DEVELOPMENT OF HIGH PRESSURE LIQUID CHROMATOGRAPHIC METHODS FOR DETERMINATION OF ANTIMALARIAL DRUGS AND THEIR APPLICATIONS IN MALARIA ENDEMIC AREAS

By

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy (Clinical Pharmacology) of the University of Dar es Salaam

University of Dar es Salaam

June, 2005

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of the university of Dar es salaam, a thesis entitled **"*Development of High Pressure Liquid Chromatographic Methods for Determination of Antimalarial Drugs and Their Applications in Malaria Endemic Areas*"**

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DECLARATION

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DEDICATION

This work is dedicated to my lovely, caring and God given wife Albina Kirango, our daughter Kweji and our sons: Joseph Buyegi, Joshua Butindi and Jeremiah Lubango-The four Minzilets-Make sure you all my children challenge me academically, I have blessed you all in the name of Jesus. Excel and fly as much as possible, my blessings from God covers your generation and other three to come.

ACKNOWLEDGEMENTS

I first thank GOD, The Lord Jesus, for giving strength and good vision to my trainers and supervisors and for the grace He gave me and established my steps on the Rock.

I would like to acknowledge my Supervisors Prof Amos Y Massele and Lars L Gustafsson for a good work they did during these 4 years of my PhD program. Without their commitment, it would not have been possible to accomplish this work. They have really made me love science. Both Prof. Lars Gustafsson and Prof. Amos Massele played major role in my training. They are the key persons in the establishment of the analytical laboratory at MUCHS in which part of my PhD work was carried out. I deeply thank Dr Orjan Ericsson for the training he gave me in the development of analytical method. He is a giant in drug analytical method development and their applications. He has a great share in my success. Through his devotion I could develop some HPLC methods which were key tools for this work. He was kind and knowledgeable. Dr Moshi Mainen was very helpful in preparation of my PhD proposal until it was accepted for registration. Further, Dr Moshi also played a great role as co-author of the second paper on the quality assessment of drugs published in 2003.

I thank Margareta Rais for technical and moral support she gave during my PhD work. She was responsible for all the technical procedures related to analytical works in the laboratory. She was also responsible for taking care of my arrivals and departures and could fit in my African way of viewing things. I deeply acknowledge all the workers in the Division of Clinical Pharmacology at Huddinge Sweden for all kinds of assistance they gave me during my training in drug analysis in their Division.

Prof Anders Rane, The head of Clinical Pharmacology at Huddinge for receiving and permitting me to work in the division as a guest researcher, he was kind and friendly, Prof Leif Bertlisson, my old friend and white African for the Clinical Pharmacokinetics book and for friendly discussions, Tomas for technical support, Margit Eriksträm for a friendly smile and administrative support, Yvonne Sjölind for accommodation arrangements for my stay in Sweden, Susanne Molander for administrative support, Moustafa for nice discussions and scientific comics, Jan-Olof Svensson for his support in analytical methods, Agneta Wennerholm and her husband Gunnar for a trip to the Baltic Sea, she was was kind and helpful, Rajaa Mirghani for good handling and asssitance in computer manipulations and other things in the laboratory and for maintaining the beauty of Africa in the laboratory in the bush of white hunters, Dr Lennart M, Stefan Lundgren for his time, Jolanta, Lilleba, Birgita, Annica S and Jenny for soothing smiles.

Jaran Eriksen for working friendly in the field, he was focused and serious on the work not pompous but simple and serious, Prof Goran Tomson for a friendly atmosphere, for moral and academic support and maintaining good collaboration between KI and MUCHS.

I thank Prof K Pallangyo who iscurrently the Principal of MUCHS for giving me a tremendous support. Both the Dean of School of Medicine Assoc. Prof Mkonyi and the Associate Dean of Research & Postgraduate Studies Assoc. Prof Lyamuya were very helpful in my work. I thank the former Principal of MUCHS, Prof Mtabaji J for supporting me both morally and administratively, the former Director for Postgraduate Studies, Prof Mhalu F for enabling prompt registration of my PhD program and for facilitating financial support.

I thank Assoc Prof S Massawe S-The current Director for Postgraduate Studies, for financial and administrative support for my PhD Program. The Registrar Prof Lembariti and Deputy Registrar Prof Manji for their administrative and moral support. Assoc. Prof. David Ngassapa, the former Assoc. Dean of Postgraduate studies School of Medicine- for timely processing my PhD documents, Dr Chambuso Mhina-Former Dean of School of Pharmacy for supporting my enrollment in the PhD Program, Dr Olipa Ngassapa –The current Dean of School of Pharmacy for moral support and caring love, Joyce Nyambelwa-Administrative officer of the Postgraduate studies and Research Directorates- For giving assistance for all PhD related issues, Assoc. Prof Mary Temu for her maternal love and care, Dr Mung'ong'o S -the Head of Medicinal Chemistry Department, Dr E. Kaale for the sulfadoxine metabolite, Kagoma, Sufi and Mubena for the support in their Microbiology laboratory, Mwenge TAG for their prayers, Jeofrey Lusibamaila and his wife for a good spiritual work and counseling-God Bless You Lusiba, Mr Butoboro and his wife for their caring love, Mr Walter Msangi for analytical work support in the laboratory and friendly co-working atmosphere, organized and keen not to waste material, Dr G Rimoy for examining volunteers for bioequivalence substudy, Prof Kongola for a friendly atmosphere and good inputs for my Thesis and Dr Mary Jande and Mr Fulgence for encouragement.

I thank Dr Jane Sayi who has been the head of Clinical Pharmacology Department during my PhD program. She gave me both moral and administrative assistance. Friendly members at Department of Clinical Pharmacology and School of Pharmacy at MUCHS are highly acknowleged.

Many contributed to this work to the success of this work: myfriends Fatuma Mhando, Jumanne, Diana, Omary, Janet, Apolo, Temba, Martin, My friend Mgeni and all volunteers for the substudies.

Last but not least I would like to thank the Director General of TFDA, M Ndomondo, for permitting me to use the analytical Laboratory of TFDA, Zena, Moshi, Danstan, Yona and Siya for their time and hard work in the analysis of SP and AQ brands.

God bless you all.

This work was financed by Sida/SAREC which is highly acknowledged.

ABSTRACT

The objective of this work was to develop high performance liquid chromatographic (HPLC) methods and apply them in assessing the pattern and extent of antimalarial drug utilization and their quality in Tanzania. The drugs investigated were amodiaquine, chloroquine and sulfadoxine/pyrimethamine.

Pyrimethamine and chloroquine filter paper methods and another HPLC method for measuring amodiaquine in human biological fluids were developed at Karolinska Institute (KI), in Sweden and were applied at MUCHS to carry out a quality assessment of antimalarials collected from wholesale importers in Dar Es Salaam and to investigate diffusion of new malaria treatment policy in a rural community in Tanzania.

The collected brands of sulfadoxine/pyrimethamine and amodiaquine tablet formulations were tested for content of active components and dissolution characteristics. Comparison of bioavailability of two SP formulations was conducted using healthy volunteers. Blood samples (100 µl) were collected and assayed for levels of sulfadoxine and pyrimethamine using HPLC methods.

In households at Kibaha district, mothers with children underfive years of age were interviewed on treatment practices of malaria. Capillary blood was collected on filter paper in duplicate from one underfive in all the households with children. The samples were assayed for chloroquine and sulfadoxine/pyrimethamine using HPLC methods. Drug levels were used to assess drug pressure and extent of diffusion of the new malaria policy in the area.

The developed analytical methods are capable of applying small volumes of blood making them suitable for field studies, in resource limited settings. By applying these analytical methods, it was possible to confirm dissolution failure rates of 13% and 44% of the collected AQ and SP samples respectively. By combining mothers' interviews and drug level analysis, it was possible to confirm that the new malaria treatment policy had diffused and there was low SP overprescribing at Kibaha district.

It can be concluded that HPLC methods applying small volume of blood samples especially dried samples on filter paper are useful tool in epidemiological studies as well as pharmacokinetic studies in resource constrained analytical laboratories of developing countries.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AQ	Amodiaquine
AT	Artemisinin/Artesunate
AUC	Area Under the Concentration Curve
BDAQ	Bisdesethylamodiaquine
Cmax	Peak plasma concentration
CMX	Co-trimoxazole
CQ	Chloroquine
DAQ	Desethylamodiaquine
DCQ	Desethylchloroquine
FDA	Food and Drug Authority
FP	Filter Paper
GIT	Gatro-intestinal tract
HCF	Health Care Facility
HIV	Human Immunodeficiency Syndrome
HPLC	High Pressure Liquid Chromatography
IS	Internal Standard
KI	Kalorinska Institute
MUCHS	Muhimbili University College of Health Sciences
PYR	Pyrimethamine
SAREC	Swedish Agency for Research Cooperation
SDX	Sulfadoxine
Sida	Swedish International Development Cooperation Agency
SM	Sulfamethoxazole
SP	Sulfadoxine/Pyrimethamine
T1/2	Half-life for drug elimination from the body
TFDA	Tanzania Food and Drug Authority
Tmax	Time required reaching peak plasma concentration
TMP	Trimethoprim
USP	United States Pharmacopea
WHO	World Health Organisation

CHAPTER 1: GENERAL BACKGROUND

1.1 THE BURDEN OF MALARIA

Malaria is a major threat to public health worldwide (Snow et al, 1999 & 2001). One third of the world population is exposed to the risk of malaria. Of the four *Plasmodium* species (*falciparum, ovale, vivax* and *malariae*), *Plasmodium falciparum* causes the most severe form of malaria with an estimated 300 to 500 million cases and 1.5 to 2.5 million deaths yearly (Snow et al, 1999, Snow et al, 2001).

Most of these deaths occur in sub-Saharan Africa and children under the age of five are at highest risk. In Tanzania, malaria is ranked as the number one cause of morbidity and mortality with an estimated annual death rate of 0.1 million (Ministry of Health, Dar Es Salaam Tanzania, 1999). Chemotherapy remains the basis for effective case management of malaria and reduction of deaths. The availability and rational use of antimalarial drugs is important for appropriate malaria treatment.

Wide-spread global resistance to chloroquine (CQ) and increasing resistance to other antimalarials such as quinine, mefloquine and sulfa-pyrimethamine (SP) drug combinations is alarming (WHO, 1996). CQ resistance became widely spread in Africa south of Sahara in 1990's causing extensive clinical treatment failure (White, 1999). Malawi was the first African country to abandon CQ as first line drug in the treatment of malaria in 1993 (Bloland et al, 1993, Shretta et al, 2000). Many other Eastern and Southern African countries have joined Malawi in changing their malaria treatment policies and switched to SP (White, 1999). Tanzania replaced CQ with SP as a first line drug for the treatment of malaria and recommended amodiaquine (AQ) as an alternative antimalarial drug of choice in the treatment of non-severe forms of *P.falciparum* malaria (Ministry of Health, Tanzania, 2000). However there were

single reports of treatment failure for this drug combination in some parts of Tanzania even before policy change (Ronn et al, 1996).

The WHO strategies aimed at malaria eradication seem to have failed and currently emphasis is put on control with a major focus on reducing morbidity and mortality with prompt treatment. In Africa, it is estimated that 40% of the fevers are caused by malaria (Brinkmann, 1991).

Prompt treatment with an effective antimalarial drug is considered as the most important means of preventing deaths from malaria (McCombie, 2002). However, in many countries where malaria occurs, health care facilities are poorly funded and a large majority of the population has difficulty in accessing health services. As the highest mortality to malaria occurs in young children in areas with poor laboratory facilities, presumptive treatment of chilhood fever has been adopted widely as a strategy in endemic areas (WHO, 1986).

The success of this strategy depends on the rational behaviour of patients and caretakers of young children as well as adequate knowledge of the health providers on how to cure malaria fevers.

1.2 FACTORS AFFECTING MALARIA TREATMENT

Effective malaria treatment is influenced by a number of factors such as the efficacy of the drug, the parasite susceptibility to the drug and adequate bioavailability of recommended drug preparations. However, prevalence of poor quality drugs and irrational use of drugs in developing countries are major problems (Abu Reid et al, 1990, Kibwage et al, 1992, Massele et al, 1993). Poor adherence to prescribed antimalarial regimens and self-medication are setbacks in achieving rational malaria treatment in endemic areas (Greenhalgh et al, 1987, Igun, 1987, Mnyika et al, 1995).

These factors are increasingly causing rapid emergence of resistant *P.falciparum* strains to many antimalarial drugs. CQ has been used in the treatment of malaria for the past 50 years, however, failure rates to CQ treatment in many endemic areas are causing great concern.

CQ was a cheap, effective drug with tolerable side effects and could be used in pregnancy. Therefore if no measures are taken to prevent development of resistance to SP, the drug will not be useful in replacing CQ in many endemic areas. Inappropriate and inadequate use of antimalarials especially in sub-optimal dosages favours development of resistance (Mnyika et al, 1995, Kun et al, 1999).

In order to promote appropriate malaria treatment, interventions aiming at improving rational use of antimalarial drugs as well as continuous monitoring of drug efficacy must be developed and implemented when proven effective.

1.3 CHALLENGES FACING ANTIMALARIAL DRUGS

1.3.1 Chloroquine (CQ)

CQ resistance was first reported almost simultaneously from South America - Colombia, Brazil and Venezuela (Moore et al, 1961, Wernsdorfer &Payne 1991) and from South East Asia (Thailand) in1961 (D'Alessandro & Buttiens, 2001).

By 1973 CQ resistance had been reported from several countries in South America (Brazil, Colombia, Guyana and Venezuela) and in Asia (Cambodia, Malaysia, Philippines, Thailand and Vietnam) but not in sub-Saharan Africa (D'Alessandro & Buttiens, 2001).

In Africa *P. falciparum* CQ resistance was first reported from the Eastern region in Kenya (Fogh et al, 1979) and in Tanzania in early 1980s (D'Alessandro & Buttiens, 2001).

A few years later, the number of African and Asian countries with CQ resistance had increased considerably (WHO 1984). By 1989, the distribution of *P. falciparum* resistant strains to CQ was almost identical in Africa, Asia and South America (Wernsdorfer &Payne, 1991).

However despite CQ resistance reports in West Africa (von Seidlein et al, 1997, Marsh, 1998), the drug is still used as a first line treatment for uncomplicated malaria. In Uganda, CQ is used with SP as combination therapy.

1.3.2 Sulfadoxine/pyrimethamine (SP) combinations:

Resistance to SP was first noted on the Thailand-Cambodian border in the mid 1960's (Bjorkman et al, 1990). Cases of parasitological failure against SP in non-immune visitors to East Africa were documented in 1982 (Timmermanns et al, 1982). Today levels of resistance vary widely within and between countries. In adults SP is given as a full dose of 3 tablets each containing 500 mg sulfadoxine (SDX) and 25 mg pyrimethamine (PYR) (WHO, 1995). SP is contraindicated in pregnant women at term, nursing mothers and infants in the first 2 months of life (WHO, 1995).

