells in seconds and can be successfully utilized in drug efficacy tests.

4.7 CORRELATIONS BETWEEN THE DIFFERENT METHODS

To evaluate the sensitivity of the different methods compared to microscope evaluation, correlation graphs with Spearman's correlation and the linear function equation was drawn up. Correlation between the theoretical and experimental values of the pLDH and FACS compared to the slides is displayed in Figure 4.15.

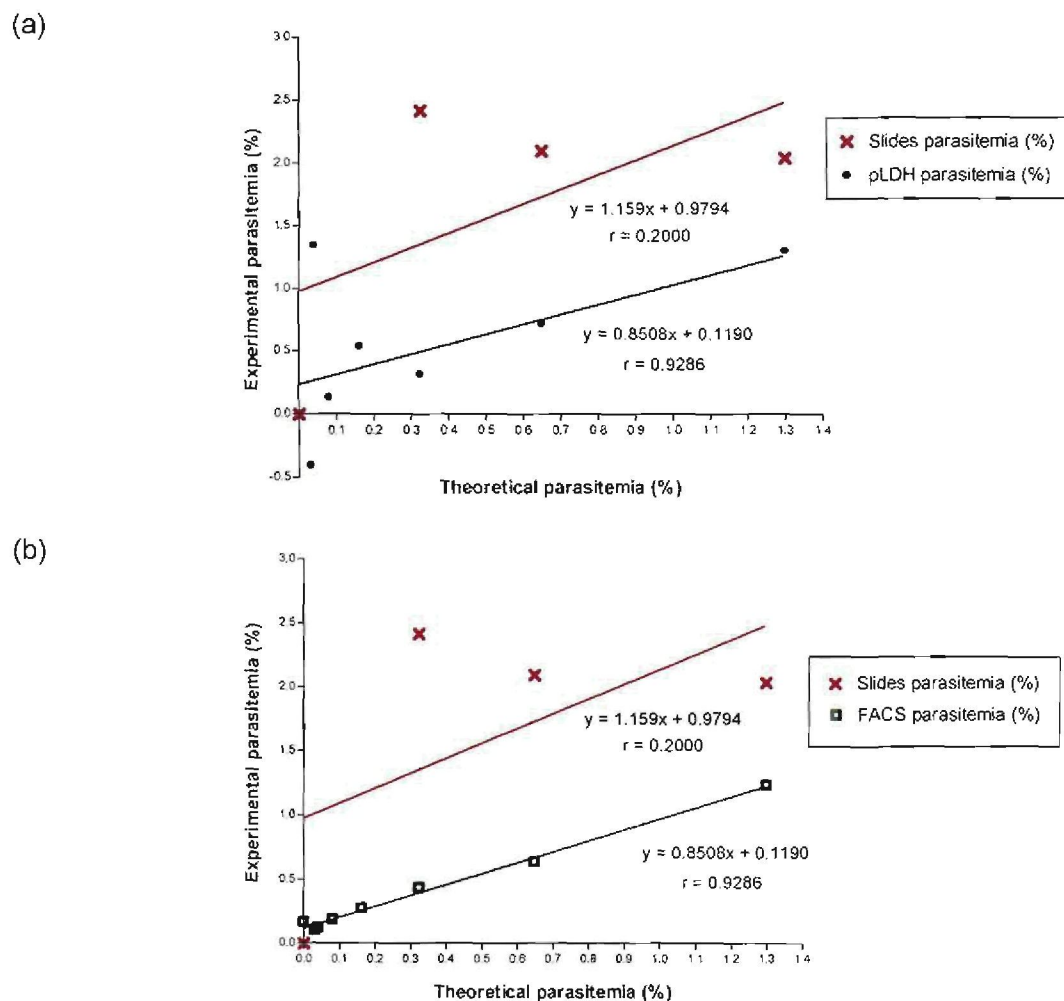


Figure 4.15 Correlation between the theoretical percentage parasitemia and the percentages parasitemia as determined by (a) the slides (red) and pLDH (blue) and (b) the slides (red) and FACS (green).

The correlation between the theoretical and experimental values of the slides is very low. This is due to the many aberrations in the method. This is an imperfect method that is subjective with a lot of room for human error. The correlation between the theoretical and experimental values for pLDH and FACS is high with the best correlation seen in the FACS. This substantiate that the FACS is the better method to determine parasite densities. When the percentage parasitemia of the FACS is compared with that determined by microscope evaluation the correlation is very poor (see Figure 4.16). This can be due to the low sensitivity of the microscope slides and the higher sensitivity of the FACS.

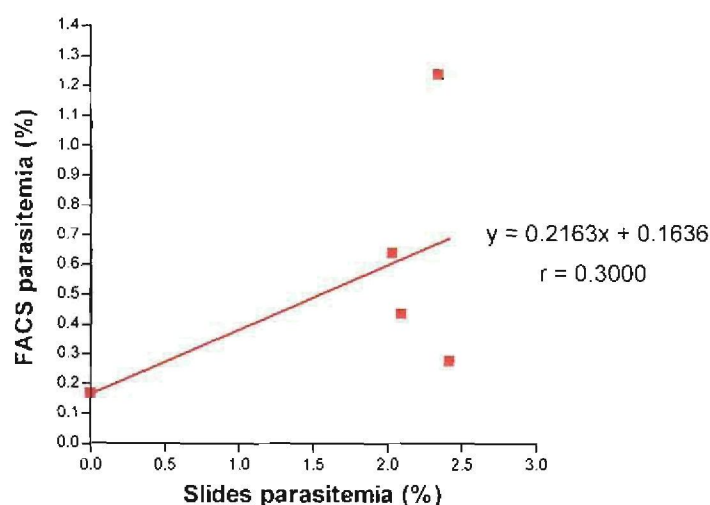


Figure 4.16 Correlation between the percentage parasitemia as determined by slides and FACS.

The correlation between the percentage parasitemia of the pLDH and slides (see Figure 4.17) has a better correlation than the FACS and slides. This is due to the fact that the pLDH measures the percentage survival and was converted to percentage parasitemia by making use of the initial parasitemia of the sample as calculated by microscope evaluation.

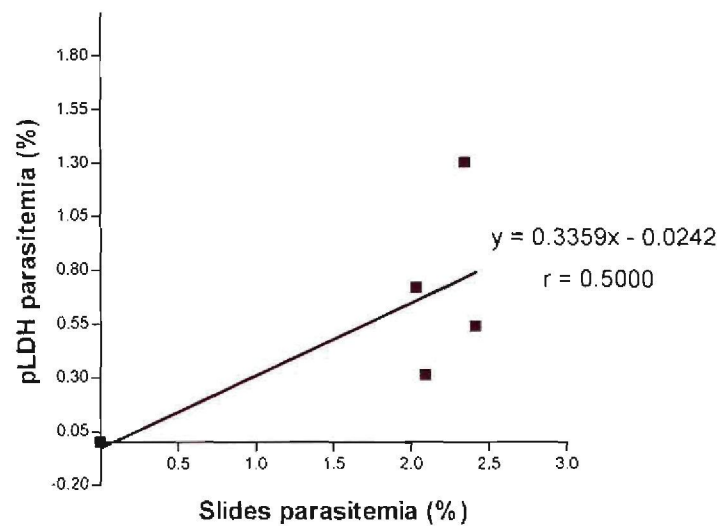


Figure 4.17 Correlation between the percentage parasitemia as determined by slides and pLDH.

The last correlation that can be made is the correlation between the percentage survival of the pLDH and FACS (see Figure 4.14). The percentage survival is used to avoid any influence from microscope evaluation. The correlation between the pLDH and FACS has a high correlation at a high parasitemia density ($X_{100\%}$ to $X_{6.25\%}$). The linear function equation shows a good correlation with the gradient near one and Spearman's correlation at exactly one. When a correlation between the pLDH and FACS is made at low parasite densities ($X_{6.25\%}$ to $X_{0.56\%}$) no correlation is present. This is due to the fact that pLDH has low sensitivity at low parasite densities. This substantiate that the FACS is the better method to determine low and high parasite densities.

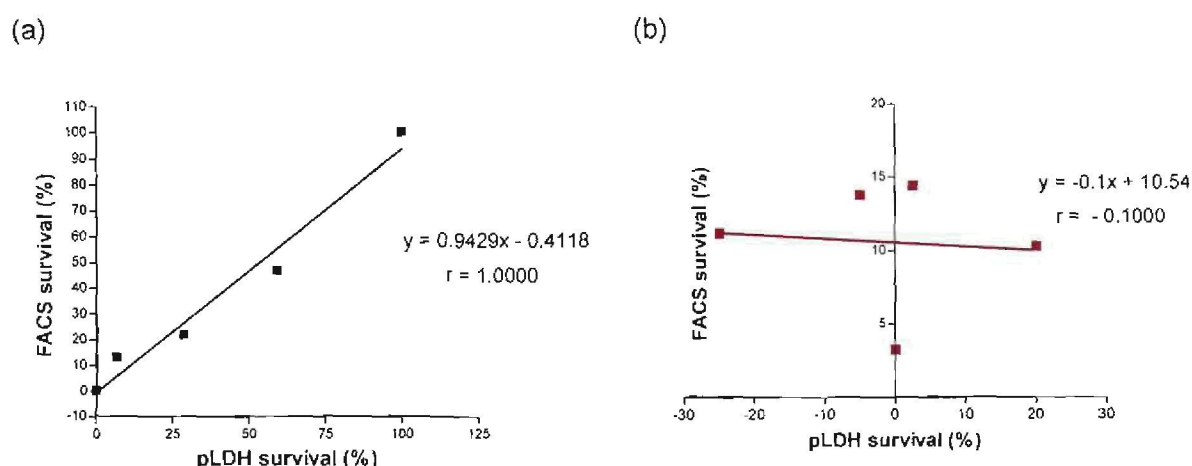


Figure 4.18 Correlation of the percentage survival between the pLDH and FACS at high parasitemia densities at a (a) high parasite density and (b) a low parasite density.

4.8 CONCLUSION

Evaluation of giemsa stain thin blood smears is the standard method in the diagnosis and drug efficacy tests. This method is also used as the basis to compare other analysis methods with each other. This method however is time consuming, subjective and had low sensitivity. Similar conclusions were made by others (Basco, 2007; Piper *et al.*, 1999; Sio *et al.*, 2006). The success of this method is based on the ability and skill of the person who is analysing the slide (Rougemont *et al.*, 2004). If the slide is not made properly, it can not be used to determine the percentage parasitemia. The amount of erythrocytes that was counted was not consistent, and this could lead to variations in the percentage parasitemia. This problem can be overcome to count an exact number of erythrocytes or to count an exact number of infected erythrocytes (Basco, 2007). The method is seen as the golden standard for evaluating the percentage parasitemia in drug efficacy test or diagnosis of infected patients (Basco, 2007; Guy *et al.*, 2007). The microscope evaluation method on the other hand is rapid, not expensive and relative simple. The FACS is able to detect low parasite densities

with high accuracy and precision. It can process bulk samples in hours and is not subjective. It can detect different phase of the parasite lifecycle as well as the species. The pLDH detection is able to detect high parasite densities but is influenced by the haematocrit and haemoglobin. Quantitatively FACS, pLDH and microscope evaluation was compared by analysing samples of *P. falciparum* infected erythrocytes. No difference were seen at high parasite densities (>1%) using FACS and pLDH. However at low parasite densities using pLDH was not sensitive compared to the FACS that was. The FACS method using AO as fluorochrome has been validated for the use in our laboratories.

CHAPTER 5

DRUG EFFICACY & FORMULATION: METHODS, RESULTS & DISCUSSION

5.1 INTRODUCTION

Malaria is one of the oldest diseases with symptoms described more than 4000 years ago (CDC, 2008). The parasitic disease kills more than 1 million people and more than 300 million are infected annually (WHO, 2007a; Worrall *et al.*, 2007). The global malaria burden has increased due to resistance against almost all of the antimalarial drugs (White, 2004; WHO, 2006) except the artemisinins (White, 2004). Chloroquine, a 4-aminoquinoline, has been the drug of choice for the treatment and prevention of malaria. Mefloquine is a quinolinemethanol structurally related to quinine (Shapiro & Goldberg, 2007). It is active against blood schizonts and used for the treatment and prevention of malaria infections caused by *P. falciparum* and *P. vivax* (Rosenthal, 2004; Shapiro & Goldberg, 2007). Pheroid™ formulations are colloidal systems (Grobler *et al.*, 2007) consisting of essential fatty acids that are a natural ingredient of the body dispersed in a liquid and nitrous oxide gas phase (Grobler, 2004). Antimalarial drugs entrapped in Pheroid™ can possibly enhance the effect of the drug, giving a better effect at lower drug concentration (Langley, 2007). This possibility needs to be investigated further. In this chapter different experiments were done to optimize the method of analysis and Pheroid™ formulation. This experiment included establishing the optimal Pheroid™ ratio, the effect the Pheroid™ has on the method of analysis and different Pheroid™ formulations in combination with mefloquine for drug efficacy tests.

5.2 MATERIALS

Materials used in the cultivation, microscopic evaluation, colorimetric evaluation and flow cytometric evaluation were also used for the drug efficacy tests. These materials for cultivation are mentioned in 4.2.1, the microscope evaluation in 4.3.1, the colorimetric evaluation in 4.4.1 and the flow cytometric evaluation in 4.5.1. Mefloquine hydrochloride used for the drug efficacy tests and formulation was purchased from Sifaviron S.p.A. (Mairano, Italy) and chloroquine phosphate from Sinoway Industrial Co. LTD. (Xiamen, China) (For certificate of analysis, see Annexure B). Pheroid™ microsphere formulations were supplied by the North West University, Unit for Drug Research and Development (Potchefstroom, South Africa). Nile Red was purchased from Molecular Probes Inc. (United States of America).

5.3 GENERAL EXPERIMENTAL DESIGN

The basic general experimental design for all the experiments was the same. The biggest difference between the experiments was the Pheroid™ microsphere formulations and antimalarial drugs used. The experiments were done in 96 well plates. For the experiments 20 µl of the drug solutions and 180 µl of infected or uninfected erythrocytes were added to the wells. A chloroquine resistant strain, RSA11, was used for the experiments. The well plates were placed in an airtight container with an atmosphere of 5% O₂, 5% CO₂ and the balance N₂. The container was placed in the incubator for 48 hours at a temperature of 37°C. After the 48 hours the plates were removed and the parasitemia determined.

