THESIS TITLE

THE ROLE OF PHARMACOKINETICS, HOST FOLATE LEVELS AND PARASITE MUTATIONS IN THE IN *VIVO* EFFICACY OF PYRIMETHAMINE-SULFADOXINE AGAINST *PLASMODIUM FALCIPARUM*

FRACTION DZINJALAMALA

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UNIVERSITY OF CAPE TOWN, DIVISION OF PHARMACOLOGY

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THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE DIVISION OF PHARMACOLOGY, FACULTY OF MEDICINE, UNIVERSITY OF CAPE TOWN

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JULY 2003

DEDICATION

This project is dedicated to my wife Alice (nee Kamanga), my son Malimau, my daddy Dickson Dzinjalamala and my mother Mary Dzinjalamala (nee Chimwala).

DECLARATION

THE ROLE OF PHARMACOKINETICS, HOST FOLATE LEVELS AND PARASITE MUTATIONS IN THE IN *VIVO* EFFICACY OF PYRIMETHAMINE-SULFADOXINE AGAINST *PLASMODIUM FALCIPARUM*

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PREFACE

The assay development, pharmacokinetics and folate studies described in this thesis were done between Feb 2000 and July 2003 in the Department of Pharmacology, University of Cape Town. All *Plasmodium falciparum* genetic analyses were performed at the Blantyre Wellcome Trust Reasearch Laboratory, Malawi. A literature review of the concepts known about factors involved in *Plasmodium falciparum* resistance to pyrimethamine-sulfadoxine and hence concepts requiring further research are presented in the introductory chapter one, with the primary scope of this project outlined in the last section of chapter this chapter.

Chapter two presents both the research methodology for genotyping *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthatase, the experimental results, and discussion of the results of the molecular work.

The development and validation of drug recovery and quantitation methods, the results of pharmacokinetic analysis of blood specimens from the in vivo pyrimethamine-sulfadoxine efficacy study subjects, a discussion and conclusions on the pharmacokinetics results, are presented in chapter three.

Chapter four describes the research methodology, the results and a discussion on the results of the investigation into the role of the nutritional substance, folate, in modulating the in vivo *Plasmodium falciparum* response to pyrimethamine-sulfadoxine antimalarial activity.

In the context of the combined observations and conclusions from the pharmacokinetic, nutritional and genetic studies, chapter five presents the overall discussion on the practical significance of results from this project. However, shortfalls are discussed at the end of each chapter since the chapters are presented and ordered by subjects Genetics, Pharmacokinetics and Nutritional studies.

This project required diverse techniques (analytical chemistry, molecular biology, pharmacokinetics) to clearly elucidate the multifactorial nature of the in vivo variation in parasitological response to standard antimalarial therapy. Here it is shown that independent of other factors, pyrimethamine pharmacokinetics, blood folate levels, transmission intensity, subject age and parasite genetic resistance, each contribute to the observed interindividual variation in parasitological and clinical response following standard treatment with the antimalarial drug, pyrimethamine-sulfadoxine.

I hope that this thesis will be valuable to its readers and will enhance their appreciation of a multidisciplinary approach to addressing scientific questions in public health.

ACKNOWLEDGEMENTS

Firstly, I thank my GOD in heaven for keeping me in perfect health throughout this project. He has been faithful to all His promises. It is true that He is no respecter of persons; what He has done for others He really does for anyone who truly seeks Him.

My heartfelt thanks go to my supervisor Peter J. Smith, Associate Professor of Pharmacology at the University of Cape Town, for his helpful academic guidance in Pharmacokinetics throughout the four years of my being his student. The skills he imparted to me will forever be remembered and used in my career. I am also deeply thankful to Dr James G Kublin, University of Malawi School of Medicine and University of Maryland Medical School, USA, for his valuable guidance in the genetic studies on *Plasmodium falciparum* resistance to antifolate drugs. Dr Kublin provided excellent supervision of all the genetics work described in this thesis.

I would like to thank my sponsor WHO/TDR/MIM who made it possible for me to undertake this PhD training in Pharmacokinetics at the University of Cape Town Medical School. Mr Allan Macheso was the owner of the MIM grant under which I got the opportunity for this postgraduate training and so I sincerely thank him for carrying out the in vivo pyrimethamine-sulfadoxine efficacy study from which I got my blood specimens for the pharmacokinetic, genetic and nutritional studies described in this thesis. Without Professor Christopher Plowe's sustained financial assistance from his NIH grant for SP resistance studies in Malawi, I would never have finished this PhD. He provided all the necessary financial support throughout the three years from 2001 to 2003. Christopher Plowe has been my mentor since February 1998 immediately after my undergraduate studies. He trained me in various molecular parasitology research techiniques in his malaria laboratory at the University of Maryland Medical School, Baltimore, USA. Quite frankly Professor Christopher Plowe has been 'the wind under my wings'.

I am deeply grateful to Professor Malcom Molyneux and Professor Terrie Taylor for the their indispensable academic, administrative and logistical support as directors of the Malawi Wellcome Trust Laboratories and Blantyre Malaria Project, respectively, where all molecular analyses of *Plasmodium falciparum* genes were done.

I thank Dr Stephen Rogerson and Dr James Beeson for onsite training (as a research scientist employed under Christopher Plowe's grant) in the pathogenesis of malaria in pregnant women and severe malaria in children.

I would like to thank Debbie Kamwendo and the rest of my colleagues at the Blantyre Wellcome Trust Research Laboratory for being such a wonderful working environment.

The assistance of Dr Karen Barnes as co-supervisor and her tolerating numerous revisions of this thesis is acknowledged with deep gratitude. My thanks go to Professor Peter Folb, the head of the Department of Pharmacology at the University of Cape Town, for his administrative support during my training.

Many thanks also go to all my fellow students and staff in the Department of Pharmacology at the University of Cape Town for being a stimulating working environment. In particular, I would like to thank Dr Helen McIlleron and Mrs Francesca Little of the University of Cape Town Departments of Pharmacology and Public Health, respectively, for their advice during the biostatistical analysis of data presented in this thesis. I thank Justin Wilkins for his unceasing selfless help with computer skills. In addition, thanks to Alicia Evans, Afia Fredericks and the entire departmental laboratory staff for always being there when I needed technical advice on the various laboratory equipments. My sincere gratitude also goes to Shirley Smith for her selfless assistance with the digital enhancement of HPLC chromatograms reported for SDX and PYR.

Finally, but not least, my very special thanks go to my wife Alice (nee Kamanga) and my son Malimau Dzinjalamala for unfailingly being supportive and patient throughout the four years of my studies. During those years I spent most of the days and nights in the

laboratory at the times when they needed my presence most. I will forever be deeply grateful for Alice's ungrudging endurance through the lonely nights in the cold Cape Town weather. Briefly, Alice was a bottomless sink for my academic stress.

ABSTRACT

Children with uncomplicated malaria and aged from 0.5 to 12 years were recruited for *in vivo* pyrimethamine-sulfadoxine (PYR-SDX) efficacy studies in low transmission (Dedza) and high transmission (Mangochi) districts of central and southern Malawi, respectively, to test the following hypotheses: that the presence of dihydrofolate reductase (DHFR)/dihydropteroate synthase (DHPS) mutations at the time of treatment is associated with recurrence of *falciparum* parasitemia; that higher host blood folate concentrations are associated with PYR-SDX therapeutic failure, and that potential differences in PYR-SDX pharmacokinetics between sensitive (ACPR) and resistant (LTF) cases and between the sites might explain observed differences in clinical response following standard therapy with PYR-SDX. Genotyping for parasite genetic mutations was done using previously described methods. Adapting the Abbot folate kit to the filter paper specimens enabled quantitation of blood folate. More sensitive and specific high-performance liquid chromatographic assays for measuring SDX and PYR recovered from filter paper samples had to be developed and validated.

PYR-SDX efficacy, at day 14, was higher in the high than low transmission area (90% vs 78%, $p<0.01$) despite similar prevalence of DHFR triple and DHFR/DHPS quintuple mutants (73% vs 74%, $p=0.64$; 61% vs 64%, $p=0.82$ respectively). The DHFR/DHPS quintuple mutant was strongly associated with recurrent parasitemia at both sites (Dedza, $p=0.004$; Mangochi, $p<0.001$). DHFR triple mutant was associated with recurrent parasitemia in Dedza only (Dedza, $p=0.039$ and Mangochi, $p=0.173$ respectively) whereas DHPS double mutant's association with parasite reappearance was true at both sites ($p=0.035$ and $p=0.022$ respectively). The combinations DHFR Arg-59 plus DHPS Glu-540, or Arg-59 plus Gly-437 predicted the presence of the quintuple mutant with 100% sensitivity and 68% and 75% specificity, respectively. This sensitivity was found to be lower than that reported from a larger study by Kublin et al. DHFR Leu-164 mutation was not detected in any of the analyzed isolate at either site.

Dedza cases had significantly higher blood folate levels than counterparts in Mangochi (38 ng/ml vs 30 ng/ml, $p<0.0001$) despite the similarity in patient demographic characteristics. In both a univariate and multivariate logistic analyis, blood folate levels were higher in cases that experienced parasite reappearance within 5 – 28 days after PYR-SDX therapy than those that showed no parasite recurrence during that period (univariate: p=0.028, n=141, multivariate: p=0.013, n=117).

The back-extrapolated peak concentration (C_0) of sulfadoxine was similar between treatment outcome groups (ACPR 79 ± 6.5μg/ml versus LTF 69 ± 6.3μg/ml, P>0.05) and between sites (Mangochi 90 ± 7.2 μg/ml versus Dedza 96 ± 6.9 μg/ml, P>0.05). Within each site the extent of exposure to SDX ($AUC_{0-\infty}$) was increased in sensitive compared to resistant cases, who experienced parasite recurrence during 4 – 28 days following PYR-SDX standard therapy (932 versus 888 μg day ml^{-1}, P=0.73 respectively); In Dedza subjects had significantly higher SDX exposure than their Mangochi counterparts (1156 versus 827 μg day ml^{-1}, P=0.021 respectively). In Dedza blood SDX elimination half-life was similar in ACPRs compared with LTFs (7.2 versus 6.4 days, P=0.42) and between sites (6.9 versus 6.6 days, P=0.44). The average day 3 PYR blood concentrations did not differ between sites and between treatment outcome groups (243 ± 31.0 versus 243 ± 15.9 ng/ml P=0.78). Similarly, Mangochi ACPRs exhibited similar day 3 PYR concentrations compared with LTFs (264 ± 27.5 versus 211± 18.4 ng/ml, P>0.05).

Following adjustment for the covariates gender, age, site, *falciparum* resistance DHFR/DHPS genotypes, pretreatment parasite density and physiological folate levels, PYR and not SDX was significantly correlated with in vivo parasite recurrence during 4 – 28 days after PYR-SDX standard therapy. Also younger age (<2.5 years) and site were independently associated with PYR-SDX therapeutic failure based on 28-day follow-up.

This is the first study to clearly demonstrate the independent role of physiological folate levels in *in vivo* PYR-SDX therapeutic efficacy with all other important covariates adjusted for. These results suggest that significant differences in folate dietary intake or supplementation among individuals living in malaria endemic areas may result in a

difference in *in vivo* PYR-SDX therapeutic response. Also the independent significant correlation of site and age with parasite reappearance underscore the importance of transmission intensity and therefore acquired immunity in determining the *in vivo* outcome of antimalarial treatment in people living in malaria endemic areas. The day-3 drug level results support previous observations of a critical and significant role of PYR in *in vivo* PYR-SDX efficacy, and are the first to characterize that role with respect to host folate levels.

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

WORKSHOP/CONFERENCE PRESENTATIONS

ORAL PRESENTATION

1. **Malaria Project and The Liverpool Wellcome Trust International Tropical Medicine Meeting, Zomba Malawi (Sept 18 – 21, 2001).**

   Pyrimethamine-Sulfadoxine efficacy and molecular markers of resistance in high and low transmission areas in Malawi.

2. **Annual Meeting of the American Society of Tropical medicine and Hygiene, Denver, Colorado, USA (November 10-14, 2002)**

   Pyrimethamine-Sulfadoxine efficacy and molecular markers of resistance in high and low transmission areas in Malawi.

POSTER PRESENTATION

3. **3rd Multilateral Initiative on Malaria (MIM) Pan-African Conference, Arusha, Tanzania (November 17 – 22, 2002):**

   Pyrimethamine-Sulfadoxine efficacy and molecular markers of resistance in high and low transmission areas in Malawi.

LIST OF ABBREVIATIONS

A	Alanine (Ala)
ACPR	Adequate clinical and parasitological response
AUC_{0-t}	Area under the curve of a concentration versus time curve from time zero to time, t.
AUC	Area under the curve of a concentration-time curve
C_0	Peak drug concentration (following an oral dose) estimated by back-extrapolation of the log-linear portion of a concentration versus time curve.
C_{max}	True peak concentration obtained directly from a linear graph of drug concentration versus time curve that includes concentration-time data in the absorption as well as the elimination phase.
CV	Coefficient of variation
CM	Centimeter
C	Cysteine (Cys)
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
DBS	Dried blood spot
ETF	Early treatment failure
gDNA	Genomic deoxyribonucleic acid
G	Glycine (Gly)
Hb	Haemoglobin
HPLC	High-performance liquid chromatography
HCL	Hydrochloric acid
HF	Haemoglobin folate
hr	The time unit, hour.
I	Isoleucine (Ile)

kg	Kilogram
K	Lysine (lys)
L	Leucine (Leu)
LPF	Late parasitological failure
LCF	Late clinical failure
LTF	Late treatment failure
mAbs	milliabsorbance
M	Molar
MM	Millimolar
MeCN	Acetonitrile
min	Minute/s
ml	Milliliter
mg	Milligram
ng	Nanogram
NaOH	Sodium hydroxide
PCR	Polymerase Chain Reaction
P. falciparum	Plasmodium falciparum
pH	Negative logarithm of the hydrogen concentration ion
PYR	Pyrimethamaine
PYR-SDX	Pyrimethamine-sulfadoxine, a combination drug
PABA	Paraaminobenzoic acid
PAHA	Paraaminohippuric acid
PAHA	Paraaminohipputic acid
R	Arginine (Arg)
Rpm	Rotation per minute
RT	Retention time
S	Serine (Ser)
SD	Standard deviation
SDX	Sulfadoxine
$t_{1/2}$	Elimination half-life associated from the terminal phase of the drug concentration versus time curve

TFA	Trifluoroacetic acid
wt	Weight
WHO	World Health Organization
1HSA	1-heptane-sulfonic acid
µM	Micromolar
µg	Microgram
µL	Microliter
λ_z	Elimination rate constant associated with the terminal phase of the drug concentration versus time curve

TABLE OF CONTENT

CHAPTER 1

INTRODUCTION: GLOBAL SCOPE OF THE MALARIA PROBLEM, PREVENTION, CHEMOTHERAPEUTIC MANAGEMENT, DRUG RESISTANCE AND THESIS RESEARCH QUESTIONS

CHAPTER 2

PYRIMETHAMINE-SULFADOXINE EFFICACY AND MOLECULAR MARKERS OF RESISTANCE IN HIGH AND LOW TRANSMISSION AREAS IN MALAWI

CHAPTER 3

THE IN *VIVO* KINETICS OF PYRIMETHAMINE-SULAFADOXINE AND CLINICAL EFFICACY

CHAPTER 4

NUTRITION AND PYRIMETHAMINE-SULAFADOXINE RESISTANCE

CHAPTER 5

OVERALL DISCUSSION AND SIGNIFICANCE OF STUDY

CHAPTER 1

GLOBAL SCOPE OF THE MALARIA PROBLEM, VECTOR CONTROL, CHEMOTHERAPEUTIC MANAGEMENT, DRUG RESISTANCE AND THESIS RESEARCH OBJECTIVES

1. INTRODUCTION

According to a recent report in Roll Back Malaria (**www.rbm.who.int**) approximately one million people are killed by malaria each year and the majority of the victims are children. Nearly 300 million people suffer from acute malaria each year. 40% of the world population lives in endemic areas and 90% of the malaria cases occur in sub-Saharan Africa (1). These malaria endemic countries are among the most impoverished nations of the world (figure 1). Their economies continue to be crippled by the direct cost of malaria control and prevention, treatment and mortality, and the indirect cost of lost productivity attributable to malaria-induced morbidity in adults and children. As chloroquine-resistant *P. falciparum* malaria spreads across Africa, the only currently available alternative for the treatment of uncomplicated malaria is the combination drug pyrimethamine-sulfadoxine (PYR-SDX).

1.1 VECTOR CONTROL

Malaria control methods have endeavored to reduce transmission of the *Plasmodium* parasite through the eradication of its anophe vector by employing insecticides like DDT, mosquito-repellents, and insecticide-impregnated bednets. These methods have not achieved a complete eradication of malaria owing to operational constraints preventing high and sustained coverage. The anopheline species are competent vectors that have become increasingly resistant to widely used insecticides, a development that complicates and frustrates malaria vector control programmes (2). In addition, DDT being non-biodegradable is an enviromental hazard.

1.2 CASE MANAGEMENT

Thus in malaria endemic countries, chemotherapy remains the mainstay for the control of malarial disease. Unfortunately, malaria parasites have developed intricate molecular mechanisms that enable them not only to resist the lethal effects of the drugs but also to circumvent their host's immune system. The latter ability has made

vaccine development difficult. Drugs widely used in Africa for the treatment of malaria include the quinoline antimalarials (quinine, chloroquine, mefloquine and halofantrine) and the antifolate drugs, sulfadoxine (SDX) and pyrimethamine (PYR).

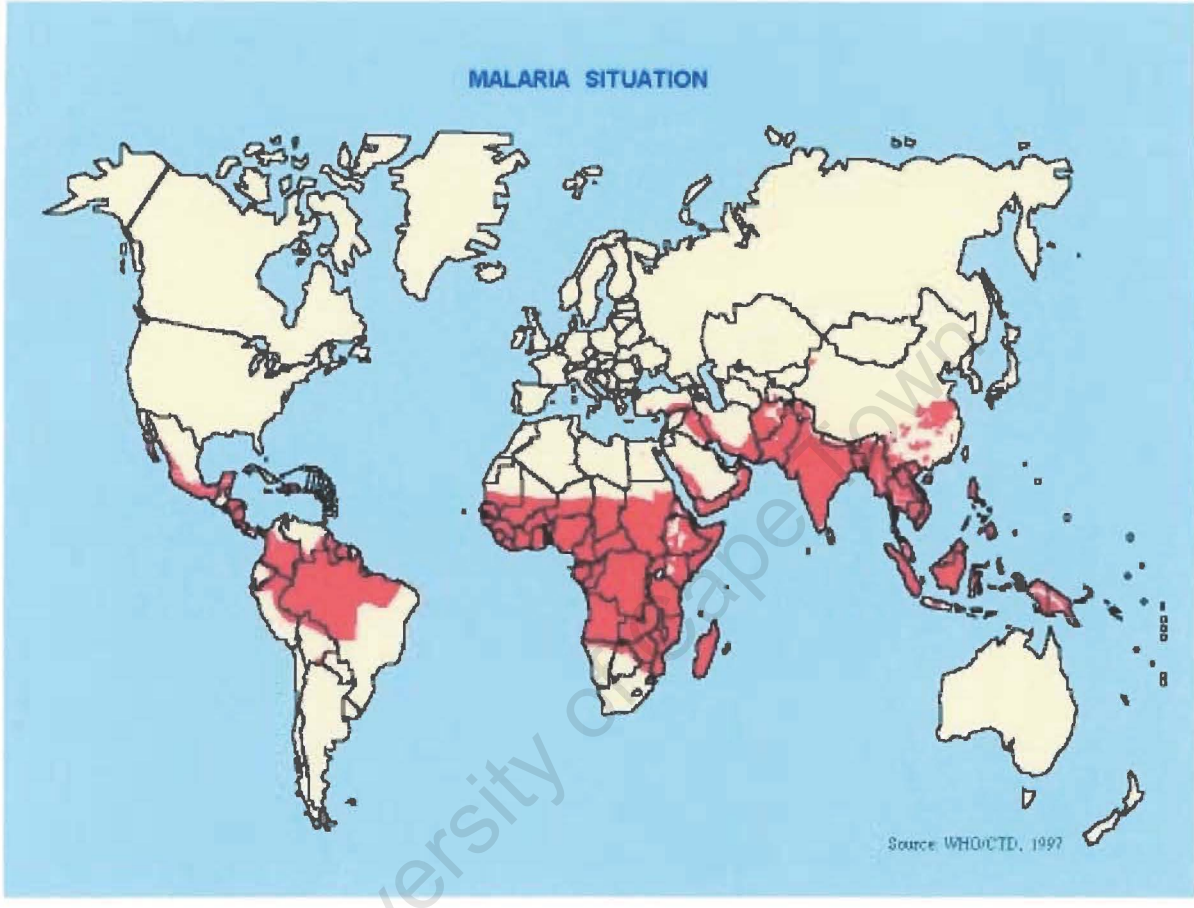


Figure 1 World Map showing parts of the world bearing the largest burden of malaria associated morbidity and mortality. Map was taken from WHO/CTD, 1997.

Currently due to the spread of antimalarial drug resistance, alternatives to commonly used drugs being considered for the management of uncomplicated and severe malaria include atovaquone-proguanil, and artemisinin derivatives (artesunate, artemether, arteether, and dihydroartemisinin) in combination with other drugs such as tetracycline, doxycycline or mefloquine. For instance in Thailand, artesunate plus mefloquine or artemether plus lumefantrine or quinine plus tetracycline are being used in the treatment of uncomplicated malaria (120,121).

1.3 PARASITE RESISTANCE TO ANTIFOLATE ANTIMALARIALS

The spread of chloroquine resistance has forced many African countries to switch to a pyrimethamine-sulfadoxine combination to treat acute, uncomplicated malaria (10,11,12). The problem, however, is that in areas where PYR-SDX has replaced chloroquine, resistance has arisen rapidly (11,13,14) leading to reliance on more expensive drugs like quinine, mefloquine or halofantrine. The cost of one treatment regimen of these alternative drugs exceeds the total per capita health care expenditure for many African countries, whereas PYR-SDX often costs only slightly more than chloroquine (15). For instance in 1987, the estimated annual direct and indirect cost of malaria in Africa was US$800 million; by 1995 this figure had increased to more than US$1,800 million (136).

The combination drug pyrimethamine-sulfadoxine (PYR-SDX), until recently, has been a cheap and relatively affordable option for almost all subSaharan African countries faced with chloroquine-resistant *falciparum* malaria. PYR-SDX administration enjoys excellent compliance due to the single dose oral therapy and is well tolerated. PYR-SDX synergy is based on the reciprocal potentiation of its two components SDX and PYR. The antimalarial action is effected by sequential blockade of the two enzymes involved in the parasite biosynthesis of folic acid. PYR inhibits dihydrofolate reductase (DHFR) whereas SDX inhibits dihydropteroate synthatase (DHPS). This results in the inhibition of the production of tetrahydrofolic acid, a cofactor for the synthesis of purines, thymidine, DNA and RNA (7).

1.3.1 *PLASMODIUM FALCIPARUM* RESISTANCE TO PYRIMETHAMINE

In settings where PYR has been widely deployed, resistance has arisen rapidly (3). The observation of focal and very rapid development of antifolate resistance in areas directly under drug pressure led to early suspicions that resistance must be due to a simple mechanism, such as point mutations in the drug target. Subsequently some studies (4,5) cloned and sequenced dihydrofolate reductase gene (a gene that encode

the target enzyme for PYR) and showed that a set of single amino acid changes at the drug-binding site of the target enzyme conferred differential resistance to PYR. Peterson et al (8,16) also produced experimental evidence that point mutations in the bifunctional enzyme dihydrofolate reductase thymidylate synthase conferred resistance to cycloguanil and pyrimethamine. Transfection experiments in P. falciparum, in which a pyrimethamine-sensitive (wild) clone was transformed with mutant dihydrofolate reductase, and the mutant transformants exhibited the predicted levels of pyrimethamine resistance, unequivocally proved that point mutations in dihydrofolate reductase confer resistance to pyrimethamine (6,9).

Thus, parasite resistance to antifolate inhibition acquired through the accumulation of point mutations in the target enzyme, dihydrofolate reductase (16), leads to reduced binding affinity of the drugs to their target. Many studies have adequately described the molecular basis of *in vitro* resistance to pyrimethamine. A serine to asparagine change at codon 108 of the *pf*DHFR results in moderate pyrimethamine resistance. The development of single point mutations, asparagine to isoleucine at codon 51 or cystine to arginine at codon 59 or both on top of the 108 mutation results in higher levels of pyrimethamine resistance. An isoleucine to leucine mutation at codon 164 in the presence of either Asn-108 and Ile-51 or Asn-108 and Arg-59 or all the three DHFR mutations Asn108, Ile51 and Arg-59 confers high-level pyrimethamine resistance (8, 5, 16, 101, 122, 123). Table 1 illustrates an analysis of all the known dihydrofolate reductase genotypes presented against the dihydrofolate reductase inhibitor, PYR. From the table notice the differential PYR resistance (IC50's) for specific genotypes. It is apparent that an increase in number of mutations confers greater level of PYR resistance. For instance the triple DHFR mutant Dd2, also common in Malawi is 2000-fold less susceptible to PYR compared with the wild 3D7 strain. The Leu-164 mutation has not yet been detected in Malawi (or Africa) where PYR-SDX efficacy rate at day 14 is above 80%. Note from table1 that the addition of the Leu-164 mutation to any one of the other DHFR mutations results in 2000-fold increase in PYR resistance.

Enzyme kinetics studies (124) using recombinant *P. falciparum* DHFR expressed in *E. coli* provided the basis for studying the effect of accumulating DHFR mutations on drug resistance and enzyme function. In these enzyme kinetics studies Asn-108

occured first and conferred moderate pyrimethamine resistance with relatively low compromise to enzyme function as measured by k_{cat}/K_m ratios for dihydrofolate. Subsequent mutations at codons 51, 59 and 164 resulted in lowered binding affinities for pyrimethamine, but caused 40-fold reductions in k_{cat}/K_m ratios for dihydrofolate, suggesting that parasites containing the more highly mutated forms of dihydrofolate reductase might be selected against in the absence of drug pressure.

	16	BR	50	51	59	108	164	PYR	Predominant distribution
3D7 (Wild type)	ala	-	cys	asn	cys	ser	ile	1	W Africa
HB3	ala	-	cys	asn	cys	**ASN**	ile	331	W Africa
FCR3	***VAL**	-	cys	asn	cys	**THR**	ile	1.2	Rare in nature
It.D12	ala	-	cys	**ILE**	cys	**ASN**	ile	755	Africa
K1	ala	-	cys	asn	**ARG**	**ASN**	ile	1048	Africa
Dd2	ala	-	cys	**ILE**	**ARG**	**ASN**	ile	2371	E Africa, SE Asia
Bolivia1	ala	-	cys	**ILE**	cys	**ASN**	**LEU**	9960	S America
Cs1-2	ala	-	cys	asn	**ARG**	**ASN**	**LEU**	6212	S America, SE Asia
V1/S	ala	-	cys	**ILE**	**ARG**	**ASN**	**LEU**	22477	S America, SE Asia
Bolivia2	ala	-	**ARG**	**ILE**	cys	**ASN**	ile	2637	S America
Bolivia3	ala	-	**ARG**	**ILE**	cys	**ASN**	**LEU**	20206	S America
Bolivia4	ala	**BR**	cys	**ILE**	cys	**ASN**	**LEU**	6210	S America

BR-Bolivia repeat; PYR-pyrimethamine; Mutations are indicated by bold capitals.
*No significant difference in IC50 from wild-type.

Table 1. Naturally occurring genotypes of *P. falciparum* dihydrofolate reductase and relative level of resistance (IC50, 50% inhibitory concentration) against dihydrofolate reductase inhibitors as determined in a yeast model. The known predominant geographic distribution for each genotype is indicated in the last column (123,125).

1.3.2 *PLASMODIUM FALCIPARUM* RESISTANCE TO SULFADOXINE

In vitro resistance to DHPS inhibitors such as sulfadoxine is conferred by single mutations of the gene encoding DHPS, the target enzyme. DHPS mutations that have been associated with resistance to sulfadoxine are as follows: Ser436Phe, Ala437Gly, Lys540Glu, Ala581Gly, Ala613Ser and Ala613Thr (7,17,18). Triglia and Cowman cloned and sequenced the *P. falciparum* bifunctional gene encoding dihydropteroate synthase and dihydro-hydroxymethylpterin pyrophosphokinase(69,70) and correlated the presence of DHPS mutations with *in vitro* resistance to sulfa drugs including sulfadoxine. Transfection studies where mutant DHPS alleles were introduced in wild parasite strains showed that the mutant DHPS alleles confer sulfonamide/sulfadoxine resistance and resulted in corresponding increases in IC50 values (126). A broad correlation between increased frequency of the aforementioned DHPS mutations and resistance to sulfa drugs, including sulfadoxine, has been observed across the world (127). The genotype Ala437Gly and Lys540Glu were selected by PYR-SDX but not chlorproguanil-dapsone in a clinical trial in Tanzania, suggesting that these mutations are specifically associated to the in vivo resistance to sulfadoxine (128). Triglia et al (18) expressed the entire dihydro-hydroxymethylpterin pyrophosphokinase-dihydropteroate synthase protein in *E. coli* and carried out enzyme kinetics studies to investigate the effects of the mutations on enzyme function and on affinity for sulfa drugs. It was hence concluded that the Ala437Gly DHPS mutation is the essential precursor to further mutations that confer higher levels of resistance. This observation is much like the Asn108 mutation in DHFR where it is the first amino acid change to occur. Plowe et al. (17) and Diourte et al (65) observed that the Gly437 mutation was common in areas of low antifolate resistance and that it did not have significant effect on enzyme function. In Triglia's (18) DHPS enzyme kinetics study the catalytic efficiency (k_{cat}/K_m) for the *p*ABA (natural substrate for DHPS), was found to vary up to 9-fold, with the wild-type and least mutated enzymes having the best catalytic activity, and the enzymes with the lowest binding affinity for the sulfa drugs (i.e. most resistance) having the lowest catalytic activity. This result suggested that the mutations in dihydropteroate synthase, that confer resistance to the sulfa drugs, compromise enzyme function, and might be selected against in the absence of drug pressure.