1.3.3 Mefloquine:

Resistance to mefloquine was reported as early as 1980s in areas near the borders between Thailand and Cambodia as well as in Thailand and Myanmar –Thai border (Nosten et al, 1991). Since then sporadic reports of resistance to the drug have been reported from a number of countries in South America, Asia, Africa, and the Middle East (D'Alessandro & Buttiens, 2001). In West Africa, resistance to mefloquine has been described from areas where the drug has not previously been used (Oduola et al, 1992).

1.3.4 Quinine:

Quinine is effective against *P.falciparum* strains resistant to CQ and SP drug combination. It has high efficacy in the treatment of malaria. However, decreasing sensitivity to quinine has been detected in areas of South-East Asia in which it has been extensively used for malaria therapy (Wongsrichanalai et al, 2002, Mohapatra et al, 2003). In many parts of high malaria endemicity, quinine is reserved as a life saving drug for treatment of severe *P.falciparum* malaria. It is recommended to be administered parenterally at a dose of 10mg base /kg 8 hourly for seven days in severe cases.

1.3.5 Artemisinin (AT) derivatives:

Synthetic analogs of Artemisinin (AT) are effective against blood stage forms of the parasite in nanomolar concentrations (Price et al, 1996). AT derivatives reduce malaria transmission and spread of resistance through their gametocytocidal properties (Price et al, 1996). Late ring-stage parasites and trophozoites are more susceptible to the drug than schizonts (Caillard et al, 1992, Ter Kuile et al, 1993, Allin & Bjorkman, 1994). The drugs also have gametocidal activity which could be important in blocking transmission of the disease (Kumar & Zhang, 1990, Mehra & Bhasin, 1993). However, AT drugs can not be used for prophylaxis since they have short half-lives and lack sporontocidal activity against the mosquito stage sporozoites (Newton et al, 2002).

AT derivatives are the most rapidly acting antimalarials todate and this class of drugs is well tolerated (White, 1999). By acting on ring stages they clear peripheral parasitaemia more quickly than other antimalarial drugs and prevent the development into mature sequestering blood stages (White, 1999). They are effective against all human malaria parasites notably multi-drug resistant *P. falciparum* and they have

another additional superiority that they reduce transmissibility (White, 1999). So far, there are no resistance reports to this group of drugs.

Synthetic AT (artesunate sodium, artemether, dihydro-artemisinin) are potential substitutes for CQ and SP in areas where these drugs have developed resistance.

To protect this class of drugs from fast losing potency, it is recommended that AT should not be used alone but always with a companion drug.

Using a combination of drugs with independent modes of action and different biochemical target is not only more effective, but also successful in preventing or slowing the development of resistance, because the probability of the parasites being simultaneously resistant to two drugs is greatly reduced. Combination of drugs is used in the treatment of tuberculosis, leprosy and HIV/AIDS.

When AT is combined with another effective antimalarial, the remaining parasites are then killed by therapeutic concentrations of this other drug. Combining AT with another drug also shortens the treatment course.

Already most African countries have changed or are on the move of changing their antimalarial treatment policies to artemisinine-based combination therapies (ACT). The most common alternatives are artemisinine + lumefantrine (Coartem®), SP plus Artesunate, piperaquine plus dihydroArtemisinin (Artekin ®) and artesunate plus mefloquine (Artecom®).

However there are still some safety concerns on this class of drugs and they are too expensive to be afforded by many in resource limited countries. AT is administered orally at a dose of 10 mg/kg once a day for 5 days, with a double dose on the first day, *i.e.*initial dose of 2 tablets of 100 mg as a single dose followed by one tablet for four consecutive days.

Very little is know about the pharmacokinetics of the ACTs and there are no reliable, field-adapted analytical methods for determination of drug concentrations of the Artemisinine derivatives.

1.3.6 Halofantrine:

Halofantrine has lost its importance in malaria control programs in endemic areas because of its high cost, its variable bioavailability, its cross-resistance to mefloquine and its fatal cardiotoxicity following standard therapy (Touze et al, 1997). It is contraindicated in pregnant women and in children under one year (WHO, 1995). Halofantrine is administered orally at a total dose of 24 mg base/kg within 24 hrs, *i.e.* 8mg base/kg in three doses at 6h intervals.

1.3.7 Amodiaquine (AQ):

AQ is a 4-aminoquinoline derivative like CQ and is a prodrug which undergoes extensive first pass metabolism in the liver. Its major metabolite desethylamodiaquine (DAQ) has been implicated for antimalarial activity (Salako et al, 1985, Winstanley et al, 1987, Sivin et al, 1993). AQ was formerly used as a prophylactic agent against malaria, but lost its importance due to the risk of toxicity like agranulocytosis and hepatitis (Maggs et al, 1987, Jewell et al, 1995) and was therefore withdrawn from the market in the early 1990's.

The mechanisms involved in these toxicities are not clear, but it has been suggested that biotransformation of DAQ to quinone imine may be responsible for granulocyte and hepatocyte toxicity (Hatton & Tea, 1986, Winstanley et al, 1990, Naisbitt et al, 1997). Early in 1990s, AQ received renewed interest as an effective and safe drug in the treatment of malaria (Sebastian et al, 1991, Fowler et al, 1993, Oliaro et al, 1996, Ndounga, 2003).

Despite these safety and efficacy reports, AQ still poses potential risks of toxicity if frequently used, therefore demanding continuous therapeutic monitoring in areas where the drug is extensively used. Some few reports on treatment failure with AQ have recently been documented (Al-Yaman et al, 1996).

AQ is given at a total dose of 25 mg base /kg over three days, i.e.10 mg base/kg for the first and second day followed by 5 mg base /kg on the third day. The drug is administered once daily.

1.4 ANALYTICAL METHODS FOR ANTIMALARIAL DRUGS

This work intends to develop and apply HPLC methods which will allow correct and easy handling of blood samples.

An overview on method development and drug measurements in biological fluids is hereby presented.

1.4.1 Measurements in biological fluids

Drug measurement is important to ensure that therapeutic agents produced by a pharmaceutical industry are safe, effective and have reproducible quality.

Drug measurements in biological fluids require sensitive and specific analytical methods so as to gain definitive information about their pharmacokinetics (Tozer & Rowland, 1995).

In human studies, blood and urine are the most commonly used biological fluids for drug analysis, but other materials like faeces, bile, cerebrospinal fluid, gastric contents, saliva and tissue biopsies can be used. In animal studies, homogenates of various organs and tissues are analysed and in studies on drug metabolism, isolated liver cells and subcellular liver cell fractions can also be used.

Within blood, a drug may bind to many constituents including plasma proteins and blood cells. Drug concentrations in whole blood and plasma can differ, thereby yielding different values for pharmacokinetic parameters. Accordingly, unlike serum and plasma, blood and plasma can not be considered equivalent, although for many applications in pharmacokinetics, the difference is relatively unimportant (Tozer & Rowland, 1995). Because of ease of clinical analysis, most measurements are made in plasma and therefore pharmacokinetics of the drug within the body is defined with reference to drug in plasma.

Other drugs like CQ strongly bind into inner compartments therefore causing variations in drug concentrations between blood/plasma and tissues and this account to long half-lives of such drugs.

1.4.2 Analytical techniques

Drug analysis has stimulated the field of analytical chemistry to devise technologies that are appropriately sensitive, precise, accurate and matched to the demands of speed and automation. Analytical techniques used in drug assays, include, chromatography, mainly high pressure liquid chromatography (HPLC) and gas chromatography (GC), immunological, microbiological and mass spectrometry techniques varying in sensitivity and selectivity. The choice of a technique depends on the nature of the analyte. Immunological and microbiological techniques suffer drawbacks of low selectivity and low precision respectively, whereas, high costs is the main drawback for mass spectrometry. High performance liquid chromatography is currently the most widely used technique because it combines specificity, sensitivity, reproducibility and cost-effectiveness.

1.4.3 Effect of endogenous molecules on drugs

Drugs and endogenous compounds participate in several equilibrium processes in biological fluids and tissues. The character of the endogenous compounds varies from inorganic salts (sodium chloride 0.1 M in urine and blood) to complex macromolecules like proteins (albumin molecular weight 60000, $6.7x10^{-4}$ M in blood) with hundreds of other compounds e.g. peptides, amino acids, lipids, nucleotides, organic acids, etc. In blood, there is binding to proteins and distribution between plasma and blood cells, primarily erythrocytes (Pahlman et al, 1999). Reversible binding of drugs to body macromolecules is one of the most important matrix effects on the biological sample in drug analysis. Cell membranes, albumin and lipoproteins are of major importance in binding of drugs.

The degree of distribution and binding is largely a function of physicochemical properties of the drug and may differ considerably from drug to drug. A drug-protein complex is formed when a drug interacts with albumin and this can affect the pharmacokinetics of the drug. Only free drug can pass through cellular membranes and reach the drug receptors or become eliminated. Usually a protein bound drug is pharmacologically inactive since large macromelcules can not diffuse across membranes. Drugs bind proteins by either a reversible or an irreversible process. When drug protein binding is reversible, the protein-bound drug acts as a depot, slowly releasing the active drug and replacing the free drug which is eliminated. A protein bound drug is unavailable for therapeutic use. A few drugs are irreversibly bound to proteins, most drugs are reversibly bound to albumin. However it should be noted that, only unbound drug can be measured by analytical methods (Gibaldi, 1984, Tozer & Rowland, 1995).

Many acidic drugs are preferentially bound to plasma proteins rather than extravascular sites. Basic drugs are more extensively bound to extravascular sites than to plasma proteins (Gibaldi, 1984).

1.4.4 Adsorption

Many drugs and related compounds suffer concentration losses due to adsorption of the molecules to different interfaces (Lindstrom et al, 1985). Since adsorptive surfaces usually have a limited capacity, the problem with adsorption losses is of increasing importance as it decreases concentration of the compounds.

Losses by adsorption may also occur during transportation of samples, evidently by adsorption to the stoppers of the tubes. If samples are allowed to thaw during transport, significant losses of the drugs will then occur according to this mechanism. Adsorption losses may be due to storage of samples in low density polyethylene containers, glass bottles and polyvinyl chloride (PVC) bags (Beitz et al, 1999, Faouzi et al, 1999). Losses of analytes due to adsorption to different interfaces during the analytical procedure, is a serious problem for many compounds, especially if they are hydrophobic and contain a polar amine group as is the case of most of antimalarial drugs. Adsorption losses may have a significant impact for low analyte concentrations especially in the order of micromole or lower.

Adsorptive loss of CQ and metabolites is significant when the drug is in contact with glass surfaces, especially in the nanomole concentration range (Staiger et al, 1981, Geary et al, 1983, Bergqvist et al, 1983). Adsorption losses of drugs increase during prolonged storage. Some measures for avoiding adsorption losses include, careful washing of glassware with acid and ethanol, silanization of glass surfaces, use of polypropylene tubes instead of glass tubes and addition of a few per cent of a polar

organic compound, like an alcohol or an aliphatic amine (isobutanol or trimethylamine).

1.4.5 Sample storage and stability

It is necessary to test whether the compounds of interest are stable during the conditions used for sampling, storage and during sample analysis. The stability of both the parent drug and the metabolite(s) in real biological samples must be checked under proposed storage conditions and should include studies on the effects of repeated thawings and freezings in order to ensure that repeated analyses of samples can be performed. There is also a risk that such procedures may give rise to concentration gradients in the tubes.

Most drugs are stable in biological fluids when stored at -20˚C but some may require much lower temperatures. The decomposition of drugs in biological samples is mostly due to enzymatic reactions, chemical reactions or both. Hydrolysis of esters is a common decomposition process that is catalysed by esterases in the samples. Aspirin for instance is easily hydrolysed by endogenous esterases during blood sampling (Shimizu et al, 2003). Enzyme catalysed decomposition may be avoided by a change of pH of the sample, addition of ezyme inhibitors or by denaturation of proteins including the enzyme (Shimizu et al, 2003).

Drugs that are prone to degradation process due to oxidation or photolability can be stabilised by addition of antioxidants to the sample and protecting the samples from light respectively, begining with sample collection to sample analysis (Grundy, 1994, Olesen et al, 1998). Sample exposure to light can be avoided by using dark rooms or wrapping the sample containers with aluminium foils during storage and sample analysis.

1.4.6 Contamination

During sampling, there is a risk for contamination by compounds released from used equipment and some of these compounds may interfere in the analytical procedure. Plasticizers are frequently responsible for such disturbances (Faouzi et al, 1995, Faouzi et al, 1999). Contamination may also originate from laboratory personnel if the same person handles tablets and draws blood samples, in this case poses appreciable risks of contaminating samples being collected (Bergqvist, 1988).

1.4.7 Sample pre-treatment

Endogenous compounds that may interfere in the analytical procedure widely differ in physico-chemical characteristics and it is impossible to design common and specific procedures for their removal. The strategy must therefore be to develop isolation procedures which are selective for the analytes. The analytical procedure will depend on the properties of both the substance and the biological sample. In some liquid chromatographic systems, it is possible to directly inject biological fluids (urine, cerebrospinal fluid) without undergoing extraction procedures. Direct injection of biological fluids on liquid chromatography requires special precautions in the form of an easily replaceable precolumn which protects and increases the lifetime of the analytical column.

The most commonly used bioanalytical sample processing is liquid extraction which involves application of different organic and inorganic solvents and may be complemented with centrifugation, evaporations and derivatization procedures.

1.4.7.1 Removal of proteins

Proteins are incompatible with most chromatographic systems used for the bioanalysis of drugs. They tend to precipitate on the chromatographic supports,

causing a pressure increase in liquid chromatographic systems, which finally get totally clogged. There are several ways to remove proteins and the most common way is to use an agent which causes the proteins to denaturate, giving a precipitate which can be separated by centrifugation.

Salts of heavy metals, (copper, zinc & lead) and organic solvents miscible with water such as methanol and acetonitrile are used for protein precipitation (Bergqvist, 1987). Strong acids, e.g. trichloroacetic acid (TCA), perchloric acid or hydrochloric acid, are also used for protein precipitation (Avgerinos et al, 1991). However it is important to ensure that the presence of these agents in the injected solution will have no influence on the retention characteristics of the sample components. Another way of removing proteins is by ultrafiltration. Plasma samples are centrifuged through a membrane which retains large molecules. The filtrate then contains free (nonbound) drug, the concentration of which, may be a relevant parameter for correlation studies of drug levels with clinical effects. Another widely used procedure for separation of proteins and other endogenous substances from soluble drugs is the application of liquid-liquid extraction.

1.4.7.2 Liquid-liquid extraction

Liquid-liquid extraction is the most preferred separation procedure as it gives high accuracy and precision. Liquid-liquid extraction methods are widely applied. Extraction conditions of drugs must be optimized to ensure high recovery of the drugs from sample matrices. Analytical processing of complicated samples by batch extraction with an organic solvent is the most common approach in the bioanalytical field. For polar compounds e.g. quaternaries, sulfates and sulfonates, which are difficult to extract, utilization of ion-pairing agents has been reported to be useful

(Lin & Jeng, 2002). Ion-pair extraction is also useful for labile compounds, which are charged at physiological pH.

The extraction of extremely hydrophilic compounds may be facilitated by salting-out techniques. High amounts of inorganic salts, e.g. sodium chloride, are added to the aqueous phase. The use of centrifuge tubes for extractions also facilitates the separation of the phases.