The drug solutions used consisted either of the antimalarial drug dissolved in water or entrapped in Pheroid™ microspheres. All serial dilutions were made by making a stock solution and diluting it to the required concentrations. Pheroid™ formulations are a ratio between Pheroid™ microspheres and nitrous oxide water (NW). The Pheroid™-drug

solution had a specific ratio and all dilutions were made with the same ratio. Dilutions of the water-drug solution were made with water. From this point forward when using the term Pheroid it will be referring to Pheroid™ microsponges.

The statistical analysis done on this data included the average, standard error of mean, t-test, relative percentage parasitemia and the 50% inhibitory concentration (IC_{50%}). The student t-test is an indication of significant differences between two sets of data. This was calculated with Statistica 8. The relative percentage parasitemia (RPP) reflects the difference in percentage between the samples with no Pheroid, the SWFI formulation (sample zero) and the other samples with the same drug concentration. This is calculated as follows:

$$\text{Relative percentage parasitemia} = \frac{\% \text{ Sample zero} - \% \text{ Sample}}{\% \text{ Sample zero}} \times 100$$

(Equation 6)

The IC_{50%} value is an indication of the antimalarial drug's ability to inhibit the growth of the parasite. This is a quantitative measurement that indicates how much drug is necessary to inhibit growth by 50% and was calculated using GraphPad Prism 4.

5.4 DETERMINING THE OPTIMAL PHEROID™ RATIO

The purpose of this part of the study was to determine an optimal Pheroid formulation for the use in *in vitro* drug efficacy studies. Because Pheroid possibly increases the drug concentration inside the cells, the optimal ratio of the Pheroid needs to be determined to maximise drug delivery. Possible toxic effects also needed to be eliminated. Microscope

evaluation was used to determine the percentage parasitemia at different drug concentration of chloroquine phosphate and different Pheroid ratios to determine the optimal Pheroid ratio.

5.4.1 Experimental design

The Pheroid formulation used in this test is a dilution of the Pheroid with the specially formulated nitrous oxide water (NW). The ratio of Pheroid to NW is the amount of Pheroid in the NW. The higher the ratio, the more Pheroid is in the formulation. The final Pheroid formulations that were used were 1:5000 (1 part Pheroid to 4999 parts NW), 1:2500, 1:1750, 1:1000 and 1:500 with 1:500 consisting of the most Pheroid. The ratios were used at different chloroquine phosphate (CQ) concentrations. The final concentration series were 0 nM, 50 nM 100 nM and 200 nM. Stock solutions of CQ in the different ratios of Pheroid were made and left for a minimum of 24 hours for entrapment. After the 24 hours a two fold serial dilution was made with each ratio. A Pheroid solution with the same ratio was used to dilute the CQ. CQ was dissolved in water as control and diluted with water just before it was used in the experiment. Slides were manually counted by taking photos of 20 random microscope fields using a Nikon DXM1200 digital camera on a Nikon ECLIPSE TE300 confocal microscope with ACT-1 software. The total amount of erythrocytes and parasites were used to determine the percentage parasitemia using Equation 1.

5.4.2 Results and discussion

The initial parasitemia was determined and found to be 10.4%. The life cycle of the parasite is 48 hours. Thus in the 48 hours of the experiment the infected erythrocytes burst releasing merozoites that reinfected other erythrocytes increasing the parasite density. The initial parasitemia for all the dilutions were the same. The percentage parasitemia as determined by microscope evaluation is displayed in Table 5.1. The control formulation with CQ resulted in a slight decrease in parasitemia at 50 nM and 100 nM. At 200 nM the parasitemia decreased to 9.87%. The lowest ratio of Pheroid, 1:5000, appeared to have no toxic effects on the cells and the starting parasitemia was similar to the control. This ratio decreased parasite levels at all CQ concentrations to levels well below the control levels. All the other

ratios also decreased parasite levels, but showed varied effects. The results indicated however that there were an overall decrease in the percentage parasitemia when the chloroquine concentration increased. To better compare the effects of the different ratios of Pheroid the RPP was calculated with Equation 6 and displayed in Table 5.2. Although all the Pheroid ratios decreased parasite levels, the best inhibition of parasite growth was observed at 1:5000 for 50 nM and at 1:1750 for both 100 nM and 200 nM (highlighted). The 1:750 ratio gave a better reduction in the percentage parasitemia in relation to the control than the other Pheroid ratios. This relation between the 1:1750 ratio and the control is illustrated in Figure 5.1. The 1:1000 and 1:500 ratio caused lysis of the erythrocytes. The optimal Pheroid ratio, appeared to be the 1:1750 because it enhances the chloroquine effect the best without any observed negative effect on the erythrocytes. The 1:1750 Pheroid ratio appears to maximise drug delivery in *P. falciparum* cultures. Because of the difficulty in the calculation when using the 1:1750 ratio, a ratio of 1:1500 was used throughout the drug efficacy studies.

Table 5.1 Percentage parasitemias as obtained from microscope evaluation to determine the optimal Pheroid ratio.

		Chloroquine concentration			
		0 nM	50 nM	100 nM	200 nM
Pheroid ratio	Control	20.19	17.46	14.09	9.87
	1:5000	21.83	9.72	7.74	4.06
	1:2500	11.73	13.71	9.65	7.94
	1:1750	15.33	13.38	6.28	2.03
	1:1000	9.57	15.62	13.55	7.05
	1:500	14.5	14.17	8.55	4.42

Table 5.2 The relative percentage parasitemia of the different Pheroid ratios compared with the control. The highlighted values indicate the highest relative percentage decrease of parasitemia.

		Chloroquine concentration		
		50 nM	100 nM	200 nM
Pheroid ratio	1:5000	44.33	45.07	58.87
	1:2500	21.48	31.51	19.55
	1:1750	23.37	55.43	79.43
	1:1000	10.53	3.83	28.57
	1:500	18.84	39.31	55.21

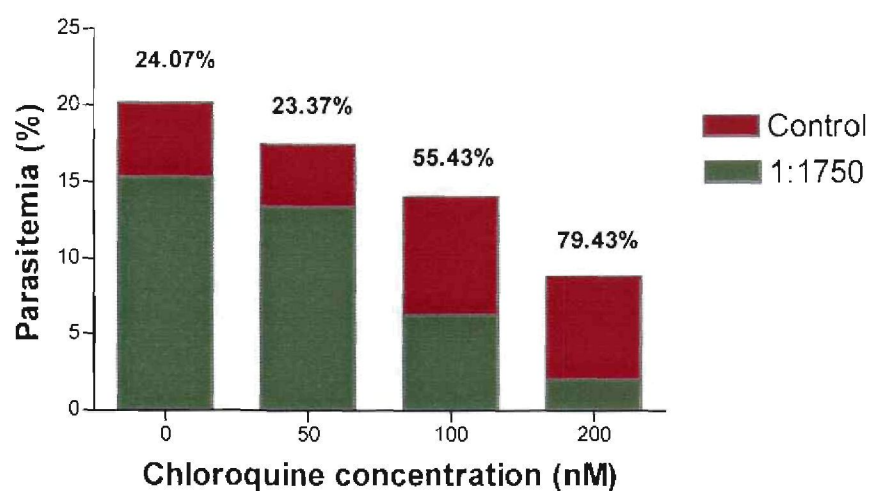


Figure 5.1 Percentage parasitemia as determined by microscope evaluation of the 1:1750 ratio of Pheroid™ microsponges to nitrous oxide water compared to the control. The percentages indicated on the bar represent the relative percentage parasitemia.

5.5 INFLUENCE OF PHEROID™ MICROSPONGES ON THE METHODS TO EVALUATE PARASITE DENSITY

To determine the influence of Pheroid on the methods used to determine the parasite densities, the percentage survival samples with and without Pheroid was determined. The same parasitemia serial dilution with and without Pheroid was used for the experiment. Firstly the pLDH method was used to determine the percentage survival and the results are explained in Figure 5.2.

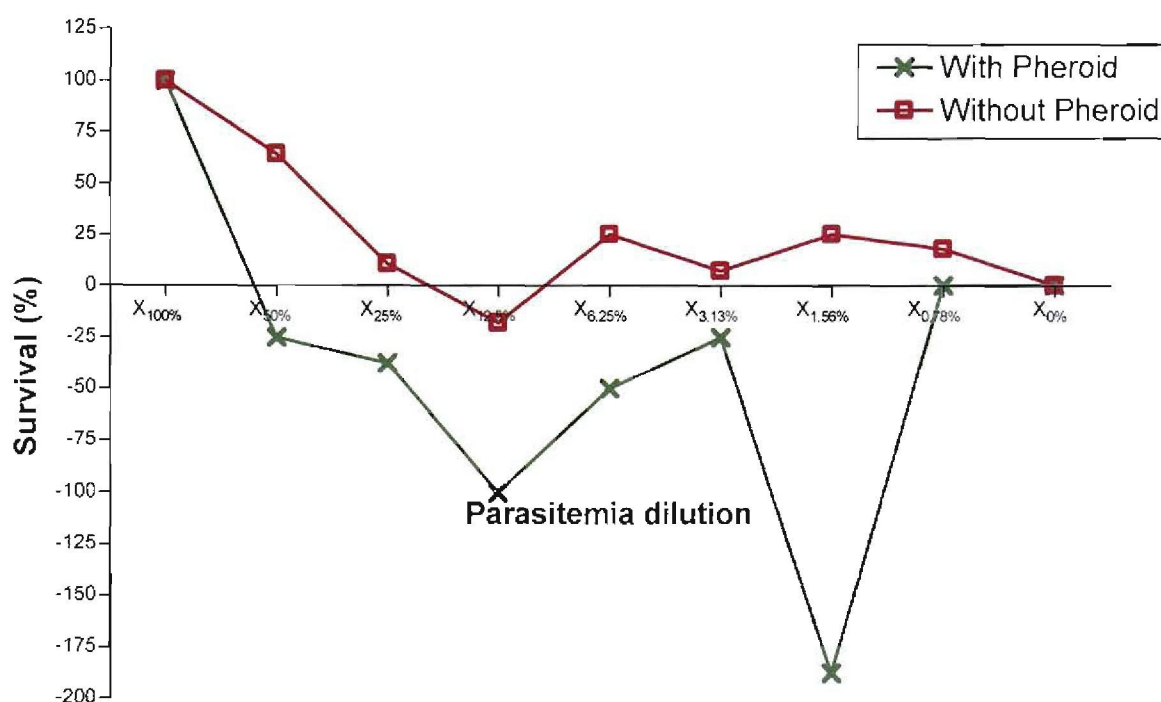


Figure 5.2 Difference between the average percentage survival of samples with and without Pheroid™ microspunge as determined by colorimetric evaluation of pLDH.

The result explained in Figure 5.2 is the average of the triplicate repeats of the different dilutions. Pheroid does have an influence on the optical densities. Pheroid has a milky appearance and this slight colour change may have an influence on the optical density

values. This will lead to lower and inconsistent percentage survival results. This method not only has a low accuracy and sensitivity, but is also influenced by the haematocrit and Pheroid. It is therefore impractical to use this method during drug efficacy tests. All subsequent experiments were performed with flow cytometry.

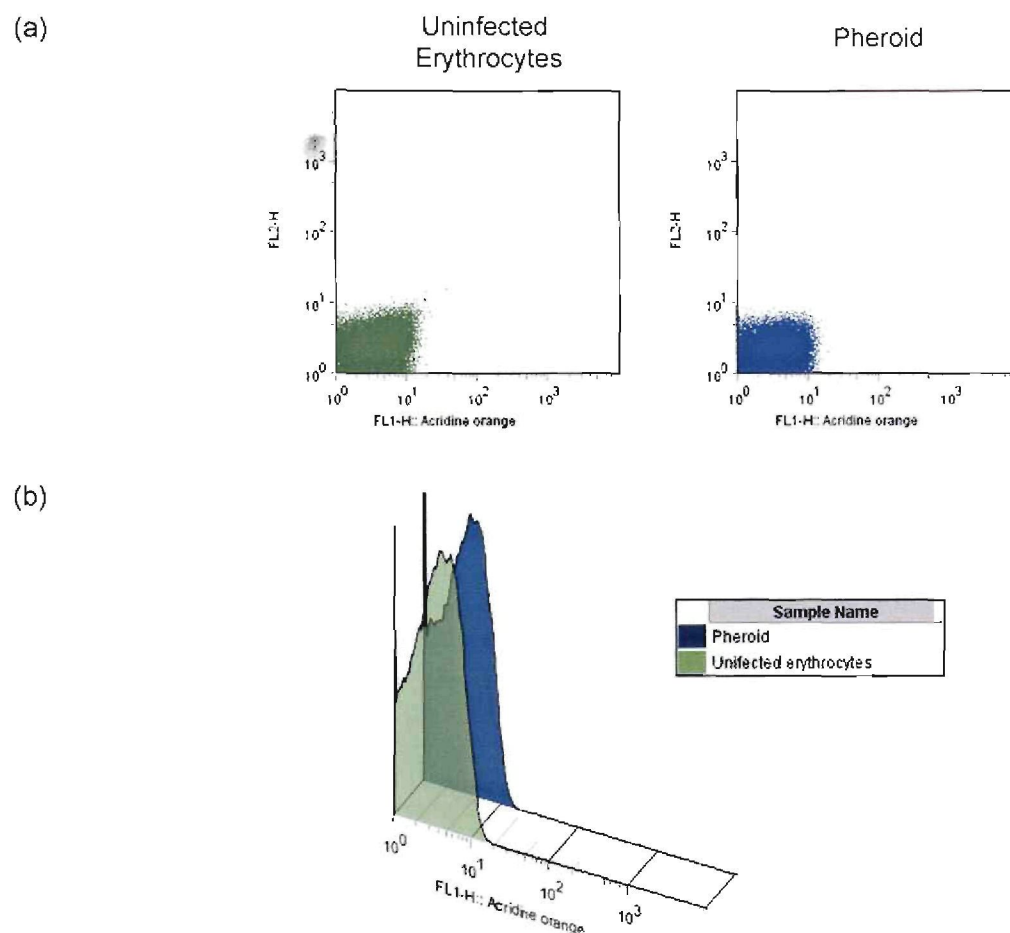


Figure 5.3 Uninfected erythrocytes without Pheroid and only Pheroid as read on the FACS with AO. (a) Dot plot of FL-2 against FL-1 and (b) overlay histogram of the uninfected erythrocytes and Pheroid.