1.4 ADDITIONAL FACTORS IN ANTIFOLATE DRUG RESISTANCE

Several categories of factors are likely to account for the ability of some individuals to clear genetically resistant infections after treatment with antifolate drugs: high host blood folate concentrations may result in increased parasitological resistance to PYR-SDX therapy (7,19,20,21,22). Since paraaminobenzoic acid [PABA] is a structural analogue of SDX, high host blood levels of the former may competitively antagonize sulfadoxine resulting in PYR-SDX therapeutic failure (22); Inter-individual variation in pharmacokinetic factors (23,24) and host immune factors (25) may account for inter-individual variation in the observed PYR-SDX therapeutic response.

1.4.1 HOST NUTRITION AND ANTIFOLATE RESISITANCE

1.4.1.1 HOST BLOOD FOLATE LEVELS

Folates serve as coenzymes in one-carbon transfer reactions of purines and pyrimidines for DNA synthesis, in the biosynthesis of methionine, serine and glycine, and in the initiation of protein synthesis. Krungkrai et al. (26) showed that Plasmodium falciparum synthesises pteroylpolyglutamate de novo from guanosine 5'-triphosphate (GTP), PABA, and L-glutamate. Chulay et al. (27) showed that folic acid antagonizes the antifolate activity *in vitro* of SDX and, to a lesser extent, of PYR. More interestingly the parasites were seen to have the capacity to synthesize pteroylpolyglutamate from both intact and degradation moeities (p-aminobenzoylglutamate and pterine-aldehyde) of exogenous folate added into the growth medium (28). This ability of malarial parasites, with or without DHFR/DHPS mutations, to salvage intact folate or its metabolites to supplement de novo synthesis may contribute to the observed in vivo parasite resistance to antifolate drugs, particulary sulfa drugs.

The intraerythrocytic stages of plasmodia possess high rates of DNA synthesis and protein synthesis, and they require a vast supply of precursor molecules in amounts that will allow the successful growth and multiplication of the parasites within the cell

(29). Although early studies showed plasmodia synthesise needed folate cofactors de novo, and that they do not utilize the exogenously supplied intact folate molecule (30), other studies found that dihydropteroate synthase from P. berghei uses para-aminobenzoylglutamate (PABG) as an alternative substrate to form dihyrofolate directly which (31), as explained, comes from the cleavage of intact folates. Folic and folinic acids have been shown to aid malarial growth or survival and to reverse inhibition by antifolates (32, 33). This is consistent with the hypothesis that cleavage products are utilized, not the intact molecules. The competitive reversal by folic acid of sulfadiazine inhibition of P.gallinaecium in vivo led Rollo (31) to conclude the host broke down folic acid to PABG. Robert (34) found that the ability of malaria parasites to utilize exogenous folates may vary with species studied and takes note of Hitching's observation that most microorganisms that synthesize folate de novo are impermeable to exogenous folates (30).

Since plasmodia can salvage folate from a host who cannot synthesize it but must get it through food or supplements, then host nutritional differences can affect blood levels of folate, which will influence plasmodial growth in vivo. Russell et al. (35) reported a very wide range (5.0-21.0 ng/mL) for serum folate in Iran, reflecting a wide variation among individuals of various populations associated with socioeconomic classes from rural and urban areas. Significant differences in folate levels in serum and erythrocytes between females and males of the same age have been described. Boys had significantly higher folate levels in serum and erythrocytes than did girls (36). Ortega RM et al. found that erythrocyte and serum folate levels are affected by smoking. Increased levels of serum and red cell folate were reported for non-smokers (37). Hasanah et al. found that reduced red cell folate associate with mania and depression (38). Variation in red cell folate with age has been documented with higher mean red cell levels (over 0.296 microgram/100mL) in the younger compared to older people (less than 0.100 microgram/100mL) (39). Studies of serum and red cell folate concentrations among normal healthy Nigerian adolescents aged between 12-17 years showed serum folate levels that were generally low with a mean of 2.8 nmol/L while red cell folate levels were much higher with a mean of 369 nmol/L (observed negative correlation r = 0.24) (40).

Watkins et al. (1985 [22]) demonstrated the antagonism of sulfadoxine and pyrimethamine antimalarial activity in vitro by p-aminobenzoic acid, p-aminobenzoylglutamic acid and folic acid (22). More recently Wang found that in parasites that use exogenous folate efficiently, SDX inhibition of plasmodial growth could be restored by levels of PYR significantly lower than those required to inhibit DHFR (19). Using isobolograms it was shown that the degree of synergy between PYR and SDX is highly dependent upon prevailing folate concentrations and are indicative of PYR acting to block folate uptake and/or utilization.

1.4.1.2 HOST BLOOD PARAAMINOBENZOIC ACID (PABA) LEVELS

Through antagonism of sulphonamide activity, the folate metabolic precursor PABA has hampered the development of in vitro tests for resistance to PYR-SDX owing to its presence in high concentration (1 mg/ml) in RPMI 1640, the medium generally used for plasmodium culture (41). Brockelman and Tan-Ariya reported that sulfonamide susceptibility was considerably enhanced in the PABA-free Waymouth formula medium (42). These studies on the sulfonamides and 4-aminobezoic acid (PABA) in malaria date back to 1960's, when sulfachrysoidine and sulfanilamide were first used to treat human malarial infections and PABA was found to reverse sulfonamide inhibition of Plasmodium gallinaceum (43). Since then there have been reports of studies on the effects of folate precursors, folate metabolites, folate endogenous products, and folate analogues on malarial growth and survival in vitro (7,19,20,21,22).

Anfinsen et al. showed that the growth of *P. knowlesi* required PABA, which prevented inhibition of growth by sulfadiazine (44). More importantly it was shown that erythrocytic forms of several species of plasmodium grew poorly or not at all in hosts fed on PABA-deficient diets (45). The requirement of *P. falciparum* for PABA has been indicated by studies of Kretschmar (46) who suggested that the low incidence of P.falciparum malaria in children fed on milk diets was due to the low content of PABA in milk. He later showed that P.falciparum did not grow in Aotus monkeys maintained on a milk diet (47). Jacobs observed increasing parasitaemia in mice infected with P.berghei with increasing PABA levels in the diet, and no growth

in its absence (48). Hawkins (49) reported the suppression of parasitemia of P. berghei in the rat and P. cynomolgi in the rhesus monkey fed on milk diets that were shown to have a low PABA content. In both cases PABA supplementation of the milk diet allowed the infections to develop.

Interestingly, a pyrimethamine resistant strain required higher levels of PABA for optimum development compared with a sensitive one (49). Plasmodia do not synthesize PABA, since nutritional studies indicate that it must be supplied in the diet (34). Differences between resistant strains in their capacity to utilize low levels of PABA have been observed (50). Highly mutated forms of parasite DHPS do not utilize PABA efficiently (18). High host levels of PABA should offset the effect of the DHPS mutations so that those parasites can survive sulfonamide pressure. The less mutated parasites die from sulfonamide competitive inhibition of PABA utilization by their efficient DHPS enzymes. On the other hand highly mutated forms will not bind the competitive inhibitor because of reduced enzyme affinity for substrate and, in addition, these parasites will require high PABA levels for survival as they utilize PABA less efficiently than their unmutated counterparts. Thus high levels of PABA should provide a selective advantage to genetically mutated parasites under drug pressure.

1.4.2 DRUG PHARMACOKINETICS IN THE HOST:

HOST BLOOD DRUG LEVELS

PYR-SDX parasitological failure may be caused not only by parasite resistance and high PABA or folate levels but also the interindividual variation in host pharmacokinetics. The rate of absorption of the dug, its distribution in the various body compartments, tissue and plasma protein binding, and the rate of metabolism and excretion of the drug all influence the steady-state level of the drug at the site where it is required (23), hence the magnitude of pharmacological activity.

When orally administered, PYR-SDX is well absorbed. Its components display peak plasma levels within 2-8 hours and are excreted mainly by the kidneys (51). Average

half-lives are about 170 hours for SDX and 80-110 hours for PYR (51). To evaluate pharmacological determinants of PYR-SDX therapeutic failure there is need to monitor the drug levels before and after therapy to verify that the treated subject had attained expected parasiticidal PYR-SDX concentrations required for permanent parasite eradication, had an adequate dose, had not vomited and that there was no poor absorption. The minimum concentrations required in vivo are, however, not established. Conditions in vivo differ considerably from those in vitro and thus concentrations in vitro and in vivo are not comparable. In vitro blood concentrations of SDX and PYR above 3.0uM and 0.38uM respectively in the blood / medium mixture inhibited all strains that had been observed to be susceptible in vivo in a study from Haiti (52). Schapira et al. estimated, from the slope of the terminal linear part of a log concentration time graph, that SDX concentrations less than 200-371 uM and PYR concentrations less than 0.2-1.40uM did not prevent a 'secondary' increase in parasitaemia in 3 of 4 non-immune patients with RII PYR-SDX resistant *P.falciparum* from Tanzania (53).

Although individual pharmacokinetic characteristics of PYR and SDX are not altered when they are used in combination as in PYR-SDX or together with mefloquine (54,55), lower serum concentrations of pyrimethamine were found in Papua New Guineans than in Caucasians, an observation that may reflect differences in the bioavailability in the two racial groups (56). Hellgren et al reported inter-individual variations in AUC for SDX that were in accordance with variation reported by others although less than what would be expected if marked inter-individual pharmacokinetic variability was present for SDX, PYR or mefloquine (57,58,59). Although Hellgren (57) did not find natural non-responders to PYR-SDX in a Tanzanian population, a rate of 10-20% 'natural' non-responders to the combination has been documented for a Thai population (60,61). Sarikabuthi et al. (59) found no difference in active unacetylated SDX concentration between responding and non-responding groups in the Thai population. This concept of a natural non-responder to PYR-SDX, being based on early Thai data, is now suspect as a result of no further supporting field evidence in separate geographical regions.

Certain disease estates cause altered drug pharmacodynamic and pharmacokinetic properties by modifying body perfusion, hepatic and metabolic function, modifying

renal function, protein binding and gastrointestinal absorption (62). For instance, the half-life of PYR has been found to be as short as 23 hour in studies in patients with AIDS suggesting variation in metabolism of PYR or altered hepatic function secondary to HIV infection (USP DI, 1995) (51).

2.0 RATIONALE FOR MORE STUDIES ON ANTIFOLATE RESISTANCE

Although the molecular basis of in vitro resistance to the antifolate drugs SDX and PYR is well described, the molecular basis in vivo is less clear. Studies in diverse settings with different malaria transmission and epidemiological patterns have shown that the prevalence of drug resistant genotypes generally exceeds the prevalence of high-level in vivo drug resistance (17, 63,64,65). For example, in Malawi, the most highly mutated form of *P. falciparum* DHFR and DHPS contains the DHFR triple mutant Asn-108/Ile-51/Arg-59 and the double DHPS mutant Gly-437 and Glu-540. In Blantyre, this "quintuple mutant" is present at a prevalence of approximately >60%, while PYR-SDX treatment failures at day 14 are present at a rate of only about 20% (66). Preliminary studies of in vivo PYR-SDX efficacy in Mangochi showed that the DHFR triple mutant Asn-108 / Arg-59 / Ile-51 was associated with in vivo resistance to PYR-SDX (67) and also found that this genotype was prevalent at a rate of about 70% while the rate of all treatment failures to day 14 was only 39%. This suggests that some infections with the quintuple mutant are still cleared by PYR-SDX treatment. Thus, factors other than parasite DHFR and DHPS must determine whether genetically resistant infections result in early treatment failure, late treatment failure, or cure. Among parasitemia with the triple mutant or the quintuple mutant, all treatment outcomes (adequate response, early, and late treatment failure) were observed. Thus a number of factors are likely to account for the ability of some individuals to clear genetically resistant infections after treatment with antifolate drugs including host blood folate, PABA levels, antimalarial drug levels achieved in the host blood, host immune factors, and possibly other parasite genetic factors not yet identified at present.

The current rapid and widespread development of P.falciparum resistance to PYR-SDX (the only remaining easily affordable alternative after chloroquine), where they have been introduced as firstline drugs, underscores the urgent need to gain a clear and thorough understanding of the determinants of resistance to these drugs. Such knowledge would be critical if the useful therapeutic life of PYR-SDX is to be prolonged and if new and effective therapeutic drugs are to be developed. Moreover if the relative roles of the determinants of in vivo PYR-SDX therapeutic outcome are understood, it will be possible to predict the PYR-SDX efficacy with reasonable accuracy and hence direct antimalarial drug distribution appropriately.

2.1 PYRIMETHAMINE SULFADOXINE IN MALAWI

The Central African country of Malawi revised its national malaria treatment policy in 1993 in response to evidence of high rates of chloroquine failure, changing the first line drug from chloroquine to PYR-SDX (12). Malawi was the first African country to effect this change. Unfortunately, just like in other areas of the world where PYR-SDX has replaced chloroquine, parasite resistance to the combination has arisen rapidly in Malawi. This phenomenon is a threat to malaria control programs not in Malawi only but also in other countries where malaria is endemic. With no easily affordable alternative antimalarial drug suitable for widespread use in Africa now available to replace PYR-SDX, an understanding of the relative role of all possible determinants of in vivo *Plasmodium falciparum* resistance to the combination is critical to malaria control programs.

As a country where after 7 years of countrywide use, PYR-SDX efficacy (at day 14) still remains above 80% in the face of a 60% prevalence rate of the highly resistant *falciparum* quintuple mutant, Malawi provided an opportunity to identify the determinants of *falciparum* resistance to this drug. To achieve a clear understanding of the determinants of antimalarial drug failure, all possible factors will need to be explored in conjunction with studies of molecular markers and in vivo resistance. This thesis describes a comprehensive analysis of the role of key factors (parasite mutations, host folate and drug pharmacokinetics) in PYR-SDX failure.

Because antimalarial treatment outcome may differ in settings with different transmission and epidemiological profiles, the thesis proposal was coordinated with field studies at two sites in Malawi with high and low malaria transmission levels. A previous study showed a strong association between long history of exposure to malaria, the presence of antimalarial antibodies and increased efficacy of PYR-SDX (68). It is well known that repeated exposure to malarial infection results in the acquisition of premunition, which protects against disease. Thus, transmission level by site as well as age could be used as a surrogate marker of partial immunity.

2.2 SCOPE OF THE STUDY

2.2.1 OVERALL AIM

The primary objective of this study was to assess the relative role of host folate levels, immunity, PYR-SDX pharmacokinetic factors and parasite resistance-conferring genotypes in pyrimethamine-sulfadoxine parasitological resistance.

2.2.2 SPECIFIC AIMS

2.2.2.1 AIM 1

1^0 Overall, measure the association of the presence of the DHFR/DHPS "quintuple mutant" *Plasmodium falciparum* genotype at the time of treatment with SP therapeutic failure

2^0 Prospectively measure the *in vivo* response to PYR-SDX and the frequency of specific point mutations in DHFR and DHPS in cases of *P. falciparum* malaria in Malawi

2.2.2.2 AIM 2

1^0 Test the hypothesis that higher host physiological blood folate levels are associated with PYR-SDX therapeutic failure.

2^0 Adapt methods of filter paper to collect and measure p-aminobenzoic acid, and folate.

2.2.2.3 AIM 3

1^0 Test the hypothesis that sub-therapeutic levels of sulfadoxine and pyrimethamine at specified post-treatment time points are associated with PYR-SDX treatment failure.

2^0 Develop and validate methods for assays for sulfadoxine and pyrimethamine levels using filter paper samples such that it will be possible to determine drug levels to assess adequacy of drug absorption.

2.2.2.4 AIM 4

Use multivariate analysis to assess if there is an independent relationship between mutations in DHPS/DHFR, host blood folate levels, PYR-SDX blood levels, intensity of transmission, age and the therapueutic failure of PYR-SDX in children diagnosed with nonsevere malaria.

CHAPTER 2

PYRIMETHAMINE-SULFADOXINE EFFICACY AND MOLECULAR MARKERS OF RESISTANCE IN HIGH AND LOW TRANSMISSION AREAS IN MALAWI

2.1 INTRODUCTION

Mutations at defined codons in DHFR have been correlated with in vivo PYR-SDX therapeutic failure in various epidemiological studies (7,66). It is now well known that the step-wise accumulation of the mutations S108N, C59R, N51I and I164L in the *falciparum* DHFR gene results in high-level pyrimethamine resistance (reviewed by Hyde J.E, 1990). Similarly, most SDX-resistant parasites that have been assayed in vitro carry the DHPS mutations, S436F, A437G, A613S and K540E (7). The role of the DHPS mutations in determining levels of SDX resistance is still less clearly understood than that of the DHFR mutations in PYR resistance (69,70).

In this study, mutation-specific nested PCR and/or restriction digestions were used to analyze DHFR and DHPS mutations (as described elsewhere [17,71]) at two sites with differing transmission intensity. The codon analysis scheme, described by Kublin et al (66) for results of the molecular assays for *Plasmodium falciparum* DHFR and DHPS mutations, was used in this study.

The SP efficacy, the prevalence of DHFR/DHPS mutations and their association with reappearance of *Plasmodium falciparum* parasitemia were compared within and between study sites. The sensitivity and specificity of single and combined DHFR/DHPS mutations in predicting the DHFR/DHPS quintuple mutant, which was previously shown to be strongly associated with PYR-SDX therapeutic failure, was also measured. The PYR-SDX efficacy was also assessed among children who had been retreated with the same drug upon failure of the initial therapy within 21 – 28 days of treatment.

2.2 PATIENTS AND METHODS

2.2.1 Study site

The study was undertaken at two sites, Dedza (low transmission, with considerable seasonal variation) and Mangochi (high transmission, with moderate seasonal variation) in central and southern Malawi, from August to October 2000 and June to September 2000 respectively. This was during a dry low malaria transmission season. The study was conducted following the WHO protocol for assessing antimalarial efficacy in highly endemic areas (72). Children aged from 6 months to 12 years, reporting at Mangochi or Dedza district hospital out-patient departments with signs and symptoms of malaria, and whose axillary temperature and parasite density at the time of the visit were $\geq 37.5^0$ Celsius and 2000/μL of blood respectively, were eligible for study. Only children whose caretakers provided informed consent were enrolled. After a supervised treatment with a standard PYR-SDX dose (dosage 1/4 tablet per 5kg wt for age ≤12, 3 tablets for age>12; 1 tablet = pyrimethamine 25mg + sulfadoxine 500mg) on the enrolment day, each subject was actively followed again on days 1, 2, 3, 7, 14, 21, 28, 35 and 42. At each of those days fingerprick blood samples were collected on 3MM Whatman filter paper, air-dried and stored in dessicated resealable plastic bags at room temperature. The ethics committee of the University of Malawi Medical School approved this study.

2.2.2 Assessment of in vivo parasite resistance

The modified WHO definition of therapeutic efficacy and parasitological resistance for areas of low to moderate transmission was used (73). Dedza (located on Dedza mountain range) is a low transmission area relative to Mangochi (situated on the shores of Lake Malawi). The study in Mangochi was done during the dry season at which time transmission is moderate.

Therapeutic efficacy was defined as follows: Early treatment failure = Development of danger signs or severe malaria on day 1, day 2, or day 3 in the presence of parasitemia;

parasitemia on day 2 higher than day 0 count irrespective of axillary temperature; parasitemia on day 3 with axillary temperature $\geq 37.5^0$C; parasitemia on day 3 $\geq$ 25% of count on day 0. Parasitological resistance was defined as follows: There are two possible categories of late treatment failure – Late parasitological failure (LPF) = presence of parasitemia on any day from day 7 to day 28 and axillary temperature $< 37.5^0$C, without meeting any criteria of early treatment failure or late clinical failure. Late clinical failure (LCF) = development of danger signs or severe malaria after day 3 in the presence of parasitemia without meeting any criteria of early treatment failure; presence of parasitemia and axillary temperature $\geq 37.5^0$C (or history of fever) on any day from day 4 to day 28, without previously meeting any of the criteria of early treatment failure.

Adequate clinical and parasitological response (ACPR) was described as absence of parasitemia on day 28 irrespective of axillary temperature without previously meeting any of the criteria of early treatment failure or late clinical failure or late parasitological failure.

2.2.3 Parasite DNA extraction

Plasmodium falciparum DNA was prepared as previously described (71). A 0.25-0.5 cm^2 piece of blood-impregnated filter paper was cut off using a pair of scissors, and wiping off the scissors on tissue paper between cuttings. The snippet was transferred to a microfuge tube to which was added approximately 50 – 100 µL methanol, making sure the paper was totally immersed in the methanol. The tube, with contents, was incubated for 15 minutes at room temperature. Methanol was then poured out while being careful to retain the snippet in the tube. The remaining methanol was allowed to evaporate for 15 minutes with tube open and lying on its side. To each tube containing filter-paper snippet was added 50 µL water and the tube heated for 15 minutes at 95-100^{0}C (not too hot to cause evaporation), vigorously vortexing the tubes every two minutes or so during the incubation. The extract was then stored at –20^{0}C until analysis. 5-10 µL of the extract was used in a 25 µL primary PCR reaction.

2.2.4 Parasite DNA amplification

Polymerase chain reaction (PCR) assays and restriction enzyme digestion have been useful and practical methods for the screening of resistance-conferring mutations in DHFR and DHPS genes (74,75). Allele-specific restriction analysis described by Plowe et al (71,17) was used to analyze DHFR and DHPS mutations at codons 108, 59, 51, 164 and 437 and 540, respectively using 5 µL of *Plasmodium falciparum* DNA extract. All PCR DNA amplifications were performed in a PTC100 thermal cycler. Gel picture 1 and 2 are examples of amplification and enzyme digestion results.

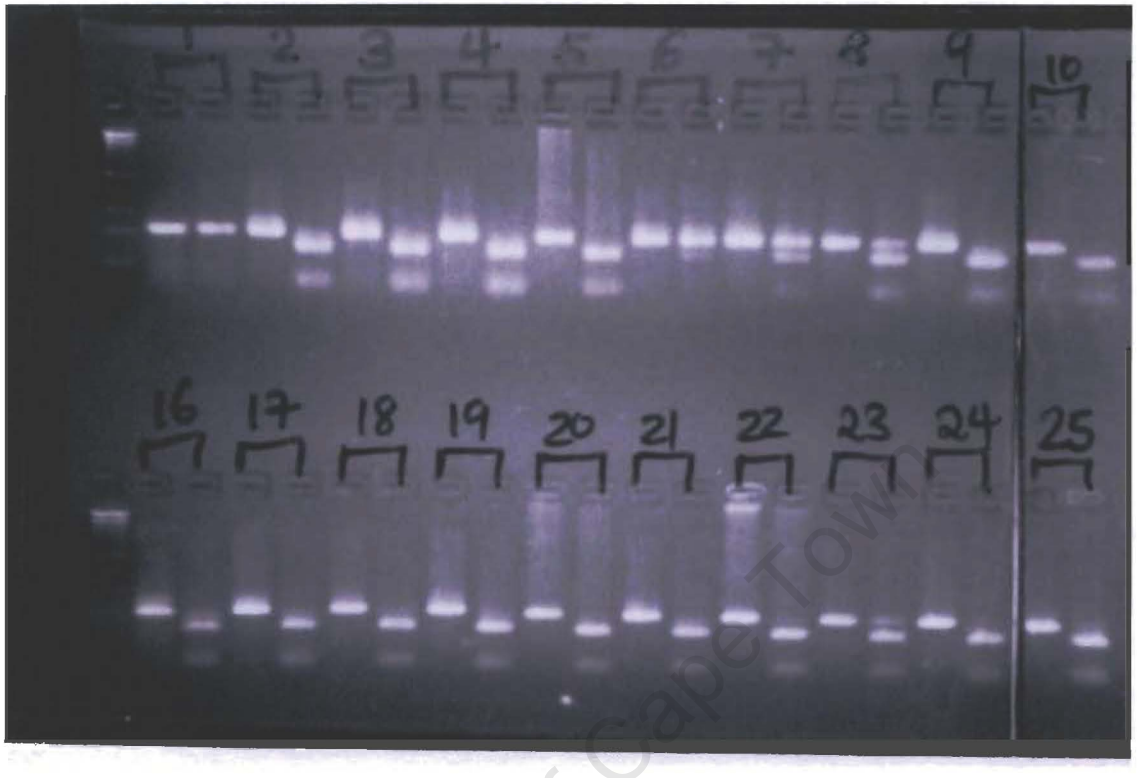
2.2.5 DHFR gene analysis

2.2.5.1 PCR for DHFR codon 51 and 59

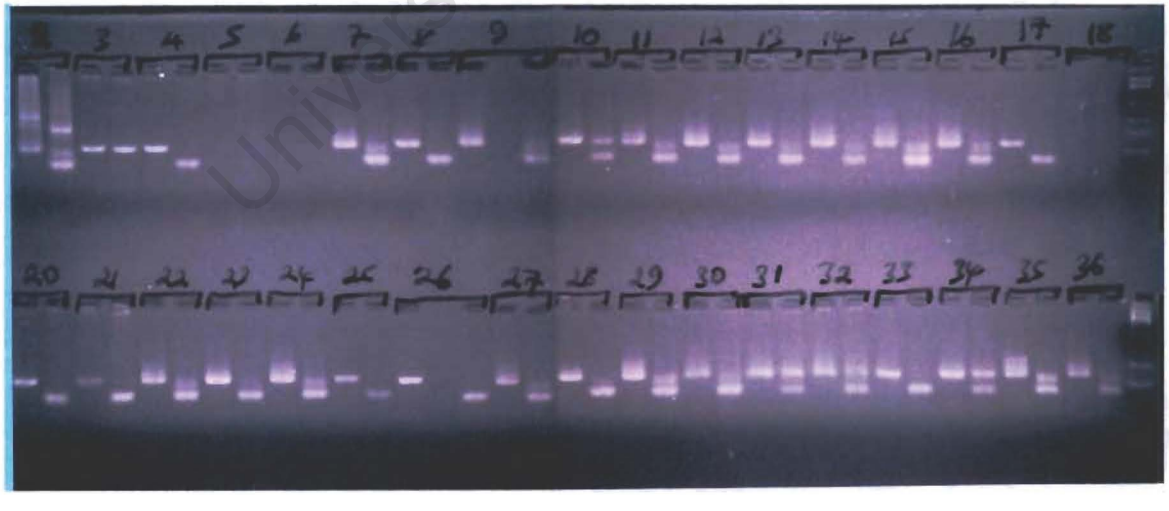
Primers used for the primary DNA amplification were FR519-A 5'**GCGCGC**TAATAACTACACATTTA3' and FR519-B 5'**CCCGGG**CTCTTATATTTCAATTT3'), yielding a product of size 147 bp. A 25µL reaction contained 1.5mM $MgCl_2$ (Gibco BRL), 200 µM dNTPs (Gibco BRL), 1 µM of each primer, 1.25 U Taq polymerase (Thermus aquaticus) enzyme (Gibco BRL). The PCR was performed using the following thermocycler conditions: Primary denaturing at 95^0C for 5 min, followed by 45 cycles at 92^0C for 30 s, at 45^0C for 30 s, at 65^0C for 45 s, and final extention (1 cycle) at 72^0C for 15 minutes.

1 µL of the primary reaction product was then used in a 25µL secondary PCR reaction. Primers used for the secondary DNA amplification were FR51-D 5'CTAGGAAATAAAGGAGTATTACCATGGAAATG**G**A3' and FR59-D 5'ATTTTTCATATTTTGATTCATTCACATATGTTGTAACTG**T**AC3'. Secondary PCR was undertaken using the following thermocycler conditions: Primary denaturing at 95^0C for 5 min, followed by 25 cycles at 92^0C for 30 s, at 45^0C for 30 s, at 65^0C for 30 s, and final extention (1 cycle) at 72^0C for 15 minutes. Secondary product size was 113 bp.

Digests for each codon were performed separately. 5 µL of the the product from the secondary reaction was digested with 1 unit enzyme overnight at 37^0C in a total of 20 µL



Gel picture 1. Results of a falciparum dihydropteroate synthatase gene digest with FokI. The enzyme cut only the mutant DHPS allele.



Gel picture 2. Results of a PCR analysis of the *Plasmodium falciparum* dihydropteroate synthetase gene (codon 437) for single amino acid changes using Allele-specific restriction analysis (ASRA) with AVAII. Isolates numbered 3, 4, 5 and 6 are the positive and negative

controls FCB (wild), PS-PERU (mutant plasmid) and NEGATIVE FOR PRIMARY REACTION, NEGATIVE FOR SECONDARY REACTION (no *falciparum* DNA added) respectively.

reaction using NE buffer #4 (New England Biolabs, Beverly, MA). For DHFR codons 51 and 59, EcoRI (New England Biolabs, Beverly, MA) and BsrGI (New England Biolabs, MA) respectively, were used to cleave the wild type codons. Sizes of the enzyme cleavage products were 35 and 78 bp, and 43 and 65 bp for EcoRI and BsrGI respectively. Products were separated by electrophoresis on 2% NewSeive gel, with a no-enzyme digest run alongside as a control. Products were visualized under UV light after ethidium bromide staining. Negative and positive controls were included in the DNA extraction and in all PCR reactions. gDNA was used as a positive control for the primary reaction. 3D7 or HB3 and DD2 or V1/S were used as positive wild and mutant controls respectively for 51 and 59 secondary PCR.

2.2.5.2 PCR for DHFR codon 108 and 164

Primers used for a 25 µL primary PCR were FR100-A 5'**GGGGGG**CAGTTACAACATATGTGA3' and FR100-B 5'**GGGGG**CACATTCATATGTACTATTT3', yielding a 414 bp product. Reagent composition of the 25 µL primary reaction and thermocycler conditions were as described for the codon 59 primary PCR above.