1.4.8 Uses of internal standards

Most analytical methods used for determinations of drugs in biological samples apply internal standards. The accuracy and precision of the quantitative data increases if an internal standard is included in the procedure. The internal standard is usually added to the samples before sample extraction. The standard curve is obtained from peak height (or area) ratios of analyte to internal standard *vs* concentration of the analyte.

The compound chosen as an internal standard should have physico-chemical properties as close to those of the analyte as possible. A good internal standard should be structurally similar to analytes. The internal standard should elute close to the retention time of the analyte of interest and if possible the internal standard should elute after the parent drug peak so that the possibility of interference with faster eluting more polar metabolites is obviated (Shah VP et al, 1991, Dagdar et al, 1995). An internal standard compensates for all variations between samples, e.g. adsorption losses, extraction yields, extent of derivatization, decomposition of analyte and detection response.

1.4.9 Method Development and Method validation

1.4.9.1 Method Development

Method development is an extensive and difficult work which begins with literature review (Skoog et al, 1992). Development of new analytical method for determination of certain substances may be necessary if:

- The existing methods are not cost effective.
- The reported method is not reproducible.
- There is a problem with sample collection, handling and stability.
- The previous methods are not satisfactory for the intended goal.
- There is no documented method for analysis of the product.

In developing analytical methods for determination of drugs and drug metabolites in biological samples, attention must be paid to the nature of the biological matrix and its influence on various steps in analytical procedures (Skoog et al, 1992).

The following are characteristics of the biological samples and must be taken into consideration:

- The drug and its metabolites are usually present in very low concentrations (usually in micromole or nanomole).
- The sample contains numerous endogenous compounds that are present in much higher concentrations than the drug.
- Components of the sample can affect the distribution properties of the drug by binding it to an aqueous phase or increasing its distribution to a lipophilic phase.
- The sample is sometimes unstable and measures must be taken to prevent decomposition.

- The sample often contains several phases with differing drug concentration.

Analytical methods employed for the quantitative determination of the drugs and their metabolites in biological samples play a significant role in evaluation and interpretation of pharmacokinetic data. It is therefore essential to employ well characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted (USP, 2000).

Validation of an analytical method includes all procedures required to demonstrate that a particular method for the quantitative determination of an analyte (or series of analytes) in a particular matrix is reliable for the intended application. Most of the principles, procedures and requirements for method validation are common to all types of analytical methods (Shah et al, 1991, Dagdar et al, 1995).

Each step in method validation should be investigated to determine an extent to which environmental matrix, material or procedural variables from time of collection of the material up to analysis including time of analysis may affect estimation of analyte in the matrix (Shah et al, 1991, Dagdar et al, 1995). Variability of matrix due to physiological state may need to be considered. Whenever possible, the same biological matrix as that in the intended samples should be used for method validation. The stability of the sample in the matrix during the collection process and sample storage period should be assessed, preferably prior to sample analysis. It is recommended that stability of analyte in the matrix from dosed subjects be confirmed (Shah et al, 1991, Dagdar et al, 1995). The most commonly applied parameters in validation of analytical methods are described below.

1.4.9.2 Parameters for validation of analytical methods:

1.4.9.2.1 Accuracy:

Accuracy is closeness of results to the true value. A drug will often be compared to pre-existing primary reference standards (USP, 2000). Such comparisons should be carefully controlled e.g by the use of spiked placebo or spiked samples of a product. In practice, accuracy can be controlled by comparing determined and added concentrations of analytes at two different levels.

1.4.9.2.2 Selectivity:

Selectivity is defined as the ability of an analytical method to discriminate the compound(s) it is aimed to measure from other sample components or closely related substances. It is recommended that selectivity of the method should be established with respect to endogenous substances, metabolite(s) and known degradation products (Shah et al, 1991, Dagdar et al, 1995). Interferences which are likely to be present in small quantities may adversely affect the quantitation of unknown samples at concentrations approaching limit of quantifications. Selectivity with respect to interferences from endogenous substances can be established by processing a minimum of six independent sources of the same blank matrix. Careful examination of chromatograms across the time windows of the peaks of interest is required to evaluate selectivity (Shah et al, 1991, Dagdar et al, 1995).

1.4.9.2.3 Precision:

Precision is defined as agreement of a set of results obtained with identical samples by the same method. Relative standard deviations are determined for 2 to 3 different concentration levels, chosen according to requirements from real samples. Such results give intra-assay precision. Variations between days are most easily obtained from quality control samples run with each series of analyses. The quality control

samples should contain two concentrations of analyte, one low and one high and they are prepared at the start of the analysis in a large batch which is divided into aliquots of a sample volume and stored. Their inter-assay variations will then demonstrate how a method works in practice, while intra-assay variations show optimal ability of the method.

1.4.9.2.4 Specificity:

Specificity is the ability to unequivocally assess presence of expected analytes. Such testing is carefully controlled to ensure that structurally similar compounds do not give similar results.

1.4.9.2.5 Detection limit

Detection limit is the lowest amount of the analyte dectable by the procedure.

1.4.9.2.6 Quantification limit:

Quantification limit is the lowest concentration on the standard curve which can be measured with acceptable accuracy, precision and variability (Shah et al, 1991, Dagdar et al, 1995).

1.4.9.2.7 Linearity range:

Linearity range refers to ability to obtain test results which are direct proportional to analyte concentration within a specified range.

1.4.9.2.8 Ruggedness:

Ruggedness (intermediate precision) refers to the degree of reproducibility of a procedure when measured by different analysts, instruments and laboratories. If it is discovered in the validation process that a method is not easily transferred between systems, analytes or analytical columns of the same type, further development and revalidation becomes necessary. Analyses should be performed on at least two columns containing two different lots of the identical packing material, preferrably

one column which was used for method development and the other previously unused. Mobile phase may also be counterchecked since a small variation in the mobile phase solvent ratio and buffer pH may account for poor ruggedness of the method.

1.4.9.2.9 Robustness

Robustness is an indicator of reliability- this is a measure of the capability of the laboratory procedures to remain unaffected by small, deliberate changes in conditions.

1.4.9.2.10 Extraction recovery

Extraction recovery is tested by comparing responses of replicates of extracted quality control samples with replicates of directly injected standard solution having the same nominal concentration. High recovery of the analyte from the matrix is desirable. Sometimes it may be necessary to intentionally sacrifice high recovery in order to achieve better selectivity and this is acceptable provided that adequate sensitivity, precision and accuracy are achieved. Solvents such as ethyl acetate normally give rise to high recovery of analyte, but this solvent also simultaneously extracts many interfering compounds. However, in some cases if an adequate and sensitive detection limit is attained with good precision and accuracy, the extent of recovery may not be considered an issue in bioanalytical method development and validation (Shah et al, 1991, Dagdar et al, 1995).

1.4.9.2.11 Stability:

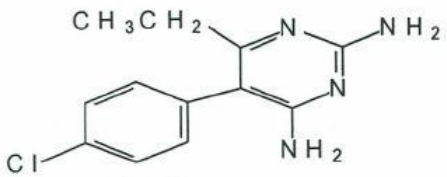
The stability of the analyte in biological matrix at intended storage condition(s) or temperature should be established. In addition the influence of freeze/thaw (at least 3 cycles) should be studied (Dagdar et al, 1995, Keil et al, 2003).

1.4.10 Analytical considerations for antimalarial drugs

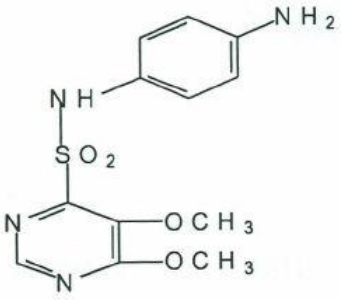
1.4.10.1 Chemical structures of the antimalarial drugs involved in this work

Below are chemical structures of compounds involved in this study.

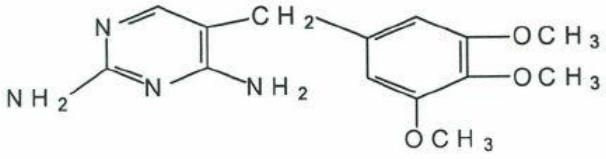
Figure 1-1 Chemical structures of pyrimethamine (PYR), sulfadoxine (SDX) and trimethoprim (TMP) and sulfamethoxazole (SM) as internal standard



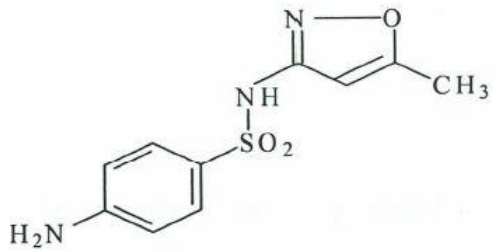
PYR



SDX

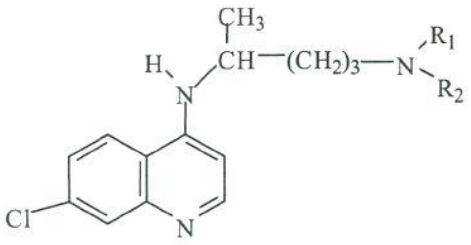


TMP



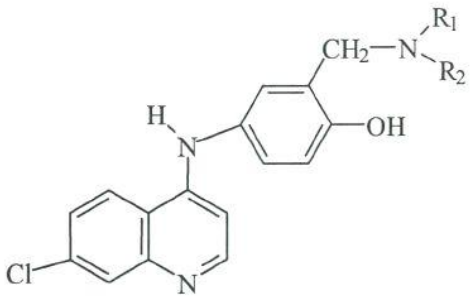
SM

Figure 1-2 Chemical structures of AQ, DAQ, BDAQ, CQ, DCQ and IS: (4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline).



Chloroquine; $R_1 = R_2 = C_2H_5$

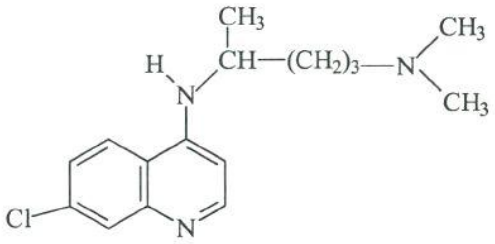
Desethylchloroquine; $R_1 = C_2H_5$,; $R_2 = H$



Amodiaquine; $R_1 = R_2 = CH_2H_3$

Desethylamodiaquine; $R_1 = CH_2CH_3$; $R_2 = H$

Bisdesethylamodiaquine; $R_1 = R_2 = H$



Internal standard

(4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline)

1.4.10.2 Detection and determination of antimalarial drugs in body fluids.

Detection and determination of antimalarial drugs in body fluids may include parent drug, its metabolite(s) or both. Therapeutic monitoring of antimalarial drugs requires analytical methods capable of determination of drug concentrations in blood, plasma and urine. Recent drug intake history is essential for correct interpretation of effectiveness *in vivo*. This information is difficult to obtain in the field, because drugs are sometimes used indiscriminately and contents of tablets are not always known (Bergqvist, 1988, Murtarda & Sessay, 1994).

1.4.10.3 Extraction of antimalarial drugs (CQ, AQ and SP) from a biological matrix

Extraction for antimalarials are done to provide high recovery and yet avoiding interference from endogenous compounds. Different extraction conditions for CQ, AQ, pyrimethamine (PYR) and SDX will be discussed in the preceeding chapters.

1.4.10.4 Analytical considerations for chloroquine

The assay results for CQ and its principal metabolite desethylchloroquine (DCQ), entirely depends on the type of the sample (serum, plasma or whole blood). CQ and its metabolite DCQ are higher in serum than in plasma (Bergqvist and Domeij-Nyberg, 1983, Rombo et al, 1985). A possible explanation for these observations is that both compounds have strong tendency to bind to blood cells especially platelets (Bergqvist and Domeij-Nyberg, 1983).

Varying plasma platelet and granulocyte count may influence whole blood concentrations as these cells contain more than half of the total CQ blood content. Because of these complications and due to difficulty in standardisation of

centrifugation process (force and duration), whole blood is mostly recommended in assays of CQ and DCQ.

1.4.10.5 Analytical considerations for amodiaquine (AQ)

AQ acts as a prodrug and is extensively metabolized to an active metabolite desethyl amodiaquine (DAQ) by liver microsomal enzymes (Salako & Idowu, 1985). Within 8-10 hours AQ is completely undetectable in blood (Salako & Idowu, 1985, Sivin et al, 1993). DAQ has long half-life and can be used to generate data in epidemiological, therapeutic drug monitoring and efficacy studies of AQ. AQ has a phenolic group which contributes to its low stability in whole blood samples. It is stable in whole blood samples stored at –70°C. AQ like CQ binds in platelets and therefore the use of whole blood instead of plasma gives more accurate data.

1.4.10.6 Analytical considerations for sulfadoxine-pyrimethamine (SP)

Difficulties arise when different antimalarials are used in combination drug. SP is composed of pyrimethamine (PYR) and sulfadoxine (SDX). PYR is a base but SDX is an acid and the concentration ratio between these drugs in plasma is high creating difficulties in obtaining a method which can give high recovery for both components in a simultaneous analysis of two substances (Bergqvist & Eriksson, 1985).

1.4.10.7 Analytical considerations for pyrimethamine (PYR)

PYR is mostly used in combination with other antimalarial drugs as in SP or SP-mefloquine preparations. Analytical methods targeting PYR must be very sensitive since when used in fixed combination formulations it is present in very small proportions. In SP tablet formulation, PYR is present as 25 mg versus 500 mg SDX. The substance is basic and this property creates difficulties in obtaining suitable methods for simultaneous extraction with acidic SDX.

1.4.10.8 Analytical considerations for sulfadoxine (SDX).

Like PYR, SDX is also used in combination with other antimalarial drugs as in SP and SP plus mefloquine as fixed dose combination. The ratio of SDX between whole blood and plasma is 0.59 (Bergqvist et al, 1987, Dua et al, 1994). This is due to the fact that about 90% of SDX is bound to albumin, which is found in plasma fraction. The rest is distributed in plasma (6%) and erythrocytes (4%) (Dua et al, 1994). Whole blood analysis is the most reliable and accurate method for SDX determination in the body.

This work is based on development of HPLC methods and their applications in studying utilisation of antimalarials in the community. Below an overview of study rationale of each sub-study is given.

1.5 STATEMENT OF THE PROBLEM.

This work was launched in October, 2000 and it addressed the below listed issues:

1.5.1 Need for developing CQ and AQ HPLC method

The new malaria treatment policy replaced CQ with SP and AQ became the second line of choice in the treatment of non-severe malaria. However, some individuals could continue using CQ in the community despite policy changes (Tarimo et al, 2000).

The long half-lives of CQ and desethylamodiaquine (DAQ) make them quantifiable in some individuals for long periods. Blood collected from these individuals would contain both CQ and DAQ. CQ and AQ and their metabolites are closely related in their structural features and the parent drugs (CQ and AQ) are both 4-aminoquinoline antimalarial drugs. Therefore a selective and sensitive chromatographic system for determination of AQ &CQ applying small volumes of blood is needed. The method must be sensitive and selective enough to enable simultaneous detection of both compounds in the same chromatographic system. The method must enable determination of these drugs and their principal metabolites from whole blood using reversed phase chromatographic system with uv detection. The method can be applied in studying metabolic pathways of these drugs and monitoring the adverse reactions caused by AQ.