To investigate whether the Pheroid had an influence on FACS a parasitemia dilution with and without Pheroid was analysed. As seen in Figure 5.3 the results obtained from flow cytometric evaluation of uninfected erythrocytes and Pheroid containing AO, the delivery system had an influence on the results. The dot plot of FL-1 against FL-2 shows that the Pheroid particles are situated in the same area of the graph than the uninfected erythrocytes. No results can be observed in the FSC/SSC plots (results not shown). The overlay histogram

shows that there was no difference between the Pheroid and uninfected erythrocytes. The Pheroid was not stained by the AO because it contains no DNA or RNA. The size of erythrocytes are between six and eight micro meters and the Pheroid is between one and a half and five micro meters. This similarity in size is the main reason for the detection of the microsphere as erythrocytes. This can have a negative influence when calculating the percentage parasitemia by underestimating the amount of erythrocytes. This would ultimately lead to lower parasitemia levels.

To compensate for this, an extra step during preparation was added to the method. Before the sample was added to a tube after the 48 hour incubation period the Pheroid and culture medium was removed. Samples were transferred to an Eppendorf-tube, it was centrifuged at room temperature for five minutes at 2250 r.p.m. and the supernatant was removed by aspiration. Samples were then transferred to tubes and analysed as described previously (4.6.2).

5.6 DRUG EFFICACY TESTS: MEFLOQUINE IN DIFFERENT PHEROID™ FORMULATIONS

This section describes the efficacy of different formulation of mefloquine and Pheroid. The 1:1500 Pheroid ratio was used for all the formulations. The efficacy of mefloquine was determined in three different formulations compared to a control using flow cytometry. The different formulations were used to determine the effect the Pheroid may have on the efficacy of mefloquine.

5.6.1 Experimental design

Mefloquine hydrochloride (MQ) was formulated in three different ways in combination with Pheroid. The MQ was either part of the Pheroid formulation during manufacturing of the Pheroid or was entrapped in the Pheroid. Pheroid has the ability to entrap hydrophobic drugs

like MQ in the membrane. In the first formulation MQ was added and did not have a chance to entrap in the Pheroid. In the second formulation MQ was added and left for 24 hours for the drug to entrap in the membrane of the Pheroid. The last formulation, MQ was incorporated into the Pheroid during the manufacturing of the Pheroid. With this formulation the Pheroid is added to the oil phase which ensures complete solubility of the drug.

A Pheroid ratio of 1:1500 was used with a series of MQ concentration. The concentration was 0 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM and 200 nM. A stock solution of MQ in the different Pheroid formulations was made. The Pheroid formulation was either used immediately with the intention that no entrapment could take place (formulation 1), left for 24 hours for entrapment (formulation 2) or MQ was incorporated into the Pheroid (formulation 3). After the solutions were made as described, a two fold serial dilution was made with each formulation. The Pheroid solution with the same ratio was used to dilute the MQ-Pheroid formulation. MQ was dissolved in water as control and diluted with water just before it was used in the experiment for each formulation. Formulation was evaluated on different days. Parasite densities were determined by flow cytometry (see 4.6.2). A total of 50000 cells were analysed. Haematocrit of 3% was used for all the formulations and the initial percentage parasitemia varied between 2 and 4%.

5.6.2 Results and discussion

The first drug efficacy test was done with the Pheroid-MQ formulation where no entrapment could take place. Table 5.3 displays the percentage parasitemia of formulation one compared with the control. With the control formulation the parasite levels showed a small increase from $5.13 \pm 0.42\%$ at 0 nM to $5.53 \pm 0.23\%$ at 200 nM. With the Pheroid-MQ formulation the parasitemia decreased from $5.93 \pm 0.25\%$ at 0 nM to $5.63 \pm 0.64\%$ at 200 nM. There was no significant difference between the Pheroid-MQ formulation and the control. With most of the Pheroid-drug concentrations, the percentage parasitemias was higher than that of the control. Pheroid consist mainly of essential fatty acids and may increase the growth of the parasites whereas water may lead to conditions that are not optimal for growth. No significant difference could be observed between the Pheroid and control as indicated by the p-values of the student's t-test.

Table 5.3 The percentage parasitemia of the different mefloquine concentration of Pheroid formulation one (no entrapment) and the control. The results are presented as the average $\pm$ SEM and the t-test values are also given.

	Control	Pheroid	T-test (p value)
0nM	5.13 $\pm$ 0.42	5.93 $\pm$ 0.25	0.297777
6.25 nM	5.31 $\pm$ 0.12	6.00 $\pm$ 0.30	0.224768
12.5 nM	6.03 $\pm$ 0.37	6.82 $\pm$ 0.53	0.073713
25 nM	5.4 $\pm$ 0.22	6.44 $\pm$ 0.43	0.082072
50 nM	6.62 $\pm$ 0.16	6.50 $\pm$ 0.20	0.567982
100 nM	6.48 $\pm$ 0.16	6.13 $\pm$ 0.78	0.699059
200 nM	5.53 $\pm$ 0.23	5.63 $\pm$ 0.64	0.872636

Table 5.4 displays the percentage parasitemia of formulation two compared with the control of water. When the formulation is left for 24 hour to allow entrapment of MQ, there is no trend seen with the Pheroid. The water shows a small decrease in the percentage parasitemia from 5.83 $\pm$ 0.66% at 0 nM to 4.12 $\pm$ 0.38% at 200 nM. The percentage parasitemia is very low at the 0 nM and high at the 50 nM in the Pheroid formulation. This can be due to experimental error. The lowest percentage parasitemia of 3.79 $\pm$ 0.10% is observed at the 200 nM concentration in the Pheroid formulation. There is however no significant difference between the Pheroid and control formulations.

Table 5.4 The percentage parasitemia of the different mefloquine concentration of Pheroid formulation two (24 hour entrapment) and the control. The results are presented as the average $\pm$ SEM and the t-test values are also given.

	Control	Pheroid	T-test (p value)
0nM	5.83 $\pm$ 0.66	3.96 $\pm$ 0.59	0.009553
12.5 nM	4.88 $\pm$ 0.06	4.11 $\pm$ 0.46	0.263475
25 nM	4.65 $\pm$ 0.06	4.19 $\pm$ 0.15	0.152430
50 nM	4.59 $\pm$ 0.23	5.10 $\pm$ 0.08	0.081292
100 nM	4.31 $\pm$ 0.17	4.13 $\pm$ 0.45	0.639004
200 nM	4.12 $\pm$ 0.38	3.79 $\pm$ 0.10	0.374981

It is evident from these two formulations that there might be another factor that contributes to the low inhibition of parasite growth. MQ is lipid soluble leading to low solubility in water. Because of this, an organic solvent was added to the formulation of Pheroid and water. The final concentration of the organic solvent, ethanol (EtOH) in the experiment was 0.05% (v/v) to minimise toxicity to the cells. Mefloquine was dissolved in 100% EtOH and then added to the water or Pheroid to obtain the required concentrations. This was left for 24 hours to entrap. The results indicated that there were no difference between the Pheroid formulation with the organic solvent and the Pheroid without the organic solvent (results not shown, see Annexure A). Other solvents can also be used to help with the low solubility of MQ. Because of the problems surrounding the solubility of MQ, MQ was incorporated into the Pheroid during manufacturing (formulation 3). The Pheroid consist of an oil and water phase. MQ was dissolved in the oil phase. During the formulation microsponges are formed and MQ is entrapped in the membrane of the microsphere.

This Pheroid formulation was characterised with confocal laser scanning microscope (CLSM). Images were captured on a CLSM with a He/Ne red laser and an argon green laser. Pheroid (100 μ l) was stained with 2 μ l Nile Red. Figure 5.4 illustrates the vesicular structures of Pheroid™ microsponges with MQ entrapped within the lipid structures. The micrographs show the Pheroid as red spheres with the green MQ molecules that has auto fluorescence. The micrographs confirmed that the drug was indeed entrapped within the Pheroid. Particle analysis and CLSM was done on all the Pheroid (for report, see Annexure C).

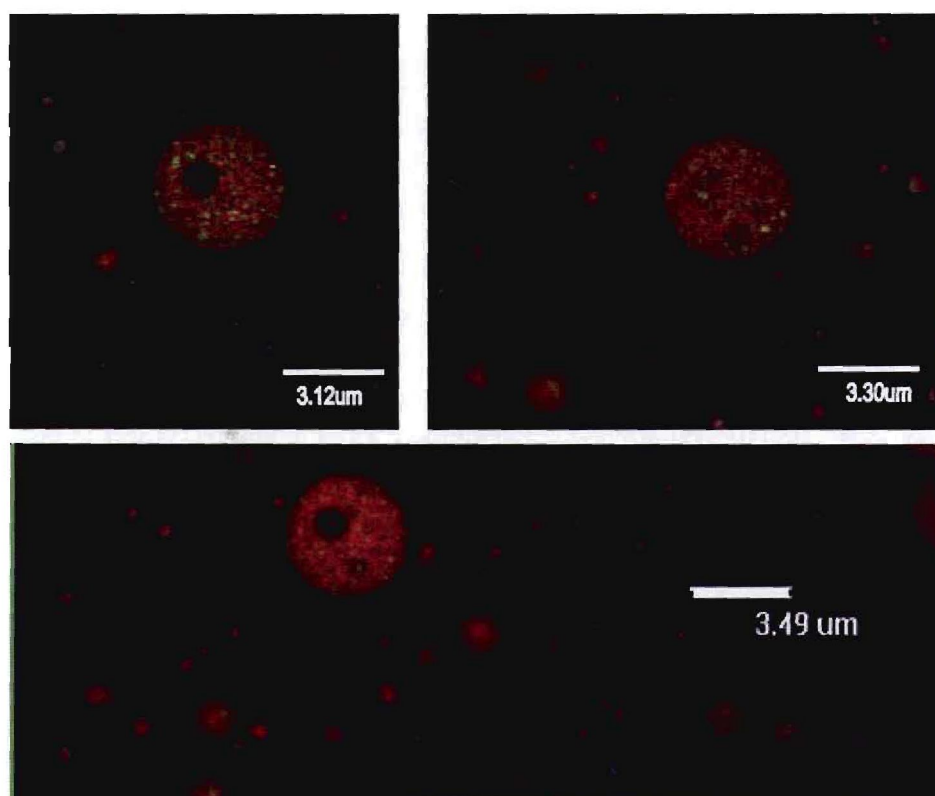


Figure 5.4 Micrographs of the Pheroid with mefloquine entrapped in the membrane. The auto fluorescent property of mefloquine gives the green colour to the mefloquine crystals and Pheroid is red in colour.

Table 5.5 shows the difference in the percentage parasitemia between formulation three and the control. There is a definite decrease in parasite levels with higher concentrations of the Pheroid-MQ formulation. The control showed a small decrease in the percentage

parasitemia from $1.93 \pm 0.10\%$ at 0 nM to $1.51 \pm 0.13\%$ at 200 nM with a spike in the percentage parasitemia at 6.25 nM. This can be due to experimental error. The lowest percentage parasitemia of $1.03 \pm 0.02\%$ is observed at the 200 nM concentration in the Pheroid formulation. There is a significant difference between the Pheroid and control formulations for 25 nM, 50 nM and 100 nM with $p \leq 0.05$. There is a statistically significant difference in the 200 nM formulations ($p = 0.078$). Figure 5.5 illustrates the decrease in average parasitemia of the control and the Pheroid-MQ formulation. The relative percentage parasitemia as determined by Equation 6 shows a noteworthy difference between the percentage parasitemias of the two formulations. There is a 27.98% reduction in growth at 25 nM MQ concentration in Pheroid. The 50 nM, 100 nM and 200 nM of the Pheroid formulation showed a 30.57%, 41.14% and 46.63% reduction in parasite growth respectively.

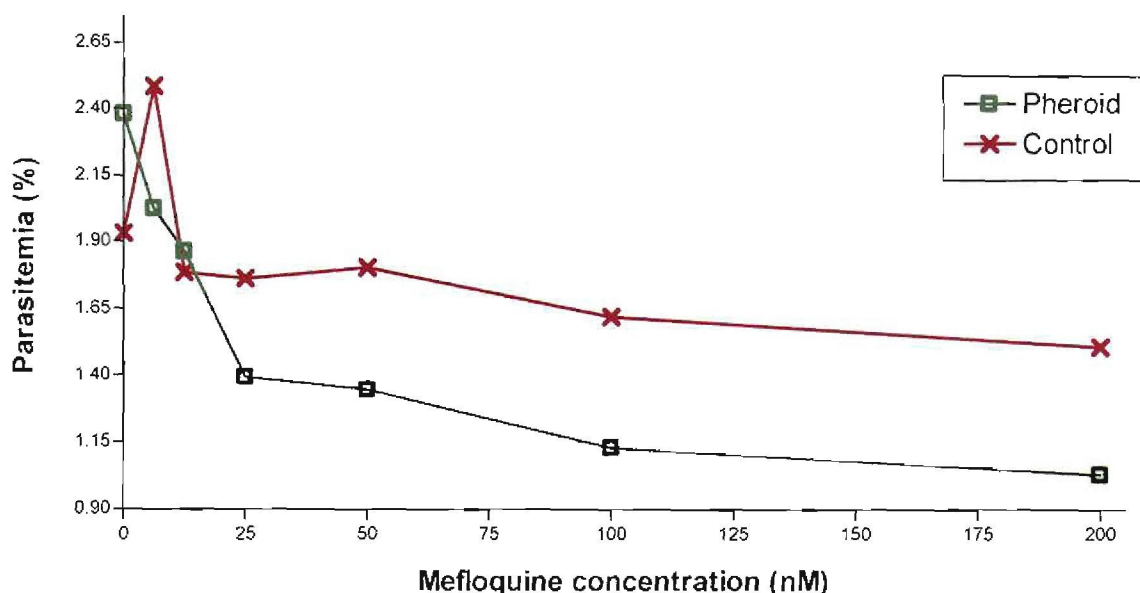


Figure 5.5 The percentage parasitemia of the control compared to the Pheroid formulation three (MQ was incorporated into the Pheroid). (The average of the triplicate was used in the graph).