The primary reaction product (1 µL) was used in a 25µL secondary PCR reaction. Primers used for the secondary DNA amplification were FR108-D 5'CTAATTCTAAAAAATTACAAAATGT3' and FR164-D3 5'TTTCTTTTCTAAAAATTCTTGATAAACAACGGAACCTCTTA3', resulting in a 254 bp secondary PCR product. Secondary PCR was undertaken using the following thermocycler conditions: Primary denaturing at 95^0C for 5 min, followed by 25 cycles at 92^0C for 30 s, at 42^0C for 30 s, at 65^0C for 45 s, and final extension (1 cycle) at 72^0C for 15 minutes. 5 µL of product from the secondary reaction was digested with 1 unit enzyme overnight at 37^0C in a total of 20 µL reaction using 0.5X NE buffer #4 (New England Biolabs, Beverly, MA) and 1x BSA (New England Biolabs). For DHFR codons

108 and 164, Alu I (New England Biolabs, Beverly, MA) and Psi I (New England Biolabs, Beverly, MA) respectively, were used to cleave the wild type codons. Approximate cleaved product sizes were 46 and 210 bp, and 42 and 214 bp for Alu I and Psi I respectively. Digest products were separated and visualized as described for DHFR codon 51 and 59. 3D7, FCR3 or DD2 were used as wild type positive control. VIS was used for a mutant positive control.

2.2.6 DHPS gene analysis

DHPS 540 Control strains: *wild* = 3D7, FCR3; *mutant* = PS-PERU control plasmid.

DHPS 437 Control strains: *wild* = PS-Mali plasmid control; FCB; *mutant* = PS-PERU control plasmid; 3D7.

2.2.6.1 PCR for DHPS codon 437

For a 25μL primary PCR, the primers PS400-A **GGGG**TATTAAATGTTAATTATGATTCT (sense) and PS400-B **GGGG**TCACATTTAACAATTTTATT (antisense) were used at 1 μM final concentration each. The primary PCR was carried out at 95^{0}C for 5 min, followed by 45 cycles at 92^{0}C for 30 s, at 45^{0}C for 30 s, at 65^{0}C for 45 s, and final extension (1 cycle) at 72^{0}C for 15 minutes. Primary product size was approximately 249 bp.

The primary reaction product (1 μL) was used in a 25μL secondary PCR reaction and primers PS400-D1 TGTTCAAAGAATGTTTGAAATGA (sense) and PS400-D2 CCATTCTTTTTGAAATAATTGTAAT (antisense) were used. The secondary PCR was carried out at 95^{0}C for 5 min, followed by 45 cycles at 92^{0}C for 30 s, at 45^{0}C for 30 s, at 65^{0}C for 30 s, and final extension (1 cycle) at 72^{0}C for 15 minutes. Secondary product size was 148 bp.

5 μL of product from the secondary reaction was digested with 1 unit enzyme overnight at 37^{0}C in a total of 20 μL reaction using NE buffer #4 (New England Biolabs, Beverly, MA). For DHPS codon 437 the enzyme Ava II (New England Biolabs, Beverly, MA)

was used to cleave the mutant type codon (Gly) and not wild (Ala). Approximate cleaved product sizes were 69 and 79 bp. After staining with ethidium bromide, digest products were separated on 2% agarose gel and visualized under UV light.

2.2.6.2 PCR for DHPS codon 540

For a primary PCR (25μL), the sense primer PS500-A **GGGCCC**AAACAAATTCTATAGTG and the antisense primer PS500-B **GGCCGG**TGGATACTCATCATATA were used. Thermocycling conditions for primary were: an initial denaturation at 95^{0}C for 5 min, followed by 45 cycles at 92^{0}C for 30 s, at 45^{0}C for 30 s, at 65^{0}C for 45 s, and final extension (1 cycle) at 72^{0}C for 15 minutes.

1 μL of the primary reaction product was then used in a 25μL secondary PCR reaction using primers PS500-D1 **GCGCGC**GTTCTAATGCATAAAAGAGG (sense) and PS500-D2 **CCCGGG**TAAGAGTTTAATAGATTGATCA*GC*TTTCTTC (antisense). The secondary PCR was carried out at 95^{0}C for 5 min, followed by 25 cycles at 92^{0}C for 30 s, at 45^{0}C for 30 s, at 65^{0}C for 30 s, and final extension (1 cycle) at 72^{0}C for 15 minutes. Secondary product size was 201 bp.

5 μL of product from the DHPS 540 secondary reaction was digested with 1 unit enzyme overnight at 37^{0}C in a total of 20 μL reaction using NE buffer #4 (New England Biolabs, Beverly, MA). For DHPS codon 540 the enzyme Fok I (New England Biolabs, Beverly, MA) was used to cleave the mutant type codon (Glu) and not wild (Lys). Approximate cleaved product sizes were 56 and 145 bp. After staining with ethidium bromide, digest products were separated on 2% NewSieve gel and visualized under UV light

2.2.7 Statistical analysis

Pyrimethamine-sulfadoxine efficacy rates were compared using the Chi square test with two-tailed significance set at P = 0.05 (EpiInfo2000, Centers for Disease Control and

Prevention, Atlanta, GA). Univariate analyses and multiple logistic regression to test for association between DHFR/DHPS mutations and parasite reappearance within 4 to 28 days after treatment were performed using STATA 7.0 (Texas, USA).

2.3 RESULTS

2.3.1 PYRIMETHAMINE-SULFADOXINE CLINICAL AND PARASITOLOGICAL EFFICACY

2.3.1.1 28-day parasitological and clinical efficacy

Based on a 28-day follow-up, parasitological outcomes could be determined for 122 and 46 participants in Mangochi and Dedza respectively. This was because temperature records had not been entered for some participants or the participant was last seen only on day 14 of post treatment follow-up.

In Mangochi and Dedza, 64 (52.4%, N=122) and 18 (39.1%, N=46) individuals, respectively, were cured with no subsequent parasitemia during four weeks of follow-up and therefore met the ACPR criteria. 49 (40.2%, N=122) and 25 (54.4%, N=46) children, in Mangochi and Dedza respectively, became parasitemic without fever (axillary temperature $\leq$ 37.5) during the follow-up period between day 4 and day 28 of initial treatment, thus meeting the criteria of LPF. Another group of children developed parasitemia accompanied with fever (axillary temperature $\leq$ 37.5) between days 4 and 28 post-treatment and thus met the criteria of LCF. There were 9 (7.3%, N=122) and 3 (6.5%, N=46) late clinical failures in Mangochi and Dedza respectively.

Overall there was no difference in the late clinical failure rates between sites (P = 1.0). Although the magnitude of the difference in ACPR rates between Dedza and Mangochi was not statistically significant (Figure 2.1, P = 0.68), it may be clinically relevant as it is clear that there was a significantly higher rate of late parasitological failure in Dedza compared with Mangochi (P = 0.010).

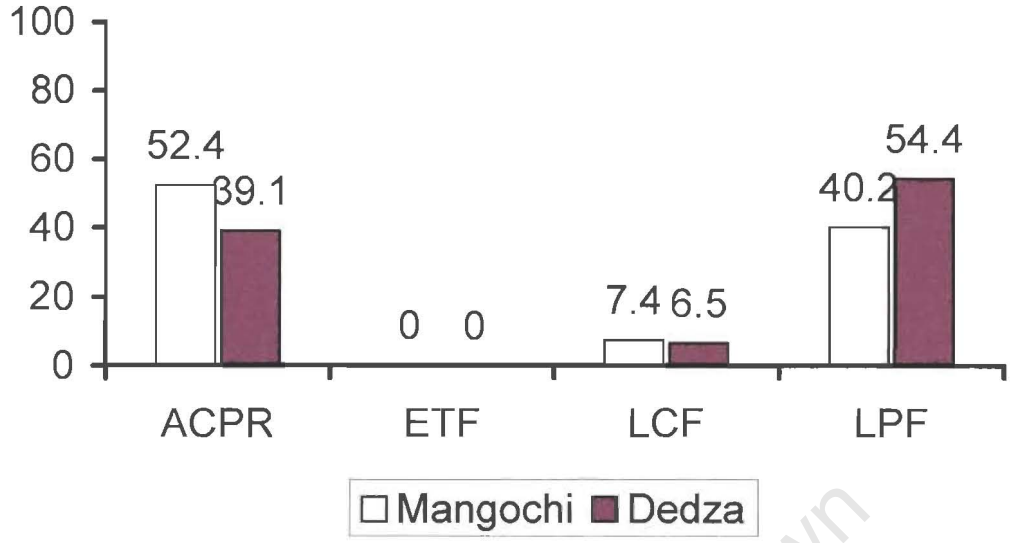


Fig 2.1 Parasitological outcomes for two areas differing in transmission intensity: Dedza (low transmission, N=46) and Mangochi (high transmission, N=122). Parasitological outcomes were based on a 28-day follow-up as defined by the WHO (Monitoring antimalarial resistance 2001, Geneva, Switzerland). ACPR =Adequate clinical and parasitological response. LPF =late parasitological failure. LCF=late clinical failure. ETF = Early treatment failure

2.3.1.2 WHO 14-day efficacy

51 and 166 patients were enrolled in Dedza and Mangochi respectively. In Mangochi 144 of the 166 (86.7%) enrolled participants completed 14 days of follow up. 21 participants (12.7%) were lost to follow up and 1 (0.60%) withdrew due to protocol violation. In Dedza, 47 of the 51 (92.2%) completed 14 days of follow up, 3 (5.9%) were lost to follow up and 1 (2%) withdrew consent to continue in the study. Figure 4.7 (Chapter 4) is a summary of the above information.

Based on the 14 day efficacy (fig 2.2) 129 (representing 90%, N=144) and 37 (representing 78%, N=47) individuals were cured with no subsequent parasitemia during

14 days of follow-up in Mangochi and Dedza respectively. The 14-day ACR rate was higher in Mangochi than Dedza (P< 0.00001). At both sites there were no children who met the criteria of early treatment failure (ETF) after the initial PYR-SDX therapy. On the other hand 15 (10.4%, N=144) children met the criteria for late treatment failure (LTF) in Mangochi and 9 (21%, N=43) met this criteria in Dedza. Figures 2.2 and 2.3 summarize the 14-day efficacy findings in Mangochi and Dedza respectively.

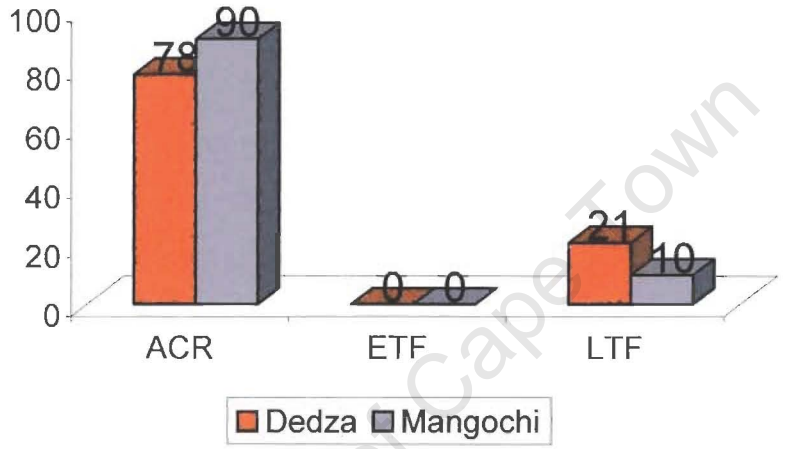


Fig 2.2 In vivo PYR-SDX efficacy findings in Mangochi, Malawi: June – September 2000 (N=144) and efficacy findings in Dedza, Malawi: August – October 2000 (N=51). Clinical efficacy was based on the WHO14-day definition.

Children (46 in Mangochi and 9 in Dedza) who failed the initial PYR-SDX treatment between day 21 and 28 were retreated with the same drug at the time when they were determined to be parasitemic again. Figure 2.3 shows the efficacy results following retreatment with the same drug. Resistance to PYR-SDX increased from 10% at the first treatment to 43% after retreatment in Mangochi and from 22% to 78% in Dedza. Following retreatment with PYR-SDX, early treatment failures observed at a rate of 2% in Mangochi and at a much higher rate, 11%, in Dedza.

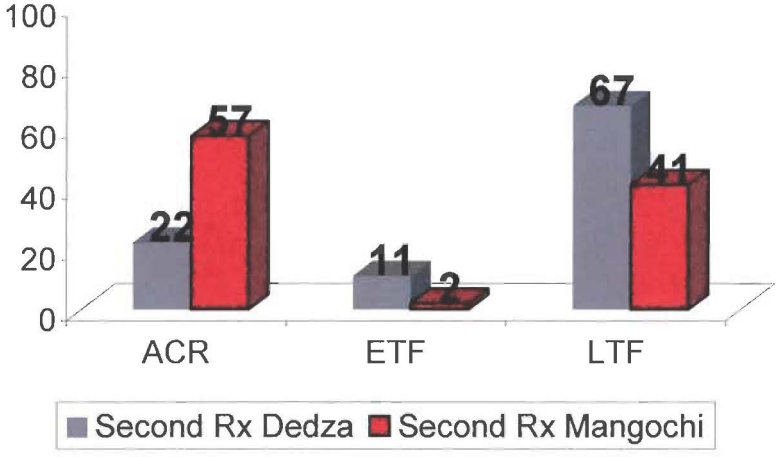


Fig 2.3 Results of in vivo PYR-SDX efficacy following retreatment with the same drug. Mangochi, N = 46 and Dedza N = 9. Clinical efficacy was based on the WHO14-day definition.

2.3.2 DHFR and DHPS genotypes

For quick visual comparison of the numbers, percentage prevalence of single nucleotide polymorphisms described below are reported in the summary table below

	DEDZA			MANGOCHI		
Prevalence (%) of single nucleotide polymorphisms	Mutant	Wild+Mutant	Wild	Mutant	Wild+Mutant	Wild
S108N	69	31	0	66	34	0
N51I	79	19	2	68	32	0
C59R	81	17	2	55	44	1
I164L	0	0	100	0	0	100
K540E	74	7	19	86	11	4
A437G	70	1	11	83	13	5

The prevalence of single amino acid changes at defined codons of the dihydrofolate reductase and dihydropteroate synthatase genes in isolates from Mangochi and Dedza malaria patients.

2.3.2.1 MANGOCHI

Of the 104 isolates successfully typed for DHFR codon 108, 69 (66%) carried the S108N mutation and 35 (34%) carried mixed alleles of the codon 108. Codon 51 PCR results were obtained for 103 isolates and 70 (68%) carried the N51I mutation. 33 (32%) carried mixed alleles at DHFR codon 51. Of the 103 typed for DHFR codon 59, 57 (55%) had the C59R mutation, 45 (44%) carried mixed alleles and 1 (1%) was wild. None of the Mangochi isolates (N=103) that were typed carried the I164L mutation, which is associated with the highest levels of PYR and cycloguanil resistance.

104 isolates were successfully typed at DHPS codons 437 and 540. For DHPS codon 540, 89 (86%) isolates had the K540E mutation, 11 (11%) were mixed and 4 (4%) were wild. At DHPS codon 437 86 (83%) isolates carried the A437G mutation, 13 (13%) were mixed and 5 (5%) were wild.

2.3.2.2 DEDZA

Of the 42 isolates successfully typed for DHFR codon 108, 29 (69%) carried the S108N mutation and 13 (31%) carried mixed alleles of the codon 108. Codon 51 PCR results were obtained for 43 isolates and 33 (79%) carried the N51I mutation. 8 (19%) carried mixed alleles at DHFR codon 51 and 1(2%) was wild. Of the 42 typed for DHFR codon 59, 34 (81%) had the C59R mutation, 7 (17%) carried mixed alleles and 1 (2%) was wild. Among all the Dedza isolates (N=43) successfully genotypes typed, none had the I164L mutation, which is associated with the highest levels of PYR and cycloguanil resistance.

43 isolates were successfully typed at DHPS codons 437 and 540. For DHPS codon 540, 32 (74%) isolates had the K540E mutation, 3 (7%) were mixed and 8 (19%) were wild. At DHPS codon 437 30 (70%) isolates carried the A437G mutation, 8 (19%) were mixed and 5 (11%) were wild. Between Dedza and Mangochi, the prevalence of single amino acid changes at critical positions of DHFR and DHPS was similar for S108N (P=0.75), N51I (P=0.21), but significantly higher for C59R (P=0.004) in Dedza.

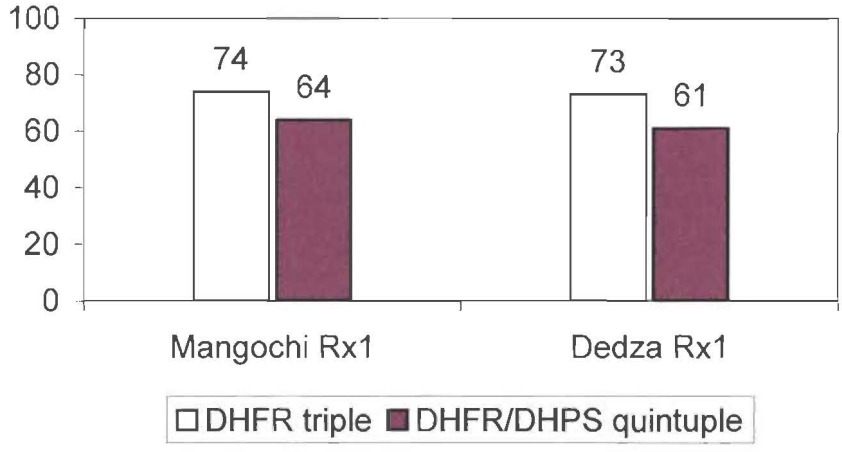


Figure 2.4 Prevalence of DHFR triple and DHFR/DHPS quintuple mutant infections at time of initial treatment. Dedza, N=33; Mangochi, N=61.

The prevalence of the triply mutant DHFR (S108N+C59R+N51I) and the DHFR/DHPS quintuple mutant (S108N+C59R+N51I+ A437G+K540E) was similar between Dedza and Mangochi (73% vs 74%, P=0.91; 61% vs 64%, P=0.86 respectively. See figure 2.4). Significant PYR-SDX selection for the triply mutant DHFR [P=0.002] and the quintuple mutant [P<0.001] was observed in Mangochi (Figure 2.5) and not Dedza (Figure 2.6) probably due to the small sample size. It was, however, apparent that even in Dedza there was a trend towards a higher proportion of resistant DHFR/DHPS genotypes in post treatment compared with pretreatment isolates.

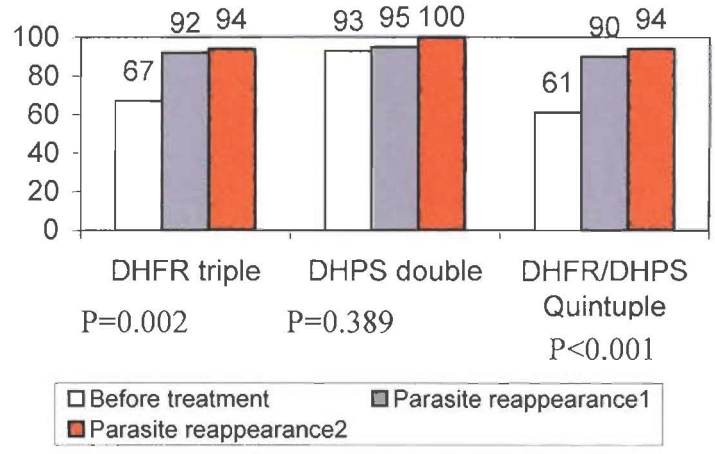


Figure 2.5 Pyrimethamine-sulfadoxine selection of resistant *P.falciparum* DHFR/DHPS genotypes in Mangochi. Parasite reappearance2 represents infections that cleared following second PYR-SDX treatment and subsequently reappeared.

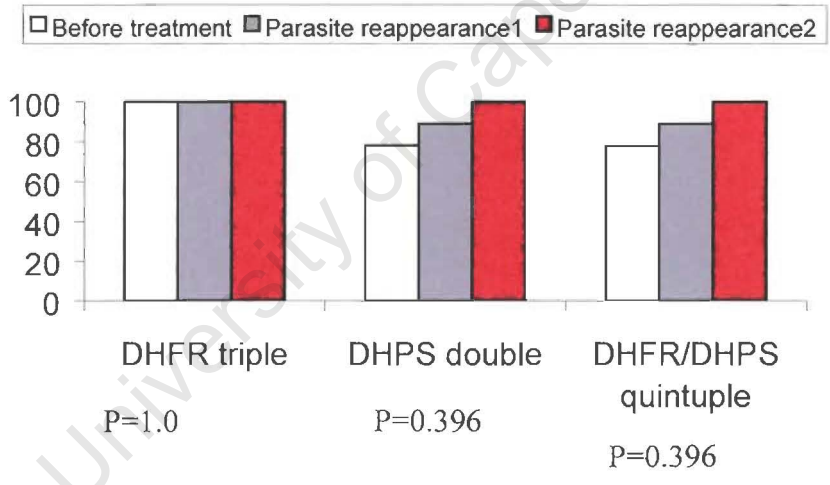


Figure 2.6 Pyrimethamine-sulfadoxine selection of resistant *P.falciparum* DHFR/DHPS genotypes in Dedza. Parasite reappearance2 represents infections that cleared following second PYR-SDX treatment and subsequently reappeared.

2.3.3 Association of DHFR/DHPS mutation with Parasitological failure

In a univariate analysis (Table 2.1), the DHFR/DHPS quintuple mutant was strongly associated with parasite recurrence within 4 – 28 days after initial PYR-SDX therapy ant both sites (Dedza, P=0.004; Mangochi, P<0.001). The triply mutant DHFR was associated with recurrent parasitaemia only in Dedza (Dedza, P=0.039; Mangochi, P=0.173). There was an association between the doubly mutant DHPS (A437G+K540E) and parasite recurrence both in Dedza and Mangochi (P=0.035, P=0.022 respectively).

Site and Year	Genotype	RR	P-value
Mangochi 2000	DHFR triple	1.94	0.173
	DHPS double	*	0.022
	DHFR/DHPS quintuple	*	<0.001
Dedza 2000	DHFR triple	1.70	0.039
	DHPS double	2.0	0.035
	DHFR/DHPS quintuple	1.89	0.004

Table 2.1 Association between DHFR triple & DHFR/DHPS quintuple mutant and parasite recurrence within 4 – 28 days (Univariate analysis). * means value infinitely large

In a multivariate analysis, only the quintuple mutant *Plasmodium falciparum* genotype was independently associated with the increased risk of parasite recurrence within 4 – 28 days following a standard PYR-SDX therapy after adjusting for PYR and SDX blood levels, age, pretreatment parasite density, gender, pretreatment physiological folate levels, study site and haemoglobin level before treatment. Complete results of the multivariate analysis are reported in Table 2.2. High collinearity was observed between the following: pretreatment parasite density and folate; quintuple and folate; pretreatment parasite density and haemoglobin; study site and folate levels. A substantial description of how the multivariate analysis was done is presented on page 84, chapter 4.

Variable	Odds Ratio	95% Conf.	Interval]	P-value
Folate	1.5	1.082469	1.975078	0.013
Day0 Hb	1.6	.287132	8.508683	0.61
Parasitemia	1.0	.9991995	1.000574	0.75
Age	32	1.190903	855.2582	0.039
PYR	0.99	.9771359	.9978644	0.018
SDX	0.98	.9674991	1.002672	0.096
Quintuple	0.0031	.0000419	.2321856	0.0091
DHPS	0.86	.0233219	32.00632	0.94
DHFR	0.00016	6.67e-11	373.1028	0.24
Site	6.0×10^6	73.04442	4.96e+13	0.010
Sex	17	1.684966	179.6801	0.016

Table 2.2 Correlation between DHFR triple & DHFR/DHPS quintuple mutant and parasite recurrence within 4 – 28 days (Multivariate analysis using logistic regression).

2.3.4 Molecular surveillance of drug resistance

The sensitivity and specificity of double mutations (C59A+A437G and C59R+K540E) as predictors of quintuple mutant genotype (S108N+C59R+N51I+ A437G+K540E) in malaria-causing *Plasmodium falciparum* was similar within each study site (Table 2.3).

Mangochi, n=102		DHFR59+DHPS540	DHFR59+DHPS437
	Sensitivity	100 (29/29)	100 (29/29)
	Specificity	77 (56/73)	81 (59/73)
Dedza, n=42	Sensitivity	100 (13/13)	100 (13/13)
	Specificity	59 (17/29)	66 (19/29)

Table 2.3 Sensitivity and specificity of double mutations as predictors of quintuple mutations in malaria-causing *Plasmodium falciparum.*

2.4 DISCUSSION

In a univariate analysis and after adjusting for interactions and confounding factors, this study found that the DHFR/DHPS quintuple mutants were strongly associated with parasite recurrence during 4 – 28 days after a standard treatment with PYR-SDX, both in a high and low transmission setting. This is consistent with a previous study conducted in Blantyre, Malawi (66), which found that infections caused by *P.falciparum* quintuple mutants (S108N+C59R+N51I+ A437G+K540E) were associated with PYR-SDX failure. However, the study herein described, is the first to clearly demonstrate the independent role of DHFR/DHPS genotype in *in vivo* PYR-SDX therapeutic failure with all other important confounding covariates (age, pre-treatment parasite density, pre-treatment Hb, blood PYR-SDX levels, physiological folate levels and transmission intensity) adjusted for.

Results from this study show that mutations in the DHFR/DHPS gene do not wholly explain the observed in vivo PYR-SDX therapeutic failure. For instance, among individuals with ACR status (based on WHO 14-day definition) 83% (25/30) and 48% (14/92) had DHPS genotypes indicative of SDX resistance in Dedza and Mangochi respectively. Similarly for DHFR, it was observed that among individuals with ACR status, 61% (20/33) and 42% (39/93) had DHFR genotypes indicative of resistance to PYR. The observation that such huge proportions of otherwise genotypically SDX-resistant or PYR-resistant isolates should be sensitive in vivo may partially be explained by acquired immunity. Interestingly, this study found that PYR-SDX efficacy at day 14 was higher in the high transmission (Mangochi) than low transmission (Dedza) setting (fig 2.2) despite a similarity not only in the general prevalence of resistant genotypes, but also blood PYR-SDX levels attained in the study subjects (See chapter 2 on results of PYR-SDX kinetics for the Dedza and Mangochi patients). Even at day 28, a higher rate of late parasitological failure was observed in the low compared to high transmission setting despite the similarities mentioned above. A previous study by Baird et al (68,25) showed a strong association between longer histories of exposure to malaria and increased efficacy of sulfadoxine-pyrimethamine, and Marsh et al (77) observed that in

areas where malaria is endemic at high levels year-round, individuals gradually develop partial immunity to malaria disease. Thus, acquired immunity to malaria certainly contributed to the higher therapeutic efficacy of sulfadoxine-pyrimethamine observed in Mangochi.

The observed higher rate of late parasite reappearance between day 4 to 28 is less likely to be due to reinfections in such a setting of low malaria transmission. In addition to Dedza being a low transmission area, the study was conducted during the dry season when malaria transmission is usually moderate. Under these conditions, the frequency of reinfection would be expected to be higher in Mangochi than Dedza. Thus, if the observed late parasite reappearance (or LPF) at both sites were due to reinfections, it would be expected that the LPF rate would be higher in Mangochi (high transmission) than Dedza (low transmission). Figures 2.2 and 2.3 shows that PYR-SDX efficacy was much lower when treating cases (LPF) that had failed the initial PYR-SDX therapy. This, plus the absence of obvious evidence for drug selection of resistant DHFR/DHPS strains when the prevalence of resistant genotypes at the time of second treatment and after that second treatment [i.e. parasite recurrence1 versus parasite recurrence2] was compared, suggested that the parasite population at the time of retreatment was predominantly recrudescent (figures 2.5 and 2.6).

In Mangochi, among individuals with the LPF outcome, 86% (43/50) carried *P.falciparum* genotypes indicative of PYR sensitivity and only 22% (11/49) had genotypes predictive of SDX sensitivity. Among individuals with an adequate clinical and parasitological response (ACPR) status, 28% (15/53) had *P.falciparum* genotypes indicative of PYR sensitivity and 81% (43/53) carried genotypes indicative of sensitivity to SDX. While the observation of significantly higher proportions (78% versus 19%, $P<0.0001$) of genotypically SDX-resistant strains in treatment failures than ACPRs was expected, the observation of significantly higher prevalence of *falciparum* DHFR genotypes predictive of PYR sensitivity (86% versus 28%, $P<0.0001$) in individuals who experienced parasite recurrence within 4 – 28 days of treatment with PYR-SDX than those that had an ACPR status was unexpected. The potential contribution of drug levels

in causing this unexpected result is discussed under 'Host-blood pyrimethamine-sulfadoxine levels and in vivo efficacy' in chapter 3.

The significantly higher rate of parasite recurrence within 4 – 28 days (or reduced PYR-SDX clinical efficacy based on 14-day follow-up) observed in Dedza compared with Mangochi (P=0.010) might, in part, be due to the 'folate effect' (7,19). The folate effect is an observation in *in vitro* studies that some *P.falciparum* strains seem to have the capacity to utilize exogenous folate thereby antagonizing the effects of sulfadoxine on DHPS (22,26). Incidentally, this thesis study found significantly higher physiological folate levels among Dedza subjects compared with their Mangochi counterparts (See folate results in chapter 4) after adjusting for possible confounders. Also in particular measured blood folate levels were generally higher in cases with LTP (i.e parasite recurrence within 4 – 28 days of initial PYR-SDX therapy) than those that had an ACPR outcome (p=0.028, see figure 4.2, n=141).