In the present study, the method was applied in an epidemiological study at Kibaha district to confirm if CQ is no longer used in malaria treatment by mothers/guardians of underfive children after change of malaria treatment policy to SP.

1.5.2 Need for developing PYR HPLC filter paper method

SP is currently used as first line drug in the treatment of uncomplicated *falciparum* malaria in Eastern and Southern African countries. An analytical method which would enable to carry out therapeutic monitoring of this drug in patients as well as pharmacokinetic and epidemiological studies is needed. It is therefore intended to develop a filter paper method for determination of PYR from whole blood which will be used in conjunction with SDX filter paper method (Bergqvist et al, 1987) to enable determination of SP in analytical laboratories in resource limited countries. In the present study, PYR filter paper method will be used in conjuction with Bergqvist SDX method to study bioavailability of two SP brands.

1.5.3 Need for conducting quality assessment of antimalarial drugs in the country

Following malaria policy change in Tanzania in 2001, there has been proliferation of marketing of many different AQ and SP brands from several countries but the quality of some of these brands remains uncertain. Data on the quality status of antimalarial drugs particularly SP on the Tanzanian market after malaria treatment policy change is important in ensuring quality malaria treatment. In the present study, quality assessment of AQ and SP brands available in wholesale pharmacies will be conducted.

1.5.4 Need for studying new malaria policy difussion in rural area communities in Tanzania

The pattern and extent of drug usage in rural communities in Tanzania after malaria policy change has not been investigated adequately. Information on whether people still used CQ, the extent of home treatment and home stocking of antimalarial drugs

in rural communities in Tanzania is missing. Reliable and accurate information on malaria treatment history is an asset in deciding the most appropriate drug use, especially in view of increasing drug resistance. Uses of antimalarial drugs prior to visits to health care facilities are a common practice in endemic areas (McCombie et al 1996). Poor correlation between mothers' reports and the actual drug taken by children before or after visiting health facilities has been observed (Nwanyanwu et al, 1996, Nsimba et al 2002).

In the present study, HPLC methods and questionnaire were used to study the pattern and extent of antimalarial uses, drug pressure and acceptability of a new malaria treatment policy as well as knowledge of mothers on the use of antimalarials at Kibaha district after malaria treatment policy change.

1.6 OBJECTIVES OF THE STUDY

1.6.1 Broad objective

To develop high performance liquid chromatographic (HPLC) methods and apply them in assessing the pattern and extent of utilization as well as quality of antimalarials in the community.

1.6.2 Specific objectives of the study

1.6.2.1. To develop a high performance liquid chromatographic method for determination of amodiaquine, chloroquine and their monodesethyl metabolites in whole blood, plasma and urine.

1.6.2.2. To develop a simple and cost-effective liquid chromatographic method for determination of pyrimethamine in whole blood samples dried onto filter paper.

1.6.2.3. To apply the developed HPLC methods in the quality assessment of amodiaquine and sulfadoxine/pyrimethamine brands sold in private wholesale pharmacies in Dar Es Salaam Tanzania.

1.6.2. 4. To apply the developed HPLC methods in the comparison of bioavailability between low quality SP brand (poor dissolution) and a good quality SP brand as part of quality assessment of sulfadoxine/ pyrimethamine brands obtained from private wholesale pharmacies in Dar Es Salaam

1.6.2. 5. To apply the developed HPLC methods in determining household adoption the new malaria treatment policy in rural communities in Tanzania using Kibaha district.

CHAPTER 2:DEVELOPMENT OF HPLC METHOD FOR DETERMINATION OF AMODIAQUINE AND CHLOROQUINE IN BIOLOGICAL FLUIDS

2.1 INTRODUCTION

AQ and CQ are both 4-aminoquinoline antimalarial drugs. AQ was formerly used as a prophylactic agent against malaria but lost its importance due to the risk of development of agranulocytosis and hepatic disorders (Hatton & Tea, 1986, Maggs et al 1987, Naisbit et al, 1987, Jewell et al, 1995). For the same reason the usefulness of AQ for treatment of malaria remained limited. However, in 1990s, AQ received renewed interest as a safe and efficacious antimalarial drug (Sebastian et al, 1991, Fowler et al, 1993, Olliaro, 1996, Ndounga, 2003).

Although CQ is officially not used in the treatment of malaria in Tanzania, the drug and its metabolites may be present in the blood of some individuals who continue using CQ even after policy changes (Tarimo et al, 2000).

Both AQ and CQ and their metabolites could be present in the blood of the individuals who will be included in AQ studies. Therefore to be able to discriminate AQ and CQ, a sensitive and selective chromatographic system capable of detecting both substances is needed. The method in turn can be used in pharmacokinetic and epidemiological studies of the two drugs.

Methods for assay of AQ and CQ have been reported, including those involving bigger volumes (≥1 ml) of blood samples (Staiger et al, 1981, Pussard et al, 1986, Winstanley et al, 1987, Houze et al 1992).

Others involve applications of expensive automated solid phase extraction techniques (Lindegårdh et al, 2002), tedious extraction procedures (Staiger et al, 1981, Mount et

al, 1986), use of laborious and less selective normal phase HPLC systems as well as expensive fluorescence detection (Alvan et al, 1982, Lindstrom et al, 1985). Most of these methods apply plasma samples instead of whole blood making it difficult to collect samples in the rural areas in developing countries.

Mihaly et al in 1985 reported an HPLC method for determination of AQ but DAQ as the principal metabolite could not be quantified (Mihaly et al, 1985).

The main objective of the present work is to develop a simple and cost-effective method to enable analysis of AQ and CQ using small volumes of whole blood samples dried on filter paper.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

AQ was obtained from Sigma, Stockholm, Sweden. DAQ and bisdesethylamodiaquine (BDAQ) were gifts from Associate Professor Yngve Bergqvist, Dalarna University College, Borlänge, Sweden. CQ was from Pharmacia, Stockholm, Sweden and DCQ was obtained from Sterling Winthrop, New York, USA. The internal standard (IS) 4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline was formerly synthesized in the Department of Medical Laboratory Sciences and Technology, Karolinska Institute (KI), SE-141 86 Huddinge, Stockholm, Sweden (Alvan et al, 1982). Solvents and chemicals (HPLC or analytical grade) were purchased from Merck, Darmstadt, Germany. Stock solutions were made by dissolving AQ, DAQ, CQ and DCQ in 0.01 M hydrochloric acid. The IS was dissolved in 0.5% diethylamine (DEA) in water.

2.2.2 Instrumentation

The method was devised using a Gilson model 231 sample injector with a 200 µl loop (Villiers le Bel, France), a Gilson model 118 UV/Vis absorbance detector and a Kontron model 422 high-pressure pump (Milan, Italy). A reversed phase column, Zorbax® SB C_{18}, 75x4.6 mm I.D, 3.5 µm (ChromTech, Hägersten, Sweden) was used. The mobile phase consisted of methanol:phosphate buffer (0.1M, pH 3): perchloric acid, (250: 747.5:2.5, v/v). Elution was carried out at room temperature using a flow rate of 1.5 ml/min. Detection was achieved at 333 nm.

2.2.3 Samples

For development and evaluation of the method, drug free whole blood, plasma and urine were spiked with AQ and its metabolite DAQ. Samples from a healthy volunteer who took a single oral dose AQ (600 mg AQ base) were also used. Urine samples were diluted 10-100 times before extraction while the blood and plasma samples were extracted directly.

Venous blood samples 10 ml in duplicate (2 x 10 ml) were obtained from a volunteer and collected in heparinised plastic tubes before the dose and at 1, 1.5, 2, 2.5, 3, 6, 8, 24, 48, 72 and 168 h after a single oral dose of 600 mg AQ base. The first (10 ml) collected blood samples were immediately centrifuged at 3500 rpm for 15 minutes to get plasma. The remaining blood samples (10 ml) and the obtained plasma samples were stored at -70°C until assay.

Urine samples were collected before drug intake and at 0-4, 4-8, 8-12 and 12-24 h after drug intake and then at 24 hour intervals for three weeks. The urine volumes from each collection were measured and aliquots of 10 ml were kept at -70°C until assay.

CQ samples were prepared by applying 100 µl aliquots of whole blood spiked with CQ and DCQ onto filter paper strips (Whatman chromatography paper, Kebo, Spånga, Sweden).

2.2.4 Analytical procedure for AQ and DAQ

To 100 µl samples (whole blood, plasma or diluted urine) 900 µl water and 50 µl of 1.6 µM IS were added, thereafter, 2000 µl of carbonate buffer pH 9.5 was added. The samples were extracted using 7 ml of diisopropyl ether on a reciprocal shaker for 20 minutes. After centrifugation for 10 min at 3500 rpm, the upper organic phase was transferred to a new tube and extracted as above for 10 min with 150 µl of 0.1 M phosphate buffer (pH 4). The phases were separated by centrifugation as above and the organic phase was removed by aspiration. Into the chromatograph, 100-120 µl of aqueous solution were injected.

To 1000 µl samples, 1000 µl water, 50 µl 1.6 µM IS (in 0.5% DEA in water) were added, thereafter, 2000 µl of carbonate buffer at pH 9.5 was added. The samples were extracted and injected into the chromatograph as described above.

2.2.5 Analysis of CQ and DCQ in samples dried on filter paper

Analysis of CQ and DCQ in blood samples dried on filter paper was carried out in a slightly different way. Filter paper containing 100 µl of dried blood spots was cut in pieces and put into centrifuge tubes. A volume of 500 µl 0.5% DEA in water, 50 µl of 1.6 µM IS (for samples with concentrations ranging between 50 and 400 nM), or 75 µl of 3.2 µM IS (for samples having concentrations of between 500 and 4000 nM), and 2000 µl of 1M KOH were added. The mixture was sonicated for 15 minutes. A volume of 7 ml of diisopropyl ether was added and extraction was made as above except that phosphate buffer with pH 2.5 instead of pH 4 was used for back

extraction. Attempts to use same phosphate buffer (pH 4) for re-extraction of CQ and metabolites could not give satisfactory extraction recovery

2.2.6 Standard curves

Standard curves were prepared by adding known amounts of AQ, DAQ (0-40 µl) standard solution containing AQ and DAQ as well as 50 µl of IS to blank whole blood, plasma and urine. The range of the standard curves used for validation of the method was 2.5-40 nM for 10 nM samples, 50-400 nM for 100 nM samples and 0.5-4 µM for 1 µM samples. The resulting peak area ratios between the internal standard and the analytes were plotted versus the concentrations. Standard curves for determination of CQ and DCQ in dried blood spots were prepared by adding known amounts of CQ, DCQ and IS to 0.5% DEA in water. The extraction procedure was carried out as described. The resulting peak area ratios were plotted versus the concentrations.

2.2.7 Extraction recovery

To determine the extraction recovery, standard solutions of AQ and DAQ were added to whole blood, plasma and urine. CQ and DCQ were added to whole blood and 100µl aliquots were dried on filter paper. Samples were extracted according to the above procedure and the peak areas were compared with those of directly injected standards.

2.2.8 Precision and accuracy

Known amounts of AQ and DAQ were added to blank whole blood, plasma, and urine. CQ and DCQ were added to whole blood and 100 µl aliquots were dried on filter paper. The samples were extracted and analyzed as described above both in one series (within assay precision and accuracy) and on different occasions (between

assay precision and accuracy). The standard deviation and coefficient of variations were calculated.

2.2.9 Interference with commonly used drugs in malaria endemic areas

Drug free whole blood samples were separately spiked with aspirin, paracetamol, sulfamethoxazole, sulfadoxine, sulfadimidine, pyrimethamine, chlorpheniramine, promethazine, quinine and trimethoprim to achieve therapeutic concentrations. Samples from patients treated with quinine were also tested. The drugs were extracted according to the method described above. The presence of peaks was monitored for interference after injection of 100-120 µl of aqueous solution.

2.2.10 Stability

The stability of AQ and DAQ in whole blood, plasma and urine samples was studied at 37°C, 20°C, 4°C, -20°C and at -70°C for 30 days. To monitor the influence of oxidation, the samples were studied in blood mixed with antioxidants (ascorbic acid or sodium thiosulfate) at different concentrations and dried. Concentration changes in plasma and urine samples stored at -20°C and -70°C were followed for 16 months. The stability of CQ blood samples dried on filter paper has been reported previously (Lindstrom et al, 1985).

2.3 RESULTS

2.3.1 Evaluation of the analytical procedure

2.3.1.1 Chromatographic condition

A chromatogram to show separation pattern of AQ, DAQ, BDAQ, CQ, DCQ and IS from spiked plasma, is shown in Figure 2-1. It can be seen that there was an excellent resolution of substances.

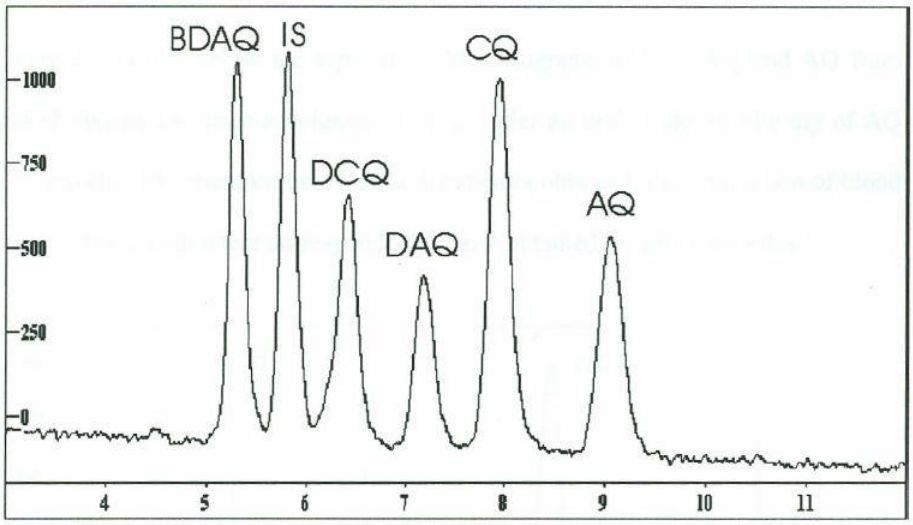


Figure 2-1 Separation chromatogram of BDAQ, IS, DCQ, DAQ, CQ and AQ in spiked blood sample.

The chromatogram from extracted blank plasma of the volunteer spiked with IS is shown in figure 2-2 below. Neither peaks of AQ and CQ nor their metabolites could be detected.