Table 5.5 The percentage parasitemia of the different mefloquine concentration of Pheroid formulation 3 (incorporated in) and the control. The results are presented as the average $\pm$ SEM and the t-test values are also given. The highlighted values indicate statistical significant results ($p \leq 0.05$).

	Control	Pheroid	T-test (p value)
0nM	1.93 ± 0.10	2.38 ± 0.15	0.211093
6.25 nM	2.49 ± 0.11	2.03 ± 0.11	0.112866
12.5 nM	1.79 ± 0.15	1.86 ± 0.11	0.614898
25 nM	1.76 ± 0.16	1.39 ± 0.10	0.044558
50 nM	1.80 ± 0.03	1.34 ± 0.03	0.014133
100 nM	1.62 ± 0.04	1.13 ± 0.04	0.019804
200 nM	1.51 ± 0.13	1.03 ± 0.02	0.078378

The IC_{50%} value is an indication of the ability of mefloquine to inhibit the growth of the parasite by 50%. The IC_{50%} of the control is 70.53 nM and of the Pheroid-MQ formulation only 15.95 nM. Lower IC_{50%} value indicates that lower concentrations are needed to inhibit the growth by 50% and thus has better efficacy. The Pheroid-MQ formulation displays better drug efficacy than the control. There is a statistical significant difference between the IC_{50%} of the Pheroid-MQ formulation and the control ($p < 0.0001$).

To compare the different formulations with each other, it was necessary to determine the percentage survival of each formulation. This was done because the initial percentage parasitemias of the three formulations were different and had to be normalised daily. Equation 5 was used to determine the percentage survival. The results are displayed in Figure 5.6. There is a distinct difference between the three formulations. MQ that was not entrapped in the Pheroid showed the least reduction in parasite density with an increase in

the drug concentrations. The best formulation was formulation three where MQ was incorporated into the Pheroid during manufacturing. The better the drug entrapment in the microsphere membrane, the better inhibition of parasite growth was observed.

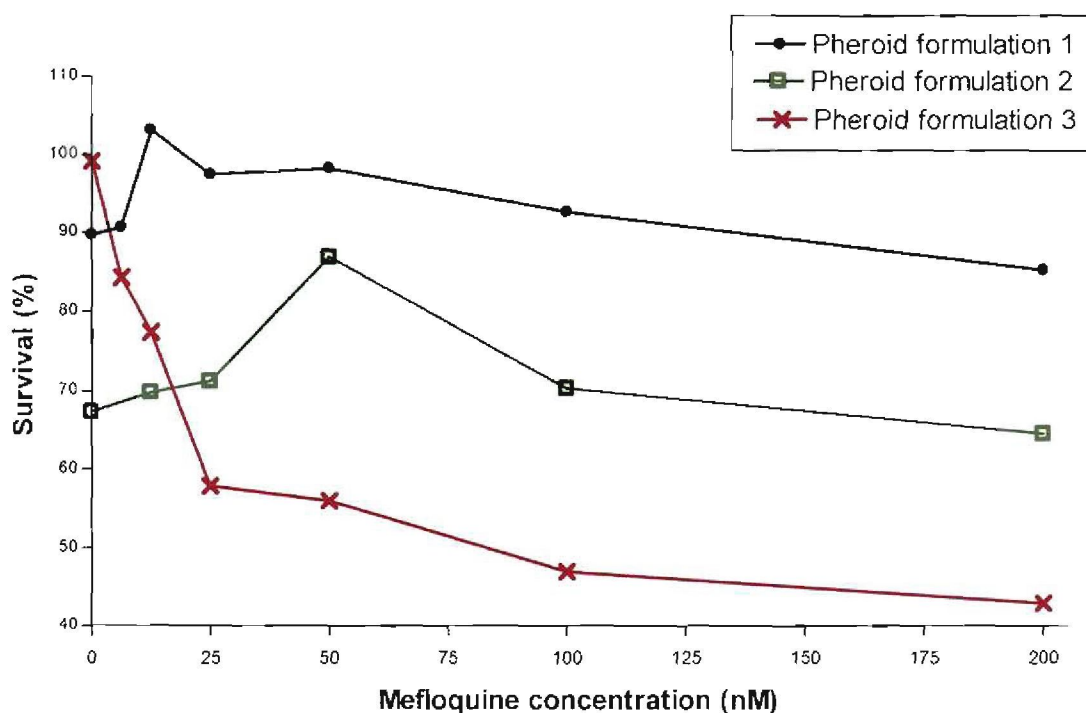


Figure 5.6 The percentage survival of the three Pheroid™ microsphere formulations at different mefloquine concentrations. (The average of the triplicate was used in the graph).

5.7 CONCLUSION

The results show that the control formulation where mefloquine was dissolved in water was not effective in decreasing parasite levels but the mefloquine incorporated into in Pheroid™ microspheres was effective. Mefloquine in formulation three, where it was incorporated into

the Pheroid™ microsponges was the most effective. The formulation where mefloquine was entrapped for 24 hours showed better efficacy than when no entrapment took place. Mefloquine build into the Pheroid™ microsponges showed a 46% inhibition in parasite growth. Mefloquine entrapped in the Pheroid™ microsponges had an increase in drug delivery and an enhanced antimalarial effect. The proposed mechanism of Pheroid is that it has a high affinity for cell membranes resulting in effective and fast delivery of the drug beyond the mechanism of drug efflux pump (Grobler, 2004). Pheroid™ can be used as a drug delivery system to enhance the effect of the antimalarial drugs, chloroquine and mefloquine.

SUMMARY & FUTURE PROSPECTS

The success in the treatment of malaria is dependent on the prompt and accurate diagnosis of malaria and effectiveness of the drug regime used. The emerging of resistance to most antimalarial drug makes the treatment of malaria difficult. The need for more effective mono and combination therapy is necessary to eradicate malaria. Pheroid™ technology was used to evaluate the drug efficacy of entrapped drugs to possibly overcome this difficulty. Pheroid™ has the ability to delivery. Pheroid™ has a high affinity for cell membranes resulting in effective and fast delivery of the drug beyond the mechanism of drug efflux pump (Grobler, 2004) enhancing the therapeutic effect. To successfully evaluate the effect of Pheroid, it was necessary to validate the methods used to determine the drug efficacy.

The methods used in this study was determining the percentage parasitemia giemsa stained thin blood smears through microscope evaluation, the colorimetric evaluation of pLDH and flow cytometric evaluation using the DNA specific dye acridine orange (AO). Even though microscope evaluation is relatively simple and a cheap method, it is time consuming, labour intensive and subjective. To overcome these limitations, this study focused on the development and validation of alternative methods to evaluate drug efficacy test in *Plasmodium falciparum* cultures. The validation is a combination of determining the selectivity, accuracy, precision, sensitivity and repeatability of these methods.

The colorimetric evaluation of pLDH is a selective method to determine the presence of *P. falciparum* in samples. The accuracy and precision is relatively good at high parasite densities but there is a decrease in the accuracy and precision at low parasite densities. This means that the method is only sensitive at a minimum parasitemia of 1%. There are a lot of different factors, like the haemoglobin, haematocrit and Pheroid™ that have an influence on this method that can possibly lead to the low sensitivity. This method doesn't comply with the

FDA standards. This method was therefore not used in determining the parasite levels in drug efficacy tests.

The flow cytometric evaluation of *Plasmodium* infected erythrocytes used the DNA specific fluorochrome AO. The absence of DNA and RNA in erythrocytes makes it possible that AO can selectively bind to the DNA and RNA of the parasite. The accuracy and precision of the method complies with the criteria of the FDA. The method is sensitive to a quantitatively parasitemia of 0.2%. The method can be repeated on different day with high accuracy. This method does meet the specific criteria of the FDA for validation of the method.

The three methods were compared with the theoretical values and with each other. The results showed that there is a very low correlation between theoretical values and the methods for the microscope evaluation ($r = 0.20$). The correlations for the pLDH were between 0.59 and 1.00 and for the FACS were between 0.95 and 1.0. This showed that the pLDH and FACS is more accurate than the microscope evaluation. The correlation between the slides and pLDH was 0.50 and for the FACS 0.30. This weak correlation is an indication of the low sensitivity of the microscope evaluation. The correlation between pLDH and FACS for high parasite levels was 1.0 and for the low parasite densities -0.10. This implies low sensitivity of the pLDH method. The FACS overall had the best correlation values. This method was able to successfully differentiate between the ring and schizont phase of the parasite. This method was used in drug efficacy tests of mefloquine in combination with Pheroid™ microsponges.

To optimize the possible enhancement of the antimalarial drug in combination with Pheroid™ different Pheroid™ ratio was tested at different chloroquine concentrations. The results showed that Pheroid™ had an overall lower percentage parasitemia compared to the control. The best inhibition of parasite growth was at the 1:1750 ratio. There were a 55.43% and a 79.43% decreases in parasite growth at the 100 nM and 200 nM concentration respectively. A 1:1500 was used in the drug efficacy tests of mefloquine and Pheroid™.

Different formulation of Pheroid™ and mefloquine was tested *in vitro* to determine the efficacy. As a control, mefloquine in water was tested and showed no significant decrease in the parasite growth. When no entrapment of mefloquine in the Pheroid occurred, there was only a slight decrease from 5.93% to 5.63% in the parasitemia. The mefloquine in Pheroid

left for 24 hours to entrap showed a decrease of 3.96% to 3.79%. There wasn't a significant difference between the Pheroid™ mefloquine formulation and the control. Mefloquine is lipid soluble and has difficulty to dissolve in water. When an organic solvent like ethanol is added, the solubility of mefloquine may increase. The efficacy of mefloquine entrapped in Pheroid™ for 24 hours in combination with ethanol showed no difference between the formulation containing the solvent and the formulation without the solvent. Pheroid™ consists of a water and lipid phase. This makes it possible to incorporate lipid soluble drugs, like mefloquine in the Pheroid™ during manufacturing. The drug efficacy test done on this formulation showed a significant difference between the Pheroid formulation and the control. The Pheroid™ formulation showed a decrease from 2.38% at a 0 nM to a 1.03% at a 200 nM mefloquine concentration. The 50 nM showed a 30.57% inhibition in growth increasing to 46.63% at 200 nM. The $IC_{50\%}$ value for the control was 70.53 nM and for the Pheroid formulation only 15.95 nM. This showed that mefloquine entrapped in Pheroid™ do have a better efficacy compared to the control. Pheroid™ entrapped with mefloquine enhances the effect of drug for better inhibition of parasite growth.

This study concluded that the percentage parasitemia can be accurately determined by flow cytometry using acridine orange. Pheroid™ entraps the mefloquine and enhances the antimalarial effect of the drug against *Plasmodium falciparum* during *in vitro* studies.

Future prospects that came to light during this study were:

- ◆ To incorporate other antimalarial drugs, mono and combination therapy, into the Pheroid™ to evaluate the drug efficacy.
- ◆ *In vivo* studies to determine the efficacy of the drugs in Pheroid™ and to determine pharmacokinetic properties.
- ◆ Toxicity studies of the drugs entrapped in Pheroid™.
- ◆ Optimization of the pLDH method and other dyes that can be used in flow cytometry to evaluate parasite densities.

All these studies are part of the process to be able to put a new product for the treatment of malaria on the market to help fight against this global killer.

ANNEXURE A
ANALYTICAL DATA
ORGANIC SOLVENT FORMULATION

Table A.1 Percentage parasitemia of three mefloquine concentration in different formulation with and without an organic solvent, ethanol.

	0 nM	2 000 nM	20 000 nM
Water no solvent	4.58	3.71	4.50
Pheroid no solvent	4.84	3.62	3.64
Water with solvent	4.22	4.54	5.46
Pheroid with solvent	5.49	3.95	3.57

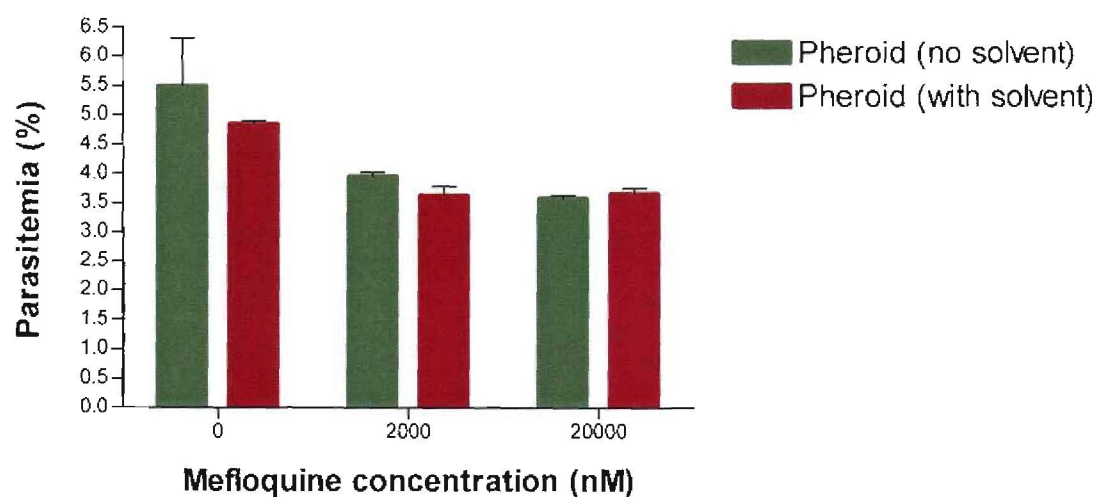


Figure A.1 Percentage parasitemia of the two Pheroid formulations, with and without an organic solvent.