The fact that the present study found 100% prevalence of DHPS genotypes indicative of SDX resistance in individuals who experienced recurrent parasitemia both in Dedza and Mangochi, suggested that if there was any drug efficacy difference due to differences in intensity of the folate effect, then it (the folate effect) might not be acting via the DHPS gene whose product is antagonized by SDX. This seems to support the findings of Wang et al (7) where the folate effect did not segregate with dihydropteroate synthase genotypes in a genetic cross between parent clones, one exhibiting the folate effect and one not. It was shown that the folate effect could be completely abolished by the addition of pyrimethamine to assay cultures at concentrations lower than those needed to inhibit dihydrofolate reductase (19). Also the folate effect showed complete linkage to the dihydrofolate reductase gene in the cross progeny, but was not linked with dihydrofolate reductase sequence in other, unrelated parasite lines, suggesting that a gene encoding the folate effect is located near but not at the dihydrofolate reductase locus on chromosome 4 (7).

Since PYR is capable of abolishing the folate effect through acting on some site closely linked to the DHFR gene, it is possible that the unexpected finding with respect to PYR might also be explained, in part, by the finding of higher blood folate levels in individuals with LPFs than ACPR status (32ng/ml vs 27ng/ml, p=0.014). It is interesting to note that the unexpected result with respect to PYR was not observed in Dedza where folate levels were similar between LPFs and ACPRs (39 ng/ml vs 38 ng/ml, respectively, p=0.76). It is possible there is an upper limit for blood folate concentrations below which PYR can abolish the folate effect. Thus, it might be that above folate-level cutoff, the action of PYR in abolishing the folate effect is more than offset by high folate levels resulting in the restoration of *falciparum* capacity to utilize the available exogenous folate. Under such conditions of characteristically high exogenous folate, it would not be unexpected to observe a higher prevalence of otherwise genotypically PYR-sensitive *falciparum* DHFR among subjects with the treatment failure (LPF) compared to ACPR status.

In this project, it was found that blood pyrimethamine levels were independently predictive of parasite reappearance within 4 – 28 days of initial PYR-SDX therapy (Table 2.2) whereas sulfadoxine was not significantly correlated. This finding underscores the relative importance of PYR in the in vivo PYR-SDX efficacy. It will be seen in chapter 3 that blood SDX levels in patients, immediately after day 3 post-treatment, dropped below concentrations required for synergy with PYR. Such a loss of synergy between PYR and SDX might have resulted in the observed high prevalence of otherwise genetically PYR-sensitive *falciparum* strains in the LPF outcome category.

The DHFR mutation I164L has been correlated with high rates of PYR-SDX therapeutic failure in South America and South East Asia (64). This study did not detect the I164L mutation in any of the isolates that were genotyped. The absence of this mutation 7 years after widespread use of the combination pyrimthamine-sulfadoxine combined with the apparent high rate of PYR-SDX efficacy (~80% at 14 days) in the face of a prevalence of the quintuple mutant of 63%, predicted that this drug would have a longer useful therapeutic life than previously anticipated. Indeed recently, Plowe et al (Submitted to BMJ, May 2003) were the first to observe that after approximately ten years of

widespread use of PYR-SDX in Malawi, the drug's therapeutic efficacy rate remains at ≥ 80%. Continued molecular surveillance of drug resistant *P.falciparum* is necessary to the understanding of the molecular mechanisms underlying resistance to antifolates. The observation of the slow rate of evolution of resistance in a high-transmission country like Malawi (in contrast to a low-transmission area like Thailand) may be partly due to the fact that in Malawi most P falciparum infections, being symptomless, do not attract treatment – therefore the proportion of the country's parasite biomass that is exposed to the drugs is much less than in Thailand.

Finding a set of mutations that can accurately predict treatment failure would greatly simplify the molecular surveillance of drug resistant malaria. A model that uses a molecular marker for chloroquine resistance was developed and used to relate the prevalence of the molecular markers to the prevalence of treatment failure (78). Although this thesis found that the mutation combinations C59R + A437G or C59R + K540E could equally predict the presence of quintuple mutations with 100% sensitivity but relatively low specificity of 71%, much larger studies by Kublin et al (66) showed, for the first time, that the presence of the single DHPS mutation K540E with a single DHFR mutation C59R accurately predicted the presence of the quintuple mutant, which is itself strongly and independently associated with PYR-SDX treatment failure. Findings from the present thesis therefore validate, at two independent sites, the use of the later combination as surrogate markers of the quintuple mutant, and they could therefore be used to predict the rate of PYR-SDX efficacy in Malawi.

This study confirms the association of the presence of the *P.faciparum* DHFR/DHPS quintuple mutant at the time of treatment with PYR-SDX failure (based on 28-day follow-up) and is the first to independently confirm the practicality of Kublin's (66) finding that a single DHFR and a single DHPS mutation can indeed be used to predict the presence of the quintuple mutant, which is associated with PYR-SDX therapeutic failure at least in Malawi. Such ability, if validated in other African countries, would be a simple and rapid molecular surveillance tool for PYR-SDX resistance.

The observation that PYR-SDX selects for DHFR/DHPS mutants (figures 2.5 and 2.6) is not new and with the well known observation that the prevalence of resistant *P.falciparum* genotypes generally exceeds the prevalence of in vivo resistance, this study is the first to perform a comprehensive analysis of the relative role of key factors (such as parasite genetic mutations, host folate levels, drug pharmacokinetics) in in vivo PYR-SDX therapeutic failure. It is herein shown that DHFR/DHPS quintuple mutants remain strongly associated with PYR-SDX failure after adjusting for all aforementioned key factors. Particularly, it has been for the first time shown that physiological (not through folic acid supplementation) blood folate levels influence the sensitivity of *P.falciparum* to PYR-SDX in vivo.

2.5 STUDY SHORTFALLS

A major shortfall in this thesis is the exclusion of work on molecular typing to distinguish recrudescence from re-infecting parasites among the LPF (i.e parasites that were detected between 4 – 28 days after treatment). It is generally agreed that reinfection in endemic areas are not a rare event so that during 28 days of follow up re-infections can not be wholly ruled out. On the other hand, the observed dramatic reduction in PYR-SDX efficacy from 90% to 57% in Mangochi and from 78% to 22% in Dedza (Fig.2.2 and 2.3) coupled with the sudden occurrence of early treatment failures at both sites (Mangochi ETF = 2% and Dedza ETF = 11) where there were none following the initial therapay, constitute a clinically direct and biochemically indirect indication that the second PYR-SDX therapy might have been acting on a parasite population predominantly recrudescent from the previous treatment. It was therefore felt that the aforementioned omitted work would not significantly alter the results reported in this thesis.

CHAPTER 3

THE *IN VIVO* PHARMACOKINETICS OF PYRIMETHAMINE-SULFADOXINE AND OBSERVED CLINICAL EFFICACY IN MALAWIAN CHILDREN

3.0 BLOOD DRUG LEVELS IN MALARIA PATIENTS

3.1 DEVELOPMENT OF ASSAY FOR RECOVERY OF DBS DRUGS

Various liquid chromatographic methods for the determination of pyrimethamine and sulfadoxine in plasma have been described (79,80,81). Whole blood concentrations of pyrimethamine and sulfadoxine are independent of variations of the erythrocyte to plasma drug concentration ratio, which may appear during treatment (82). Measuring PYR and SDX in whole blood may thus be more relevant in assessing the antimalarial efficacy of pyrimethamine-sulfadoxine. A chromatographic method for quantifying PYR and SDX in whole blood (82) and one quantifying PYR in whole blood blotted on filter paper (DBS) have been described (83). In the method described by Bergqvist, the limit of SDX determination using 100 µl of capillary blood was found to be 7.8 µg/ml (84). The observed achievable blood levels observed after administration of a single standard dose of pyrimethamine-sulfadoxine ranges from 50 – 750 ng/ml and 15 – 100 µg/ml respectively when followed over a period of 10 days (85). In that study (85) PYR-SDX were recovered from plasma samples spotted on filter paper and quantitated by high-performance liquid chromatography. Recently Michael et al. described solid-phase extraction and an HPLC assay for simultaneous determination of SDX and PYR (86). However, extraction recoveries for both drugs were only about 60% and the limit of quantitation for PYR was only 1 µg/ml, well above the therapeutic range for the drug, 25 – 150 ng/ml.

In this thesis, SDX and PYR were measured separately from dried blood spots on filter paper using a method that combines the advantage of DBS collection (simple, economical) and the ability to detect whole blood PYR and SDX levels, obtained by venipuncture and dried on filter paper, in the observed therapeutic concentration range. In this method, the practically convenient drug retention times (<10 min), the higher drug extraction recoveries combined with the fact that after elution of the drugs from DBS no solid phase extraction was required, offer a feasible method for reliably measuring SDX and PYR separately in DBS. Additionally, PYR determination in DBS was done in a

smaller volume of capillary blood than a previously described (100 µl versus 150 µl) method (83).

3.1.1 MATERIALS AND METHODS

3.1.1.1 REAGENTS AND CHEMICALS

A mobile phase of HPLC grade acetonitrile (BDH Laboratory Supplies) and HPLC grade water purified through a Millipore ion exchange system was filtered using a 0.45 µm filter (Millipore HALP filter disk) and subsequently degassed by bubbling helium gas for ten minutes at ambient temperature. Sulfadoxine was kindly obtained as a gift from Wellcome Trust Laboratories, College of Medicine in Blantyre, Malawi. Pyrimethamine and the sodium salt of 1-heptane-sulfonic acid (1HSA) were obtained from Sigma. Trifloroacetic acid (TFA) was from Merk-Schuchardt. n-hexane was from Scharlau Chemie S.A. Ethanol and ethylacetate were obtained from Merck Laboratory supplies. Sodium hydroxide, concentrated HCL, chloroform and anhydrous sodium acetate were from BDH Laboratory supplies. Ammonia was from Saarchem (PTY) Ltd. Whatman 3MM filter paper was from Whatman International Limited. Calibrated capillary tubes (100µl) were from the Drummond Scientific Company.

3.1.1.2 APPARATUS

Two solvent pumps (Shimadzu LC-10AS liquid chromatograph pumps) were connected to an automatic injection valve (SIL-10A Shimadzu Auto Injector, model SIL-10A) equipped with a variable injection loop linked to a UV Diodearray Detector (Shimadzu, model SPD – M10A). The detector was coupled to a Professional Computer (Professional Computer Manufacturers, model 486 GV), which was linked to a printer (Hewlett Packard DeskJet 670c). The computer recorded and stored all charts/chromatograms. A Phenomex 5µm C18 reverse-phase analytical column 150× 4.6 mm was used for the sulfadoxine assay with a C18 guard column placed before it to prolong its lifespan. The UV detector was set at a wavelength of 254 nm for both sulfadoxine and pyrimethamine.

Detector sensitivity was 150mAbs and 6mAbs (A.U.C) for SDX and PYR respectively. A Haisil 100 C8 5μm analytical column 250 × 4.6mm was used for PYR analysis. All liquid chromatographic determinations of the drugs SDX and PYR were performed utilizing the above-mentioned apparatus.

3.1.1.3 EXTRACTION OF PYRIMETHAMINE FROM DBS OF WHOLE BLOOD

To the filter paper DBS snippet in a glass test tube 3.5 ml of NaOH (1M) were added and incubated for 15 minutes at ambient temperature with intermittent vigorous vortexing (three times for 1 minute). Chloroform (5.0 mL) was then added and PYR was extracted into the organic phase as a neutral base with vigorous agitation (1 min). The aqueous phase containing the DBS was extracted a second time with chloroform and the combined chloroform extract evaporated to dryness with an RC10.10 vacuum rotary evaporator connected to a cold trap (-180 ^{o} C) and a pump. The residue was resuspended in 120 μl acetonitrile (MeCN). 50 μl of the extracted PYR was injected directly into the chromatographic column. The mobile phase was 30mM acetonitrile + 1-heptane-sulfonic acid (65 + 35, v/v), pH 2.68 at a flow rate of 0.5mL/min, and absorbance at 254nm. Spiking both whole blood and the mobile phase with known concentrations that covered the range 1 μg/ml to 0.015 μg/ml, and drying 100 μl aliquots on filter paper prepared calibration samples.

3.1.1.4 EXTRACTION OF SULFADOXINE

To extract SDX from DBS the method described by Winstanely et al (85) for its recovery from plasma spotted on filter paper was adopted and modified. The filter paper strips blotted with whole blood spiked with known SDX concentration were cut up, sonicated in acetate buffer (0.1 M; pH 3.48; 15 min), and extracted by vortexing (1 min), into hexane/ethyl acetate (1:1, v/v; 5 ml) in glass test tubes. This extraction phase was done twice. After centrifugation (4000 rpm, 15 min) the pooled organic phase was transferred to clean tubes and evaporated to dryness as described for PYR. Samples were

reconstituted in mobile phase (120 µl acetonitrile/water, 1:1, v/v) and 50 µl aliquots injected into the chromatograph at a flow rate of 0.5 ml/min with detection at 254nm. Calibration samples were prepared by spiking both whole blood and the mobile phase with known concentrations that covered the range 200 µg/ml to 12.5 µg/ml, and drying 100 µl aliquots on filter paper.

3.1.2 PRACTICAL APPLICATION OF THE METHODS

In order to demonstrate that the developed methods are of practical use the drug kinetics for 7 cases of uncomplicated malaria were characterized in a pilot study. These cases were given a standard dose of pyrimethamine-sulfadoxine and actively followed up to 28 days in an ongoing PYR-SDX in vivo efficacy study in Malawi. 100µl of capillary blood was collected onto filter paper at each of the post-treatment days 3, 7, 14, 21, 28.

3.1.3 ANALYSIS OF DATA

Data for AUC-Drug Concentration curves were fitted in a regression analysis using GraphPad Prism® (GraphPad software version 2.01). P-values were calculated using the non-parametric method of Mann-Whitney. Errors were reported as standard error of mean. Recovery was calculated using spiked controls and standards in mobile phase. %Recovery = {(Sample Peak area) ÷ Standard in mobile phase Peak area}× 100. A minimum of 5 determinations per concentration level was used to assess precision and accuracy. At each level in one run (within-run) the mean value had to be ≤ 15% deviation from the nominal concentration for that point.

3.1.4 RESULTS OF ASSAY DEVELOPMENT

SDX and PYR retention times were 6.7 min and 4.3 min respectively, and there was no evidence of significant interference at those retention times as determined from the predosing HPLC trace.

3.1.4.1 SULFADOXINE

A mobile phase of MeCN/water in the ratio 75:25 (v/v) resulted in chromatograms that exhibited good selectivity, separation and resolution. Extraction of SDX from DBS was found to be critically dependent on the inclusion of a sonication step during incubation in acetate buffer. Omission of sonication led to poor recoveries at lower concentrations. The assay was linear (r^2 = 0.994, Table 3.1) in the concentration range 12.5 –200 µg/ml. Overall SDX mean % recovery was 77± 3.2%, inter- assay CV = 12.2% and, mean intra-assay CV was 13.5% for the range (Table 3.1).

	Concentration µg/ml	Inter-assay C.V. (%)	Intra-assay C.V. (%)	Recovery (%±SE)	R^2
Sulfadoxine	200	8.3	9.3	82 ± 2.4	0.994
	100	11.7	13.8	80 ± 3.3	
	50	11.9	12.7	67 ± 3.6	
	25	16.2	18.8	82 ± 5.9	
	12.5	13.3	13.3	70 ± 3.8	
Pyrimethamine	1.000	6.5	9.7	101 ± 3.0	0.998
	0.500	11.9	14.6	96 ± 5.1	
	0.250	11.5	8.8	91 ± 4.3	
	0.125	8.9	17.1	85 ± 3.1	
	0.0625	16.9	13.7	72 ± 6.1	
	0.0313	12.9	14.0	63 ± 5.5	
	0.0151	-	-	ND	

Table 3.1 Percentage recoveries of sulfadoxine and pyrimethamine from 100 µl of whole blood, which was spotted onto a filter paper. (SDX Mean recovery =77 ± 3.2%, C.V.=9.4; Mean PYR recovery=85 ± 6.0%, C.V.=11%). ND = not detected.

SDX STABILTY

The stability of a drug can be influenced by several parameters including pH, heat, light, gases and storage time. Because the DBS preparations collected in the field must be dried overnight at ambient temperature, heat was the most relevant parameter to be investigated. Thus SDX DBS specimens were stored at three temperatures 4°C, 37°C and room temperature (~22°C) for a period of one month to assess the rate of degradation. All DBS specimens had the same SDX concentration (200µg/ml). Weekly, one sample from each storage condition was extracted and the sulfadoxine and pyrimethamine in the extract quantitated. Over this period, no evidence of significant loss of SDX was observed when amount of parent SDX remaining was compared with that recovered from freshly made and extracted DBS (Table 3.2) at the expiration of the storage period.

Condition	Obs	Mean(µg/ml)	Std. Dev.	Min	Max	P-value
Fresh	4	191.0	12.05	178.6	206.9	
Room Temp	4	183.9	21.85	164.5	213.9	0.65
4 Deg C	4	159.2	29.03	122.2	192.7	0.096
37 Deg C	4	198.0	51.34	122.2	235.1	0.77

Table 3.2: Mean SDX recoveries at the three temperatures over 21 days under different storage conditions.

3.1.4.2 PYRIMETHAMINE

Acceptable PYR solubility was obtained in MeCN/1HSA/5%HCL (2:2:1, v/v/v). Serial dilutions in the range 1 µg/ml – 0.015µg/ml injected into HPLC resulted in a good linearity (r^2 = 0.9972, Table 3.1).

Adopting the method described by Winstanely (85) for the extraction of PYR from plasma spotted on filter paper did not work under our laboratory conditions. Non-linearity and low drug recoveries were encountered. It was found that incubation in 1M sodium hydroxide followed by extraction into chloroform gave excellent recovery of the

drug. To check the PYR extraction efficiency from DBS and its reproducibility in this protocol, samples were prepared with each dried blood spot comprising 100 µl of whole blood at a range of PYR concentrations. The percentage recoveries for PYR are reported in Table 3.1. The inter-assay coefficient of variation (CV) at 1 µg/ml is 6.5% and at 31 ng/ml it is 12.9%. Overall PYR % recovery was 85.67 ± 6.0% in the concentration range considered. The mean CV for the recoveries in the entire concentration range was 11%.

PYR STABILITY

In PYR stability studies all DBS specimens had the same PYR concentration (1µg/ml). Weekly one sample from each storage condition was extracted and the pyrimethamine in the extract quantitated. Over this time period the average amount of pyrimethamine remaining in the filter paper specimen after 4 weeks was determined in 50 µl of reconstituted DBS extract. Over this period, no evidence of significant loss of PYR was observed when amount of parent PYR remaining was compared with that recovered from freshly made and extracted DBS (Table 3.3) at the expiration of the storage period.

Condition	Obs	Mean(ng/ml)	Std.Dev.	Min	Max	P-value
Fresh	4	1029	233.6	711.7	1204	
Room Temp	4	1099	219.0	852.5	1345	0.68
4 Deg C	4	976.0	67.42	922.9	1063	0.67
37 Deg C	4	1011	225.5	852.5	1345	0.92

Table 3.3: Stability data for pyrimethamine over a period of three weeks

3.1.5 METHOD APPLICATION TO IN VIVO STUDY SAMPLES

These methods were used to determine SDX and PYR levels in patients a sample of patients with uncomplicated malaria, who received a standard oral dose of PYR-SDX (1.25 mg pyrimethamine and 25 mg sulfadoxine per kg of body weight) to establish feasibility of methods for field samples. PYR-SDX kinetics was as expected from an oral route of administration in such cases. Observed drug concentrations were in the range

found by previous independent studies using different methods (Winstanley et al 1992 [85]). Few studies have been done on PYR-SDX disposition in malaria patients. Figures 3.1, 3.2 and 3.3 show the mean concentration-time curves of pyrimethamine and sulfadoxine.

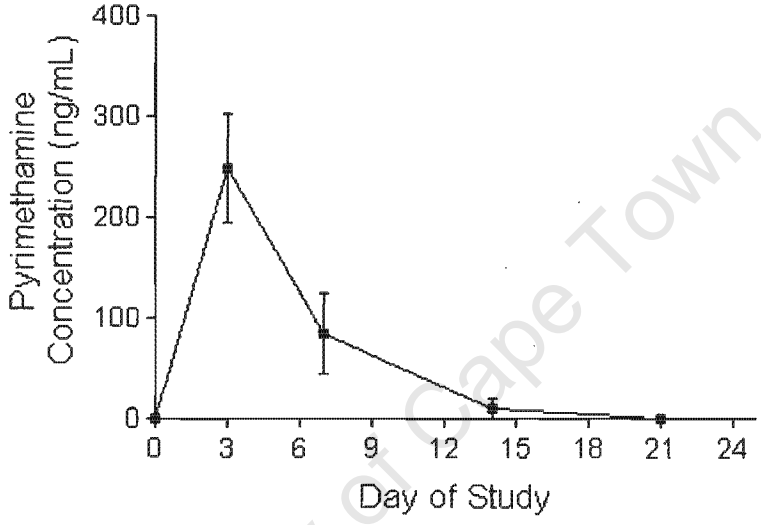


Figure 3.1 Average blood pyrimethamine levels for patients with uncomplicated malaria (N= 7, a random selection) following a supervised pyrimethamine-sulfadoxine standard oral dosing (1.25 mg/kg PYR + 25 mg/kg SDX). The median age for patients was 12.5 months (range11 – 26 months); median weight was 10 kg (range 8 – 10.5 kg). Two were male and the other two female.

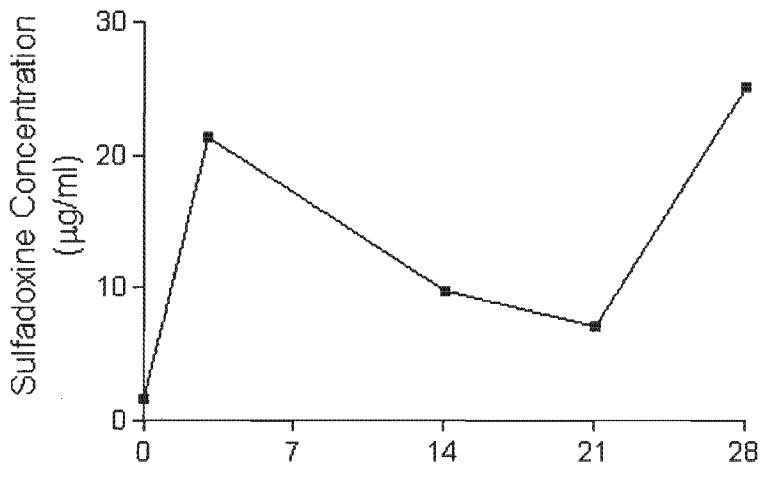


Figure 3.2 The concentration-time profile for sulfadoxine in a malaria patient following the administration of a standard oral dose (1.25 mg/kg PYR + 25 mg/kg SDX) of pyrimethamine-sulfadoxine. The patient was retreated with the same drug for presenting with parasitaemia on day 21 after intial PYR-DSX therapy. The patient was male, had a body weight of 12 kg and was 33 months old.

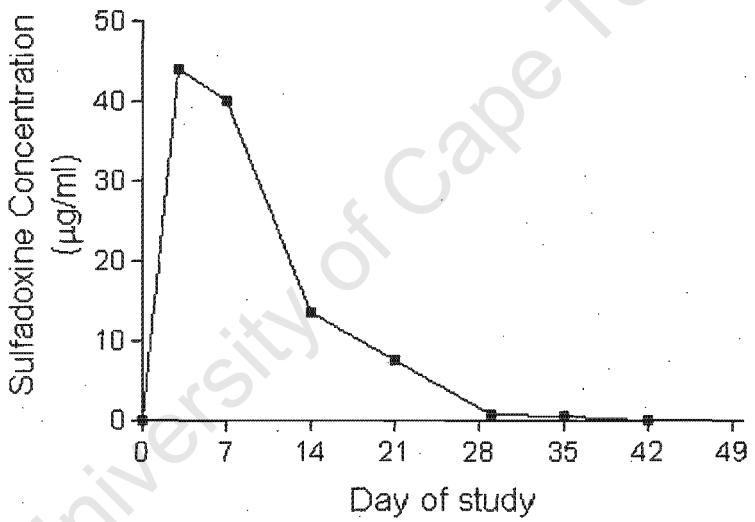


Fig. 3.3: The concentration-time profile for sulfadoxine in a malaria patient following the administration of a standard oral dose (1.25 mg/kg PYR + 25 mg/kg SDX) of pyrimethamine-sulfadoxine. The patient was male, had a body weight of 10 kg and was 21 months old.

	Actual Drug concentration	Measured Drug concentration ± SD	Min – Max
PYR in spiked Dried Blood Spot (ng/mL)	1000 (N=4)	809.9 ± 44.57	743.4 – 836.1
	31 (N=3)	28.75 ± 1.394	27.77 – 29.74
SDX in spiked Dried Blood Spot (µg/ml)	200.0 (N=3)	200.7 ± 26.20	164.5 – 226.4
	6.125 (N=3)	5.929 ± 0.5021	5.545– 6.497
	3.063 (N=3)	2.378 ± 0.7563	1.892 – 3.249

Table 3.4 Accuracy of pyrimethamine recovery from DBS

Results of studies on accuracy of the methods for SDX and PYR recovery from filter paper are shown in Table 3.4. Randomly chosen representative SDX and PYR chromatograms are reported in the figures below: SDX chromatograms for a malaria patient before (Figure 3.4a) and after taking (Figure 3.4b) the standard oral dose of pyrimethamine-sulfadoxine. Figure 3.4 shows the PYR chromatograms for a malaria patient before (3.4c) and after taking (3.4d) the standard oral dose of pyrimethamine-sulfadoxine.

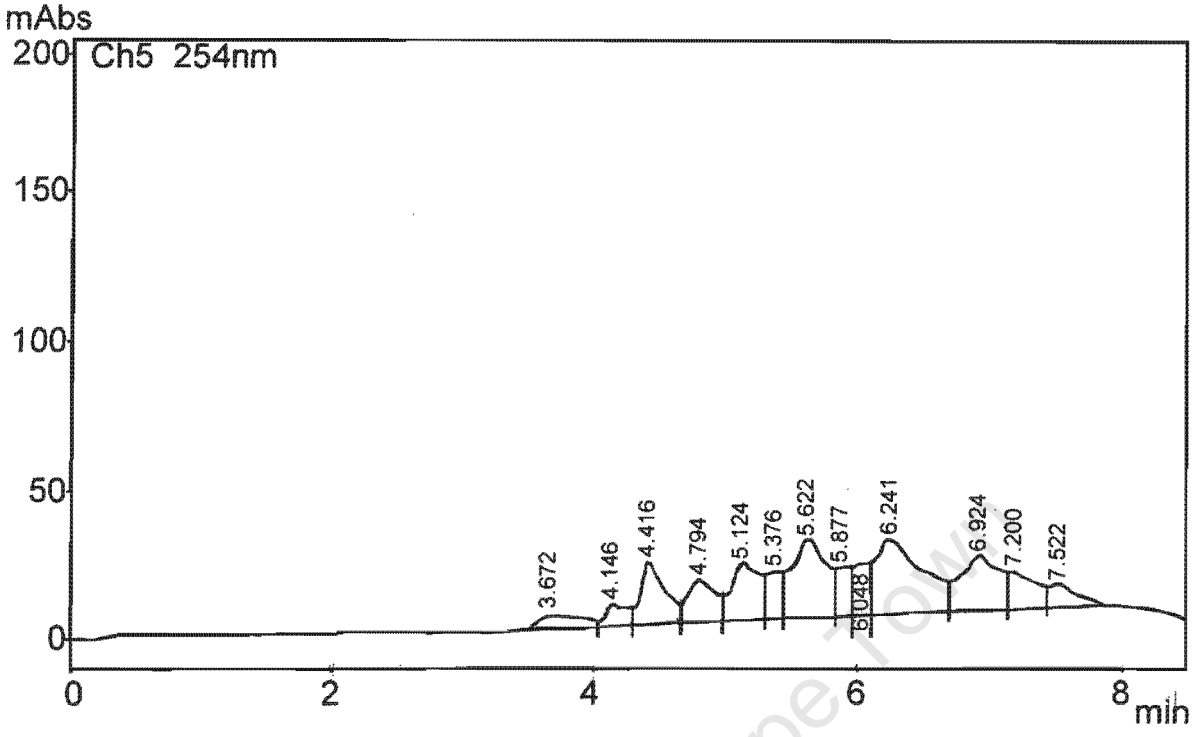


Figure 3.4a Chromatogram of DBS extract from a malaria patient before taking a standard oral dose of pyrimethamine-sulfadoxine. Dried blood spot was extracted with method for SDX recovery from filter paper. SDX (RT) = 6.7 min.