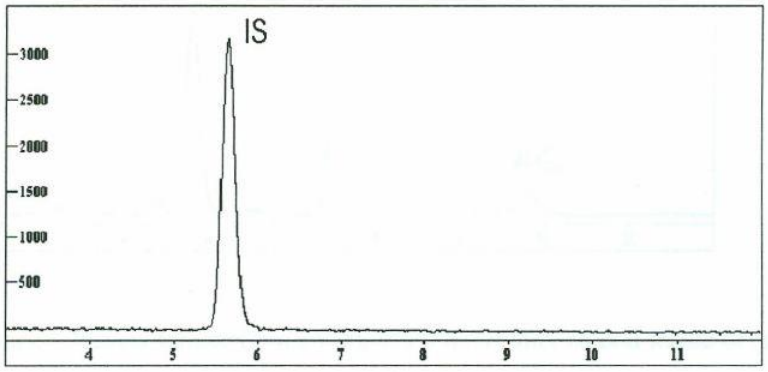


Figure 2-2 Blank plasma (Spiked with internal standard) from a volunteer

Figure 2-3 & 2-4 below are separation chromatograms of IS, DAQ and AQ from spiked plasma and from a volunteer, 6 hours after an oral intake of 600 mg of AQ respectively. The retention times of the substances obtained after extraction of blood samples from a volunteer corresponded to those obtained in spiked samples.

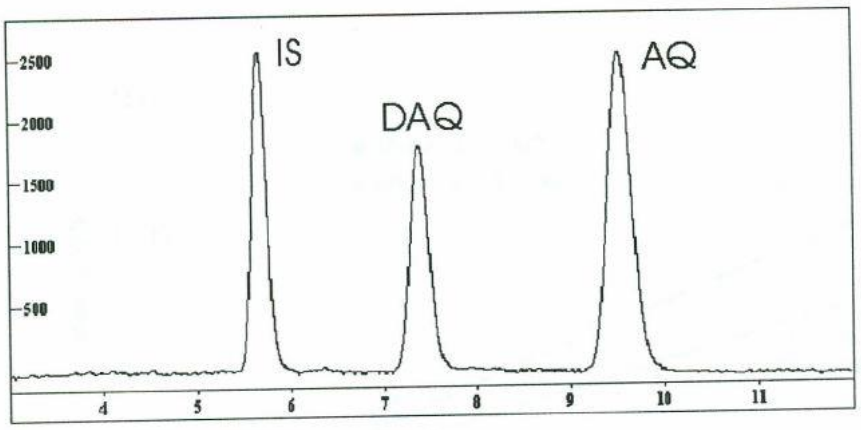


Figure 2-3 Separation chromatogram of IS, DAQ and AQ in spiked plasma.

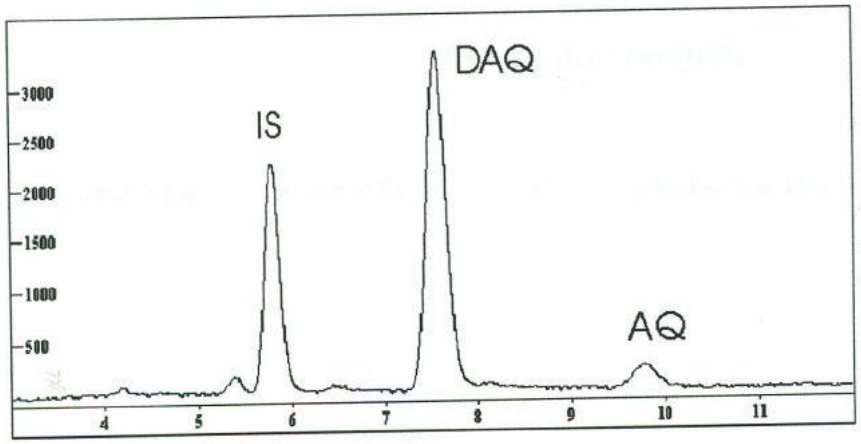


Figure 2-4 Plasma sample from a volunteer 6 hours after an oral intake of 600 mg of AQ

2.3.1.2 Calibration curves

Calibration curves for AQ, DAQ, CQ and DCQ in plasma, blood and urine samples over the concentration range studied are depicted in Figure 2-5 up to Figure 2-7 below. The correlation coefficients were between 0.991 and 0.999 in all runs.

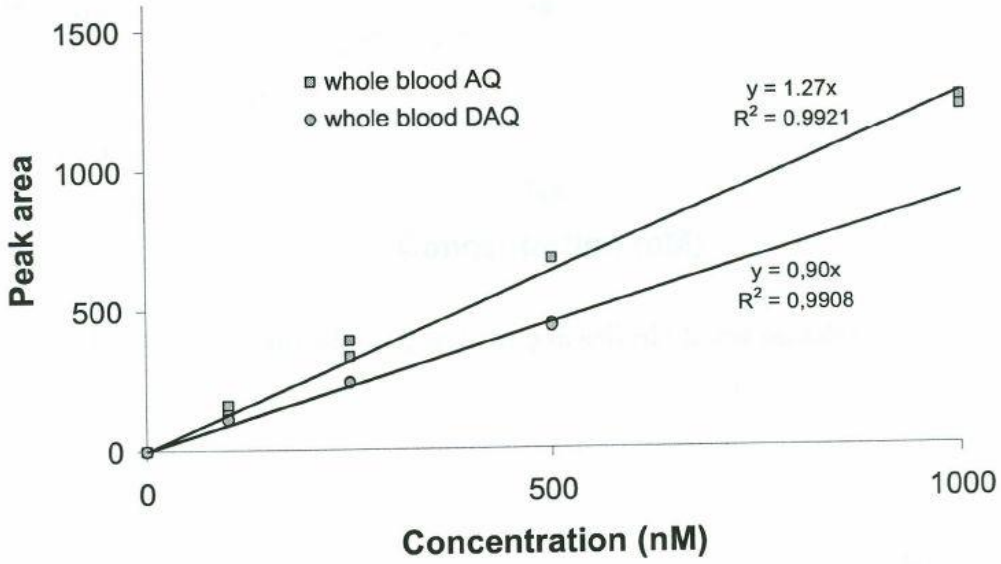


Figure 2-5 Calibration curve for AQ and DAQ in spiked whole blood samples

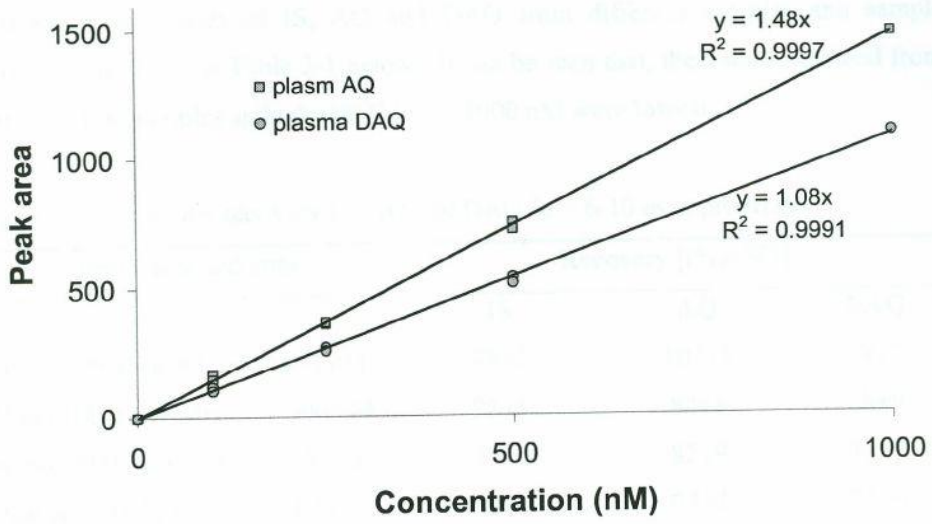


Figure 2-6 Calibration curve for AQ and DAQ in spiked plasma samples

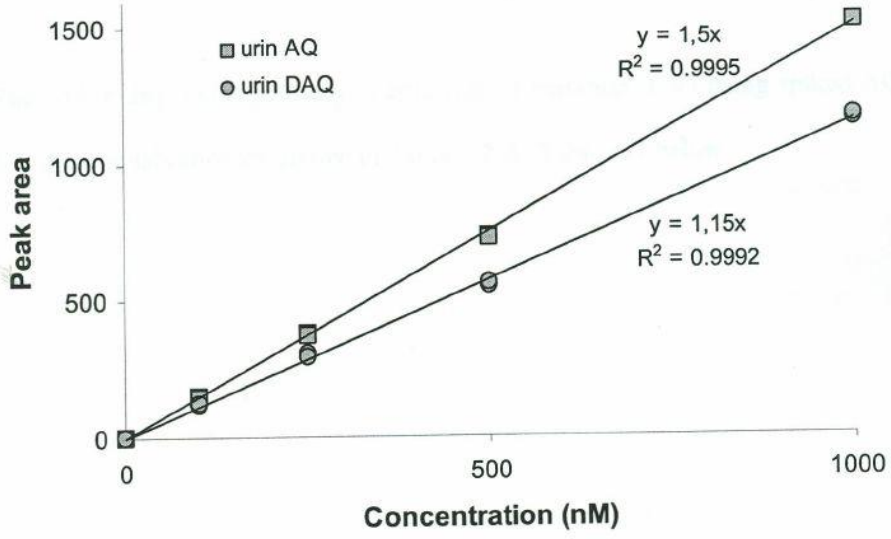


Figure 2-7 Calibration curve for AQ and DAQ in spiked urine samples

2.3.1.3 Extraction recovery & accuracy

Extraction recoveries of IS, AQ and DAQ from different samples and sample volumes are given in Table 2-1 below. It can be seen that, the values obtained from 100 µl blood samples spiked with DAQ at 1000 nM were lowest.

Table 2-1 Extraction recovery for AQ and DAQ (n = 6-10 as appropriate).

Sample size and conc.		Recovery [(%)±SD]		
		IS	**AQ**	**DAQ**
Blood 100 µl (n=6)	100 nM	83±2	103±2	78±7
Blood 100 µl (n=10)	1000 nM	92±5	84±6	68±9
Blood 1000 µl (n=10)	10 nM	73±9	82±9	71±7
Plasma 100 µl (n=8)	100 nM	88±6	97±4	75±6
Plasma 100 µl (n=8)	1000 nM	84±6	87±2	77±6
Plasma 1000 µl (n=9)	10 nM	88±5	70±8	81±6
Urine 100 µl (n=9)	100 nM	93±6	94±5	73± 9
Urine 100 µl (n=9)	1000 nM	97±7	91±7	73±8

The within day and day to day coefficients of variation (CV) using spiked AQ, CQ and their metabolites are shown in Table 2-2 & Table 2-3 below.

Table 2-2 Accuracy, within- and between-assay precision (n=7) for AQ and DAQ.

Sample size and conc		Found* [(nM) ±SD]		Within-assay CV(%)		Between-assay CV(%)	
		AQ	DAQ	AQ	DAQ	AQ	DAQ
Blood 100 µl	100 nM	97±7	98±4	7	4	9	5
Blood 100 µl	1000nM	1023±31	1005±20	3	2	4	5
Blood 1000 µl	10 Nm	11±1	10.5±1	8	8	10	9
Plasma 100 µl	100 nM	102±4	98±7	4	7	5	4
Plasma 100 µl	1000nM	984±30	990±30	3	3	2	3
Plasma1000 µl	10 nM	10±1	10±1	4	5	8	5
Urine 100 µl	100 nM	102±4	108±8	4	7	9	6
Urine 100 µl	1000nM	980±20	972±19	2	2	6	5

= mean±SD

Within day and day to day variations were always ≤10% demonstrating that the methods exhibit good precision under the validated conditions. Assays of spiked samples always gave good results, confirming good accuracy of the methods.

Table 2-3 Accuracy, within-assay precision and extraction recovery for CQ and DCQ in blood samples dried on filter paper (n=10).

Samples 100 µl	Added nM	Found* [(nM)±SD]	Within-assay CV(%)	Recovery (%)±SD)
CQ	100	96±2	2	84±6
	200	198±8	4	72±8
	2000	2002±100	5	92±4
DCQ	50	49±2	5	104±2
	100	99±2	2	101±5
	1000	990±40	4	105±6

*** = mean±SD**

The limits of quantification were 100 nM and 10 nM for all analytes using 100 µl and 1000 µl biological samples, respectively, with an intra- and inter-assay variation of <10%.

2.3.1.4 Interferences

The drugs tested in section 2.2.8, were selected due to their importance in treating different diseases in tropical regions. There was interference of AQ with quinine but other drugs and endogenous substances did not show any interference.

2.3.1.5 Stability

AQ and DAQ were not stable in spiked blood samples and blood samples from one volunteer at any of the temperatures studied except at -70°C. Whole blood samples were more stable at +4°C in a refrigerator than at -20°C. Whole blood samples containing AQ or DAQ may be kept for a maximum of one week in refrigerator. AQ and DAQ concentrations showed a slow decline in plasma and urine samples stored

at -20°C but remained stable at -70°C for more than 12 months. Since whole blood samples were not stable at -20°C, it is recommended that they should be frozen at -70 °C immediately after collection especially if the study requires determination of the actual drug levels. However, AQ blood samples may be stored in refrigenator for not more than 7 days for those studies simply targeting detection of the drug in the body. In the same reasoning, plasma and urine samples should not be stored at -20°C for more than 3 months. Standards and stock solutions were always stored at -70°C and were found to remain stable for more than a year.

2.4 DISCUSSION

An HPLC system for quantification of AQ and CQ using small volumes of whole blood samples is reported. The chromatographic system used affords complete selective separation of AQ, CQ and their active metabolites. Despite good chromatographic selectivity, simultaneous determination of AQ and CQ is not possible since different pH has to be used for the liquid-liquid extraction. The peaks present in the chromatograms of samples from the healthy subject correspond to those observed in the spiked samples.

The developed method is simple, cost-effective and sensitive enough to enable analysis of antimalarial CQ and AQ using small volumes of blood or dried blood onto filter paper (for CQ). There was no intereference with tested drugs neither endogenous substance did not show any interference at the wavelength used. However quinine interfered with AQ peaks. Even though there is no separation between AQ and quinine an interference by quinine can in most cases be revealed by inspection of the chromatograms since a peak due to dihydroquinine is always present. In studies involving determinations of AQ, only volunteers without quinine intake prior to the study should be recruited.

The stability of AQ and DAQ in whole blood is poor even at -20°C. Repeated freezing and thawing did not markedly affect stability. Whole blood samples can be kept in a refrigerator for one week, but it is recommended that plasma is separated and frozen as soon as possible. Plasma should thus be used for analysis of AQ and DAQ in studies in areas where -70°C facilities are not accessible. AQ and DAQ blood samples were more stable at +4°C than at -20 °C and it was not possible to explain the reason behind this strange phenomenon.

It is recommended that AQ plasma samples should not be stored for more than three months at -20°C.

Attempts to extract dried EDTA or heparin whole blood samples spiked with AQ and DAQ on filter paper did not yield any peaks. However, it was possible to detect peaks of these substances after extraction from plasma samples. Standard solution dried on filter paper and in polypropylene tubes also gave peaks. It was suspected that oxidation in the process of drying the blood could be the major problem. Use of antioxidants (ascorbic acid and thiols) to protect AQ and DAQ did not give positive results.

It is most likely that AQ and DAQ undergo complexation with constituents of blood particularly divalent and trivalent cations present in blood especially during the drying process. Another problem could be the type of filter paper used in the sample collection as in this work only Whatman No 3 was tested. The compounds may bind or form a complex with Whatman paper material.