<i>ANNEXURE B</i> <i>CERTIFICATE OF ANALYSIS</i>
--

Mod.05/01-8 REV.2 del 22/04/2004

Sifavor S.p.A. Via Livelli 1 - 26052 Casaleto Lodigiano (Pav. - Mantova) (ITALY) Tel. 0371-73991 - Fax 0371-73108 - e-mail: sifavor@mediolan.com	CERTIFICATE OF ANALYSIS
---	--------------------------------

MEFLOQUINE HYDROCHLORIDE

Revision: 24/02/2005 Page 1 of 1

Analysis Number 60604	Batch: 8700/03/06	Kg: 232.00	Formula: $C_{17}H_{19}F_3N_2O \cdot HCl$	M.W: 414.80
Analysis Date 03/04/2006	Manufacturing Date 17/03/2006	Retest Date 03/2009	Complies with: Ph.Eur.-BP - USP	

TEST	RESULTS	SPECIFICATIONS
DESCRIPTION Aspetto	complies	white or slightly yellow crystalline powder
IDENTIFICATION Identificazione	complies complies	(1): IR spectrum (2): chlorides test
APPEARANCE OF SOLUTION Aspetto della soluzione	complies	a 5% solution in methanol is clear and not more intensely coloured than reference solution BY7
OPTICAL ROTATION Rotazione ottica	- 0.02°	-0.20° to +0.20°
WATER Acqua	2.04%	not more than 3.0%
SULPHATED ASH Ceneri solforiche	0.00%	not more than 0.1%
HEAVY METALS Metalli pesanti	complies	not more than 20 ppm
RELATED SUBSTANCES (HPLC) Impurezze organiche (HPLC)	(1): < LOD (2): < LOD (3): 0.03% (4): RRT 0.91: 0.01% RRT 2.03: 0.01% (5): 0.05%	(1) impurity A nmt 0.10% (2) impurity B nmt 0.10% (3) impurity C nmt 0.20% (4) each unknown impurity nmt 0.10% (5) sum of all impurities nmt 0.50%
ASSAY Titolo	100.43%	btw.99.0% and 101.0% with reference to the anhydrous, solvent-free substance (pol.)
RESIDUAL SOLVENTS Solventi residui	29 ppm	acetone nmt 200 ppm

REPRESENTED BY:

D E Fine Chemicals (Pty) Limited

 P.O. BOX 706
 RIVONIA 2126
 Johannesburg
 South Africa


Issue: QUALITY CONTROL <i>[Signature]</i> 03/04/06	Control: QUALITY ASSURANCE <i>[Signature]</i> 03/04/06	Approval: PLANT MANAGER <i>[Signature]</i> 03.04.2006
---	---	--

SINOWAY INDUSTRIAL CO., LTD

16/F., HUICHENG COMM. COMPLEX, 839 XIAHE RD., XIAMEN, CHINA

TEL: 0086-592-5854962

POSTCODE: 361004

FAX: 0086-592-5854960

E-MAIL: sale3@china-sinoway.com

Certificate of analysis

NAME OF PRODUCT: CHLOROQUINE PHOSPHATE

BATCH NO: 0602026

QUANTITY: 0.5KG

MFG DATE: MAR.2006

REP. DATE: MAR.3, 2006

EXP. DATE: FEB.2010

ITEMS	SPECIFICATIONS	RESULTS
CHARACTERS	A WHITE OR ALMOST WHITE CRYSTALLINE POWDER ONE MELTING AT ABOUT 195°C	CONFORMS 195.5°C
IDENTIFICATION	POSITIVE	CONFORMS
SOLUTION S	AS STIPULATED	CONFORMS
APPEARANCE OF SOLUTION	≤BY ₂ OR GY ₂	<QY ₂
PH(10% W/V)	3.8-4.3	4.06
RELATED SUBSTANCE	ONE SPOT ≤1.0% ANY OTHER SPOT ≤0.5%	CONFORMS CONFORMS
HEAVY METALS	≤20PPM	<10PPM
LOSS ON DRYING	≤2.0%	0.42%
ASSAY	98.5-101.0%	100.14%
CONCLUSION	CONFORMS TO BP2003/USP28	

REPRESENTED BY:

D B Fine Chemicals (Pty) Limited

P.O. Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS
1995 2005

SINOWAY INDUSTRIAL CO., LTD

REPRESENTED BY:

D B Fine Chemicals (Pty) Limited

P.O. Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS
1995 2005

ANNEXURE C
PHEROID™ PARTICLE ANALYSIS
&
CONFOCAL LASER SCANNING
MICROSCOPY MICROGRAPHS

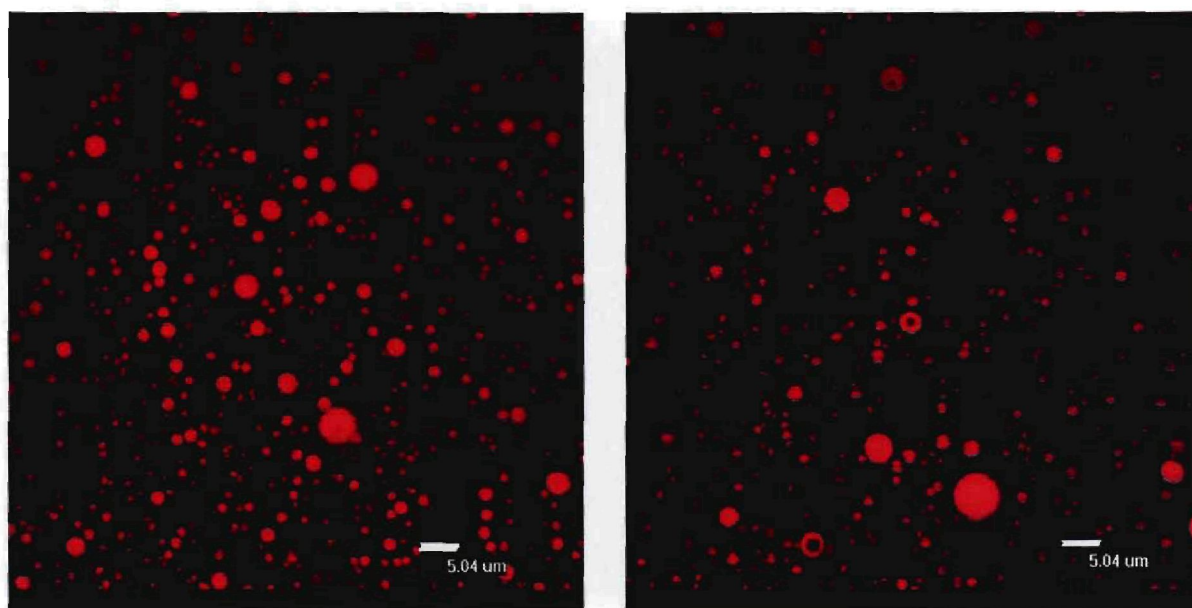


Figure C.1 Micrographs of Pheroid™ microsponges (Batch number: S08001).

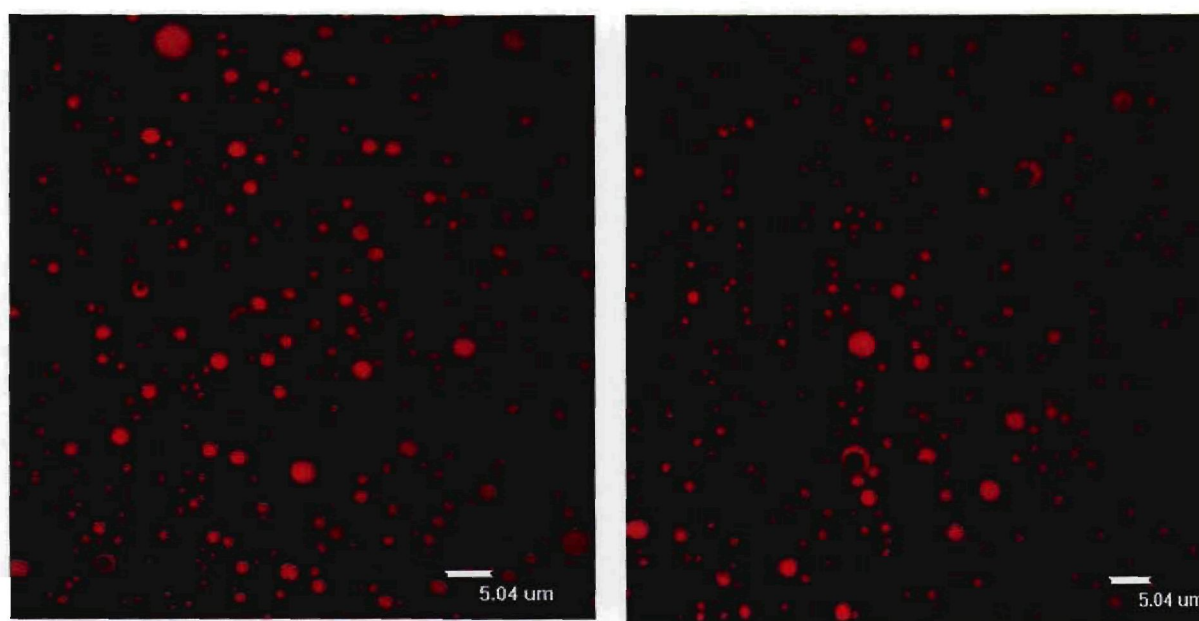


Figure C.2 Micrographs of Pheroid™ microsponges (Batch number: S08010).



MASTERSIZER

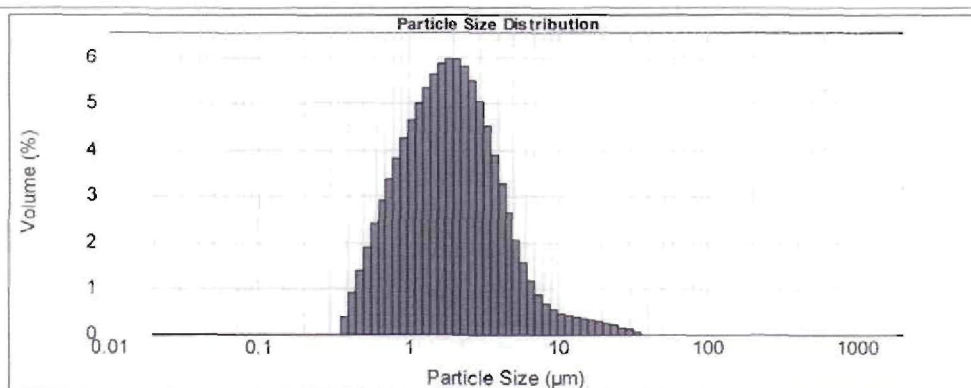


Result Analysis Report

Sample Name: G08001
Sample Source & type: Isotec
Sample bulk lot ref:
SOP Name: Isotec Nutrition Samples using Hydro 2000
Measured by: Micron Scientific
Result Source: Measurement
Measured: 18 February 2008 09:26:00 AM
Analysed: 18 February 2008 09:26:01 AM

Particle Name: Oleic Acid	Accessory Name: Hydro 2000MU (A)	Analysis model: General purpose	Sensitivity: Enhanced
Particle RI: 1.458	Absorption: 0.1	Size range: 0.020 to 2000.000 um	Obscuration: 26.46 %
Dispersant Name: Water	Dispersant RI: 1.330	Weighted Residual: 3.777 %	Result Emulation: Off
Concentration: 0.0086 %Vol	Span : 2.292	Uniformity: 0.854	Result units: Volume
Specific Surface Area: 1 m ² /g	Surface Weighted Mean D{3,2}: 1.463 um	Vol. Weighted Mean D{4,3}: 2.705 um	

d(0.1): 0.713 um d(0.5): 1.864 um d(0.9): 4.984 um



G08001, 18 February 2008 09:26:00 AM

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	0.00	1.002	4.63	7.096	0.67	50.236	0.00	355.666	0.00
0.022	0.00	0.159	0.00	1.125	4.99	7.962	0.66	56.368	0.00	399.062	0.00
0.025	0.00	0.178	0.00	1.262	5.33	8.904	0.53	63.246	0.00	447.744	0.00
0.028	0.00	0.200	0.00	1.416	5.62	10.024	0.45	70.963	0.00	502.377	0.00
0.032	0.00	0.224	0.00	1.589	5.84	11.247	0.40	79.621	0.00	563.677	0.00
0.036	0.00	0.252	0.00	1.783	5.96	12.619	0.38	89.337	0.00	632.466	0.00
0.040	0.00	0.283	0.00	2.000	5.96	14.159	0.35	100.237	0.00	709.627	0.00
0.045	0.00	0.317	0.00	2.244	5.79	15.867	0.32	112.468	0.00	796.214	0.00
0.050	0.00	0.356	0.38	2.518	5.48	17.825	0.29	126.191	0.00	893.367	0.00
0.056	0.00	0.399	0.92	2.825	5.03	20.000	0.25	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	1.38	3.170	4.49	22.440	0.20	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	1.90	3.557	3.68	25.179	0.16	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	2.41	3.991	3.25	28.251	0.13	200.000	0.00	1415.862	0.00
0.089	0.00	0.632	2.90	4.477	2.63	31.696	0.04	224.404	0.00	1588.666	0.00
0.100	0.00	0.710	3.37	5.004	2.08	35.596	0.00	251.785	0.00	1782.932	0.00
0.112	0.00	0.796	3.82	5.637	1.57	39.905	0.00	282.506	0.00	2000.000	0.00
0.126	0.00	0.893	4.23	6.325	1.17	44.774	0.00	316.979	0.00		
0.142	0.00	1.002		7.096		50.236		355.666	0.00		

Operator notes: 12/02/2008

Malvern Instruments Ltd
 Malvern, UK
 Tel: +44(0)1691 692496 Fax: +44(0)1691 692786

Mastersizer 2000 Ver. 5.31
 Serial Number: MAL1007548

File name: Examples
 Record Number: 298
 18 Feb 2008 09:27:46 AM



MASTERSIZER 2000

Result Analysis Report

Sample Name:
MICROSPONGES WITH MEF

Sample Source & type:
Isotec

Sample bulk lot ref:

SOP Name:
Isotec Nutrition Samples using Hydro 2000

Measured by:
Micron Scientific

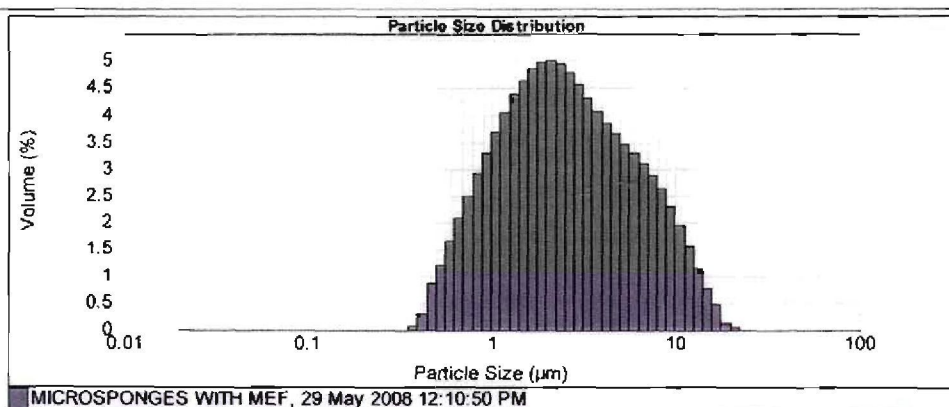
Result Source:
Measurement

Measured:
29 May 2008 12:10:50 PM

Analysed:
29 May 2008 12:10:51 PM

Particle Name: Oleic Acid	Accessory Name: Hydro 2000MU (A)	Analysis model: General purpose	Sensitivity: Enhanced
Particle RI: 1.458	Absorption: 0.1	Size range: 0.020 to 2000.000 um	Obscuration: 28.48 %
Dispersant Name: Water	Dispersant RI: 1.330	Weighted Residual: 3.064 %	Result Emulation: Off
Concentration: 0.0110 %Vol	Span: 3.036	Uniformity: 0.915	Result units: Volume
Specific Surface Area: 3.27 m ² /g	Surface Weighted Mean D[3,2]: 1.834 um	Vol. Weighted Mean D[4,3]: 3.625 um	

d(0.1): 0.843 um d(0.5): 2.447 um d(0.9): 8.272 um



MICROSPONGES WITH MEF, 29 May 2008 12:10:50 PM

Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %
0.020	0.00	0.142	0.00	1.002	3.67	7.066	2.89	50.238	0.00	365.656	0.00
0.022	0.00	0.159	0.00	1.125	4.03	7.962	2.62	56.368	0.00	399.052	0.00
0.025	0.00	0.178	0.00	1.282	4.35	8.904	2.30	63.246	0.00	447.744	0.00
0.028	0.00	0.200	0.00	1.476	4.63	10.024	1.93	70.963	0.00	502.377	0.00
0.032	0.00	0.224	0.00	1.689	4.85	11.247	1.54	79.621	0.00	553.677	0.00
0.036	0.00	0.252	0.00	1.783	4.97	12.619	1.13	89.337	0.00	602.456	0.00
0.040	0.00	0.283	0.00	2.000	5.00	14.159	0.79	100.237	0.00	709.627	0.00
0.045	0.00	0.317	0.00	2.244	5.00	15.867	0.44	112.498	0.00	796.214	0.00
0.050	0.00	0.356	0.00	2.518	4.92	17.825	0.13	126.191	0.00	893.367	0.00
0.056	0.00	0.399	0.00	2.825	4.56	20.000	0.03	141.580	0.00	1002.374	0.00
0.063	0.00	0.448	0.84	3.170	4.31	22.440	0.00	158.895	0.00	1124.683	0.00
0.071	0.00	0.502	1.21	3.567	4.06	25.179	0.00	178.250	0.00	1251.915	0.00
0.080	0.00	0.564	1.64	3.991	3.84	28.251	0.00	200.000	0.00	1415.892	0.00
0.088	0.00	0.632	2.07	4.477	3.64	31.688	0.00	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	2.49	5.024	3.46	35.666	0.00	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	2.90	5.637	3.29	39.905	0.00	282.508	0.00	2000.000	0.00
0.128	0.00	0.893	3.29	6.325	3.10	44.774	0.00	316.979	0.00		
0.142	0.00	1.002	3.29	7.066	3.10	50.238	0.00	365.656	0.00		

Operator notes: BN: S 08008
PD: 27/05/2008

Malvern Instruments Ltd.
Malvern, UK
Tel: +44(0)1452 869400 Fax: +44(0)1452 869789

Mastersizer 2000 Ver. 5.31
Serial Number: MAL1007548

File name: Examples
Record Number: 1016
29 May 2008 12:11:59



MASTERSIZER



Result Analysis Report

Sample Name:
MICROSPOGES FOR MALARIA TC

Sample Source & type:
Isotec

Sample bulk lot ref:

SOP Name:
Isotec Nutrition Samples using Hydro 2000

Measured by:
Micron Scientific

Result Source:
Measurement

Measured:
29 May 2008 11:58:09 AM

Analysed:
29 May 2008 11:58:10 AM

Particle Name:
Oleic Acid

Particle Rf:
1.458

Dispersant Name:
Water

Accessory Name:
Hydro 2000MU (A)

Absorption:
0.1

Dispersant Rf:
1.330

Analysis model:
General purpose

Size range:
0.020 to 2000.000 μm

Weighted Residual:
3.099 %

Sensitivity:
Enhanced

Obscuration:
29.64 %

Result Emulation:
Off

Concentration:
0.0119 %Vol

Span :
2.702

Uniformity:
0.828

Result units:
Volume

Specific Surface Area:
3 m^2/g

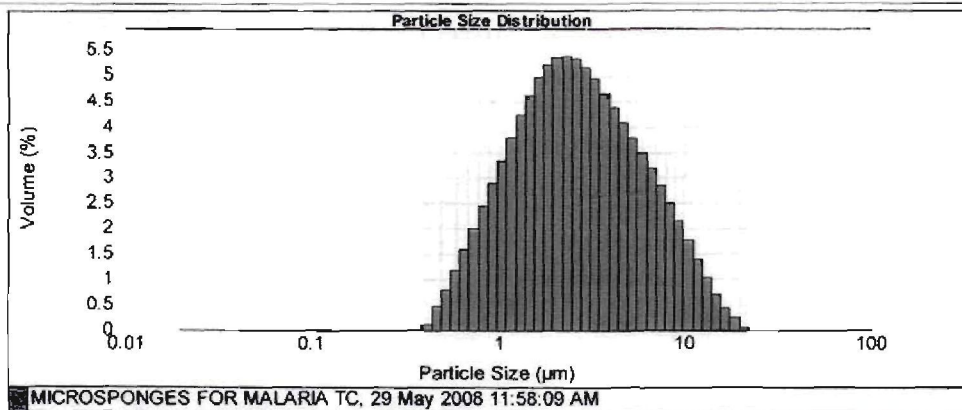
Surface Weighted Mean D[3,2]:
2.002 μm

Vol. Weighted Mean D[4,3]:
3.695 μm

d(0.1): 0.950 μm

d(0.5): 2.822 μm

d(0.9): 8.033 μm



MICROSPOGES FOR MALARIA TC, 29 May 2008 11:58:09 AM

Size (μm)	Volume in %	Size (μm)	Volume in %	Size (μm)	Volume in %	Size (μm)	Volume in %	Size (μm)	Volume in %	Size (μm)	Volume in %
0.020	0.00	0.142	0.00	1.002	3.33	7.095	2.85	50.238	0.00	355.656	0.00
0.022	0.00	0.159	0.00	1.125	3.77	7.982	2.50	56.368	0.00	399.052	0.00
0.025	0.00	0.178	0.00	1.262	3.77	8.904	2.13	62.249	0.00	447.744	0.00
0.028	0.00	0.200	0.00	1.416	4.20	10.024	2.13	70.963	0.00	502.377	0.00
0.032	0.00	0.224	0.00	1.598	4.60	11.247	1.75	79.621	0.00	563.677	0.00
0.036	0.00	0.252	0.00	1.783	4.94	12.619	1.38	89.337	0.00	632.456	0.00
0.040	0.00	0.283	0.00	2.000	5.19	14.159	1.02	100.237	0.00	709.627	0.00
0.045	0.00	0.317	0.00	2.244	5.30	15.887	0.44	112.408	0.00	796.214	0.00
0.050	0.00	0.355	0.00	2.518	5.29	17.825	0.25	126.191	0.00	893.367	0.00
0.056	0.00	0.399	0.00	2.825	5.13	20.000	0.05	141.589	0.00	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	4.90	22.440	0.00	158.866	0.00	1124.633	0.00
0.071	0.00	0.502	0.45	3.557	4.64	25.179	0.00	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.70	3.991	4.36	28.251	0.00	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	1.18	4.477	4.07	31.668	0.00	224.404	0.00	1588.606	0.00
0.100	0.00	0.710	2.00	5.024	3.78	35.905	0.00	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	2.43	5.637	3.48	39.905	0.00	282.509	0.00	2000.000	0.00
0.125	0.00	0.893	2.88	6.325	3.17	44.774	0.00	316.979	0.00		
0.142	0.00	1.002	2.88	7.095	3.17	50.238	0.00	355.656	0.00		

Operator notes: BN: S08010
PD: 27/05/2008

Malvern Instruments Ltd.
Malvern, UK
Tel: +44(0)1684-892200 Fax: +44(0)1684-892200

Mastersizer 2000 Ver. 5.31
Serial Number: MAL1007548

File name: Examples
Record Number: 1012
29 May 2008 11:58:10 AM

<i>ANNEXURE D</i> <i>CONFERENCE PARTICIPATION</i>

THE 28TH ANNUAL CONFERENCE OF THE ACADEMY OF PHARMACEUTICAL SCIENCES

~ Poster Presentation ~

An *in vitro* evaluation of PheroidTM microsponges for anti-malarial drug delivery

Chrizaan Slabbert, Marguerite Pretorius, Wilna Liebenberg, Lissinda du Plessis, Awie Kotze

Unit for Drug Research and Development, Faculty of Health Science, North-West University,
Potchefstroom, 2531

Introduction

Malaria is a leading cause of death in Africa and other tropical and subtropical regions in the world. More than 300 million people are infected by the parasite and more than 1 million people are killed by the disease annually. Children and pregnant women is most at risk in the developing countries of Africa. The occurrence of resistance among parasites to commonly used antimalarial drugs (chloroquine) is causing a progressive increase in the severity and mortality of the disease. Drug delivery systems can be used to overcome the problem of drug resistance. The PheroidTM system is a patented drug delivery system which entraps drugs with high efficiency and delivers it to target sites in the body. It consists of a unique submicron emulsion type formulation.

Purpose

The purpose of this study was to determine an optimal PheroidTM microsphere formulation for the use in *in vitro* studies. Because microspheres possibly increase the drug concentration inside the cells, the optimal concentration of the PheroidTM microspheres needs to be determined to maximise drug delivery.



Method

PheroidTM microsphere formulations were diluted with specially formulated water in different ratios (1:5000; 1:2500; 1:1750; 1:1000; 1:500). Chloroquine concentrations of 0nM, 50nM, 100nM and 200nM were prepared with these PheroidTM microsphere dilutions. A Chloroquine resistant strain of *P. falciparum* at 5% hematocrit and 0.5% parasitemia was used as model. Parasite red blood cells were incubated with the PheroidTM-drug combinations at 37°C in an atmosphere of 5% O₂ 5% CO₂ and 90% NO₂ for 48 hours. Inhibition of parasite growth was measured in relation to the control without drugs, by counting the parasitemia in Giemsa-stained thin blood smears.

Results

PheroidTM microsphere formulations were evaluated at various drug concentrations. The antimalarial activity of 200nM and 50nM chloroquine combined with the PheroidTM at a 1:2500 dilution in relation to that of the equivalent dose of chloroquine alone inhibited parasite growth by 304% and 200% respectively. The more concentrated PheroidTM drug dilutions inhibited parasite growth to a greater extent than a corresponding dose of chloroquine alone. This resulted in a reduction of the minimum effective concentration and increased the therapeutic efficacy of chloroquine. The results of this study can be used for further *in vitro* studies to ensure that the drugs incorporated in the PheroidTM delivery system enhance the effect of the drugs to its full potential.

THE 29TH ANNUAL CONFERENCE OF THE ACADEMY OF PHARMACEUTICAL SCIENCES

~ Poster Presentation ~

Alternative methods to determine Malaria parasitemia levels for drug efficacy tests

Chrizaan Slabbert, Lissinda du Plessis, Awie Kotze

Unit for Drug Research and Development, Faculty of Health Science, North-West University,
Potchefstroom, 2531

Introduction

Malaria is a leading cause of death in Africa and other tropical and subtropical regions in the world. There is an urgent need for new drugs and vaccine development. The effect of these drugs is often investigated *in vitro*. Microscopic analysis of blood smears is currently the most frequently used method to measure parasitemia levels in experiments of drug efficacy studies. Manual counting of the Giemsa stained thin blood smears makes this analysis method subjective, time consuming and labour intensive. Several new methods to determine parasitemia levels have been developed, but these are only used in diagnostics and not in research laboratories. Alternative methods for the determination of parasitemia levels include methods utilizing flow cytometry and colorimetry.

Purpose

The purpose of this study is to develop a faster and more effective analysis method to determine parasitemia levels in drug efficacy studies for research purposes.

Method

Parasitemia levels were determined with flow cytometry and colorimetrically and compared to microscope counting. Microscopically, parasitemia were determined by counting the amount of red blood cells that was infected over the uninfected red blood cells in ten random fields. For the flow cytometry method the cells were stained with Acridine Orange and the specific properties of the cells suspended in PBS were measured by an argon laser as they passed through a column one by one. NBT were used in the colorimetric method and absorbance was measured at 630nm with a microplate reader. To determine the sensitivity of the different methods a serial dilution of the parasite red blood cell were made in triplicate. Correlation graphs between the different methods were drawn in GraphPad Prism 4. Intra-/Interday tests were done on all the methods.

Results

The parasitemia levels determined by counting microscopically the amount of parasites in the fields showed a clear correlation with the theoretic values. There was a correlation between the microscope and flow cytometry analysis methods. The flow cytometry was more sensitive at high starting parasite levels but failed at very low parasitemia levels. The colorimetric method showed a correlation with the theoretic values. The intra-/interday tests showed repeatability. On the basis of data reported here, rapid and sensitive alternative methods have been established for determining parasitemia. These methods can be used for *in vitro* and *in vivo* drug efficacy test.