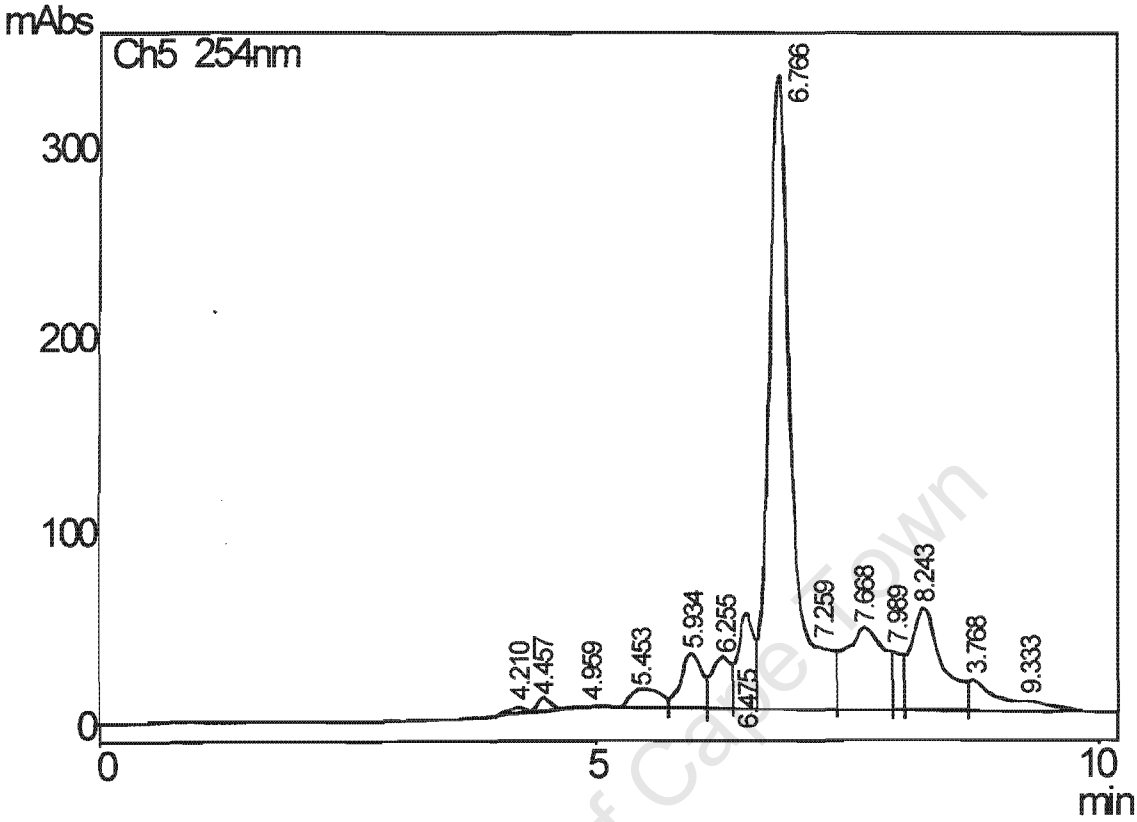


Figure 3.4b Chromatogram of DBS extract from a malaria patient after taking a standard oral dose of pyrimethamine-sulfadoxine. Dried blood spot was extracted with method for SDX recovery from filter paper. SDX (RT) = 6.7 min.

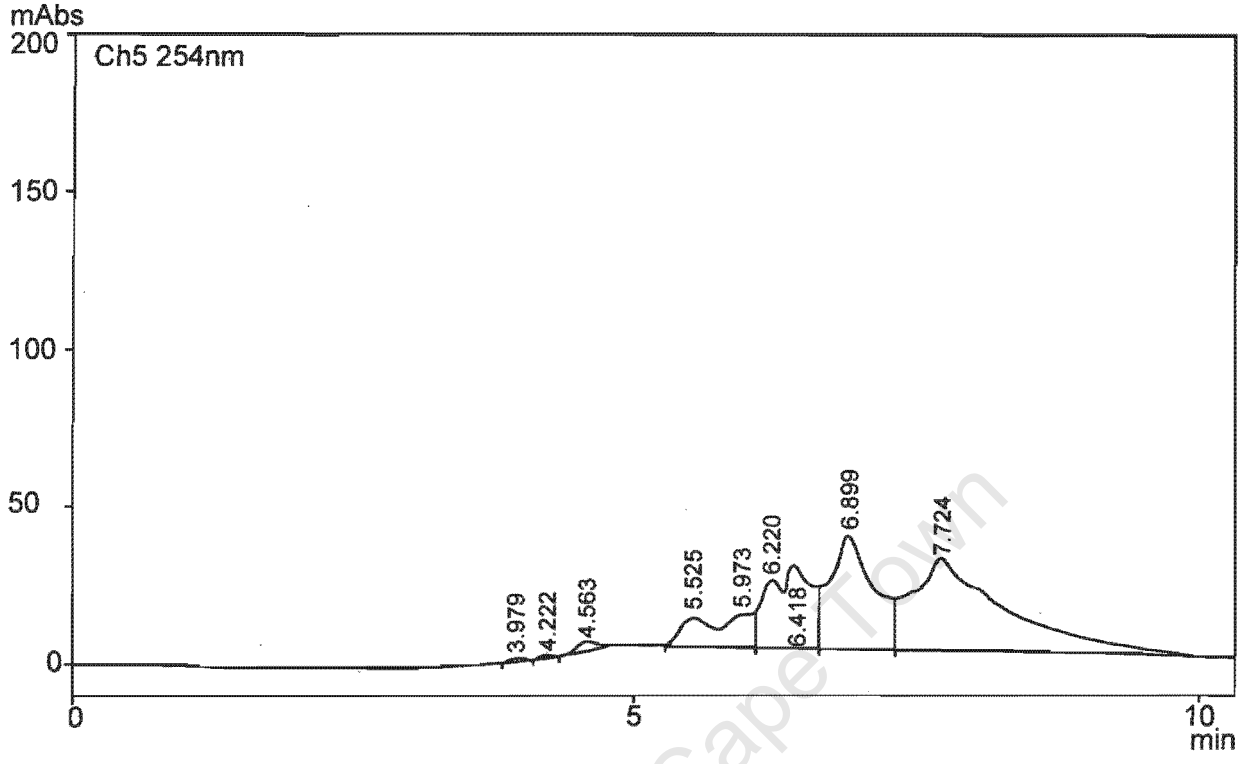


Figure 3.4 c Chromatogram of DBS extract from a malaria patient before taking a standard oral dose of pyrimethamine-sulfadoxine. Dried blood spot was extracted with method for PYR recovery from filter paper. Highlighted area is PYR (RT) = 4.3 min.

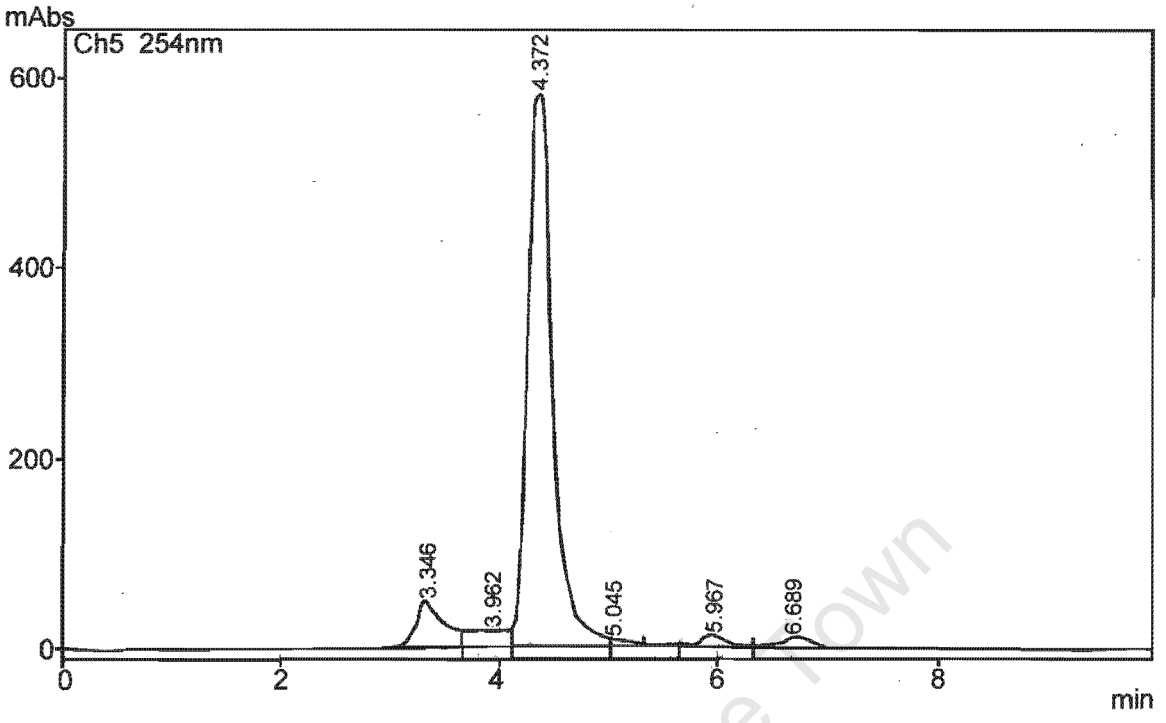


Figure 3.4d Chromatogram of DBS extract from a malaria patient after taking a standard oral dose of pyrimethamine-sulfadoxine. Dried blood spot was extracted with method for PYR recovery from filter paper. PYR (RT) = 4.3 min.

3.2 SDX AND PYR BLOOD LEVEL MEASUREMENTS FROM THE IN VIVO SP EFFICACY STUDY IN MALAWI

3.2.1 SUBJECTS AND STUDY DESIGN

166 (80 females, 86 males) and 51 (24 females, 27 males) children with uncomplicated *falciparum* malaria were enrolled in *in vivo* therapeutic efficacy studies in Mangochi and Dedza respectively. Figure 4.7 in chapter 4 shows why of the 209 enrolled subjects only 171 completed the study and were eligible for analysis. Demographic data of the study participants is summarized in Table 3.5. Subjects gave written informed consent before participation in the study, which was approved by the independent ethics committee of the University of Malawi Medical School and the University of Cape Town.

Venous capillary blood samples (100 µL) were spotted on Whatmann No.3 filter paper and immediately air-dried on a clean bench. The blood specimens were collected as follows: before dosing and 3, 7, 14, 21and 28 days after a single oral PYR-SDX dose (1.25 mg/kg PYR+ 25 mg/kg SDX). The air-dried filter papers were stored at 4 °C until analysis. The HPLC assay described in section 3.1 was used to determine the blood concentrations of PYR and SDX.

	DEDZA (N=50)	MANGOCHI (N=159)
Gender	Male 27 Female 24	Male 86 Female 80
Age (months)	13.0 – 120 (35.9)	6 – 100 (28.4)
Body Weight (Kg)	7 – 23 (11.2)	6 – 26 (10.6)
Day 0 Parasitemia (parasite/µL)	2065 – 57180 (13,704)	2083 – 33850 (9534)
Day 0 Haemoglobin (g/L)	6 – 11 (7.3)	3 – 12 (8.2)
Axilliary tempertature (°C)	35.5 – 40.7 (38.4)	36 – 41.3 (38.4)
Dose (mg, single and oral)	250 – 750 (342)	250 – 3752 (358)

Table 3.5 Summary of whole patient population demographic characteristics (i.e all study participants). Data are range (mean) Statistical comparison: age (p=0.12), parasitemia (p=0.013), Haemoglobin (p=0.10).

3.2.2 PHARMACOKINETICS AND STATISTICAL ANALYSES

Mean values of C_0 were obtained by back extrapolation of the log-linear portion of the concentration-time profile. As PYR-SDX blood concentrations peak within 2 – 3 hours of administration, days 3, 7, 14, 21 and 28 were considered data points in the terminal phase. Data points day 3, day 7, day 14 and day 21 were used to calculate the terminal rate constant (λ_z) by log-linear regression. The drug half-life ($t_{1/2}$) was estimated as $\ln 2/\lambda$. The area under the concentration-time curve from time 0 to infinity ($AUC_{0-\infty}$) and from time 0 to the last quantifiable concentration observed at time t (AUC_{0-t}) was estimated by linear trapezoidal rule.

Noncompartmental analysis with WinNonlin V4.0.1 (Pharsight Corporation, Mountain View, Califonia, USA.) was used to obtain pharmacokinetic parameters. Blood concentrations below the limit of quantitation of the assay were set to a value of 0.

Differences in PYR-SDX blood concentrations and pharmacokinetic parameters between treatment outcomes, sites and gender were assessed by logistic regression analysis.

3.2.2.1 RESULTS

Neither SDX nor PYR was detected by HPLC analysis in any of the subjects on day 0 (pretreatment) samples. Patient demographic characteristic were similar between and within sites (Table 3.5).

3.2.3.1 SULFADOXINE

The blood SDX concentration-time curves (presented as mean ± SE) for ACRs and TFs are illustrated in figure 3.4e (See concentration-time data in the summary tables 3.7 and 3.8). Sensitive (ACPR) cases had similar blood sulfadoxine concentrations compared to cases who failed (LTF) PYR-SDX therapy (At day 3: 76.6 µg /ml vs 68.5 µg /ml, P=0.68; at day 7: 40.9 µg /ml vs 35.6 µg /ml, P=0.31; at day 14: 25.1µg /ml vs 20.9 µg

/ml, P=0.34; at day 21: 14.5µg /ml vs 14.6 µg /ml, P=0.80 for ACPR and LTF respectively). The concentration of SDX apparently declined with a first order process in all subjects (Dedza and Mangochi) as illustrated by the semilogarithmic plot of the concentration-time data for the two sites (Figures 3.5 and 3.6).

Summary Table 1

Time day	Conc. ug/mL	SEM ug/mL	Residual ug/mL	AUC day*ug/mL	AUMC day*day*ug/mL	Weight
0.0000	0.0000	0.00		0.0000	0.0000	
3.000 *	70.48(n=33)	11.18	8.881	105.7	317.2	1.000
7.000 *	42.29(n=27)	6.119	-5.984	331.3	1332.	1.000
14.00 *	29.04(n=25)	4.940	-2.470	580.9	3791.	1.000
21.00 *	22.27(n=19)	4.097	1.697	760.5	6851.	1.000
28.00	28.89(n=15)	4.156		939.6	1.132e+004	

Blood SDX concentration-time data from Dedza (n=35). Starred points were used for determination of rate constant associated with the terminal elimination phase. Under the concentration column figures in brackets are subjects used to obtain the mean concentration mean concentration at that time point. Note that the number of cases with detectable drug concentration per time point decreased with increasing post-treatment time.

Summary Table 2

Time day	Conc. ug/mL	SEM ug/mL	Residual ug/mL	AUC day*ug/mL	AUMC day*day*ug/mL	Weight
0.0000	0.0000			0.0000	0.0000	
3.000 *	69.78(n=94)	4.366	7.337	104.7	314.0	1.000
7.000 *	35.44(n=76)	2.407	-5.724	315.1	1229.	1.000
14.00 *	20.13(n=59)	1.979	0.2752	509.6	3084.	1.000
21.00 *	9.821(n=29)	1.127	0.2412	614.5	4792.	1.000
28.00	28.60(n=27)	4.930		748.9	8317.	

Blood drug concentration-time data from Mangochi (n=98). Starred (*) points were used for determination of rate constant associated with the terminal elimination phase. Under the concentration column figures in brackets are subjects successfully used to obtain the mean concentration at that time point. Note that the number of cases with detectable drug concentration decreased with increasing post-treatment time.

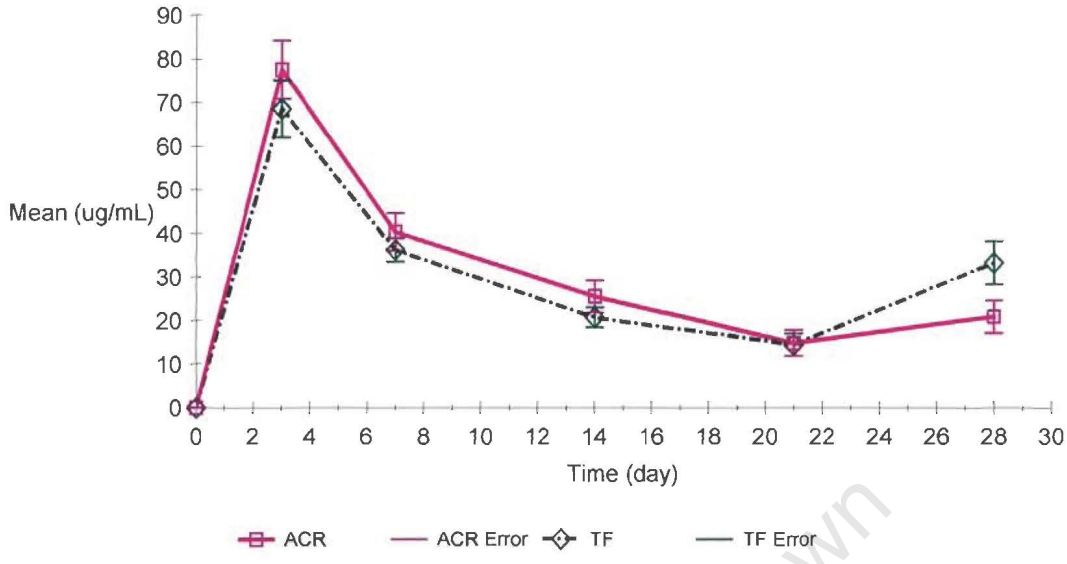


Figure 3.4e Blood sulfadoxine concentration-time profiles for ACR (solid line) and TF (dashed line) subjects. Some patients were retreated with PYR-SDX on day 21 and that is why drug levels rise after day 21. ACR represents subjects with an adequate parasitological and clinical response (ACPR). TF represents subjects who had a late treatment failure outcome (LTF).

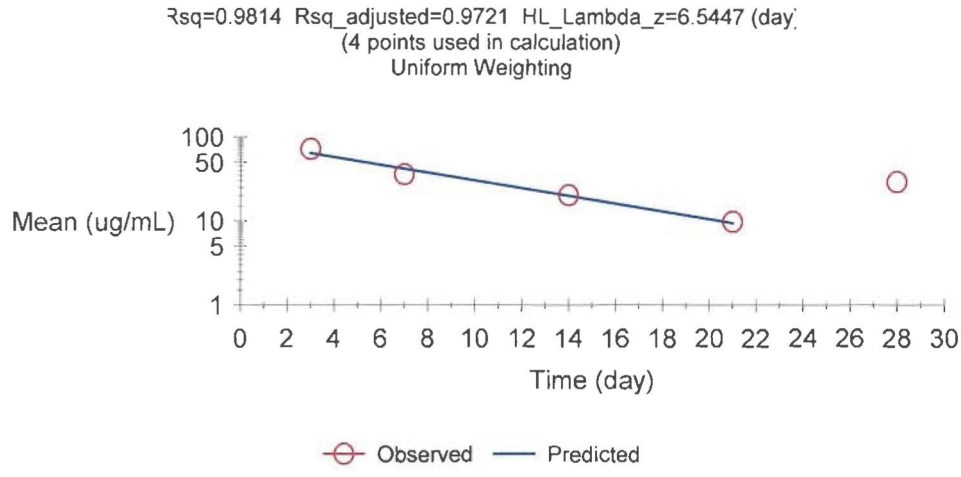


Fig 3.5 Semilogarithmic plot of the blood sulfadoxine concentration-time data reported in Summary Table 1 (Dedza, n = 35).

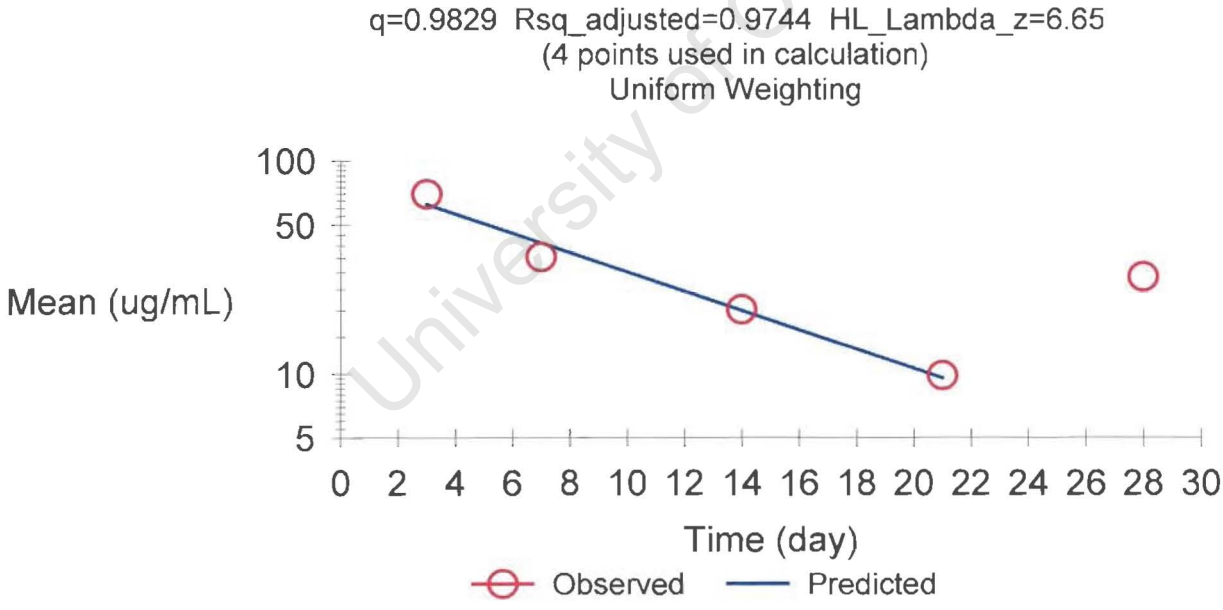


Fig 3.6 Semilogarithmic plot of the blood sulfadoxine concentration-time data reported in Summary Table 2 (Mangochi, n=98)

	AUCinf (ug day mL^{-1}) ± SE	$Et_{1/2}$ (day) ± SE	~ C_0 (µg /ml) ± SE	~ C_{d3} (µg /ml) ± SE
Dedza (n=27)	1156 ± 182	6.54 ± 0.96	96 ± 6.90	70 ± 11
Mangochi(n=84)	827 ± 54.5	6.65 ± 0.76	90 ± 7.20	75 ± 4.9
ACPR (n=48)	932 ± 100	7.15 ± 1.17	79 ± 6.52	77 ± 6.6
LTF (n= 62)	888 ± 78.9	6.41 ± 0.619	69 ± 6.27	69 ± 6.6

Table 3.6 Estimates of sulfadoxine pharmacokinetic parameters

Patients in Dedza had higher levels of drug (SDX) exposure than their Mangochi counterparts irrespective of their treatment outcome (1156 versus 827 ug day mL^{-1}, p=0.021, Dedza and Mangochi respectively). Table 3.6 shows that, overall, blood SDX elimination half-life was similar in ACPRs compared with LTFs (7.15 versus 6.41 days, p=0.42) and there was no difference in the mean half-life observed for subjects in Dedza compared with those in Mangochi (6.54 versus 6.65, p=1.0). The extent of exposure to SDX (reported as $AUC_{0-\infty}$) was similar in ACPRs compared with LTFs (932 versus 888 ug day mL^{-1}, p=0.72).

Similarly, within each site the extent of SDX exposure was similar between ACPRs and LTFs (832 vs 821, p=0.92 and 1311 vs 1065, p=0.52 for Mangochi and Dedza respectively) although Dedza ACPRs tended to have greater SDX exposure (1311 vs 832 respectively, p=0.051, almost reaching significance). However, LTFs had similar exposure (1065 vs 821, p=0.17, Dedza and Mangochi respectively). The back-extrapolated C_0 was similar between sites and between treatment outcome groups.

SDX concentration-time profiles were similar between males and females as illustrated in figure 3.7.

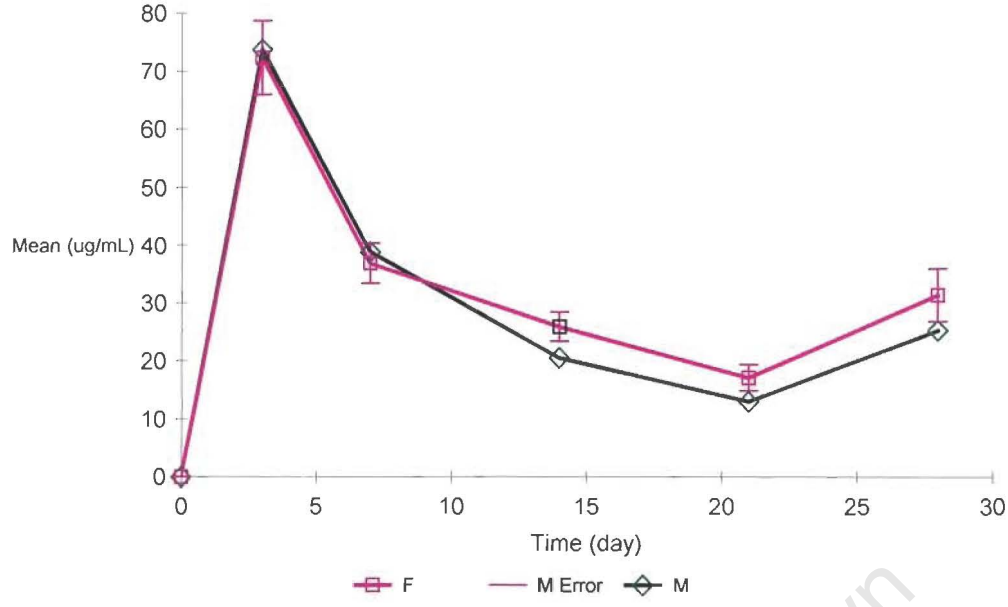


Figure 3.7 SDX blood concentration-time profile for female (F) and male (M) patients with *P.falciparum* uncomplicated malaria following standard PYR-SDX oral therapy.

3.2.2.2 PYRIMETHAMINE

Since beyond day 7 post-therapy PYR concentrations tended to fall below the assay limit of quantification (Figure 3.1 in section 3.1.5), day 3 was used to assess potential differences in blood concentrations of the drug between subjects and between sites. Of 10 randomly selected cases, only 7 had detectable PYR concentration both on day 3 and 7 (see summary Table 3 below). Thus, with only two concentration-time data points, reliable estimation of the PK parameters K_{el}, $t_{1/2}$ and extent of drug exposure ($AUC_{0\text{-}inf}$) for PYR, as was done with SDX, became impossible.

Summary Table 3

Time (day)	Conc.	Pred.	Residual	AUC	AUMC	Weight
0.0000	0.0000			0.0000	0.0000	
3.000 *	247.7	259.2	-11.47	371.6	1115.	1.000
7.000 *	83.46	77.73	5.731	1034.	3770.	1.000
14.00 *	9.205	9.446	-0.2412	1358.	6265.	1.000
21.00	0.0000			1390.	6716.	

Blood pyrimethamine concentration-time data for 7 patients from Mangochi

Starred (*) values were included in the estimation of the terminal elimination phase.

Average day 3 PYR concentrations did not differ (P=0.84) between sites (irrespective of treatment outcome): Dedza 243 ± 31.0 ng/ml, 95% CI 179 – 307, N= 29 and Mangochi 234 ± 15.9 ng/ml, 95% CI 202 – 266, N=75. In Dedza, ACRs achieved similar day 3 PYR concentrations compared with LTFs (228 ± 34.6 ng/ml, 95% CI 155 – 300, N=9 versus 276 ± 65.3 ng/ml, 95% CI 125 – 427, P=0.48, N=20 respectively). Similarly, Mangochi ACPRs exhibited similar day 3 PYR concentrations compared with TFs (264 ± 27.5 ng/ml, 95% CI 207 – 320 versus LTFs 211± 18.4 ng/ml, 95% CI 174 – 248, P= 0.10, N=75 respectively).

Figures 3.8(a) and 3.8 (b) illustrate the interindividual variation on measured day 3 PYR concentrations. The concentration of PYR apparently declined with a first order process as illustrated by the semilogarithmic plot of the concentration-time data for the 4 subjects for whom measurements were done (Figure 3.9).

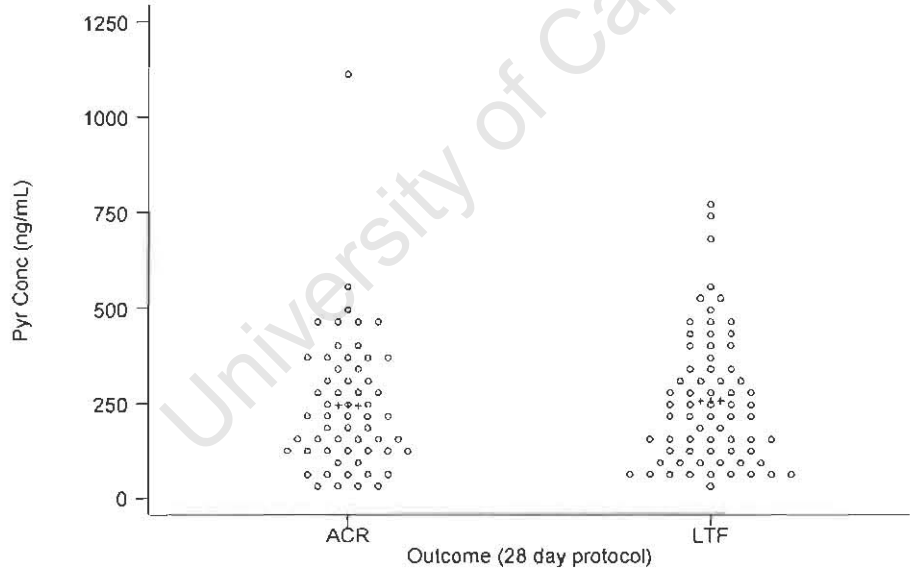


Figure 3.8 (a) Blood PYR levels dotplotted by treatment outcome (Dedza and Mangochi combined). LTF=Late treatment failure (N=69), ACPR= sensitive treatment outcome or adequate clinical and parasitological response (N=58). The plus-lines mark the mean PYR level for each outcome group. Each dot represents an individual subject.

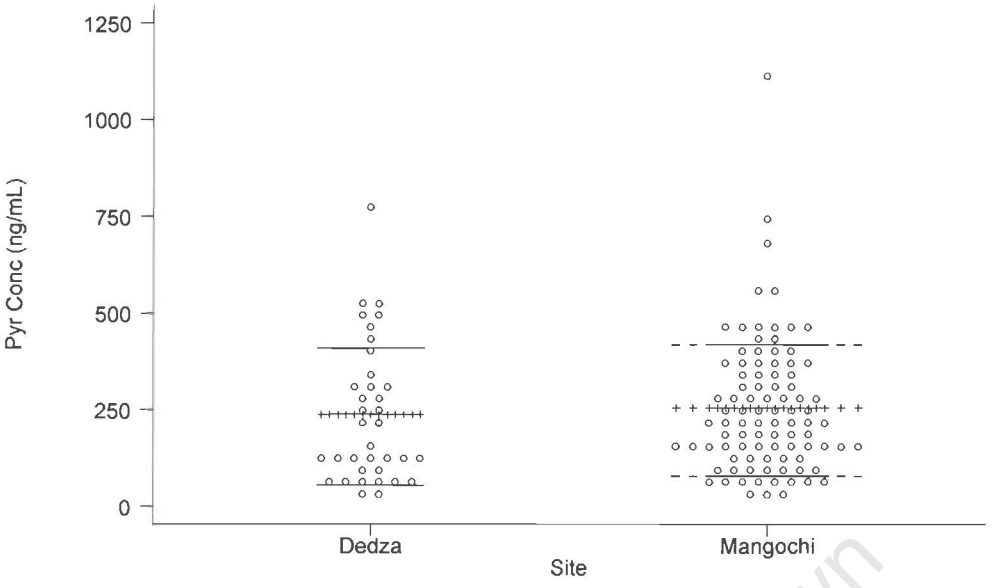


Figure 3.8 (b) Blood PYR levels dotplotted by site (irrespective of therapeutic outcome). The plus-lines mark the mean PYR level for each site. Each dot represents an individual subject. N=36 and N=95 for Dedza and Mangochi respectively.