An automated solid-phase extraction method for determination of AQ and CQ from blood samples dried on filter paper published by Lindegårdh et al, 2002, is expensive to be adapted in analytical laboratories of resource limited countries where malaria is endemic. A simple field adapted method is still needed. The method must ensure

sample stability for more than a month and should involve simple extraction procedures. CQ and DCQ have been reported to be stable in whole blood samples dried on filter paper (Lindstrom et al, 1985).

The major achievement in this work was an analytical method which applies small volume of blood samples (100 µl) in studying the pharmacokinetic parameters of DAQ after an oral intake of 600 mg of AQ base. However, it was not possible to assay AQ for more than 8 hrs after AQ intake by using this sample size since the drug is rapidly metabolized to DAQ (Salako et al, 1985, Mihaly et al, 1985, Winstanley, 1987). To enable quantification of AQ up to 12 hrs with coefficient of variation of <10%, 1ml whole blood and plasma samples were used and gave precise and accurate results (Table 2-2) It was possible to follow the concentrations of DAQ for up to 240 hours in 100 µl blood samples. DAQ was observed shortly after intake of tablets. In clinical studies involving AQ, DAQ could be used in producing reliable data than the parent compound. BDAQ could be detected using

blood samples of 100 µl (Figure 3-1), but no attempts to quantify this compound were made due to lack of adequate reference standard.

An HPLC method for measuring CQ in whole blood samples dried on filter paper is good achievement of this work. Filter paper methods are field adapted for collecting blood samples dried on filter paper. Lindström et al, reported afilter paper method for CQ and its metabolite DCQ in 1985. The method applies a normal phase chromatographic system which is difficult to set and takes time to stabilise. In addition it involves the use of expensive fluorescence detection system (Lindström et al, 1985). The new method developed in this work, applies reversed phase C-18 column with commonly applied UV detection.

The ability of this method to detect AQ and BDAQ and its applicability to study the pharmacokinetics of AQ and DAQ after a single oral dose of 600 mg AQ base suggests that it is more sensitive than the methods previously published (Mihaly et al, 1985, Pussard et al, 1986, Mount et al, 1986, Winstanley et al, 1987).

The method can be applied in studying metabolic pathways of the two drugs and for therapeutic drug monitoring of AQ in patients. It can also be used in epidemiological studies in malaria endemic areas.

2.5 CONCLUSION:

Cost-effective and more reliable HPLC method applying UV detection and a reversed phase system for determination of AQ and CQ using a small volume of blood sample (100µl) has been developed.

CHAPTER 3:DEVELOPMENT OF LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF PYRIMETHAMINE FROM BLOOD SAMPLES DRIED ON FILTER PAPER

3.1 INTRODUCTION

Pyrimethamine (PYR) is an inhibitor of dihydrofolate reductase enzyme in *Plasmodium falciparum.* PYR is a component of sulfadoxine-pyrimethamine drug combination (SP) which is an antifolate used in the treatment of uncomplicated *falciparum* malaria, in many endemic areas with high clinical failure of chloroquine (CQ) against *Plasmodium falciparum* strains (Bloland et al, 1999).

The substance has a relatively low bioavailability due to its poor solubility in solid dosage form. There is a need to continuously monitor the effectiveness of the components of SP, especially that of PYR which is present in a small amount as compared to sulfadoxine (SDX) in a fixed drug combination (500 mg SDX and 25 mg PYR).

PYR is also currently used in combination with sulfadiazine and folinic acid in the treatment of toxoplasmosis as one of the opportunistic infections in HIV-AIDS patients.

Pharmacokinetic and epidemiological studies focusing on PYR alone or in combination with other drugs require a suitable blood sampling method and sample storage for subsequent transportation to analytical laboratories.

To be able to determine PYR from blood samples collected in the field, especially in rural areas in poor countries, filter paper sampling procedure is highly desirable.

Existed liquid chromatographic methods for determination of pyrimethamine alone or simultaneously with sulfadoxine (SDX) in biological samples were tested, but none of them were completely satisfactory (Edstein, 1983, Midskov, 1985, Bergqvist& Eriksson, 1985, Winstanley et al, 1992). A simple and cost effective method which would enable the quantification of PYR using small volumes of blood (100µl) dried on filter paper was needed. Ronn et al developed a PYR filter paper method using synthetic monopropionyldapsone (MPD) as internal standard which is not accessible (Ronn et al, 1995). The method involves a time consuming nitrogen evaporation, whose availability may also be an issue in low income countries.

The SP filter paper method reported by Green et al in which both SDX and PYR could be simultaneous determined from dried blood samples is a great achievement (Green et al, 2002). However, the method is expensive in some laboratories of developing countries as it involves the use of solid-phase extraction and a photodiode detector.

The aim of this work was to develop a filter paper HPLC method which enables quantification of pyrimethamine by using simple extraction procedure and an accessible internal standard.

3.2 MATERIALS AND METHOD

3.2.1 Chemicals

SDX and PYR were provided by Hoffman-La Roche (Basel, Switzerland). N_4-acetylsulfadoxine (N4-ASDX) was a generous donation from Prof. A. Van Schepdael and Dr E. Kaale of the Laboratory of drug analysis at Katholieke Universitet, Leuven, 3000, Belgium.

TMP and sulfamethoxazole (SM) were purchased from Sigma Chemical Co. (St Louis, MO, USA). SP tablets (Fansidar®, Hoffmann La Roche, Basel, Switzerland) were purchased locally from one wholesale pharmacy in Dar Es Salaam.

Solvents and chemicals (HPLC or analytical grade) were obtained from Merck (Darmstadt, Germany). Filter papers (Whatman® chromatography paper) were purchased from Kebo, Spånga, Sweden.

Stock solutions were made by dissolving PYR and TMP in about 5 ml methanol in 10 ml volumetric flasks followed by sonication for 10 minutes. Dilution to volume was made using deionised water. All dilutions of stock solutions to working standards were always done with water. The stock solutions were kept in a deep-freezer at -20°C and were also stable in a refrigerator at +8°C.

3.2.2 Instrumentation

The chromatographic system used in developing the PYR method, was devised with a Gilson model 231 sample injector with a 200 µl loop (Villiers le Bel, France), a Gilson model 118 uv/vis absorbance detector and a Kontron model 422 high-pressure pump (Milan, Italy).

Method development was carried out in the Unit of Tropical Pharmacology, Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska Institute (KI), SE-141 86 Huddinge, Stockholm, Sweden and was further tested in the laboratory of the Department of Clinical Pharmacology, Faculty of Medicine, Muhimbili University College of Health Sciences (MUCHS), Dar Es Salaam Tanzania where also the PYR bioequivalence studies were conducted.

In the laboratory of the Department of Clinical Pharmacology at MUCHS, an HPLC equipped with 7125 Rheodyne manual injector with a 100 µl loop (Cotati, California USA), a Shimadzu solvent delivery module LC-10AD VP pump (Shimadzu

Corporation Kyoto Japan) and a Shimadzu UV-Visible spectrophotometric detector SPD-10A (Shimadzu Corporation Kyoto Japan) was used. The results obtained both at KI and MUCHS did not show any significant variation.

A reversed phase column, Zorbax® SB C_{18}, 150x4.6 mm I.D, 5 µm (ChromTech, Hägersten, Sweden) was used and the mobile phase consisted of phosphate buffer (0.05M, pH 5):acetonitrile: concentrated perchloric acid, (750:300:2.5, v/v).

The phosphate buffer was prepared by mixing 4.5 ml of 1M NaH_2PO_4 and 1 ml of 0.5 M Na_2HPO_4, diluting to a litre with water and adjusting the pH to 5. Elution was carried out at room temperature using a flow rate of 1.2 ml/min. Detection was achieved at 270 nm.

3.2.3 Biological samples

Drug free whole blood (blank) was spiked with PYR to obtain final concentrations of 40 - 527.2 ng/ml and the resulting solutions were used in developing and evaluating the method. Aliquots of 100 µl whole blood were frozen into polypropylene tubes at -20˚C until assay. Other aliquots of 100 µl were transferred onto filter paper strips. The blood spots were left to dry at room temperature and kept in polythene folders until assay.

Samples from a single healthy volunteer who took a single oral dose of three SP tablets of 525 mg each (500 mg SDX and 25 mg PYR) were also used. Blood from finger-prick 100 µl in duplicate was collected using heparinised precision capillaries. The samples were collected before and at different time intervals after an oral tablet intake as described previously (Winstanley et al, 1992). The blood samples were dried in spots onto filter paper and kept in polythene folders until assay.

3.2.4 Analytical procedure for PYR

The dried blood spots on the papers were cut into small pieces and put into 12 ml polypropylene tubes. To each tube, 50 µl of the internal standard (6-8µg/ ml) were added followed by 2.0 ml of 0.2 M sodium hydroxide. The samples were gently shaken for 30 minutes. The samples were extracted using 5 ml of diisopropyl ether on a reciprocal shaker for 25 minutes.

After centrifugation for 10 min at 3500 rotations per minute (rpm), the upper organic phase was transferred to a new tube and 150 µl of the mobile phase were introduced. Sample extraction was carried out for 10 minutes as described above. The phases were separated by centrifugation as above and the upper organic phase was removed by aspiration. Into the chromatograph 80-100 µl of the aqueous solution were injected. Similar procedures were conducted in 100µl whole blood samples frozen in polypropylene tubes after thawing.

3.2.5 Standard curves

Standard curves were prepared by adding known amounts of PYR (0-60 µl) standard solution and IS (50 µl) to blank dried blood samples on filter paper. The standard curves were prepared by duplicate analysis of 100µl heparinised blank whole blood spiked with working solution to obtain final concentrations of 44-704 ng/ml of PYR (n=5) and dried on filter paper strips. Aliquots of 100 µl spiked whole blood was transfered on the Whatman® filter paper strips and dried. The standard curves were prepared by duplicate analysis of 100 µl heparinised blank whole blood spiked with PYR at concentrations of 44-704 ng/ml and was dried on filter paper strips.

One quality control sample (in duplicate) containing PYR at a concentration of 50.6 or 527.2 ng/ml was always included in each analysis. Calibration curves for whole

blood samples frozen in polypropylene tubes were prepared analogously using blank liquid whole blood.

The resulting peak area ratios between the internal standard and the analyte were plotted versus concentrations.

3.2.6 Extraction recovery

To check the extraction recovery, standard PYR was added to whole blood and 100 µl aliquots were dried onto filter paper. Samples were prepared as above and 50 µl of TMP as internal standard (6µg/ml) were added. The samples were assayed as described previously. Samples were extracted according to the above procedure and the peak areas were compared with those of directly injected standards. For whole blood samples frozen in polypropylene tubes, the extraction recovery was assessed analogously.

3.2.7 Precision and accuracy

Known amounts of PYR were added to blank whole blood and 100 µl aliquots were dried on filter paper. For whole blood samples frozen in polypropylene tubes same procedure was followed. The samples were extracted and analyzed as described earlier both in one series (within assay precision and accuracy) and on different occasions (between assay precision and accuracy). The standard deviations and coefficients of variation were calculated.

3.2.8 Interference

SDX, N_4-acetylsulfadoxine (N4-ASDX), the main metabolite of SDX, SM, amodiaquine, chloroquine, paracetamol, acetyl salicylic acid and other commonly used drugs were studied for interference by spiking the drugs in blank whole blood

followed by extraction as described above. Endogenous substances were also monitored for interference.

3.2.9 Stability

The stability of PYR blood samples dried on filter paper was not tested due to adequate information available in the literature (Ronn et al, 1995, Green et al, 2002).

3.3 RESULTS

3.3.1 Evaluation of the analytical procedure

3.3.1.1 Chromatograms

The chromatographic system used exhibited a good separation for PYR and TMP (the IS) after injection of extracted spiked samples and samples from the volunteer. Neither SDX nor SM could show any interference. The chromatographic systems applied both at KI and MUCHS gave the same separation patterns, confirming ruggedness of the method.

The separation chromatogram of standard solution containing a mixture of TMP, SDX, SM and PYR is shown in Figure 3-1 below.

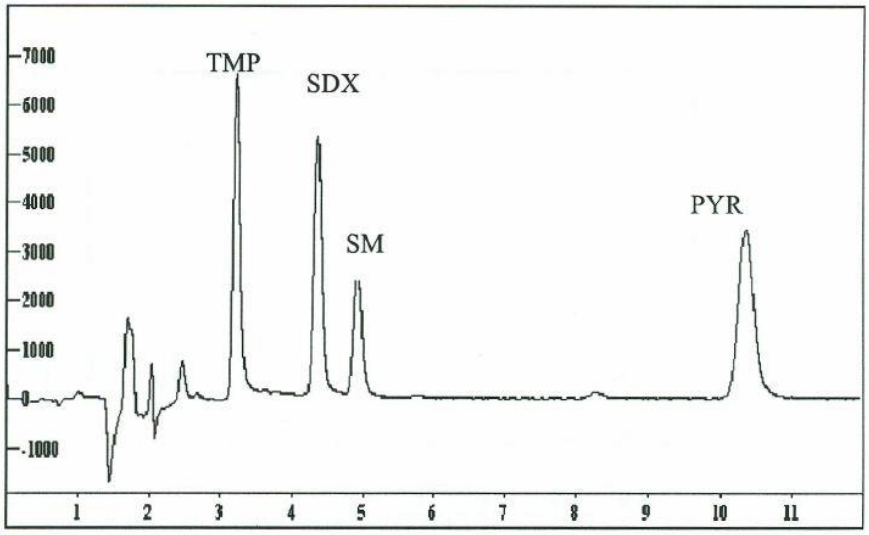


Figure 3-1 Separation chromatogram of TMP, SDX, SM and PYR in an aqueous mixture of the 4 substances.

The retention times of PYR and IS in spiked samples were compared with those obtained from blood samples of the healthy volunteer, 24 hours after an oral dose of three tablets of SP. The chromatograms are shown in Figure 3-2 & Figure 3-3 below.

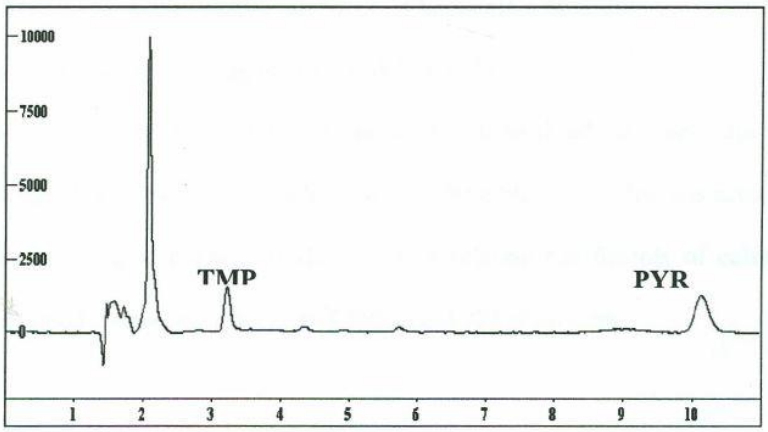


Figure 3-2 Separation of TMP (IS) and PYR in spiked whole blood dried on filter paper

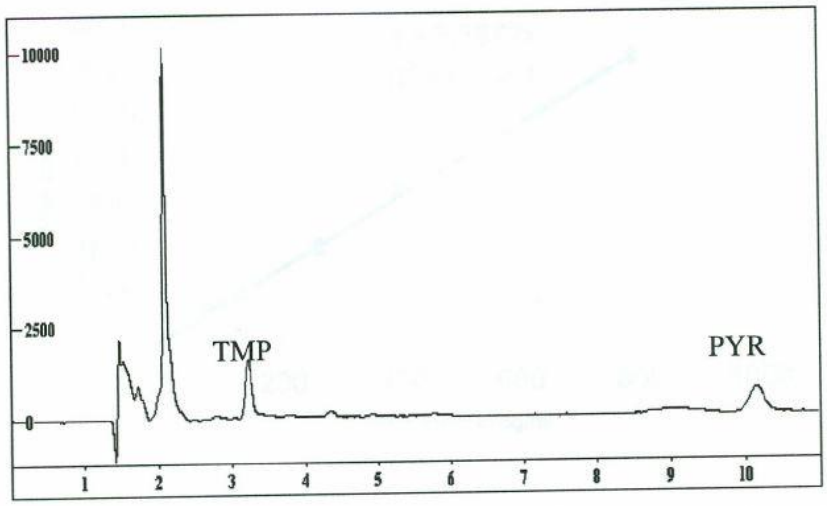


Figure 3-3 A separation chromatogram of TMP (IS) and PYR in whole blood of a subject on filter paper 24 hours after ingestion of 3 SP tablets.