REFERENCES

- APPAWU, M.A., BOSOMPEN, K.M., DADZIE, S., MCKAKPO, U.S., ANIM-BAIDOO, I., DYKSTRA, E., SZUMLAS, D.E., ROGER, W., KORAM, K. & FRYAUFF, D.J. 2003. Detection of malaria sporozoites by standard ELISA and VecTest™ dipstick assay in field-collected Anopheline mosquitoes from a malaria endemic site in Ghana. *Tropical Medicine and International Health*, 8(11):1012-1017.
- ATTWOOD, D. 2003. Disperse systems (In Aulton, M.E. ed. *Pharmaceutics The Science of Dosage Form Design*. Edinburgh: Churchill Livingstone, p. 70-100).
- BACON, D.J., JAMBOU, R., FANDEUR, T., LE BRAS, J., WONGSRICHANALAI, C., FUKUDA, M.M., RINGWALD, P., SIBLEY, C.H. & KYLE, D.E. 2007. World antimalarial resistance network (WARN) II: In vitro antimalarial drug susceptibility. *Malaria Journal*, 6:120-127.
- BASCO, L.K. 2007. Field application of *in vitro* assays for the sensitivity of human malaria parasites to antimalarial drugs. Switzerland: World Health Organization. 191p.
- BASCO, L.K., MARQUET, F., MAKLER, M.M. & LE BRAS, J. 1995. *Plasmodium falciparum* and *Plasmodium vivax*: Lactate dehydrogenase activity and its application for *in vitro* drug susceptibility assay. *Experimental Parasitology*, 80:260-271.
- BEERS, M.H. & BERKOW, R. 1999. Parasitic infections. Beers, M.H. & Berkow, R. The Merck manual of Diagnosis and Therapy. Merck Research Laboratories. p. 1241-1247.
- BHAKDI, S.C., SRATONGNO, P., CHIMMA, P., RUNGRUANG, T., CHUNCHARUNEE, A., NEUMANN, H.P.H., MALASIT, P. & PATTANAPANYASAT, K. 2007. Re-evaluating acridine orange for rapid flow cytometric enumeration of parasitemia in Malaria-infected rodents. *Cytometry Part A*, 71A:662-668.

BIO MEDICINE http://www.bio-medicine.org/biology-dictionary/Giemsa_stain/ [Date of access: 6 October 2008].

BLOLAND, P.B. 2001. Drug resistance in malaria. Switzerland: World Health Organization. 27 p.

BLOOM, M.V. Polymerase Chain Reaction.
http://www.accessexcellence.org/RC/CT/polymerase_chain_reaction.php [Date of access: 02/10/2008].

BLUMBERG, L. & FREAN, J. 2007. Malaria control in South Africa – challenges and successes. *South Africa Malaria Journal*, 97(11):1193-1197.

BOSCH, I., BRACHO, C. & PÉREZ, H.A. 1996. Diagnosis of malaria by acridine orange fluorescent microscopy in an endemic area of Venezuela. *Memórias de substituição iswakdi cruz*, 91(1):83-86.

BOTES, A. 2007. Transdermal delivery of isoniazid and rifampicin by Pheroid™ technology. Potchefstroom: NWU. (Dissertation – M.Sc) 147p.

BOTHA, M.M. 2007. Pre-clinical evaluation of the possible enhancement of the efficacy of antiretroviral drugs by Pheroid™ Technology. Potchefstroom: NWU. (Dissertation – M.Sc) 155p.

BROCKMAN, A., NAKACHE, S., PHIAPHUM, L., LOOAREESUWAN, S., WHITE, N.J. & NOSTEN, F. 2004. Field evaluation of a novel colorimetric method – double-site enzyme-linked lactate dehydrogenase immunodetection assay – to determine drug susceptibilities of *Plasmodium falciparum* clinical isolates from Northwestern Thailand. *Antimicrobial Agents and Chemotherapy*, 48(4): 1426-1429.

CASSIM, R. 2007. Method development and stability of Pheroid™-based anti-retroviral formulations. Potchefstroom: NWU. (Dissertation – M.Sc) 161p.

CDC. 2008. <http://www.cdc.gov/malaria/> [Date of access: 25 August 2008].

CHEOYMANG, A., HUDCHINTON, D., KIOY, D. & NA-BANGCHANG, K. 2007. Bioassay for determination of fosmidomycin in plasma and urine: Application for pharmacokinetic dose optimization. *Journal of Microbiological methods*, 69:65-69.

COETZEE, H. 2007. The transdermal delivery of arginine vasopressin with Pheroid™ Technology. Potchefstroom: NWU. (Dissertation – M.Sc) 128p.

COHEN, W.D. 1982. The cytomorphic system of anucleate non-mammalian erythrocytes. *Protoplasma*, 113:23-32.

CORBETT, Y., HERRERA, L., CONZALEZ, J., CUBILLA, L., CAPSON, T.L., COLEY, P.D., KURSAR, T.A., ROMERO, L.I. & ORTEGA-BARRIA, E. 2004. A novel DNA-based microfluorimetric method to evaluate antimalarial drug activity. *American Journal of Tropical Medicine and Hygiene*, 70(2):119-124.

DAILY, J.P. 2006. Antimalarial Drug Therapy: The role of parasite biology and drug resistance. *Journal of Clinical Pharmacology*, 46:1487-1497.

DATE, A.A., JOSHI, M.D. & PATRAVALE, V.B. 2007. Parasitic diseases: Liposomes and polymeric nanoparticles versus lipid nanoparticles. *Advanced Drug delivery reviews*, 59:505-521.

DE BRUYN, T. 2006. Nasal delivery of insulin with Pheroid technology. Potchefstroom: NWU. (Dissertation – M.Sc) 117p.

DE RIDDER, J. 2007. Malaria manual, revised edition. Pretoria:University of Pretoria. 22p.

DESJARDINS, R.E., CANFIELD, C.J., HAYNES, J.D & CHULAY, J.D. 1979. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrobial Agents and Chemotherapy*, 16(6):710-718.

DOH, NATIONAL DEPARTMENT OF HEALTH. 2002. Guidelines for the treatment of malaria in South Africa. 18p.

DRUGBANK. 2008. <http://www.drugbank.ac/> [Date of access: 4 August 2008].

DUA, V.K., DEV, V., PHOOKAN, S., GUPTA, N.C., SHARMA, V.P. & SUBBARAO, S.K. 2003. Multi-drug resistant *Plasmodium falciparum* malaria in Assam, India: Timing of recurrence and antimalarial drug concentrations in whole blood. *American Journal of Tropical Medicine and Hygiene*, 69(5):555-557.

- EDL. 2003. Standard treatment guidelines and essential drugs list. Essential Drugs Programme South Africa. Department of Health. 365p.
- EDWARDS, C.M.B., COHEN, M.A. & BLOOM, S.R. 1999. Peptides as drugs. *QJM:An international journal of medicine*, 92:1-4.
- EICKSTEIN-LUDWIG, U., WEBB, R.J., VAN GOETHEN, I.D.A., EAST, J.M., LEE, A.G., KIMURA, M., O'NEILL, P.M., BRAY, P.G., WARD, S.A. & KRISHNA, S. 2003. Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature*, 424:957-961.
- EKLAND, E.H. & FIDOCK, D.A. 2008. *In vitro* evaluations of antimalarial drugs and their relevance to clinical outcomes. *International Journal of Parasitology*, 38(2008):743-747.
- EL BISSATI, K., ZUFFEREY, R., WITOLA, W.M., CARTER, N.S., ULLMAN, B. & MAMOUN, C.B. 2006. The plasma membrane permease PfNT1 is essential for purine salvage in the human parasite *Plasmodium falciparum*. *PNAS*, 103(24):9286-9291.
- ELGAR, D. 2008. Evaluation of the preclinical effects of perorally administered pro-Pheroids. Potchefstroom: NWU. (Dissertation – Ph.D) 139p.
- FCP. 2008. Flow cytometry principles. http://biology.berkeley.edu/crl/flow_cytometry_basic.html [Date of access: 17 October 2008].
- FOLEY, M. & TILLEY, L. 1998. Quinoline Antimalarials: Mechanisms of action and resistance and prospects for new agents. *Pharmacological Therapy*, 79(1):55-87.
- GALLUP, J.L. & SACHS, J.D. 2001. The economic burden of malaria. *American Journal of Tropical Medicine and Hygiene*, 64(1,2)S:85-96.
- GERBER, M. 2007. Synthesis and transdermal penetration of selected lamivudine derivatives. Potchefstroom: NWU. (Dissertation – PhD) 156p.
- GERRITSEN, A.A.M., KRUGER, P., ACHIM VAN DER LOEFF, M.F. & GROBUSCH, M.P. 2008. Malaria incidence in Limpopo Province, South Africa, 1998-2007. *Malaria Journal*, 7:162-169.

GUERRA, C.A., GIKANDI, P.W., TATEM, A.J., NOOR, A.M., SMITH, D.L., HAY, S.I. & SNOW, R.W. 2008. The limits and intensity of *Plasmodium falciparum* transmission: Implication for malaria control and elimination worldwide. *PLOS Medicine*, 5(2):300-311.

GEYER, M. 2001. Antimalarial drug combination therapy report of a WHO technical consultation. Switzerland: World Health Organization. 36 p.

GIBBON, C.J. 2005. South African Medicines Formulary. Division of Clinical Pharmacology. Cape Town. 581p.

GOLLENSER, J., WAKNINE, J.H., KRUGLIAK, M., HUNT, N.H. & GRAU, G.E. 2006. Current perspective on the mechanism of action of artemisinins. *International Journal for Parasitology*, 36:1427-1441.

GROBLER, A., KOTZE, A. & DU PLESSIS, J. 2007. The design of a skin-friendly carrier for cosmetic compounds using Pheroid™ technology. (In Wiechers, J., ed. Delivery system technologies. Wheaton, IL.: Allured Publishing Corporation).

GROBLER, A.F. 2004. Emzaloid™ Technology. Potchefstroom: NWU. 20p. (Confidential Concept Document).

GUY, R., LIU, P., PENNEFATHER, P. & CRANDALL, I. 2007. The use of fluorescence enhancement to improve the microscopic diagnosis of falciparum malaria. *Malaria Journal*, 6:89-95.

HANG, V.T., BE, T.V., TRAN, P.N., THANH, L.T., HIEN, L.V., O'BRIEN, E. & MORRIS, G.E. 1995. Screening donor blood for malaria by polymerase chain reaction. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 89:44-47.

HARE, J.D. & BAHLE, D.W. 1986. Analysis of *Plasmodium falciparum* growth in culture using acridine orange and flow cytometry. *The Journal of Histochemistry and Cytochemistry*, 34(2):215-220.

HASTINGS, I.M. & DONNELLY, M.J. 2005. The impact of antimalarial drug resistance mutations on parasite fitness, and its implications for the evolution of resistance. *Drug Resistance Updates*, 8:43-50.

HAYNES, R.K. & KRISHNA, S. 2004. Artemisinins: Activities and actions. *Microbes and infection*, 6:1339-1346.

HOEK, K.G.P., GEY VAN PITTIUS, N.C., MOOLMAN-SMOOK, H., CARELSE-TOFU, K., JORDAAN, A., VAN DER SPUY, G.D., STREICHER, E., VICTOR, T.C., VAN HELDEN, P.D. & WARREN, R.M. 2008. Fluorometric assay for testing rifampin susceptibility of *Mycobacterium tuberculosis* complex. *Journal of Clinical Microbiology*, 46(4):1369-1373.

HOLMES, E. 2006. The synthesis and transdermal delivery of stavudine derivatives. Potchefstroom: NWU. (Dissertation – M.Sc) 69p.

HONEYWELL-NGUYEN, P.L & BOUWSTRA, J.A. 2007. Vesicles as skin delivery vehicles. (In Wiechers, J., ed. Delivery system technologies. Wheaton, IL.: Allured Publishing Corporation. *In press*).

HOPSKINS, H., KAMBALE, W., KAMYA, M.R., STAEDKE, S.G., DORSEY, G. & ROSENTHAL, P.J. 2007. Comparison of HRP2- and pLDH-based rapid diagnostic tests for malaria with longitudinal follow-up in Kampala, Uganda. *American Journal of Tropical Hygiene*, 76(6):1092-1097.

HYDE, J.E. 2005. Drug-resistant malaria. *TRENDS in Parasitology*, 21(11):494-498.

HYDE, J.E. 2007. Drug-resistant malaria – an insight. *The FEBS journal*, 274:4688-4698.

IQBAL, J., HIRA, P.R., SHER, A. & AL-ENEZI, A.A. 2001. Diagnosis of imported malaria by *Plasmodium* lactate dehydrogenase (pLDH) and histidine-rich protein 2 (PfHRP2) based immunocapture assays. *American Journal of Tropical Medicine and Hygiene*, 63(1,2):20-23.

JIMÉNEZ-DÍAZ, M.B., RULLAS, J., MULET, T., FERNÁNDEZ, L., BRAVO, C., GARGALLO-VIOLA, D. & ANGULO-BARTUREN, I. 2005. Improvement of detection specificity of *Plasmodium*-infected murine erythrocytes by flow cytometry using autofluorescence and YOYO-1. *Cytometry Part A*, 67:27-36.

JOHNSON, O.F.L. & TRACEY, M.A. 1999. Peptide and protein drug delivery. (In Mathoiwitz, E., ed. Encyclopaedia of controlled drug delivery. Vol. 2. New York: Wiley. P816-883).

- KADDOURI, H., NAKACHE, S., HOUZÉ, S., MENTRÉ, F. & LE BRAS, J. 2006. Assesment of the drug susceptibility of *Plasmodium falciparum* clinical isolates from Africa by using a *Plasmodium* lactate dehydrogenase immunodetection assay and an inhibitory maximum effect model for precise measurement of the 50-percent inhibitory concentration. *Antimicrobial Agent and Chemotherapy*, 50(10):3343-3349.
- KICKS, G.A., TYLER, P.C., EVANS, G.B., FURNEAUX, R.H., SCHRAMM, V.L. & KIM, K. 2001. Purine-less death in *Plasmodium falciparum* induced by immucillin-H, a transition state analogue of purine nucleoside phosphorylase. *The Journal of Biological Chemistry*, 277(5):3226-3231.
- KOTZÉ, J.C. 2005. Nasal drug delivery of calcitonin with Pheroid technology. Potchefstroom: NWU. (Dissertation – M.Sc) 125p.
- KÜHN, A. 2008. Pro-Pheroid-based antiretroviral formulations: HPLC method development and stability studies. Potchefstroom: NWU (Dissertation – M.Sc) 108p.
- LAMBROS, C. & VANDERBERG, J.P. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *Journal of Parasitology*, 65(3):418-420.
- LANGLEY, N. 2007. Preclinical evaluation of the possible enhancement of the efficacy of anti-malarial drugs by Pheroid™ technology. Potchefstroom: NWU. (Dissertation – M.Sc) 106p.
- LEWISON, G. & SRIVATAVA, D. 2008. Malaria research, 1980-2004, and the burden of diseases. *Acta Tropica*, 106:96-103.
- LI, Q., GERENA, L., XIE, L., ZHANG, J., KYLE, D. & MILHOUS, W. 2007. Development and validation of flow cytometric measurement for parasitemia in cultures of *P. falciparum* vitally stained with YOYO-1. *Cytometry Part A*, 71A:297-307.
- LUBBE, J. 2007. Pheroid™ technology in peptide drug delivery. Potchefstroom: NWU. (Dissertation – Ph.D) 123p.
- MAKLER, M.T. & HINRICHS, D.J. 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *American Journal of Tropical Medicine and Hygiene*, 48:205-210.