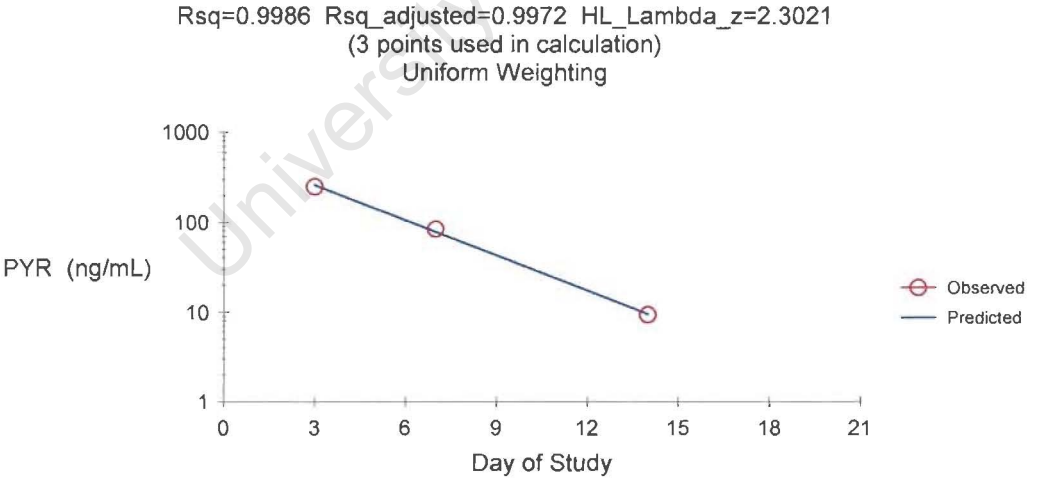


Figure 3.9 Semilogarithmic plot of the blood pyrimethamine concentration-time data from 4 malaria patients in Mangochi. See concentration-time data in Summary Table 3. Patients received a standard oral dose of pyrimethamine-sulfadoxine.

3.3.1 DISCUSSION

3.3.1.1 DRUG ASSAY DEVELOPMENT

The SDX and PYR concentration range used for the method development spans the observed blood concentrations achieved after administration of a single standard oral dose of pyrimethamine-sulfadoxine (750 – 50 µg/L and 100 – 15 mg/L respectively) and followed over a period of 10 days (85).

Bergqvist's SDX recovery at 155 µg/L was only 1.92% (intra- and inter-assay CVs: 2.0 – 2.3% and 3.9 –6.1%). He later found the CV to range from 3 – 6% for the SDX concentration range 16 – 310 µg/ml (88). Winstanley's lower limit for SDX from 50 µl plasma spot was 1000µg/L (intra-assay CVs were 3.6 and 4.3% at 50 and 100 mg/L, respectively; inter-assay CVs were 9.9 and 2.2% respectively). In this thesis the limit of quantitation for SDX from whole blood spotted on filter paper was approximately 6 µg/mL (See Table 3.4 in section 3.1.5). The method was very sensitive for SDX assays, which will mostly be in the µg/ml concentration range for the first 14 days after administration of a single SDX-PYR dose.

Overall mean SDX recovery was 77± 3.2%, with inter- assay CV = 9.4, in contrast with Bergqvist's recovery (2%) and Winstanley's for SDX recovery from plasma other than whole blood spots. The method described in this thesis is simple and quick, allowing the quantification of SDX from at least 30 samples within 12 hours.

In this thesis, the mean recovery of PYR from DBS was 86%. The method developed for PYR thus compares favorably to Bergqvist's method for plasma samples and obtained higher PYR recoveries than those previously reported by Michael et al (86). Yngve Bergqvist recovered PYR from plasma (not on filter paper) at an efficiency of 74% for a concentration of 124 µg/L (Inter-assay CV= 3.5 – 7.8% and intra-assay CV= 3.4 – 5.7%) (87). In the method described, PYR recovery from dried blood spots was ≥ 80% at 125 µg/L. Winstanley found that the lower limit for PYR from 200 µl plasma spot was 20

µg/L (intra-assay CVs were 8.9 and 2.7% at 50 and 600 µg/L, respectively; inter-assay CVs were 8.9 and 3.3% respectively) (85). This thesis study found that the limit for reliably measuring PYR from 100 µl of whole blood spots was 31 µg/L, a value comparable to that obtained by Winstanley (85) for plasma extracts (See Table 3.1 and 3.4 for accuracy and precision).

Sulfadoxine has been found to be stable for at least 15 weeks at 37°C(84). In this thesis study it was also found that both sulfadoxine and pyrimethamine were stable for at least 4 weeks at 37°C. Thus kept away from direct light and heat and at room temperature the DBS filter paper PYR and SDX should remain stable for a convenient length of time. These methods that are herein described are suitable for the determination of PYR and SDX levels in dried spots of whole blood collected on filter paper after administration of therapeutic doses of SDX-PYR.

3.3.1.2 BLOOD DRUG LEVEL MEASUREMENT IN MALARIA PATIENTS

The observation that none of the patients studied in Dedza or Mangochi gave positive HPLC result for pretreatment SDX or PYR concentrations may not rule out the presence of residual concentrations of the drugs since concentrations below the limit of quantitation for the HPLC method would not have been detected. What may be said, however, is that if any of the patients had residual SDX or PYR in their blood, then the drug concentrations were less than 6µg/ml and 31 ng/ml respectively, the concentrations said to be responsible for selection of resistant *falciparum* parasites.

3.3.1.2.1 SULFADOXINE

PHARMACOKINETICS

In the present study, back-extrapolated SDX concentration (C_0) was found to fall within the previously observed range of Cmax, 51 – 169 µg/mL, following oral administration

of the standard PYR-SDX dose (85,89,90). The apparent elimination half-life of SDX agreed with previous reports of the range 4 – 11 days (85,90,91). The observed range of day 3 SDX (59 – 75μg/mL) in this study was similar to the range (mean 73.2 μg/mL, range 51 – 89 μg/mL) found elsewhere by Hellgren (91). Bustos (90) found a mean SDX day 3 concentration of 184 ± 40μg/mL.

Clearly, PK parameters for the patient group studied in Malawi are in broad agreement with reports from previous pharmacokinetic studies carried out in other malaria endemic countries. It is, nonetheless, also clear that there is substantial interindividual variation in SDX disposition both in the present study and the above-cited previous studies. The cause of these differences is unknown, but they may reflect the effect of disease. It is well known that disease severity may alter the disposition of some drugs (27,62,51). For instance small intestinal disease alters gastric emptying and gastric emptying is an important determinant of the rate and sometimes the extent of drug absorption. In the present study, age differences do not explain the observed wide interindividual variation since the age was similar between the groups studied (Table 3.5). Moreover, PYR-SDX kinetics has been reported in children and plasma concentrations were similar to those in adults (92). This thesis study did not find evidence of differences in SDX concentration-time profile between ACPRs and LTFs, females and males, between day 3 and 28 post-therapy (See figures 3.4, 3.7).

Despite the aforementioned similarity in demographic characteristics and despite a similar prevalence of resistant *falciparum* genotypes, it was observed that the in vivo PYR-SDX efficacy (chapter 2) was higher in Mangochi (high transmission area) than Dedza (low transmission area). This observation suggests that the development of malaria-specific partial immunity acquired from repeated exposure to infective mosquito bites may explain the observed disparity in PYR-SDX efficacy between the two sites. Moreover, the fact that in Dedza, where PYR-SDX efficacy was lower, subjects exhibited a significantly higher extent of exposure to SDX compared with subjects in Mangochi where PYR-SDX efficacy was higher (1156 vs 827 μg day mL^{-1}, p=0.021), underscores the importance of differences in levels of malaria-specific immunity. Differences in blood

folate levels also needed to be considered. Multivariate logistic regression was used to measure the relative role of SDX after adjusting for age, pretreatment parasite density, *falciparum* resistant genotype, pretreatment haemoglobin, site, blood folate and pyrimethamine levels. This analysis found that SDX levels were not correlated with PYR-SDX therapeutic failure.

DISCUSSION OF SDX RESULTS BASED ON PREVIOUSLY REPORTED IN VIVO SENSITIVITY OF *P.FALCIPARUM*

Although the clinically relevant therapeutic range for SDX is not well established, a study found that nonimmune patients with RII SP resistant *P.falciparum* from Tanzania, who attained SDX concentrations below the range 62 – 115 μg/mL developed secondary parasitemia (53). Also Chulay (27) found that in vitro SDX concentrations greater or equal to 60 μg/mL were synergistically effective against the resistant *P.falciparum* strain, K39, which correlates with the in vivo inhibitory range of 62 – 115μg/mL. Although patients, until day 3 post-treatment, sustained SDX levels that could kill both resistant and sensitive parasites, it is clear that after day 3 post-treatment (see summary table 1 and 2) SDX concentrations decreased rapidly (in both ACR and TF, and at both study sites) to levels that were below those required to kill resistant *falcparum* isolates in vivo (i.e below the range 62 – 115 μg/mL).

Although in vivo tests are a much more direct measure of parasite sensitivity, the mean inhibitory concentration values obtained are at best accurate for the estimation of drug efficacy only in the population used for the field study. This is because the level of malaria-specific acquired immunity varies from one area to another depending on differences in transmission intensity. Hence minimum cut-off values for parasite growth inhibitory concentrations for resistant or sensitive *falciparum* isolates obtained in one field study may not always be an accurate reference for assessing the drug's efficacy in another population living in a different geographical location with a different transmission intensity. Moreover, as observed in this thesis, PYR-SDX pharmacokinetics is often highly variable among patients within and between populations.

In this thesis, with field data for SDX or PYR scarce despite extensive search of the literature, it was not possible to find data related to the minimum SDX or PYR concentration in human blood that is effective in inhibiting a parasite strain susceptible in vitro to the respective drug. However, by logistical regression, optimal blood concentration breakpoints for SDX and PYR, which best separate ACPR from LTFs were determined. Blood concentrations higher than a breakpoint of 348 ng/ml for PYR were associated with 94% reduced risk of late treatment failure (P=0.010). Similarly, blood concentrations higher than a breakpoint of 80µg/ml for SDX were associated with 90% reduced risk (respectively) of late treatment failure (P=0.050). A study in Gabon found that blood concentrations higher than 175 ng/ml and 100 µg/ml for PYR and SDX respectively were associated PYR-SDX treatment success (Aubouy et al. 2003 [129]).

DISCUSSION OF SDX RESULTS BASED ON PREVIOUSLY REPORTED IN VITRO SENSITIVITY OF *P.FALCIPARUM*

A previous study (91) found that SDX levels in the range 0.993 – 1.55 µg/mL (minimum range) effected 100% in vitro inhibition of *P.falciparum* that were susceptible in Tanzanian children who had received a standard dose of PYR-SDX. Another study in Haiti children found that SDX concentrations greater or equal to 0.390 µg/mL resulted in 100% in vitro inhibition of *falciparum* strains that were susceptible in vivo (52).

From the studies in Haitian and Tanzanian children, it may be anticipated that a 1µg/mL SDX blood concentration could kill all susceptible malaria parasites whereas resistant strains would require a 60-fold higher concentration to be fully inhibited. Hence, for Dedza and Mangochi (Malawi), under the SDX levels observed between day 3 and day 28, exclusive drug selection of the resistant *P.falciparum* strains would not be unexpected. It may be recalled from chapter 2 that PYR-SDX strongly selected for resistant parasite genotypes as evidenced by the observation that there was a greater proportion of resistant genotypes in post treatment than pretreatment patient samples

(P=0.002 for DHFR triply mutant *falciparum* and P<0.001 for the quintuple mutant). Furthermore, the observation that PYR-SDX efficacy was, at both sites, significantly reduced in cases that were retreated with the same drug following the development of parasitemia within 28 days of the initial treatment seems to suggest that at the time of the second treatment the drug was dealing with a predominantly genotypically resistant *P.falciparum* population. Thus, although this thesis project did not attempt to distinguish recrudescent parasitemia from reinfections, it is unlikely a high proportion of susceptible malaria parasites could survive the sulfadoxine concentrations observed in Dedza and Mangochi. Therefore, the late parasite recurrence observed in the Dedza and Mangochi studies comprise true parasitological recrudescence as well as reinfections that are expected to have some level of resistance to PYR-SDX to survive at these concentrations.

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3.3.1.2.2 BLOOD PYRIMETHAMINE CONCENTRATIONS AND PREVIOUS IN VITRO PYR ACTIVITY AGAINST *FALCIPARUM*

The apparent elimination PYR half-life (2.3 days) was in the range of previous reports, 1.5 – 7.4 days (79,89,93,94). Back-extrapolated PYR concentrations (C_0=700ng/mL) were comparable to previously reported peak serum levels 130 – 590 g/mL (85,89,90) observed following an oral administration of a PYR-SDX standard dose.

As with SDX there was wide inter-individual variation in day 3 PYR blood concentrations probably for the same reasons including (for both PYR and SDX) differences in the times at which post-treatment day 3 blood samples were collected when patients were seen again at the clinic or when they had to be followed up to their home for not showing up.

Results from the day 3 PYR-level studies did not provide evidence for concentration differences between ACRs and TFs both within and between sites (section 3.2.3.1). Similarly, no such differences were, overall, apparent between day 3 PYR concentrations observed in Dedza and Mangochi subjects. What is clear, however, is that all subjects (on

day 3 only) irrespective of treatment outcome and study site sustained not only therapeutic PYR concentrations (mean 290 ng/ml, 95% CI 182 – 398) but also levels synergistically effective against resistant *falciparum* strains in *in vitro* studies. A previous study found that PYR ≥ 15 ng /ml was synergistic against K39, a resistant strain of *P.faciparum* in vitro (27). A study found that PYR levels in the range 9.95 – 15.5 ng/mL (minimum range) effected 100% in vitro inhibition of *P.falciparum* that were susceptible in Tanzanian children who had received a standard dose of PYR-SDX (91). Another study in Haiti children found that PYR concentrations greater or equal to 9.32 ng/mL resulted in 100% in vitro inhibition of *falciparum* strains that were susceptible in vivo (52). An in vivo study found that nonimmune patients with RII PYR-SDX resistant *P.falciparum* from Tanzania, who attained PYR concentrations ≤ 49.7-348 ng/ml developed recrudescent parasitemia (53). It is important to note that the in vitro mean inhibitory concentrations reported from the studies cited above are consistent with full sensitivity in vivo in a non-immune population, such as the patients studied in Tanzania.

The cited observations suggest that as at day 3 post-therapy, PYR concentrations in ACRs and TFs, and at both study sites were sufficiently above those required to kill both susceptible and resistant *falciparum* parasites. However, it is impossible from the present study to know whether such effective PYR concentrations had been maintained for at least three parasite life cycles for an ACPR outcome to be achieved. When drug levels fall below the minimum effective concentration there is a net parasite growth, the rate of which being relatively slower in semi-immune people, results in late treatment failure (76). There were no cases of early treatment failure in Dedza and Mangochi at first treatment suggesting that *falciparum* isolates were predominantly of RI level of resistance. Even if it were assumed PYR concentrations indeed remained above those required for effective synergy against resistant parasites, the rapid decline of SDX levels below concentrations required for synergy immediately after day three would result in an overall loss of the required effective synergy between PYR and SDX as a combination. Such a situation would result in differential pyrimethamine susceptibility among parasites that would otherwise be susceptible to the combination, resulting in selection of the low-grade resistant parasites observed in this study.

This thesis found that day 3 PYR was associated with PYR-SDX failure only after adjusting for age, pretreatment parasite density, *falciparum* resistant genotype, pretreatment haemoglobin blood folate and SDX levels. This result agrees with a study that found a significant association between PYR blood concentrations and parasite clearance times (95) although in that study physiological folate levels and other DHFR/DHPS parasite mutations relevant in Africa were not considered.

3.3.2 CONCLUSION

Although it is likely that some degree of resistance to both sulfadoxine and pyrimethamine is necessary for *in vivo* PYR-SDX resistance, the relative importance of sulfadoxine vs. pyrimethamine *in vitro* resistance in causing *in vivo* PYR-SDX failure has not been clearly resolved. While some studies have observed therapeutic success of PYR-SDX in the face of *in vitro* resistance to pyrimethamine, suggesting that sulfadoxine resistance is key to PYR-SDX failure, others have found similar sulfadoxine inhibitory concentrations (IC50's) and differential pyrimethamine susceptibilities among isolates with *in vivo* sensitive vs. resistant phenotypes, suggesting that pyrimethamine resistance is paramount (53,96). Recent *in vitro* pharmacokinetic studies of the synergistic action of pyrimethamine and sulfadoxine done under physiologic folate and PABA conditions indicate that the *in vivo* response to PYR-SDX is likely to be determined primarily by parasite sensitivity to pyrimethamine (97). An in vivo PYR-SDX efficacy in Buenaventura, Colombia was recently the first to confirm this *in vivo*. They found that an increase of 1 μg/ml in the pyrimethamine concentration was significantly associated with longer parasite clearance times. No significant association was found for sulfadoxine in that study (95). DHPS 540 and DHFR 59 mutants were not detected in the Colombia study. In contrast, all DHFR and DHPS mutations known to be prevalent and important in Africa were used in this thesis in order to determine the relative role of PYR in *in vivo* PYR-SDX therapeutic failure.

A regression analysis in this thesis found, after adjusting for folate levels, age, pretreatment parasite density, *falciparum* resistant genotype, pretreatment haemoglobin and SDX levels, a significant association between day 3 pyrimethamine levels and parasite recurrence within 28 days of PYR-SDX treatment, and is the first to confirm the primary role of pyrimethamine in *in vivo* PYR-SDX therapeutic failure in an African country where malaria is endemic. Blood folate levels were not included in the Colombia study.

3.4 STUDY LIMITATIONS

The PYR assay limit of quantitation did not permit a full pharmacokinetic analysis of the majority of patients. Such an analysis would have given better insights into the relationship between the time course of PYR concentrations and the observed in vivo PYR-SDX efficacy. The single time point measurements herein used for comparison between two treatment outcome groups, gender groups and sites, only allowed the estimation of the minimum bioavailable PYR (i.e. drug remaining in the systemic circulation).

For both SDX and PYR it was not possible to obtain time points in the absorption phase because of patient compliance problems owing to discomfort with frequent finger pricking. Thus, data points were restricted to day 0, 3,7, 14, 21, 28. Even then some patients withdrew their consent to continue in the study because they felt the procedure was too invasive. Since from a finger prick (at each time point) blood was needed for genetic anaysis (100µL), for SDX (100µL), for PYR (100µL) and for folate (50µL), it was sometimes necessary to sample repeatedly. Such situations reduced patient compliance with follow up.

The SDX inhibitory concentrations used for reference were previously reported in vitro measurements which may not always reflect the human in vivo situation where, immunity, nutrition, disease status may alter/influence the therapeutic response of the

drug in question. However, since non-immune patients with RII PYR-SDX resistant *P.falciparum* from Tanzania, who attained SDX concentrations below the range 62 – 115 µg/mL developed secondary *falciparum* parasitaemia, this thesis considered that range as the best available in vivo therapeutic range estimate for SDX. As for PYR the clinically relevant concentration range is 25 – 150 ng/mL as cited elsewhere in this chapter.

In the four subjects for whom sufficient concentration-time data was generated, observed mean PYR blood concentrations remained within the clinically relevant range, 25 – 150 ng/mL, for at least 12 days (see figure 3.1). In the present study, blood levels (40 ng/mL) observed 10 days post therapy compared favorably with those (52 ± 39ng/mL) that Winstanley (85) reported for children with non-severe *falciparum* malaria. With PYR kinetics in only 4 of the 146 cases (for whom only day 3 PYR concentration was determined) it would be inaccurate to extrapolate the results to the rest of the patients. Comparison of ACPRs with LTFs, male with female and site with site was, therefore, based on measured day 3 PYR blood concentrations. Comparisons based on the time period during which inhibitory concentrations of PYR were present in the blood would have been more informative than results from single point measurements.

In vivo and in vitro methods have, for many years, been used to assess the activity of drugs against *P.falciparum* (130). Each of these tests has inherent drawbacks. In areas of intense transmission of malaria, the majority of the infections are due to multiple parasite lines. The magnitude of multiplicity of infection may vary from one depending on the level of transmission and from individual to individual. A time-dependent clonal fluctuation in peripheral blood, observed both in low-transmission (131) and holoendemic (132) areas suggested that some of the parasites were hiding in deep vasculature at day 0. Thus, in vitro tests may not be able to detect minor parasite clonal lines that are resistant to PYR-SDX and that are at the origin of treatment failure. Similarly, in vitro methods may not be able to detect sensitive clones that emerged from deep vasclature, multiplied under growth non-inhibitory drug pressure and then reappeared at the time of treatment failure as new infections or 'resistant' parasites. This situation leads to underestimated minimum inhibitory drug concentration for resistant

isolates since at the time of treatment failure the parasite may comprise the aforedescribed sensitive clones, the truly resistant clones and newly reinfecting clones. Likewise, the minimum growth inhibitory concentrations for sensitive clones estimated from day 0 isolates that resulted in ACPR would in fact be a summation of inhibitory concentrations for the sensitive as well as that for resistant *falciparum* clones which (the resistant parasites) resulted in an ACPR due to the host immunity adding its activity to that of the antimalarial drug. In vitro methods do not account for the influence of malaria-specific acquired immunity on the drug's ability to clear parasitemia. The result of the foregoing discussion is that parasite growth inhibitory concentration values derived from in vitro tests reflect the mean susceptibility of the multiple parasite clonal lines present in an individual. Therefore, in vitro and in vivo data from high-transmission areas may not correlate in the case of PYR-SDX (133).

Although in vivo tests are a much more direct measure of parasite sensitivity, the mean inhibitory concentration values obtained are at best accurate for the estimation of drug efficacy only in the population used for the field study. This is because the level of malaria-specific acquired immunity varies from one area to another depending on differences in transmission intensity. Hence minimum cut-off values for parasite growth inhibitory concentrations for resistant or sensitive *falciparum* isolates obtained in one field study may not always be an accurate reference for assessing the drug's efficacy in another population living in a different geographical location with a different transmission intensity. Moreover, as observed in this thesis, PYR-SDX pharmacokinetics is often highly variable among patients within and between populations.

Recrudescence and reinfections are discussed in chapter 2 section 2.5.

CHAPTER 4

HOST PHYSIOLOGIC FOLATE CONCENTRATIONS AND THE *IN VIVO* PYRIMETHAMINE-SULFADOXINE EFFICACY AGAINST *PLASMODIUM FALCIPARUM*

4.1 FOLATE

4.1.1 DRIED BLOOD FOLATE ASSAY DEVELOPMENT

Dried blood spots (DBS) have been used in the analysis of amino acids (98), glucose (99), therapeutic drugs (100) and DNA (71,102). In these methods of sample analysis, the dried blood spot is punched out and eluted in the chosen solvent(s) and the analyte in the eluate is then determined using appropriate methods.

Two methods have been described for the determination of blood folate in dried blood spots, one using acid extraction and internally standardised gas chromatography-mass spectrometry detection (103), and another that quantitates red cell folates by stable isotope dilution gas chromatography-mass spectrometry (104). These methods, though reported to be sensitive, specific and accurate, require a mass spectrometer which is not affordable by most developing countries. In addition, these methods require cold facilities to transport and store the samples. Although O'Broin described an excellent method for recovering folate from DBS, he uses a microbiological assay for the quantitation of folate in extracts (105), an approach that is labor intensive, requiring culturing of Lactobacillus casei. Such whole blood folate assays have limited use in assessing folate nutritional status and a more rapid and reliable assay system is required. A folate assay system that adapts the DBS matrix to the Abbot IMx folate kit, using alkaline phosphatase to enhance specificity was developed in this thesis.

4.1.2 MATERIALS AND METHODS

Materials comprised an IMx Automated Immunoassay Analyser (Abbot Laboratories), Technicon H-2 System for Haemoglobin analysis, F200 sonicator, IMx folate module (Abbot Laboratories), IMx folate assay kit, borosilicate tubes, resealable plastic bags, desiccant packets or silica gel, folic acid (pteroylglutamic acid) from BDH laboratory Suppliers, 1.5 ml eppendorf tubes, Whattman No.3 filter paper, pipette tips (1000 µl and 200 µl), ascorbic acid and Triton X-100. Venous blood was collected into heparinized vacutainers.

4.1.3 ELUTION OF DBS FOR FOLATE AND HB ANALYSIS

DBS were prepared by blotting 50 µl whole blood on Whattman No.3 filter paper (Sigma). Since using an in-house eluant (an ascorbate-detergent solution of composition: 5g ascorbic acid/L containing 0.1%(by vol) Triton X-100) made no difference in the initial analyses, folate (BDH Laboratory Suppliers) and haemoglobin were therefore conveniently coeluted into an ascorbate-detergent solution provided with the IMx Folate kit (Abbot Laboratories). DBS were punched out and DBS material placed into a round-bottomed disposable borosilicate tube, covered in eluant (folate lysis reagent, 1 ml). This was vortex mixed, and sonicated for 30 minutes. This extraction of whole DBS into 1000 µl of the lysis reagent provided sufficient eluate for both haemoglobin spectrometry and folate assay by ion capture reaction. The hemolysate was stored at –20°C.

DBS eluate (150 µl) was pipetted into the sample well of an IMx ion capture cell and the run initiated (on the IMx Automated Immunoassay Analyser) within 30 minutes following extraction or thawing. Hb values were measured using the Technicon H-2 System for Haemoglobin analysis where the remainder of eluate from the folate assay was used. Much less than 150 µl was required for the Hb assay. Whole blood controls were used to monitor DBS extraction efficiencies. To assess the linearity of the DBS extraction method whole blood from normal individuals was spiked with known concentration of folic acid spanning the range 0 – 20 ng/mL and quantitation results were corrected for endogenous blood folate level before generating the calibration curve (figure4.1). The IMx Folate kit also comes with folate calibrators and controls, which allow accurate quantitation of folate in eluates of either whole blood or DBS. Haemoglobin-folate (HF) values were calculated by dividing the whole blood folate concentration by the sample haemoglobin concentration: HF (nmol/g) = [WB, whole blood folate (nmol/L)]/Haemoglobin (g/L). IMx folate results were calculated as ng/ml and converted to nmol/L or pmol/g (1nmol/L=2.265ng/ml) [105]. Results are reported as haemoglobin folate because it correlated well with the erythrocyte folate concentration of normal Americans as measured by conventional methods (106).

4.1.4 RESULTS

Over 75 % of DBS folic acid was eluted by a combination of 30 min simultaneous incubation and sonication followed by another 30 min incubation at room temperature. The DBS folate reproducibility was acceptable (within run CV= 7.7% at 20ng/ml and CV= 4.8 at 5ng/ml). There was a significant positive correlation between detector response and increasing DBS folic acid concentration (R^2 = 0.998, P = 0.0001, Figure 4.1). This was comparable to the level of correlation obtained for the IMx folate kit folate calibrators (R^2 = 0.83, P=0.0114). In both cases the range of folic acid concentration studied was 0 – 20 ng/ml. The mean folate concentration for 1ml DBS (50 µl blood spot) was 66.0 ± 1.20 ng/ml. The mean HF was 3209 ± 204.3 (95% CI = 2684 – 3734) and the mean Hb for the DBS was 163 ± 6.15g/L (95% CI = 142 – 184 g/L). Folate and Hb folate assay results are summarized in table 4.1. It should be noted that DBS were eluted in 1mL of eluant, which effectively diluted the folate concentration down to the standard curve range. Thus, the reported values are folate concentrations obtained after multiplication by the dilution factor. Below is a typical calculation for original DBS folate concentration.

For specimen 1, standard curve-derived concentration = 3.3 ng/mL (This was the folate concentration for the 1mL eluate). The amount of folate in eluate = 3.3 ng/mL*1mL= 3.3 ng (This was the amount of folate recovered from the 50 µl DBS). Hence folate concentration in the original blood = (3.3 ng/mL*1000µl)/50µl*1mL= 66 ng/mL.

Specimen No	1	2	3	4	5	6
Folate ng/ml	66	68	64	52	48	56
HF (pmol/g)	3238	3753	3532	2551	2649	3532
Hemoglobin (g/L)	180	160	160	180	160	140

Table 4.1: Summary of results for the Hb spectrometry and folate ion capture assays on DBS elutes. Each specimen was run in duplicate (n = 12). Blood was obtained from healthy individuals. DBS (50µl) was eluted into 1 ml of lysis reagent.