By careful inspection of the two chromatograms, it can be seen that the retention times of PYR and IS in spiked samples corresponded with those from blood samples obtained from the healthy volunteer 24 hours after an oral dose of three tablets of SP each containing 500 mg of SDX and 25 mg PYR.

The calibration curve for PYR in whole blood dried on filter paper is shown in Figure 3-4 below. Excellent linearity in whole blood samples was seen for PYR over the concentration range studied. The correlation coefficients of calibration curves obtained were always between 0.995 and 0.998 in all runs.

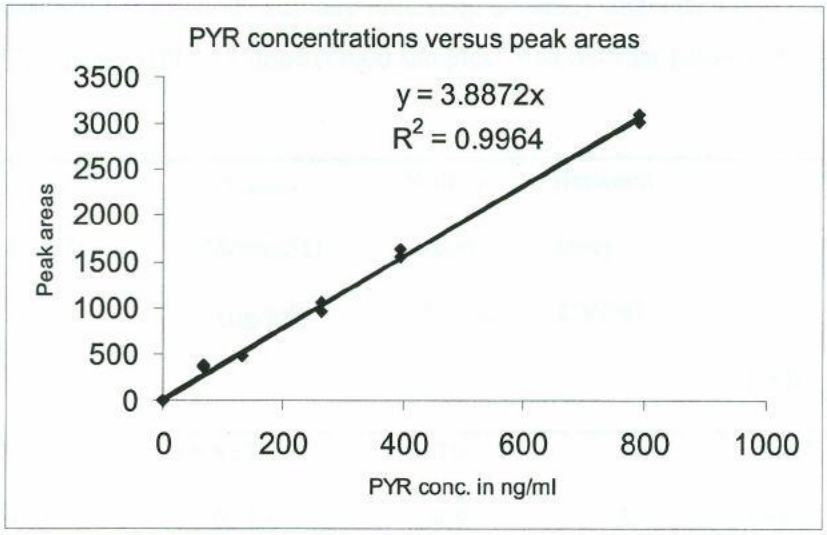


Figure 3-4 Calibration curve for PYR assay in whole blood dried on filter paper.

Intra and inter-day variations of both whole blood and dried samples were always ≤10% demonstrating that the precision of the method is good under the selected conditions. Assays results of spiked PYR in whole blood and in blood samples dried on filter paper are shown in Table 3-1 & 3-2.

Table 3-1 Intra- and inter-day precision, accuracy and extraction recoveries of PYR whole blood HPLC method (whole blood samples n=6).

C_{spiked}, (ng/ml)	C_{found}, Mean±SD (ng/ml)	Within-assay CV (%)	Between-assay	Recovery Mean±SD (%)	
				PYR	TMP
40	41.0±5.1	12	15	33±5	41±7
100.1	97.1±4.0	8.6	4.1	46±7	42±3

Table 3-2 Intra- and inter-day precision, accuracy and extraction recoveries of PYR filter paper HPLC method (blood samples dried on filter paper n=8).

C_{spiked}, (ng/ml)	C_{found}, Mean±SD (ng/ml)	Within-assay CV (%)	Between-assay CV(%)	Recovery Mean±SD (%)	
				PYR	TMP
40	36.8±3.7	10	15	27±2	30±7
50.6	52.9±2.6	4.9	5.5	38±5	33±3
100.1	99.1±8.5	8.6	3.1	42±2	40±4
527.2	478.7±9	1.9	7.3	40±4	39±4

The calculated concentrations of the quality control samples used in the analysis always gave results ranging between 90-110% of the expected concentration confirming the reliability of the method. The limit of quantification was 40 ng/ml with intra- and inter-assay variations of ≤10%. Extraction recoveries for PYR and TMP are low and the peaks are quite far apart (Figure 3 1 & Figure 3 3). However, pattern of peak separation and the extent of extraction recovery should not be an issue in bioanalytical method development and validation as long as adequate separation, sensitivity, precision and accuracy are achieved (Dadgar et al, 1995).

3.3.1.2 Interferences

Neither SDX nor its metabolite N4-ASDX interfered with the peaks of PYR and its IS. Other tested drugs also did not exhibit any interference to PYR and IS nor there was interference with endogenous substances.

3.3.1.3 Pharmacokinetics

Figure 3-5 below illustrates a concentration - time curve of PYR after an oral intake of three SP tablets by a healthy volunteer. Following ingestion of 3 SP tablets each containing 500 mg SDX and 25 mg PYR, the peak blood concentration (C_{max}) of 470 ng/ml was reached after 2hrs. The calculated elimination half-life ($t_{1/2}$) was 170 h^{-1}hrs and the area under the curve AUC_{0-240} was 55103 $ng.h.ml^{-1}$. The blood concentration of PYR three days after drug administration was 236 ng/ml.

However it should noted that the data obtained from a single volunteer are not adequate to project the actual pharmacokinetic profiles of the drug. At least six healthy volunteers should be involved.

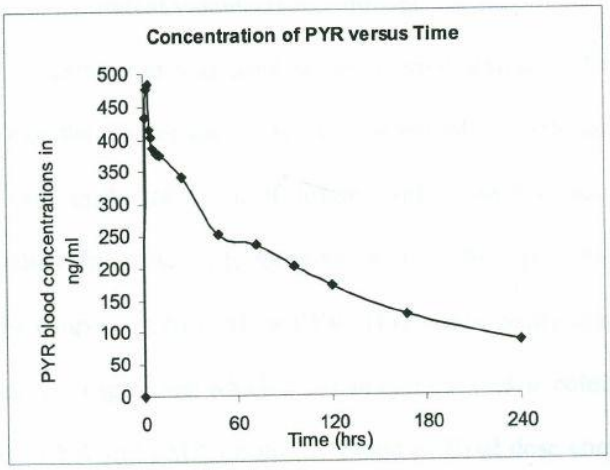


Figure 3-5: Pharmacokinetic profiles of PYR after a single oral intake of 3 tablets of SP (500mg SDX and 25 mg PYR) of SP by healthy subject in a pilot study.

3.4 DISCUSSION

Filter paper methods are suitable in field studies since they require small volumes of blood (100 µl) making it possible to apply them even in small children. Dried blood samples have been shown to have a dramatically reduced amount of pathogenic

organisms including HIV (CDC, 1997). The method hereby described involves simple extraction steps followed by direct injection of the extract in the reversed phase chromatographic system.

Synthetic internal standards which are neither drugs nor metabolites of commonly used drugs are convenient to apply in determination methods since there is rarely likelihood of interference with drugs pre-taken by the volunteers or patients. They only have a setback that, most of these substances cannot always be accessed even from the author of the method.

Synthesis facilities are not always at hand, neither in developing countries, nor in the industrialised world. On the other hand, compounds which are commonly used in drug formulations are always available in the market and could be used as internal standards but under controlled volunteers recruitment.

In this method trimethoprim was used as an internal standard. The substance is widely available on the market since it is a component of co-trimoxazole (CMX), an antifolate drug used in the treatment of different infectious diseases. However it is important to exclude from the study those volunteers who report that they recently have been on the course of CMX, SP or PYR. This can be partly done by screening urine samples using a Lignin test which gives an orange- yellow colour reaction with all sulfonamides (PYR and TMP are mostly found as fixed dose combinations with sulfonamides).

However, it should be noted that Lignin test is just a preliminary test. Volunteers should be asked to state clearly whether they recently took those drugs. Otherwise, if doubtful information is given, a confirmatory test using an HPLC technique involving the comparison of the retention times of PYR and TMP in urine samples of the volunteers with that of spiked urine samples may be applied.

However, an HPLC screening method is time consuming and neither is field adapted since it cannot be used in study areas far from the analytical laboratory.

Exclusion of volunteers with significant levels of these compounds in blood is important since the presence of PYR or TMP as internal standard in blood before intake of the tested drug will interfere with the actual drug levels leading to confounding results.

The applicability of this method in studying the pharmacokinetics of PYR from healthy volunteer suggests that the method is suitable in malaria endemic area. The concentrations of PYR obtained from a volunteer were within the calibration range (44 -704 ng/ml) with Cmax being 470 ng/ml.

In addition, the ability to quantify PYR from whole blood samples dried on filter paper makes this method a useful tool in epidemiological studies as well as therapeutic drug monitoring in rural areas where PYR is widely used, since huge number of samples can be collected and transported to an analytical laboratory for drug analysis. The method avoids fridge availability problems which is an issue in rural areas of many developing countries. The method also does not require a time consuming organic solvent evaporation making it fast, simple and cost-effective and can be applied to study PYR alone *in vivo* or in conjunction with Bergqvist SDX method (Bergqvist et al, 1987) to study SP drug combination.

3.5 CONCLUSION:

An HPLC method for determination of pyrimethamine in dried whole blood has been developed.

CHAPTER 4: APPLICATIONS OF HPLC METHODS FOR QUALITY ASSESSMENT OF AMODIAQUINE AND SULFADOXINE/PYRIMETHAMINE TABLET FORMULATIONS

4.1 INTRODUCTION

The availability of quality antimalarial drugs is important for effective and safe treatment of malaria. Use of fake and low quality antimalarial drugs is a major cause of treatment failures, toxicity or side effects (Shakoor et al,1997, Menkes, 1997). Frequent use of such drugs result in sub-therapeutic plasma concentrations and eventually favors the emergence of resistant *P. falciparum* strains (Petralanda, 1995, Kun et al, 1999, Macarthur et al, 2001).

Marketing of poor quality drugs in developing countries has been widely reported (Abu et al, 1990, Kibwage et al, 1992, Mahamood et al, 1994, Murtarda et al, 1994, Menkes, 1997, Shakoor et al, 1997, Ogwal-Okeng et al, 1998, Stenson et al, 1998, Taylor et al, 2001, Alw, 2001, Newton, et al, 2001, Rimoy et al, 2002, Risha et al, 2002). Drugs with incorrect amount of active contents as well as drugs devoid of active ingredient(s) are found in these markets (Alw, 2001, Taylor et al, 2001, Newton, et al, 2001).

The generic antimalarial tablet formulations tested by different authors were frequently found to elicit lower bioavailability than the corresponding innovators' products (Abu et al, 1990, Mahamood et al, 1994, Weidekamm et al, 1998, Ogwal-Okeng et al, 1998, Na-Bangchang et al, 2000, Jungheinrich et al, 2002, Rimoy et al, 2002).

In Tanzania in 2000, SP and AQ became first and second line antimalarial drugs respectively. Since then, there has been intensified marketing of many different

amodiaquine (AQ) and sulfadoxine/Pyrimethamine (SP) generics from different sources.

The present study aimed at investigating the quality of SP and AQ generic tablet formulations marketed by wholesale pharmacies in Dar Es salaam after malaria treatment policy changes.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

SDX and PYR were provided by Hoffman-La Roche (Basel, Switzerland). N_4-acetylsulfadoxine (N4-ASDX) was a generous donation from Professor AV Schepdael and Dr Kaale E of the Laboratory of drug analysis at Katholieke Universitet Leuven, 3000, Belgium.

Trimethoprim (TMP) and sulfamethoxazole (SM) were purchased from Sigma Chemical Co. (St Louis, MO, USA). AQ and SP tablets were purchased locally from wholesale pharmacies in Dar Es Salaam. Acetonitrile and diisopropyl ether (HPLC and analytical grade respectively) were obtained from Merck (Darmstadt, Germany). Filter papers (Whatman® chromatography paper) were purchased from Kebo, Spånga, Sweden. Stock solutions were made in 10 ml volumetric flasks by dissolving PYR and TMP in about 5 ml methanol followed by sonication for 10 minutes. The dilution to volume was made using water. The stock solutions were kept in a deep-freezer at -20°C but were also stable in a refrigerator at +8°C.

4.2.2 Instrumentation

An HPLC equipped with 7125 Rheodyne manual injector with a 100 µl loop (Cotati, California USA), a Shimadzu solvent delivery module LC-10AD VP pump (Shimadzu Corporation Kyoto, Japan) and a Shimadzu UV-Visible

spectrophotometric detector SPD-10A (Shimadzu Corporation Kyoto Japan) was used.

A reversed phase column, Zorbax® SB C_{18}, 150x4.6 mm I.D, 5 µm (ChromTech, Hägersten, Sweden) was used and the mobile phase consisted of phosphate buffer (0.05M, pH 5):acetonitrile: concentrated perchloric acid, (750:300:2.5, v/v). The phosphate buffer was prepared by mixing 4.5 ml of 1M NaH_2PO_4 and 1 ml of 0.5 M Na_2HPO_4, diluting to a litre with water and adjusting the pH to 5. Elution was carried out at room temperature using a flow rate of 1.2 ml/min. Detection was achieved at 270 nm.

4.2.3 Study area

Dar Es Salaam, is the commercial center of Tanzania with more than 250 retail pharmacies, 20 wholesale pharmacies and medical stores which are currently estimated to be more than 1000. There are also more than 4 private pharmaceutical industries in the city. Dar Es Salaam is principal center of importation and distribution of drugs in the country.

4.2.4 Tablet sampling

AQ and SP tablet samples were collected from wholesale pharmacies which hadimport licenses. Wholesale importers are key providers of drugs for hospitals, dispensaries, retail pharmacies and medical stores in the country. SP and AQ are included in the essential drug list of Tanzania. The National Drug Regulatory Agency provided a list of wholesale importers and list of SP and AQ brands approved to be used in the country. There were 12 registered wholesale importers in Dar Es Salaam, whereas only 8 were active.

At each of these 8 pharmacies, all available AQ and SP brands were bought. The exclusion criteria were samples with a shelf-life of less than a year from the day of collection.

A sample was an intact box of 100 tablets or a sealed tin of 100 tablets. In cases where a brand existed in both tins and boxes, all of them were bought. A summary and list of collected AQ and SP tablet samples is shown in Table 4-1 & 4-2 below. Fifteen and eighteen respectively AQ and SP samples were collected.