- MAKLER, M.T., RIES, J.M., WILLIAMS, J.A., BANCROFT, J.E., PIPER, R.C., GIBBINS B.L. & HINRICHS, D.J. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *American Journal of Tropical Medicine and Hygiene*, 48:739-741.
- MAKLER, M.T., PIPER, R.C. & MILHOUS, W.K. 1998. Lactate dehydrogenase and the diagnosis of malaria. *Parasitology Today*, 14(9):376-377.
- MARITZ, F. 2008. *In vivo* evaluation of the efficacy of nasal Pheroid™ base calcitonin formulations. Potchefstroom: NWU. (Dissertation – M.Sc) 134p.
- MARTIN, A. 1993. Physical Pharmacy, physical chemical principles in the pharmaceutical sciences. 4th ed. Philadelphia: Lippincott Williams & Wilkins. 622p.
- MATTHEE, L.I. 2007. A preclinical evaluation of the possible enhancement of the efficacy of antituberculosis drugs by Pheroid™ technology. Potchefstroom: NWU. (Dissertation – M.Sc) 150p.
- MENS, P.F., SCHOONE, G.J., KAGER, P.A. & SCHALLIG, H.P.F.H. 2006. Detection and identification of human *Plasmodium* species with real time quantitative nucleic acid sequence-based amplification. *Malaria journal*, 5(80):1-6.
- MENS, P.F., VAN AMERONGEN, A., SAWA, P., KUGER, P.A. & SCHALLIG, H.D.F.H. 2008. Molecular diagnosis of malaria in the field: development of a novel 1-step nucleic acid lateral immunoassay for the detection of all 4 human *Plasmodium* species. And its evaluation in Mbita, Kenya. *Diagnostic Microbiology and Infectious Diseases*, 61:421-427.
- MULLIS, K. 1990. The unusual origin of the polymerase chain reaction. *Scientific America*, 262(4):56-61, 64-65.
- 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. *Antimicrobial Agents and Chemotherapy*, 44(4):972-977.

- NOEDL, H., WERNSDORFER, W., MILLER, R.S. & WONGRISCHANALAI, C. 2002. Histidine-Rich Protein II: A novel approach to malaria drug sensitivity testing. *Antimicrobial Agents and Chemotherapy*, 46(6):1658-1664.
- NOEDL, H., WONGSRICHANALAI, C. & WERNSDORFER, W.H. 2003. Malaria drug-sensitivity testing: new assays, new perspectives. *TRENDS in Parasitology*, 19(4):175-181.
- NOEDL, H., TEYJA-ISAVADHARM, P. & MILLER, R.S. 2004. Nonisotopic, semiautomated *Plasmodium falciparum* Bioassay for measurement of antimalarial drug levels in serum or plasma. *Antimicrobial Agents and Chemotherapy*, 48(11):4485-4487.
- NOEDL, H., BRONNERT, J., YINGYEUN, K., AHLMAYR, B., KOLLARITSCH, H. & FUKUDA, M. 2005. Simple Histidine-Rich Protein 2 double-site sandwich enzyme-linked immunosorbent assay for use in malaria drug sensitivity testing. *Antimicrobial Agents and Chemotherapy*, 49(8):3575-3577.
- NOEDL, H., KRITSANAI, K., YINGYEUN, K., LAOBOONCHAI, A., FUKUPA, M., SIRICHAISINTHOP, J. & MILLER, R.S. 2006. Sensitivity and specificity of an antigen detection ELISA for malaria diagnosis. *American Journal of Tropical Medicine and Hygiene*, 75(6):1205-1208.
- NOLLA, H. 2008. Basic Principles in flow cytometry. biology.berkeley.edu/crl/FlowBasics2.ppt [Date of access: 17 October 2008].
- OHRT, C., PURNOMO, SUTAMIHARDJA, M.A., TANG, D. & KAIN, K.C. 2002. Impact of microscopy error on estimates of protective efficacy in malaria-prevention trials. *The Journal of Infectious Diseases*, 186:540-546.
- PALMER, T. 2001. Enzymes biochemistry, biotechnology, clinical chemistry. Horwood publishing: Chichester. 402p.
- PERSSON, K.E.M., LEE, C.T., MARSH, K. & BEESON, J.G. 2006. Development and optimization of high-throughput methods to measure *Plasmodium falciparum*-specific growth inhibitory antibodies. *Journal of Clinical Microbiology*, 44(5):1665-1673.

- PIPER, R., LEBRAS, J., WENTWORTH, L., HUNTCHOOKE, A., HOUZE, S., CHIODINI, P. & MAKLER, M. 1999. Immunocapture diagnostic assays for malaria using plasmodium lactate dehydrogenase (pLDH). *American Journal of Tropical Medicine and Hygiene*, 60(1):109-118.
- PRETORIUS, M., LIEBENBERG, W., WESSELS, A. & KOTZE, A. 2008. Stability tests on anti-malarial drugs encapsulated in Pheroid™ and Pro-Pheroid drug delivery systems. (In Posters presented at the 29th Annual Conference of the Academy of Pharmaceutical Sciences held in Hunters Rest on the 22 to 26 September 2008).
- PRUDHOMME, J.G. & SHERMAN, I.W. 1999. A high capacity *in vitro* assay for measuring the cytoadherence of *Plasmodium falciparum*-infected erythrocytes. *Journal of Immunological Methods*, 229:169-176.
- RAYNES, K. 1999. Bisquinoline antimalarials: their role in malaria chemotherapy. *International Journal for Parasitology*, 29:367-379.
- REID, I.A. 2004. Vasoactive Peptides. (In Katzung, B.G., ed. Basic and Clinical Pharmacology. 9th ed. Boston: McGrawHill. p. 281-297).
- RILEY, R.S. & IDOWU, M. 2008. Principles and applications of flow cytometry. <http://www.patholgy.vcu.edu/education/lymph/documents/flow.pdf> [Date of access: 17 October 2008].
- RINGWALDE, P., MECHE, F.S., BICKEL, J. & BASCO, L.K. 1999. In vitro culture and drug sensitivity assay of *Plasmodium falciparum* with nonserum substitute and acute-phase sera. *Journal of Clinical Microbiology*, 37(3):700-705.
- ROSENTHAL, P.J. 2004. Antiprotozoal Drugs. (In Katzung, B.G., ed. Basic and Clinical Pharmacology. 9th ed. Boston: McGrawHill. p. 864-875).
- ROUGEMONT, M., VAN SAANEN, M., SAHLI, R., HINRIKSON, H.P., BILE, J. & JATON, K. 2004. Detection of four *plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species specific real-time PCR assays. *Journal of Clinical Microbiology*, 42(12):5636-5643.

- RUSSELL, B.M., UDOMSANGPETCH, R., RIECKMANN, K.H., KOTECKA, B.M., COLEMAN R.E. & SATTABONGKOT, J. 2003. Simple *in vitro* assay for determining the sensitivity of *Plasmodium vivax* isolates from fresh human blood to antimalarials in areas where *P. vivax* is endemic. *Antimicrobial Agent and Chemotherapy*, 47(1):170-173.
- SAITO-ITO, A., AKAI, Y., HE, S., KIMURA, M. & KAWABATA, M. 2001. A rapid, simple and sensitive flow cytometric system for detection of *Plasmodium falciparum*. *Parasitology International*, 50:249-257.
- SCHUSTER, F.L. 2002. Cultivation of *Plasmodium* spp. *Clinical Microbiology Reviews*, 15(3):355-364.
- SHAPIRO, T.A. & GOLDBERG, D.E. 2007. Chemotherapy of protozoal Infections: Malaria. Goodman & Gilman's pharmacology.
- SHARGEL, L. & YU, A. 1999. Applied Biopharmaceutics & Pharmacokinetics. 4th ed. New York: McGraw-Hill. 768p.
- SIO, S.W.S., SUN, W., KUMAR, S., BIN, W.Z., TAN, S.S., ONG, S.H., KIKUCHI, H., OSHIMA, Y. & TAN, K.S.W. 2006. MalariaCount: An image analysis-based program for the accurate determination of parasitemia. *Journal of Microbiological Methods*, 68:11-18.
- SMILKSEIN, M., SRIWILAIJAROEN, N., KELLY, J.X., WILAIRAT, P. & RISCOE, M. 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrobial Agents and Chemotherapy*, 48(5):1803-1806.
- STEYN, J.D. 2006. Nasal delivery of recombinant human growth hormone with Pheroid technology. Potchefstroom: NWU (Dissertation – M.Sc) 115p.
- STRATTON, L., O'NEILL, M.S., KRUK, M.E. & BELL, M.L. 2008. The persistent problem of malaria: Addressing the fundamental causes of a global killer. *Sosial Science & Medicine*, 67:854-862.
- STRAUSS, T. 2005. Peroral delivery of calcitonin with Pheroid technology. Potchefstroom: NWU. (Dissertation – M.Sc) 118p.
- SWAN, H., SLOAN, L., MUYOMBIVE, A., CHAVALITSHEWINKOON-PETMITR, P., KRUDSOOD, S., LEOWATTANA, W., WILAIRATANA, P., LOOAREESUWAN, S. &

- ROSENBLATT, J. 2005. Evaluation of a real-time polymerase chain reaction assay for the diagnosis of malaria in patients from Thailand. *American Journal of Tropical Medicine and Hygiene*, 73(5):850-854.
- TOMAR, D., BISWAS, S., TRIPATHI, V. & RAO, D.N. 2006. Development of diagnostic reagents: raising antibodies against synthetic peptides of PfHRP-2 and LDH using microsphere delivery. *Immunobiology*, 211:797-805.
- TRAGER, W. & JENSEN, J.B. 1976. Human malaria parasites in continuous culture. *SCIENCE*, 193:673-675.
- TREN, R. & BATE, R. 2004. South Africa's War against Malaria Lessons for the Developing World. *Policy Analysis*, 513:1-20.
- TRIPATHI, R.P., MISHRA, R.C., DWIVEDI, N., TEWARI, N. & VERMA, S.S. 2005. Current status of malaria control. *Current Medicinal Chemistry*, 12(22):2643-2659.
- TULANE. 2008. Biochemistry of *Plasmodium* – Brief Overview. <http://www.tulane.edu/~wiser/malaria/Summary.html#sugar>. [Date of access: 13/20/2008].
- UYS, C.E. 2006. Preparation and characterization of Pheroid vesicles. Potchefstroom: NWU. (Dissertation – M.Sc). 147p.
- VAN DER MERWE, H., GROBLER, A.F., LIEBENBERG, W. & KOTZÉ A.F. 2008. Enhancement of cell viability by a Pheroid™ formulation. (In Posters presented at the 29th Annual Conference of the Academy of Pharmaceutical Sciences held in Hunters Rest on the 22 to 26 September 2008).
- VAN DER WALT, M.E. 2007. Transdermal delivery of acyclovir and ketoconazole by Pheroid™ technology. Potchefstroom: NWU. (Dissertation – M.Sc) 131p.
- VOET, D. & VOET, J.G. 1995. Biochemistry. 2nd ed. John Wiley & Sons, Inc. New York. 1360p.
- VOGELSON, C.T. 2001. Advances in drug delivery systems. *Modern drug discovery*, 4(4):49-52.

- WALLIKER, D., HUNT, P. & BABIKER. H. 2005. Fitness of drug-resistant malaria parasites. *Acta Tropica*, 94:251-259
- WHAUN, J.M., RITTERSHAUS, C. & IP, S.H.C. 1983. Rapid identification and detection of parasitized human red cells by automated flow cytometry. *Cytometry*, 4:117-122.
- WHITE, N.J. 2004. Antimalarial drug resistance. *The Journal in Clinical Investigation*, 113(8):1084-1092.
- WHITE, N.J. 2008. *Plasmodium knowlesi*: the fifth human malaria parasite, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 46(2):172-173.
- WHO. 2002. Monitoring Antimalarial Drug Resistance, report of a WHO consultation. WHO: Switzerland. 30p.
- WHO. 2006. Guidelines for the treatment of malaria. WHO Library Cataloguing-in-Publication Data. Switzerland. 253p.
- WHO. 2007a. World Health Organization Fact sheet no 94. <http://www.who.int/mediacentre/factsheets/fs094/en/print.html> [Date of access: 5 Feb. 2007].
- WHO 2007b. World Health Organization. *In vitro* micro-test (Mark III) for the assessment of the response of *Plasmodium falciparum* to chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine and artemisinin. 21p.
- WONGSRICHANALAI, C., PICKARD, A.L., WERNSDORFER, W.H. & MESHNICK, S.R. 2002. Epidemiology of drug-resistant malaria. *THE LANCET Infectious Diseases*, 2(4):209-218.
- WORRAL, E., MOREL, C., YEUNG, S., BORGHI, J., WEBSTER, J., HILL, J., WWISEMAN, V. & MILLS, A. 2007. The economics of malaria in pregnancy – a review of the evidence and research priorities. *THE LANCET Infectious Diseases*, 7:156-168.
- ZAKERI, S., NAJAFABADI, S.T., ZARE, A. & DJADID, N.D. 2002. Detection of malaria parasites by nested PCR in south-eastern, Iran: Evidence of highly mixed infections in Chahbahar district. *Malaria Journal*, 1(2):1-6.