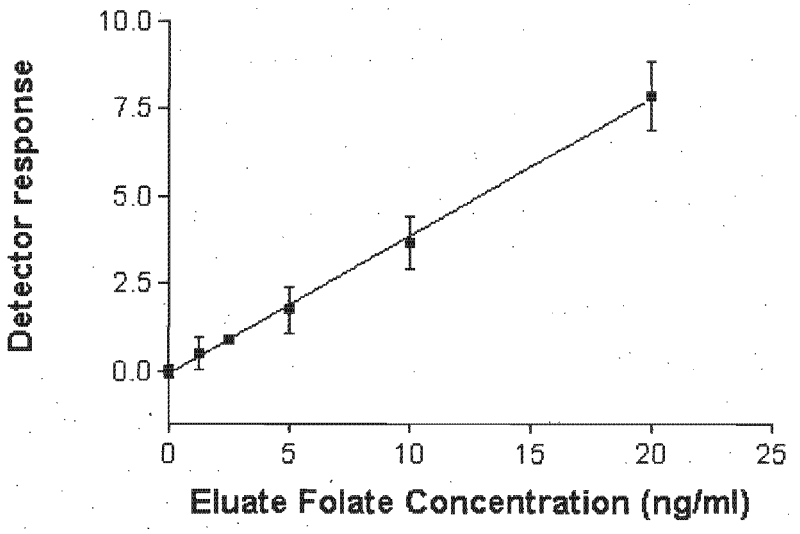

Fig.4.1 Linear plot of folic acid from dried blood spots versus detector response. $R^2 = 0.998$, $P = 0.0001$. For each concentration data point DBS were prepared in triplicate from drug-free whole blood spiked with known amounts of folic acid in the range 0 – 20 ng/ml.

4.1.5 FOLATE AND THE IN VIVO PYR-SDX EFFICACY STUDY

PYR-SDX efficacy studies were conducted following the WHO definition (72,73) for assessing antimalarial efficacy in areas of high malaria endemicity and details are in chapter 2. Children aged between 6 months and 12 years reporting at Mangochi and Dedza district hospital out patient departments with signs and symptoms of malaria, and whose axillary temperature and parasite density at the time of the visit were $\geq 37.5^0$ Celsius and 2000/μL of blood respectively, were eligible for study. Only children whose caretakers provided an informed consent were enrolled. After a supervised treatment with PYR-SDX on the enrolment day, the child was followed again on days 3,7,14,21,28,35 and 42.

209 cases of uncomplicated malaria met the inclusion criteria for the PYR-SDX efficacy study which took place at the two places, Dedza and Mangochi; high and low

transmission intensity areas respectively. 50 and 159 cases were recruited in the Dedza and Mangochi study respectively. Excluding cases lost to follow up, cases that violated study protocol and cases without known therapeutic outcome, we measured the pretreatment blood folate levels of 41 and 102 cases in Dedza and Mangochi respectively. Figure 4.7 summarizes the study design.

4.1.6 RESULTS (UNIVARIATE ANALYSIS)

Irrespective of study site, measured whole blood folate levels were higher in cases that failed PYR-SDX therapy that those that had a successful clinical outcome (p=0.028, see figure 4.2, n=141). However, in Dedza alone there was no difference in blood folate levels between resistant and sensitive cases (39 ng/ml vs 38 ng/ml, respectively, p=0.76). This could be due to a small sample size at this site (n=41). In contrast, Mangochi (n=102) resistant cases had higher folate levels than the sensitive (32ng/ml vs 27ng/ml, p=0.014).

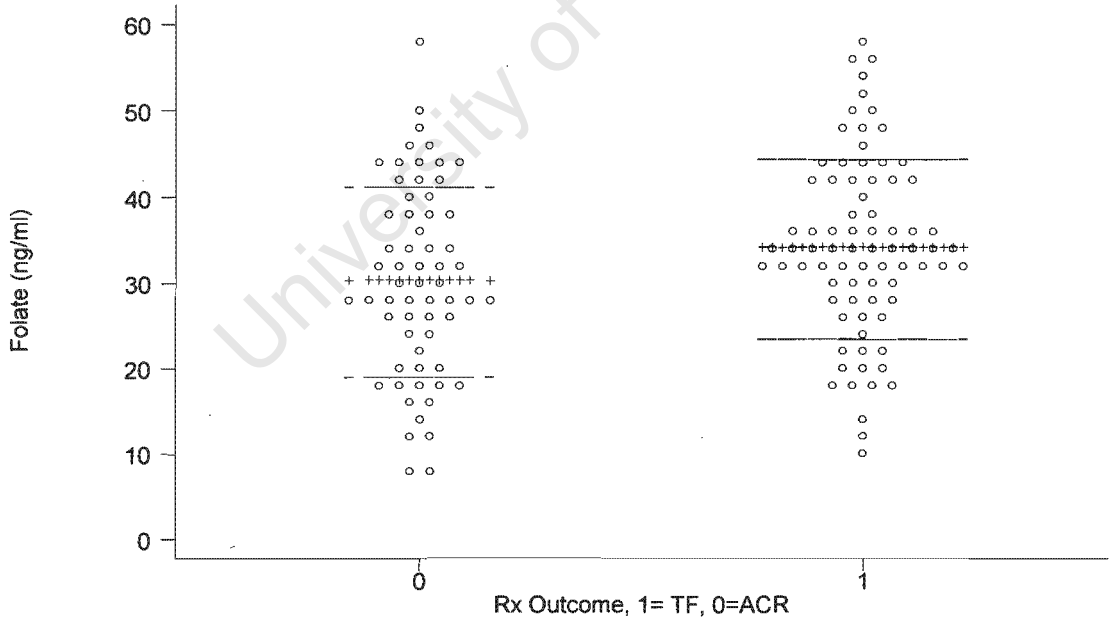

Fig 4.2 Blood folate levels dotplotted by treatment outcome (Dedza and Mangochi combined). Each dot represents an individual case. TF=treatment failure, ACR= sensitive

treatment outcome which in this case means ACPR. The dashed lines are the mean plus or minus the standard deviation whereas the plus-line marks the mean folate level for each outcome group.

Generally, Dedza cases had significantly higher blood folate levels than their counterparts in Mangochi (See figure 4.3, 38 ng/ml vs 30 ng/ml, $p<0.0001$). It is interesting to note that the PYR-SDX efficacy rate was lower in Dedza than in Mangochi (65% vs 80%) despite similar prevalence of DHFR and DHPS resistance-conferring mutations. It was observed that folate levels tended to be higher in boys than girls but the difference was not statistically significant (34 ng/ml vs 31 ng/ml, $p=0.092$, See fig 4.4). Whereas parasite density was positively associated with host-blood folate concentrations (Fig 4.5), there was a negative association between age and parasite density (Fig 4.6). There was a weak positive association between age and blood folate levels ($p = 0.57$).

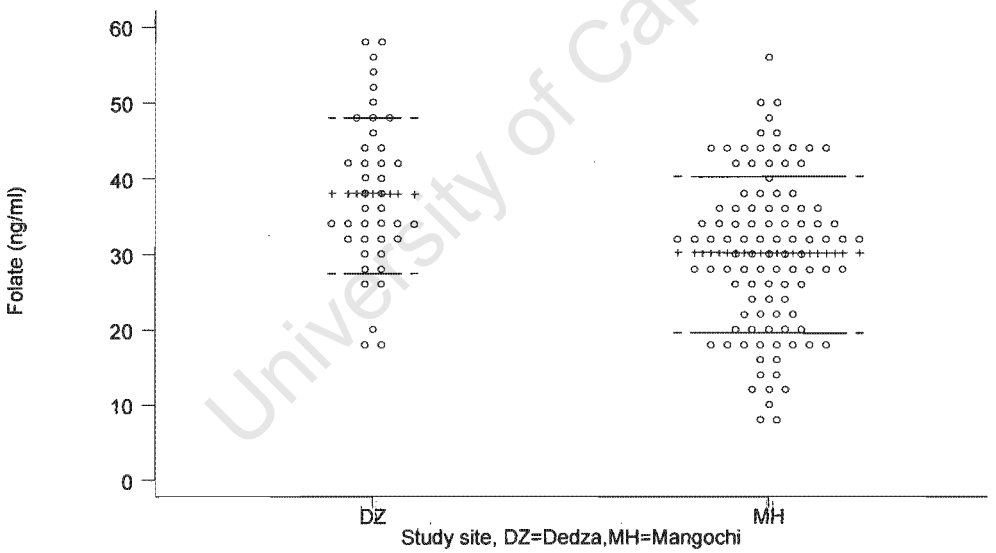

Fig.4.3 Blood folate levels dotplotted by site (Dedza and Mangochi, N=102 and N=41 respectively). DZ=Dedza, MH= Mangochi. The dashed lines are the mean plus or minus the standard deviation whereas the plus-line marks the mean folate level for each outcome group.

Within each site, for example Mangochi, there was no difference in folate levels between males and females (31 vs 28 ng/mL, p=0.20, no. of males =44 vs no. of females =40 respectively). However, males tended to have higher levels at that site. Similarly, there was no difference in folate levels between males and females (41 vs 36 ng/mL p=0.13, n=19 vs n=14 respectively) in Dedza although within this site males tended to have higher levels.

Fig. 4.4 Blood folate levels dotplotted by sex (Dedza and Mangochi combined). F= female, M= male. . Each data point represents an individual patient. The dashed lines are the mean plus or minus the standard deviation whereas the plus-line marks the mean folate level for each outcome group.

Parasite density at baseline was not different between ACPRs and Failures (9848 versus 8948parasites/μL p=0.49). Parasitaemia was not associated with treatment outcome in a univariate analysis.

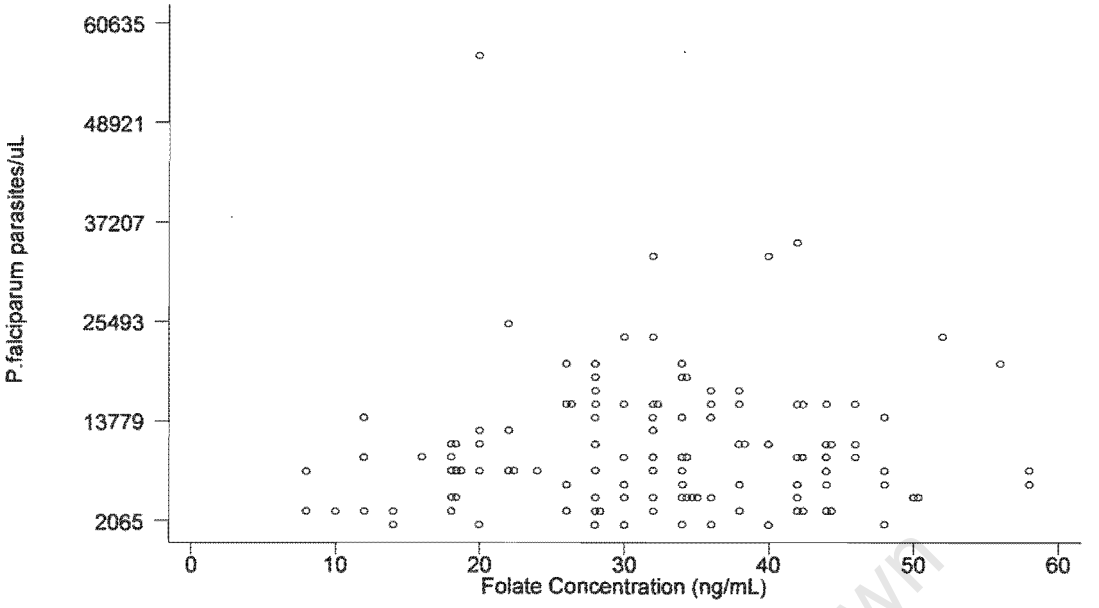

Fig 4.5. Plot of malaria parasite density as a function of folate concentration (r = 0.05, p=0.32, n=143).

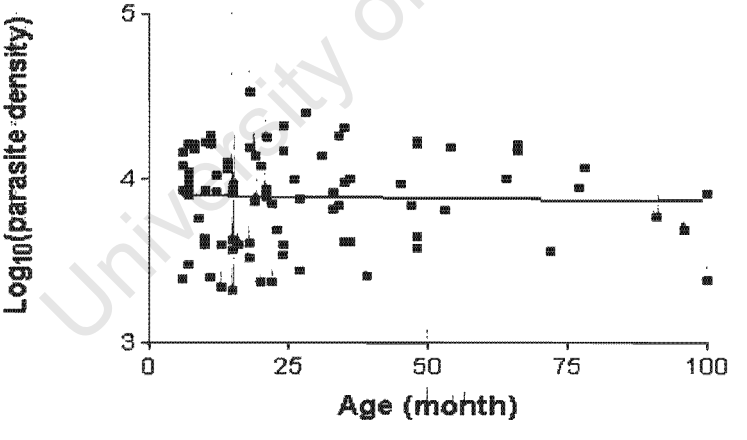

Fig 4.6. A linear plot of $\log_{10}$ parasite density versus age (month) of children sick from uncomplicated Plasmodium falciparum malaria (r = -0.03, p=0.61). There was a weak trend towards decreasing parasite density with inceasing age. Each dot represents an individual (n=87, age was not recorded for some cases).

4.1.7 RESULTS (MULTIVARIATE ANALYSIS)

Good predictor variables that are independently associated with treatment outcome would be useful in predicting the treatment outcome in field PYR-SDX efficacy studies. Previous studies found an independent association between treatment outcome and age, parasite density at the time of treatment, and haemoglobin. SDX and PYR Blood drug levels achieved, as at day 3, have been incorporated in the model.

In the univariate analysis above, a sex-based difference in folate levels among the subjects was found. Sex was thus included in the overall model just in case that could explain the difference in treatment outcome.

Data was input and analyzed in Stata 7.0 multilogistic regression program. A correlation matrix for the treatment outcome and all the proposed predictor variables is reported in table 4.2. A multivariate regression model incorporating all important interaction terms was run and the results are reported in table 4.3.

	Outcome	Folate	hbd0	Sex	Par	Age	PYR	SDX
Rx Outcome	1.0000							
Folate*	0.2396	1.0000						
Day0 Hb	0.0186	0.1263	1.0000					
Sex*	0.3157	-0.0123	-0.0434	1.0000				
Parasitaemia	-0.0767	0.0953	-0.3442	-0.2992	1.0000			
Age	-0.1245	0.1088	0.4844	-0.0834	-0.1347	1.0000		
PYR*	-0.161	-0.0567	-0.1199	-0.1691	0.1603	0.2436	1.0000	
SDX*	-0.1482	0.1360	-0.0588	-0.0337	0.1121	-0.1014	-0.1664	1.0000

Table 4.2 Correlation Matrix for the PYR-SDX Efficacy Studies.
SDX =sulfadoxine, PYR=pyrimethamine, Rx=treatment, Hb=haemoglobin, Par=parasitaemia (day0), Covariates that suggest a strong correlation (r) with Treatment outcome are marked with *. This correlation matrix was used only for a rough determination of which valuables were to be included in the multivariate regression model. Also initially, potential correlation among explanatory variables were noticeable from the table. P-values for each of the r-values are left out for convenience of presentation.

Variable	Odds Ratio	95% Conf.	Interval]	P-value
Folate	1.5	1.082469	1.975078	0.013
Day0 Hb	1.6	.287132	8.508683	0.61
Parasitemia	1.0	.9991995	1.000574	0.75
Age	32	1.190903	855.2582	0.039
PYR	0.99	.9771359	.9978644	0.018
SDX	0.98	.9674991	1.002672	0.096
Quintuple	0.9969	.7678144	.9999581	0.0091
DHPS	0.86	.0233219	32.00632	0.94
DHFR	0.00016	6.67e-11	373.1028	0.24
Site	6.0×10^6	73.04442	4.96e+13	0.010
Sex	17	1.684966	179.6801	0.016

Table 4.3 multivariate logistic regression analyses for association between different predictors variables and SP efficacy in Mangochi. Of the 105 cases who had all data on PYR, SDX, FOLATE, DHFR /DHPS genotype, 34 cases (all from Mangochi had no corresponding day 0 Hb records in the case record forms. These cases could not be used in the multivariate analysis, which led to n=71 for that purpose. Low iron/haematocrit status has been correlated with longer parasite clearance time in malaria patients (25). Thus given the fact that folate may enhance parasite life, it was imperative to include an adjustment for haemoglobin to see a clear effect of folate on treatment failure. The odds ratio reported for site is for Dedza, for sex=female, for age ≥ 2 years and for the presence of the quintuple.

4.1.8 DISCUSSION

4.1.8.1 ASSAY DEVELOPMENT

The analysis of folate in DBS matrix using the method described here offers a faster, less labour intensive and accurate option for folate screening in blood specimens. The calculation of folate level is dependent upon the Hb level, and differences in Hb levels are multifactorial (107). The hemoglobin concentration of the DBS elute was consistent with that reported [105] by O'Broin (140 – 180 g/L versus 148 – 179 g/L; mean=163 ± 6.15g/L). Both these ranges also agree with that reported in 'Scientific Tables': range = 134 –173 g/L with a mean of 158.5 g/L (107). The mean hemoglobin-folate (HF) for a 50 µl dried blood spot reported in this study was consistent with that measured using

microbiological assay quantitation (50 – 100 µl venous blood DBS, HF = 2607 ± 1195 pmol/g), the current gold standard assay (105).

Different ranges of whole blood folate levels have been previously reported (107), mean 12.0ng/ml, range 3.0 – 20.0 ng/ml; mean 6.35 ng/ml, range 1.5 – 25 ng/ml; and mean 89 ng/ml, range 47 – 149 ng/ml as determined by different methods. The former two are quantitated microbiologically using Streptococcus faecalis and pediococcus cerevisiae respectively and the later by using Lactobacillus casei (108). The normal physiological level of folate in human plasma is approximately 12 – 45 nM (5.3 – 20 ng/ml), mainly in the form of 5-methyltetrahydrofolate (109,110). The huge range of whole blood or serum folate levels may be explained by differences in methods of measurement as well as differences in dietary patterns. In humans folate is entirely diet-derived. It would therefore be expected that members of a certain population might have low folate dietary intake. In populations that consume folic acid-containing supplements or eat fortified cerials, certain members might exhibit higher blood folate levels. Folate results obtained in the population studied for the DBS assay development (mean folate concentration for whole blood was 66.0 ± 1.20 ng/ml) are within previously above-reported ranges for other populations.

The whole blood folate level is a superior indicator of folate nutritional status and consists of the polyglutamates and monoglutamates. The erythrocyte 5-methyltetrahydrofolate polyglutamates are hydrolyzed to assayable folate monoglutamates by endogenous DBS plasma γ-glutamyl hydrolase during elution. The method adapted and described for this thesis measures total whole blood folate with reasonable sensitivity (limit of quantitation 1.25 ng/ml) and will permit large-scale blood folate screening on DBS for the prevention and control of neonatal neural tube defects associated with folate status.

Since plasmodia can salvage folate (7,19) from a host who cannot synthesize it but must get it through food, then host nutritional differences can affect blood levels of folate, which will influence plasmodial growth in vivo. Russell et al. reported a very wide range

(5.0-21.0 ng/mL) for serum folate in Iran, suggesting a wide variation among individuals of various populations representing various socioeconomic classes from rural and urban areas (111). To achieve a clear understanding of the determinants of antimalarial drug failure, all possible factors including blood folate levels need to be explored in conjunction with field studies of molecular markers of resistance. Host-blood folate levels must be measured by reliable methods that are also suitable for large-scale field studies. The folate effect (folate antagonizing antifolate drug treatment) has been observed over comparable concentrations of folate measured in the assay described in this thesis (19). Thus it was possible to characterize the relationship between poor treatment outcome and physiological levels of folate in malaria cases who received a standard treatment of pyrimethamine-sulfadoxine in Malawi.

The method herein described combines the advantage of DBS collection (simple, economical) and rapidity of quantitation without culturing of organisms. 100 specimens can be run per folate kit and 24 eluted DBS specimens are analysed within 90 minutes. This method does not require discontinuation of PABA supplementation for accuracy as in Dueker's method and it uses relatively affordable instruments. Further, it does not require isotope-labelling of the folate for internal standard as with Santhosh-Kumar' method.

4.1.8.2 PYR-SDX EFFICACY STUDIES

Natural differences in host immunity (25), efficacy of absorption and metabolism of the drugs (23), serum folate levels, parasite genotypes (102) and their efficiency in using exogenous folates (7) have hampered the prediction of PYR-SDX therapeutic failure. The relative role of these factors in predicting PYR-SDX clinical efficacy has not been clearly characterized. For instance, the antagonistic role of physiological folate in PYR-SDX efficacy, although demonstrated in vitro, has not been clearly characterized using in vivo PYR-SDX efficacy studies where adjustments for host drug absorption, age, parasite density and genotype have been made. However, it is known that supplementation with folate resulted in a significantly higher rate of treatment failure among Gambian children

who received PYR-SDX (21). In contrast, folate supplementation in pregnant women showed no adverse effect on *falciparum* parasitemia in pregnant women (112).

A highly positive relationship between treatment failure and physiological folate levels found in Malawian children (table 4.2) should, therefore, not be unexpected. Moreover in the univariate analyis it was observed that measured whole blood folate levels were higher in cases that failed PYR-SDX therapy than those that had a successful clinical outcome (p=0.028, see figure 4.2, n=141). This association held up even after adjusting for age, pre-treatment haemoglobin, pre-treatment parasite density and, parasite DHFR/DHPS genotype (table 4.3). Malawian children exhibited increasing parasite densities with increasing physiological folate levels (figure 4.5) but this trend was not statistically significant (p=0.32). This observation is not unexpected because certain Plasmodium falciparum genotypes are efficient at utilising exogenous folate (19,21). The study described here is the first to clearly demonstrate the independent role of physiological folate levels in *in vivo* PYR-SDX therapeutic efficacy with all other important confounding covariates adjusted for.

The observed independent association between site of study and PYR-SDX treatment failure was surprising since the prevalence of Plasmodium falciparum DHFR and DHPS genotypes was the same for the two sites Dedza and Mangochi. Furthermore the mean age for children at both sites was not different (36 ± 27 vs 28 ± 24 months, p=0.12 respectively). This could in part be explained by differences in transmission intensity and therefore acquired malaria-specific immunity, and the higher folate levels in the Dedza subjects.

Partial immunity to malaria is thought to develop over years of repeated infections. Spontaneous elimination of parasitemia and treatment response was found to improve with age in malaria-endemic areas (113). The present study found an independent association between age and PYR-SDX therapeutic efficacy. This observation is in agreement with previous studies (113). It is important to mention that the mean ages

(month) for sensitives and failures were not different in a univariate analysis (28 versus 27, p=0.84).

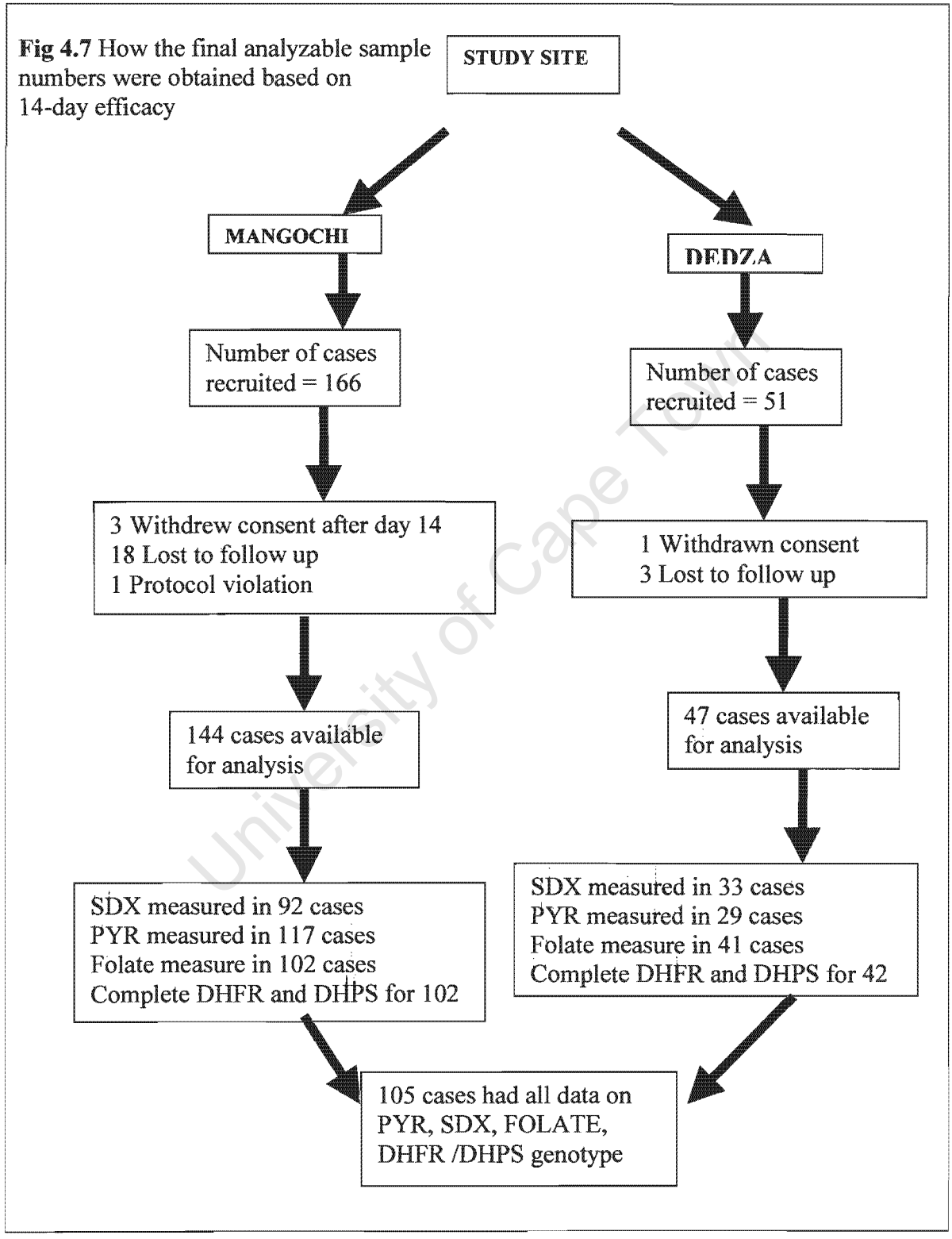

Fig 4.7 How the final analyzable sample numbers were obtained based on 14-day efficacy

4.2 PARAAMINOBENZOIC ACID

4.2.1 PABA ASSAY DEVELOPMENT

P-aminobenzoic acid (PABA) is a folate metabolic precursor. It is present in an unphysiologically high concentration (1 mg/ml) in RPMI 1640, the medium generally used for plasmodium culture and for this reason it has made difficult, through antagonism to sulfonamide activity, the development of in vitro tests for resistance to PYR-SDX (41). Brockelman and Tan-Ariya reported that sulfonamide susceptibility was considerably enhanced in the PABA-free Waymouth formula medium (42). In humans PABA is supplied nutritionally. Recently (1985) Watkins et al. demonstrated the antagonism of sulfadoxine and pyrimethamine antimalarial activity in vitro by p-aminobenzoic acid, p-aminobenzoylglutamic acid and folic acid (22). Plasmodia do not synthesize PABA, since nutritional studies indicate that it must be supplied in the diet (34). Many methods of assessment of pancreatic exocrine function measure PABA levels in serum after ingestion of the appropriate probe drug.

Various investigators (114, 115) have measured paraaminobenzoic acid (PABA) in blood by chemical methods. Liquid chromatographic procedures for determination of PABA in urine and serum have also been described (116). Colorimetric methods that have been developed are based on the reactions of aromatic acids where PABA is diazo-coupled or determined by the dimethylaminocinnamaldehyde method. Thus, patients who are taking some drugs may give false high results and some food products may also interfere with determination of aromatic amines (117,118,119). A method for quantifying PABA in whole blood or plasma blotted on filter paper has not been described. Such a method will facilitate the study of PABA disposition in humans and animals. In this project we determined PABA levels in whole blood spots by a high performance liquid chromatography method.

4.2.2 MATERIALS AND METHODS

4.2.2.1 REAGENTS

HPLC grade acetonitrile (MeCN, BDH Laboratory Supplies) and HPLC grade water purified through a Millipore ion exchange system was filtered using a 0.45 μm filter (Millipore HALP filter disk) and subsequently degassed by bubbling helium gas for ten minutes at ambient temperature.

The sodium salt of 1-heptane-sulfonic acid (1HSA) was obtained from Sigma. Trifloroacetic acid (TFA) was from Merk-Schuchardt. Eppendorfs (1.5 ml) were from Abbot Laboratories, USA. PABA (4-aminobenzoic acid) and PAHA were from BDH chemicals Ltd and Fluka Biochemica. n-hexane was from Scharlau Chemie S.A. Ethanol and ethylacetate were obtained from Merck Laboratory supplies. Sodium hydroxide, concentrated HCL, and chloroform were from BDH Laboratory supplies. Whatman 3MM filter paper was from Whatman International Limited.

4.2.2.2 APPARATUS

Two solvent pumps (Shimadzu LC-10AS liquid chromatograph pumps) were connected to an automatic injection valve (SIL-10A Shimadzu Auto Injector, model SIL-10A) equipped with a variable injection loop linked to a variable UV Diodearray Detector (Shimadzu, model SPD – M10A) by a Communication Bus Module (Shimadzu, model CBM-10A). The detector was coupled to a Professional Computer (Professional Computer Manufacturers, model 486 GV), which was linked to a printer (Hewlett Packard DeskJet 670c). The computer recorded and stored all charts/chromatograms. A Haisil 100 C8 5μm analytical column 250 × 4.6mm was used for PABA assay with a C18 guard column placed before it to prolong its lifespan. PABA detection was at 274 nm at a sensitivity of 6mAbs.

4.2.2.3 PABA EXTRACTION AND ANALYSIS

The mobile phase, 30mM 1-heptane-sulfonic acid/acetonitrile (MeCN/1HSA, 50:50, v/v, pH2.68), was spiked with PABA and PAHA to assess linearity in the concentration range, 15 – 500 ng/ml. Drug-free whole blood or serum was spiked with PABA and PAHA in quantities covering this concentration range. The spiked blood (200 µl) was then spotted on whatman no.3 filter paper and air-dried overnight. To the filter paper DBS snippet in a glass test tube, 2 ml of NaOH (1 M, pH 13.59) was added and incubated for 15 minutes at ambient temperature. The incubation was done simultaneously with a sonication process. TFA (0.1%, 2 ml) was added to the sonicated DBS specimens and vortexed twice (1 minute each time) to acidify. Ethanol (3 ml) was then added to the acidified DBS, vortexed vigorously for 1 min and the mixture spun at 4000 rpm for 10 min. The supernatant was aspirated into a clean tube. Chloroform (5.0 mL) was then added to the acidic water/ethanol supernatant. With vigorous agitation (1 min) PABA was extracted into the chloroform phase as a neutral molecule. The aqueous/ethanol phase containing the DBS snippets was extracted a second time with chloroform and the combined chloroform extract evaporated to dryness with an RC10.10 vacuum rotary evaporator connected to a cold trap (-180 ° C) and a pump. The PABA residue was resuspended in 0.1% TFA and 100 µl of extract injected directly into the chromatographic column. The metabolite p-amino-hippuric acid (PAHA) was taken through the whole procedure to determine if it would interfere with PABA determination. To assess PABA stability in DBS stored at room temperature, 4 ° C and 37 ° C for 1 month, specimens were prepared and stored appropriately. DBS PABA level was measured at baseline and after 1 month.

4.2.3 STATISTICAL ANALYSIS

Data for AUC-Drug Concentration curves were fitted in a regression analysis using GraphPad Prism® (GraphPad software version 2.01). P-values were calculated using F test. Errors were reported as standard error of mean Recovery was calculated using spiked controls and standards in mobile phase.

%Recovery = {(Sample Peak area) ÷ Standard in mobile phase Peak area}× 100

4.2.4 RESULTS

4.2.5 ASSAY DEVELOPMENT AND VALIDATION

Good chromatograms and a practical PABA retention time (RT) were obtained when the PABA stock was made by dissolving the drug in either 0.1% TFA or 1HSA and injecting 100µl of the sample in a mobile phase of MeCN/1HSA (50:50, v/v, pH 2.68) at a flow rate of 0.5 ml/min. Concentration ranged from 12.5 ng/ml to 500 ng/ml. Figure 4.8(a) shows the PABA calibration curve in spiked mobile phase and the chromatogram is reported in figure 4.8(b).

Concentration (ng/ml)	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
0.0	0.0	0.0	0.0	0.0	0.0	0.0
15.125	20458.0	14735.0	13143.0	16690.0	5224.0	9294.0
31.25	19720.0	12484.0	28469.0	23123.0	13698.0	13698.0
62.5	23111.0	17660.0	39176.0	23470.0	31676.0	24336.0
125.0	33181.0	43820.0	73529.0	70906.0	59993.0	56039.0
250.0	118400.0	104860.0	143290.0	163364.0	147058.0	140264.0
500.0	233627.0	218680.0	318623.0	308146.0	319166.0	320518.0

Table 4.4 Data for figure 4.8

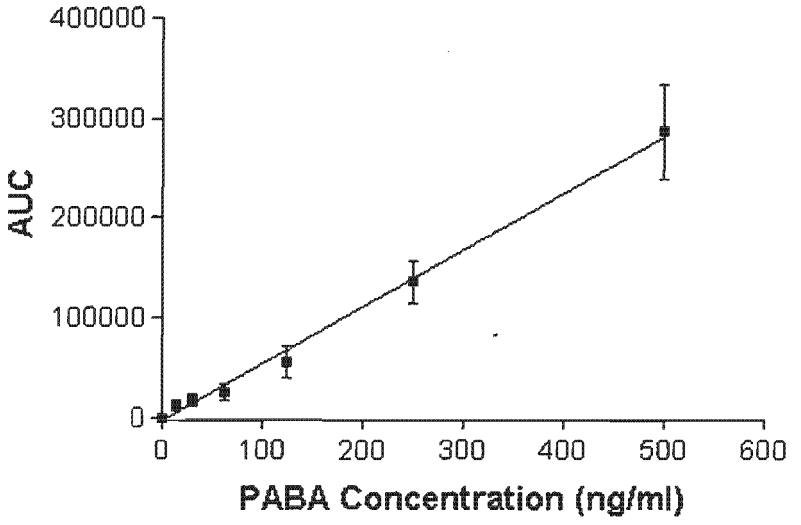

Figure 4.8(a) Graph of AUC (Peak Area) plotted against concentration of PABA spiked in mobile phase (MeCN: 1HSA, pH 2.68, 50:50, v/v, 0.5ml/min). This graph is constructed from data shown in Table 4.2.1. Equation: $y = 571x + 3446$, $r^2 = 0.9950$

Specimens of PABA were prepared in triplicate at concentrations of 500ng/ml and 31 ng/ml by either spiking known amounts of the drug in mobile phase or spiking drug-free whole blood and blotting 100µl on filter paper. Results are reported in Table 4.4.

	Actual PABA Concentration ng/mL (No. of Samples)	Measured Mean PABA concentration (ng/mL) ± SD	95% CI
PABA in spiked mobile phase	500 (N=3)	444.0 ± 94.44	209.4 – 678.6
	31 (N=3)	22.17 ± 8.959	-58.33 – 102.7
PABA in spiked Dried Blood Spot	500 (N=3)	437.9 ± 88.49	218.1 – 657.7
	31 (N=3)	20.78 ± 2.786	-4.251 – 45.81

Table 4.5 Studies for accuracy and precision

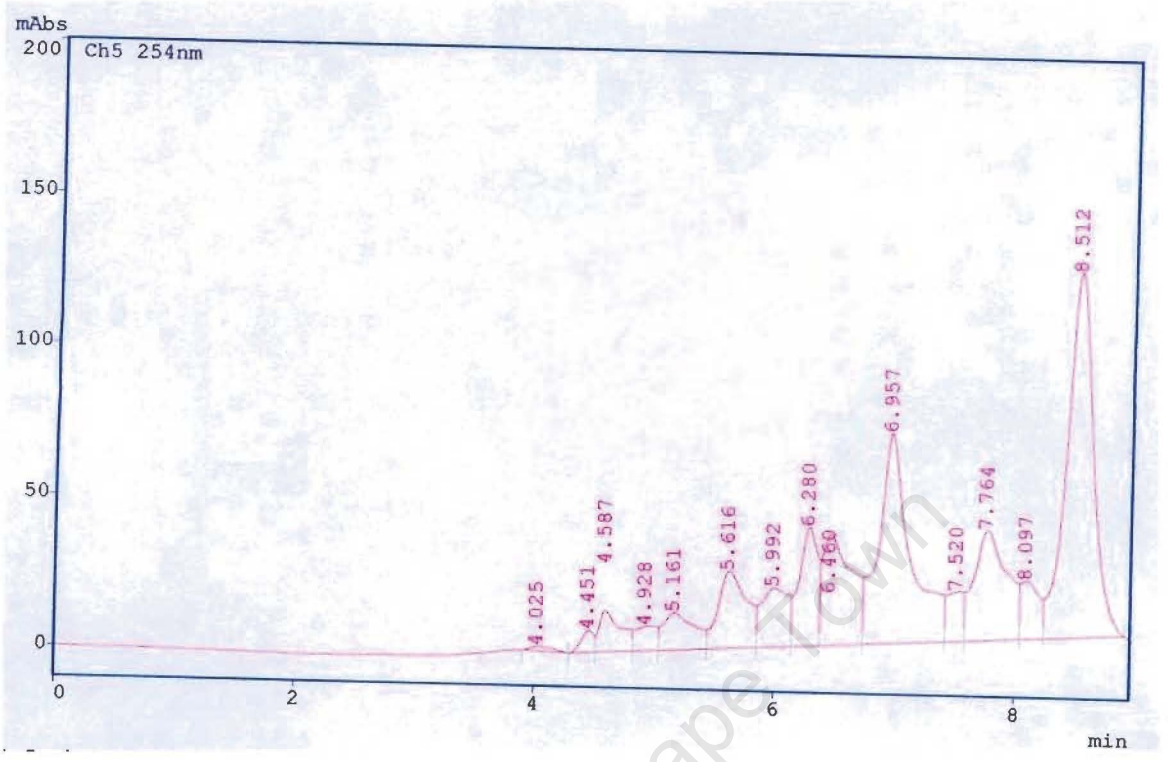

Figure 4.8(b) The HPLC trace for PABA (8.5 min.) and PAHA (paraaminohippuric acid, 6.9 min) in acetonitrile/1HSA (50:50, v/v, pH 2.68) solvent system. This was obtained by injection of 100µl of a 1 µg/ml each of PABA and PAHA onto a 5µm C8 (Haisil) reverse chromatographic column. PAHA (RT=5.5 min.) did not interfere with PABA analysis under the buffer conditions used.

More experiments were done to assess the efficiency of the extraction method over the entire PABA concentration range of interest, 500 ng/ml – 15 ng/ml and figure 4.9 shows the calibration curve from analyzed DBS extracts.

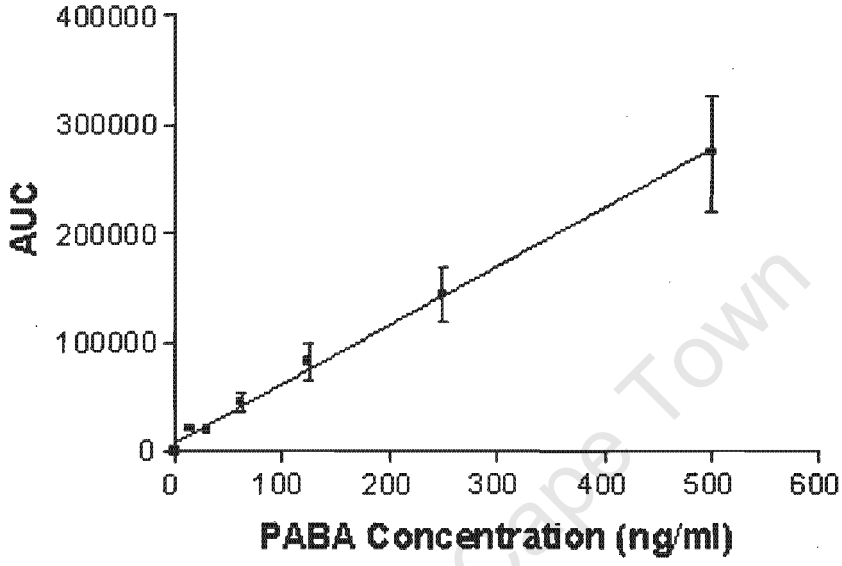

Figure 4.9 Linear plot of paraaminobenzoic acid recovered from DBS. Mobile phase was MeCN: 1HSA, pH 2.68, 50:50 (v/v), and 0.5ml/min.
Equation: $y = 538x + 8291$, $r^2 = 0.9966$.

In Table 4.6 below, PABA recoveries from DBS are reported. The mean recovery over the entire concentration range was 84.00 ± 4.041% (95% CI: 66.0 – 101). Inter-assay CV for recoveries in this concentration range was 8.33 %. Inter-assay CV was 3.41% and 16.06% at 500 ng/ml and 62 ng/ml respectively. The mean intra-assay was 8.22% for the range.

PABA DBS Concentration (ng/ml)	Trial 1	Trial 2	Trial 3
500.0	74	79	74
250.0	80	87	74
125.0	88	91	85
62.50	106	100	77
31.25	113	ND	67
15.13	230	ND	ND
Mean % Recovery for Concentration Range	87	89	76

Table 4.6 Recoveries (%) of paraaminobenzoic acid from dried blood spots on filter paper. ND = Not detected

After 1 month the mean PABA content recovered from filter paper stored at room temperature, 4°C and 37°C was not different (357.5 ng/mL, 488.3ng/mL, 354.1 ng/mL respectively) from the PABA content as determined on day 0 (524.4 ng/mL). Although there was no statistically significant difference in recovered amounts of PABA, it appeared that at room temperature and 37°C some of the drug was lost as evidenced by the relatively lower recoveries 357.5 and 345.1 g/mL (cf. 488.3 ng/mL recoverd at4°C) Storage of PABA at 4°C, and away from light and heat for periods longer than 1 month may hence be recommended.

4.2.6 PABA AND THE IN VIVO PS EFFICACY STUDY

At the time of pretreatment sample collection 100µL each for SDX, PYR, Folate and PABA respectively had to be obtained per subject. In addition, enough blood for parasite PCR genotyping had to be collected. All subjects were children aged between 6 months and 12 years. Due to the difficulty of having to obtain four spots of capillary blood specimens from a subject and due to consent withdrawal associated with repeated finger pricking (where necessary) and squeezing, we did not manage to obtain specimens for the PABA analysis. The field PS efficacy study started well after the assay described here had already been developed. The assay is reported for future studies that may need its use.

4.2.7 DISCUSSION

The method described, can reliably measure PABA levels in 200 µl of whole blood or serum spiked with PABA in the concentration range 31 ng/ml – 500 ng/ml. PABA recoveries from filter paper were good with mean % recovery of 84.00 ± 4.041% in the aforementioned concentration range. It should therefore be possible to measure blood levels of PABA in parasitologically resistant and sensitive malaria cases. Reasonable estimates of the physiological levels of PABA through backward-extrapolation, towards the origin, may be also obtained using a linear graph of the studied concentration range (15 – 500 ng/ml). The method is sensitive with a limit of quantitation of 31 ng/ml. Extracts obtained using our method may be subjected to a HPLC – Mass Spectrometer quantitation of the PABA content. These detection methods being sensitive would be of great value if coupled to our efficient extraction method. This would more than offset the sensitivity limit of the method herein described. Thus, it would be possible to estimate p-aminobenzoic acid values between 0 and 15 ng/ml.

Peerapan Tan-ariya (42) found that 25 ng/ml is the lowest concentration of PABA that would support normal growth for the SDX-sensitive strains. Using the method herein described concentrations above 25 ng/ml can be reasonably estimated. At PABA concentrations greater than 25 ng/ml, otherwise sensitive *falciparum* strains would have a survival advantage due to PABA antagonism of sulfadoxine, which acts to inhibit DHPS utilization of PABA. Under these circumstances high PABA concentrations would effectively compensate for the through-mutation-compromised DHPS functionality. This means that with the methods herein described it is possible to estimate PABA concentrations that would cause otherwise PYR-SDX sensitive *falciparum* strains to fail PYR-SDX therapy. This PABA assay could be important for future studies that may seek to investigate the relative role of PABA in PYR-SDX therapeutic failure.

Although it was not possible to obtain specimens from malaria patients for PABA blood level analysis, this assay has other potential applications. For instance, methods for testing pancreatic function (116) use PABA excretion index to investigate pancreatic

disease. People are given n – benzoyl – tyrosyl para – amino benzoic acid (NBT – PABA) to ingest and after the appropriate time serum or urine levels of PABA are measured (118). Plasma / urine PABA concentration increases in health people whose pancreas can cleave PABA from NBT – PABA. Levels rise very slowly in people with pancreatic disease. Thus the PABA assay offers a convenient method for collecting plasma specimens on filter paper matrix using small a amount of plasma (200 µl). It may be relatively easy to adapt the DBS PABA method to urine with success. Investigations into PABA disposition in humans or animals may need to be done using whole blood in order to determine the blood to plasma distribution ratio of the drug.

A high performance liquid chromatographic method that allows determination of filter-paper-collected capillary blood PABA in the concentration range 15 – 500ng/mLwas thus developed and validated in this project.

4.3 STUDY SHORTFALLS

The major shortfall of this thesis study is that physiological paraaminobenzoic acid (PABA) was not measured. It is suspected that PABA may influence in vivo PYR-SDX efficacy independently of folate levels in the light of the fact that Krungkrai et al. showed that *Plasmodium falciparum* synthesizes pteroylpolyglutamate de novo from guanosine 5'-triphosphate (GTP), p-aminobenzoate (PABA), and L-glutamate (28). In addition, since in another study these parasites were seen (30) to have the capacity to synthesise pteroylpolyglutamate from both intact and degradation moeities (p-aminobenzoylglutamate and pterine-aldehyde) of exogenous folate added into the growth medium, it would have been appropriate to adjust for physiological PABA levels. This study did not have sufficient blood specimens to allow the inclusion of PABA in the analysis. Thus, it cannot be known from this study whether folate level influenced PYR-SDX levels independently of PABA levels.

However, since the 'folate effect' did not segregate with dihydropteroate synthase genotypes (genes encoding DHPS, an enzyme whose substrate is PABA) in a genetic cross between parent clones, one exhibiting the folate effect and one not (18), and since the folate effect showed complete linkage to dihydrofolate reductase gene in the cross progeny, it seems inconceivable that folate would not influence PYR-SDX efficacy independent of PABA which must exert its influence via DHPS, which is not linked to the folate effect.

CHAPTER 5

OVERALL DISCUSSION AND SIGNIFICANCE OF STUDY

5.1 DISCUSSION

It has been shown for the first time in this thesis that DHPS/DHFR mutations, independently of folate, drug levels and acquired immunity, determine the clinical outcome of antimalarial treatment with PYR-SDX. More importantly it has been demonstrated that patients infected with the most highly mutated forms (parasites with 3DHFR and 2 DHPS mutations) of *P.falciparum* are more likely to fail PYR-SDX treatment than those infected with the less mutated forms. Nevertheless, it was found that a large proportion of patients, though infected with this genotype, were treated successfully with the drug. This suggested that parasite mutations, although they may predict the therapeutic outcome with reasonable accuracy, might not be the only determinant of the treatment outcome in infections with *P.falciparum*. By conducting the PYR-SDX in vivo efficacy study at two sites differing in transmission intensity it was designed that site (thus transmission intensity and hence acquired immunity), would be used as another surrogate marker of malaria-specific immunity besides the routinely used marker, age. Indeed it was found that PYR-SDX efficacy was higher in the high than low transmission area despite not only a similar prevalence of DHFR triple and DHFR/DHPS quintuple mutants but also similarity of drug concentration-time profiles. This result suggested that transmission intensity and hence immunity may indeed play an important supportive/modulating role in the therapeutic response to antimalarial drug like PYR-SDX.

Results of the PYR-SDX efficacy study were as expected for each of the sites based on their relative transmission levels. Blantyre city is a lower transmission setting compared to Mangochi. Despite the apparent similarity in the prevalence of the quintuple mutants (60% versus 64 % respectively) Blantyre had a treatment failure rate twice that of Mangochi. This may be explained by the development of partial immunity in the Mangochi population owing to the people's repeated exposure to malaria antigens. This kind of non-sterile partial immunity aided in the clearance of *P.falciparum* parasitaemia. The two low transmission sites, Dedza and Blantyre had similar prevalence of quintuple

mutants (61% versus 60% respectively) and the rate of PYR-SDX failure (22% versus 20% respectively).

Although there was no difference in the mean child age between Dedza and Magochi cases (24 months versus 23 months, range for both sites: 6 to 120 months), at both sites, children under five years of age had a higher rate of treatment failure than older ones but without achieving statistical significance. Interestingly age was indeed independently associated (P=0.049) with PYR-SDX therapeutic failure after adjusting for site, parasite density, hemoglobin, drug levels, sex, physiological folate levels and parasite resistance genotype. Thus this result also seemed to support the conclusion that repeated exposure to infective mosquito bites in a high transmission setting (and longer exposure by reason of age) may have resulted in the development of malaria-specific partial immunity. This specific premunition (and not exclusively parasite genetic resistance) may thus influence the therapeutic response to antimalarial drugs. Indeed two previous studies (Baird et al., 1991 and Mayxay et al., 2001) showed a strong association between long history of exposure to malaria, the presence of antimalarial antibodies and increased efficacy of PYR-SDX (68, 137).

This thesis study is the first to demonstrate the independent role of physiological folate levels in influencing sulfadoxine therapeutic efficacy. The antagonistic role of physiological folate in PYR-SDX efficacy, although demonstrated in vitro, had not been clearly characterized using in vivo PYR-SDX efficacy studies with proper adjustments for the covariates, host drug absorption, age, gender, pretreatment parasite density and hemoglobin, DHFR/DHPS genotypes, and immunity. Previous studies (very few) assessed the role of folate supplementation and not physiological level (i.e. blood folate levels attributable to natural food sources other than medication). Furthermore, those studies did not measure the level of folate attained in the blood so that they could therefore not assess a quantitative association with parasite resistance. Also in those studies the individual contribution of critical *falciparum* DHFR/DHPS mutations and drug levels were not adjusted for.

The PYR-SDX oral disposition results revealed a wide interindividual variability in blood PYR-SDX levels. At day 3 post-treatment about 48% of the patients appeared to have blood SDX levels that were below those required for effective synergy against resistant parasites in vitro – an in vivo situation most certainly facilitating drug selection of resistant *falciparum* genotypes. Also, by day seven, pyrimethamine levels appeared far below those required for synergy with sulfadoxine. It was apparent that there was some degree of variability in the length of time parasites were, per patient, exposed to PYR-SDX concentrations above the level effective against resistant strains. These observations could partly account for differences amongst patients in their ability to clear resistant parasites. Quite intriguingly, only pyrimethamine and not sulfadoxine was associated with PYR-SDX treatment failure after adjusting for all confounding covariates. This constitutes a first strong demonstration of the critical role of pyrimethamine in the therapeutic efficacy of the combination PYR-SDX where physiological folate levels and all critical DHFR/DHPS mutations (in Africa) were included in the analysis. It must be emphasized that this study did not find any patients with sub-therapeutic drug levels defined as concentrations required to kill genetically susceptible *falciparum* strains in vitro or concentrations required to prevent the development of secondary parasitaemia in non-immune subjects. What was found, rather, was that the drug levels beyond day 3 could not be effective against genetically resistant parasites, thus warranting parastological resistance. Such 'sub-therapeutic' drug levels coupled with the ability of certain *falciparum* strains to utilize exogenous folate (which in this study was shown to be significantly correlated with parasite recurrence) would probably result in the late recrudescence of even otherwise genetically susceptible parasites. Indeed a high proportion of such genotypes were found in isolates among individuals with a sensitive (ACPR) PYR-SDX treatment outcome. A recent study demonstrated in vitro the recrudescence of susceptible FCR3 strain in cultures containing inhibitory PYR concentration (74).

5.2 SIGNIFICANCE OF THESIS RESULTS

5.2.1 NUTRITION AND ANTIFOLATE EFFICACY

Results from the folate study suggest that significant differences in folate dietary intake among individuals living in malaria endemic areas may result in a corresponding difference in in vivo PYR-SDX (or other antifolate drugs) therapeutic response. Since folic acid is entirely diet-derived, it may thus be one of the critical determinants of parasitological resistance to antifolates in malaria endemic areas, where individuals have a high dietary in-take of folates. It is important to note that no subject recruited in the study described in this thesis received any folic acid or iron supplementation. In one study, no adverse effect, due to folic acid supplementation, was observed on parasitemia in pregnant women whereas supplementation in children resulted in a higher frequency of therapeutic failure among those that received folic acid supplementation. These contradictory findings on the role of folates in malarial disease highlight the need to thoroughly understand the effect of folic acid supplementation in pregnancy on malarial disease and to further assess the effect of dietary-derived folate.

5.2.2 MOLECULAR MARKERS OF RESISTANCE

Apart from aiding the rational designing of a new generation of antifolates effective against resistant *falciparum* strains, studies of the molecular markers of resistance have been aimed at finding a limited set of parasite mutations that could conveniently be used as surveillance tools to predict the efficacy of antimalarial drugs like PYR-SDX and chloroquine. Such ability would enable national treatment policy makers to quickly assess antimalarial efficacy and through epidemiological mapping of resistance foci, deploy effective drugs to the appropriate areas. According to the most recent observations (66), the best marker of sulfadoxine-pyrimethamine resistance in the field is the presence of quintuple mutants (S108N + C59R + N51I + A437G + K540E). In this thesis study it was demonstrated that the quintuple mutant *P.falciparum* was strongly predictive of

treatment failure. More importantly it was shown that the presence of this genotype could be predicted with the combination of only a single DHFR and a single DHPS mutation. This meant that treatment failure could be predicted using a set of only two mutations. This would be a resistance surveillance tool that allows a rapid assessment of drug efficacy at a minimum laboratory cost. As demonstrated in this thesis, the independent association of the quintuple mutant with therapeutic failure even after adjusting for folate, means that the quintuple mutant can predict treatment failure without necessarily determining blood folate or drug levels. This renders credence to the practicality of using molecular markers as large-scale PYR-SDX resistance surveillance tools.

Results of this thesis suggest that mutations in the dihydropteroate synthatase gene may not be a prerequisite for resistance and that they play only a minor role in the early stages of PYR-SDX resistance in Africa although the presence of such mutations augments the parasite's level of resistance. This agrees with previous suggestions in an excellent review by Sibley et al (135) (135).

5.2.3 MALARIA VECTOR CONTROL PROGRAMMES

The observed higher efficacy of PYR-SDX in a high transmission setting again raises the question whether measures that reduce parasite transmission and thus immunity may lead to reduced PYR-SDX clinical efficacy in an environment with an increasing prevalence of antifolate resistance-conferring mutations. An excellent review on the relationship between malaria control/transmission intensity and the evolution of drug resistance was done by Ian Hastings (134). It is well known that repeated exposure to malarial infection results in the acquisition of antiparasite immunity, which protects against clinical disease (76). This immunity is partial and non-sterilizing. The majority of people in malaria endemic areas are parasitaemic but asymptomatic and in these people parasite levels are maintained below threshold levels. The rationalization of malaria interventions that reduce exposure in people living in endemic areas might therefore be warranted seeing that this partial immunity appears to augment antimalarial drug efficacy. It may be

perceived from this study that there is reason for optimism in the development of a malaria vaccine that elicits some degree of protective immune response.

5.2.4 CHEMOTHERAPEUTIC MANAGEMENT OF MALARIA

This study showed that parasite recurrence might not always be as result of sub therapeutic drug concentrations but that it seems to be due, in part, to the length of the time during which parasites were exposed to synergistically effective concentrations. In this study it was demonstrated that although predicted therapeutic levels had been sustained until day 3 post therapy, immediately thereafter levels dropped below those synergistic against resistant *falciparum* isolates. This meant that only one parasite growth cycle had been exposed to an inhibitory concentration. This observation highlights the need to devise ways of keeping synergistic in vivo PYR-SDX concentrations beyond day 3 such that at least 3 *falciparum* growth cycles would be exposed to effective drug levels.

5.2.5 DEVELOPMENT OF DRUG ASSAY PROCEDURES

A more sensitive reversed phase high performance liquid chromatographic method was developed for the analysis of pyrimethamine (PYR) and sulfadoxine (SDX) in 100 µl venous dried blood spots (DBS) on filter paper. Recovery of PYR from spiked blood applied to filter paper and dried was 85 ± 4.508%. The coefficient of variation at 1.000 µg/ml – 0.015 µg/ml was 10.3 % and the limit of quantitation was 31 ng/ml. The DBS SDX recovery was 78 ± 3.8% with an inter-assay variation at 12.5 – 200 µg/ml of 11.8% (CV < 10% at 200µg/ml). The limit of SDX quantitation was 6 µg/ml.

A reliable method for measuring whole blood folate from dried blood spot (DBS) was developed. Greater than 75% DBS folic acid was eluted by single cycle sonication in the IMx folate lysis reagent. Within run CV was 7.7% at 20 ng/ml and 4.8% at 5 ng/ml. The correlation between detector response and DBS folic acid concentration was positive (r^2 =

0.97, P = 0.0003) in the range studied (0 – 20 ng/ml). Only 50 µl of capillary blood per dried blood spot was used. The method agreed well with that using *Lactobacillus casei*, the current gold standard reference assay. By adapting DBS specimens to the IMx folate kit, this method facilitates accurate folate analysis using a commercially available kit and does not require a microbiological assay to quantitate the folate. This method is appropriate for detecting folate deficiency and assessing factors associated with antifolate.

A chromatographic method was developed for the quantitation of PABA in dried blood specimens. The method could reliably measure PABA levels in 200 µl of whole blood or serum spiked with PABA in the concentration range 31 ng/ml – 500 ng/ml. PABA recoveries from filter paper were good with mean % recovery of 84.00 ± 4.041% in the aforementioned concentration range. The method was sensitive with a limit of quantitation of 31 ng/ml.

5.3 FUTURE RESEARCH WORK

The development of a malaria vaccine being a priority for improved and sustained malaria control, an effective sub-unit vaccine to be developed would have to encompass a wide variety of *P.falciparum* genotypes infecting the most venerable group of people, pregnant women and children.

It will thus be interesting to measure the malaria-specific antibody profiles, including any other immune markers that correlate with protection), in our cases of *P.falciparum* (sensitive and resistant cases). This will determine the relative role of host partial immunity in augmenting antimalarial therapeutic efficacy and address the question 'why are some individuals able to clear otherwise drug resistant parasite genotypes?'
A comparison of immunoglobulin level and composition between treatment failure and sensitive cases will also more clearly assess the relative contribution of malaria-specific acquired immunity in the clinical and parasitological resolution of malarial disease.

Malawi is a potential and prospective vaccine-testing site in central Africa (Dr. JG Kublin and Prof. C.V. Plowe, University of Maryland, Malaria Section, Center for Vaccine Development, USA; personal communication). I suspect that humoral responses to candidate vaccine antigens may be modulated by the host's history of exposure to the parasite in question. Thus a knowledge of the baseline repertoire of specific, protective antibodies directed against common *P.falciparum* variants in Malawi is crucial to the accurate assessment of protective potential of various analogues of recombinant *P.falciparum* proteins that may be tested in Malawi in a bid to develop viable malaria vaccine.

Studies in immune adults living in areas (Malawi) endemic for *P.falciparum* transmission, investigating the IgG1 and IgG3 antibody responses to various analogues of recombinant *P.faciparum* Merozoite Surface Protein-1 and how seasonality of transmission modulates the immune responses, would be critical to any attempt to a vaccine-based malaria control strategy. Malaria continues to be one of the greatest contributor diseases to the high mortality and morbidity rate among infants and pregnant women in Malawi and worldwide. This proposed future research work is pertinent to the quest for a viable and lasting solution to this global malaria problem.

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