Table 4-1: A summary of the wholesale pharmacies from which the sampled sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ) were sourced.

Pharmacy identity	Available SP samples of adequate shelf-life	Available AQ samples of adequate shelf-life
I	2[1]	2
II	3[1]	1[1]
II	3[1]	2[1]
IV	2[1]	2
V	2[1]	2
VI	2[1]	2
VII	2	2
VIII	2	2

[1] Another AQ and SP was present at the pharmacy but with expired date.

None of the listed pharmacies stored all brands, mostly 2 or 3 different brands of either AQ or SP. Only one sample of Fansidar® (Hoffmann La Roche, Switzerland) was collected in a single pharmacy since samples found in other pharmacies had due short expiry dates (Table 4-1). Similarly only one sample of Camoquine® an AQ brand (Park-Davis, USA) was available in a single pharmacy. No more than 7 AQ and 8 SP brands were found at the time of sampling.

Table 4-2: Summary of sulfadoxine-pyrimethamine (SP) and amodiaquine AQ samples collected and their quality in 8 wholesale pharmacies in Dar Es Salaam, Tanzania.

Brand name	Samples (n)	Country of origin	Failed in quality test (n)
	Sulfadoxine-pyrimethamine		
Falcidin	2	Kenya	0
Falsitat	2	Cyprus	2
Fansidar	1	Switzerland	0
Malaridox	2	India	2
Malostat	2	India	0
Orodar	3	Kenya	0
SP	2	Tanzania	2
Sulphadar	4	Tanzania	4
	Amodiaquine		
Amobin	1	Kenya	0
Amodiaquine Nemi	1	India	0
Camoquin	1	USA	0
Emoquin blister	3	Kenya	0
Emoquin tin	2	Kenya	2
Malaratab	2	Kenya	0
Malaridose	3	India	0
Malatab	2	India	0

Most of the SP collected samples originated from Tanzania, Kenya and India. No locally manufactured AQ sample was found during sample collection.

The drug sellers were not given the reasons for buying the samples. The simulated client was given the drugs in their original containers as they do routinely. The name of the brand, strength, batch number, manufacturer's name and the expiry dates were coded. Sample collection was conducted between Jan-February 2002

4.2.5 Sample analyses

To blind the drug analyst, 30 tablets of each sample were removed from their initial packages, coded and sent to the quality control laboratory for analysis. Only the code, name and strength of each sample were provided to the laboratory to ensure confidentiality. All drugs were analyzed at the Pharmacy Board (currently known as

Tanzania Food, Drug and Cosmetics Authority -TFDA) analytical laboratory in Dar Es Salaam.

Drug quality was determined according to the standards of United States Pharmacopoeia (USP), 24th edition (USP, 2000). The identity was confirmed by high-performance liquid chromatography (HPLC).

For assay of SP and AQ tablet samples, an HPLC method described in the USP with minor modifications were used respectively (USP, 2000). The HPLC method was slightly modified to improve the resolution between sulfadoxine (SDX) and pyrimethamine (PYR) and to enable determination of AQ using the same chromatographic system. The mobile phase used consisted of 1% acetic acid in water and acetonitrile in the ratio of 3:2 respectively. Perchloric acid was added drop by drop to the resulting mixture so as to improve resolution of the peaks.

Precision and accuracy of AQ and SP assays were determined and the results are shown in Table 4-3 below.

Table 4-3: Performance for amodiaquine (AQ), Sulfadoxine (SDX) and pyrimethamine (PYR) in spiked samples of AQ (n=8) and sulfadoxine-pyrimethamine (SP, n=7).

Sample	% Recovery(±SD)		Within-assay SD (%)		Between-assay SD (%)	
AQ	97± 8		6		9	
SP	PYR	SDX	PYR	SDX	PYR	SDX
	97±9	102± 5	6	4	9	5

Dissolution of the tablets was carried out using a dissolution apparatus with six wells. The sampled solutions were quantified for released SDX and PYR using previously described method. Sampled solutions for dissolution tests were quantified for AQ using another method described in the USP (USP, 2000). All dissolution tests were conducted in 6 individual tablets of each product.

Sample preparation for assay involved weighing and crushing the tablets followed by dissolving, diluting and filtration prior to analysis. Quantitative determination of the active principles was based on 20 tablets of each sampled brand, whereas for identity tests 4 tablets of each sample were used.

4.2.6 Bioequivalence investigation of 2 SP tablet formulations

4.2.6.1 Selection criteria of SP Interchem from Moshi, Tanzania

In the dissolution test performed above, eight (8/33) SP samples failed in the dissolution test. Of the failed samples, six (from three brands) were local products manufactured by pharmaceutical industries in Dar Es Salaam and Moshi.

SP brand manufactured by Interchem-Moshi gave the highest dissolution failure rate for both SDX and PYR so it was selected for further bioavailability comparison with Fansidar®.

The test formulation (brand A) was compared with B, an innovator product-Fansidar® (Hoffmann La Roche, Basel, Switzerland) which earlier gave excellent dissolution rate performance. Both tests and reference formulations involved the same batches initially subjected to dissolution tests.

4.2.6.2 Subjects recruitment for bioequivalence study

Seventeen healthy volunteers (7 males and 10 females) were initially recruited. The mean age, body weight, height, and body mass index of the subjects were determined.

4.2.6.3 Clinical procedure

This was a single blind, randomized two-way cross-over study with a 2 months wash-out period separating the two administrations (Winstanley et al, 1992). To

avoid bias, the investigators were blinded for the formulation taken by volunteers and the blood samples were coded. The study protocol was approved by National Institute of Medical Research (NIMR) ethics Committee in Dar Es Salaam, Tanzania. An informed consent to particiicipate in the study was also obtained from each subject following a full explanation of the design and the objectives of the study. Carefully selected inclusion/exclusion criteria were applied in order to ensure that the clinical and pharmacokinetic data obtained could be evaluated.

The inclusion criteria was a healthy volunteer while exclusion criteria included any serious disease including pulmonary tuberculosis, any significant deviation in parameters of clinical chemistry, renal and liver dysfunction as well as known history of allergy to sulfa drugs or failure to complete the study. Subjects below 18 and above 45 years were also excluded.

Subjects whose blood samples prior to drug administration contained active components of co-trimoxazole and SP as confirmed by Lignin colour test and by HPLC method (Bergqvist et al, 1987) were excluded. The volunteers were instructed to refrain from eating grape fruit and drinking grape juice or alcohol, two days before and during the study period (Lilja et al, 2000). They were also instructed not to take any medications two weeks before as well as during the study period. Those who got malaria during the study period were treated with AQ and neither with SP nor any other sulfonamides. Screening before the start of the study included medical history, physical examination and laboratory tests (blood chemistry –ALAT &ASAT, and a complete haematology status). Blood pressure and pulse rate were determined before medication and 8 hours after drug intake. Physical examination and laboratory tests were repeated during the last week of wash-out.

The volunteers were informed in advance about the possibility of such side effects like nausea, dizziness, vomiting, diarrhea, headache, skin reaction, loss of appetite and general malaise and were advised to report to the physician in case of any serious side effects. To avoid risk of disease transmission, sterile single use prickers and collecting capillary tubes were used.

4.2.6.4 Drug administration and blood sample collection:

In African settings, volunteer subjects may deny having taken a certain drug for the fear of being excluded from a study. In this study, the levels of SDX and SMTX from blood samples in volunteers prior to drug administration were determined. Among the 17 recruited volunteers, 2 of them were found to have quantifiable levels of SDX in their blood despite denying to have taken SP very recently. The two were therefore excluded from the study.

Following an overnight fast (Milton et al, 1989), 12 healthy volunteers were randomised to receive a single oral dose of 3 SP tablets (each containing 500 and 25 mg of SDX and PYR) in a form of either A as test drug or B as reference drug. The tablets were administered under supervision. The A and B tablets administered were taken from same batches used for dissolution rate tests.

The tablets were swallowed with a glass of water (200 ml) in the morning. A low fat content breakfast and standardized lunch was served 3 hrs and 5 hrs after drug intake respectively. The second drug administration was done after a wash-out period of 8 weeks (Winstanley et al, 1992). Blood samples were collected pre-dose and at 1, 2, 3, 4, 5, 6, 8, 10, 24, 48, 72, 96,120, 168 and 240 hrs after drug intake (Winstanley et al, 1992). The blood samples (100µl) were obtained from a finger prick in duplicate into heparinised precision capillaries transferred on to filter paper and dried at room temperature. To avoid sample contamination, the blood samples were kept in

polythene folders until assay. Adverse events were recorded during the whole study period.

4.2.6.5 Analytical methods.

Blood samples were assayed for SDX using an HPLC method described by Bergqvist et al, 1987 with minor modifications. PYR was quantified using another HPLC method developed as part of this work. The method has been described in detail in Chapter 3. The methods were validated prior to carrying out assays of the samples from volunteers.

4.2.6.6 Pharmacokinetic parameters

The pharmacokinetic parameters of sulfadoxine (SDX) and pyrimethamine (PYR) were estimated using single compartment model. The areas under the plasma drug concentration- time curve up to the last sampling time (AUC_{0-240}), total AUC ($AUC_{0-infinity}$) and terminal elimination half-life ($t_{½}$) for SDX and PYR were determined by a computer software (Kinetica, Huddinge Stockholm, 2001). Peak blood concentrations (C_{max}) and the time required to achieve peak concentrations (T_{max}) were obtained from the blood concentration-time data. The relative bioavailability (fRel) of test formulation A with respect to SDX and PYR were calculated by dividing AUC_{0-240} to the corresponding AUC_{0-240} of reference formulation B.

According to international guidelines the two formulations are bioequivalent if the relative bioavailability of A at a 90% confidence interval lied within the range of 0.80-1.25 and if the ratio of C_{max} of A formulation to C_{max} of B formulation was found between 70-143% (FDA, 1992).

4.2.6.7 Statistical analysis

Data from the two SP formulations was analysed for SDX and PYR based on C_{max}, $AUC_{0\text{-last}}$, $AUC_{0\text{-inf}}$, T_{max} and $t_{½}$. Assessment of difference between the test A and reference B formulation for these pharmacokinetic parameters was performed using Student's *t* test. Results are presented as mean ± standard deviation (SD).

4.3 RESULTS

4.3.1 *In vitro* quality assessment of tablet samples

4.3.1.1 Validation of the analytical methods used for content dermination and dissolution tests

Intra and inter-assay coefficients of variation of the method were always <10%. The results demonstrated good precision and accuracy for AQ, SDX and PYR in AQ and SP spiked samples. The results are shown in Table 4-3 above.

4.3.1.2 Assay for content and dissolution rate tests

Of the 33 collected samples twelve of them (36%) were found to be of poor quality and the results are summarized in Table 4-4: below.

Table 4-4: Summary of substandard sulfadoxine-pyrimethamine (SP) and amodiaquine AQ samples classified according to the analysis they failed (% are given as proportion of the failed samples and the total collected samples for both SP and AQ n=33)

Reasons for being Substandard	SP	AQ	%
Active ingredients are below	2[a]	0	6
Low amount released in the dissolution medium after 30 minutes	8[b]	2[c]	30
Total	10	2	36

[a] All from Cyprus, [b] 6 from Tanzania and 2 from India, [c] All from Kenya.

The major reason for a drug being a substandard product was due to poor dissolution rate of the active ingredients. Based on USP specifications, 2/18 (11%) SP samples (Falcistat®, Remedica, Cyprus) had slightly low dose content. The samples contained 88% and 87% of PYR as opposed to the pharmacopoeial specifications (USP, 2000).

There was an excellent dissolution conformity for Orodar®, Falcidin®, Malostat®, Fansidar®, Emoquine blister packed and Camoquine® (USP, 2000). Figure 4-1 shows dissolution characteristics of different amodiaquine samples. A slight dissolution failure was observed in two AQ tablet formulations which originated from Kenya.

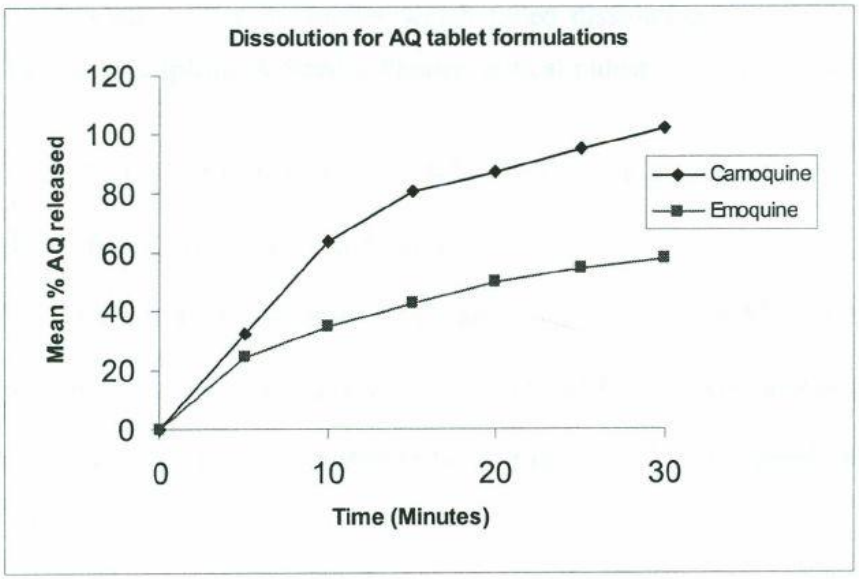


Figure 4-1 : Dissolution profiles of two amodiaquine brands: Camoquine®, (Parke-Davis, USA), as reference sample compared to Emoquine®, Ellys, Nairobi-Kenya) tablet samples in tins.

On the contrary, a tremendous dissolution failure of all locally produced SP samples [6/6, (100)%] and 2 samples from India (Figure 4-2) was observed.

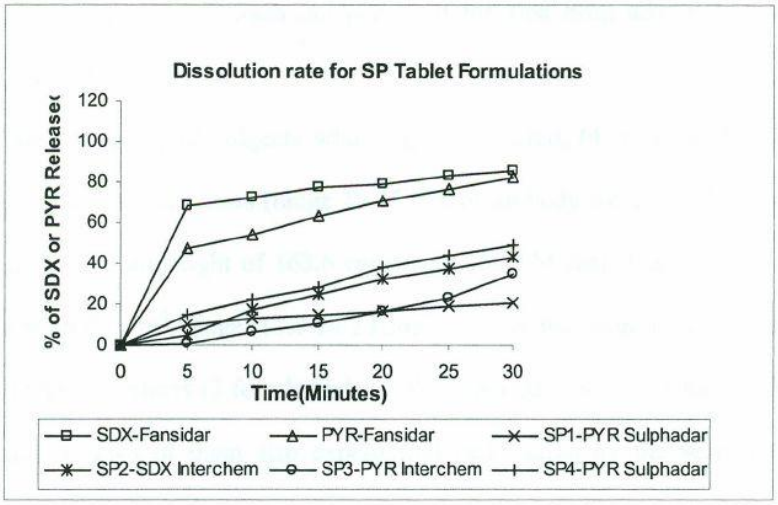


Figure 4-2 Dissolution profiles of sulfadoxine (SDX) and pyrimethamine (PYR) for Fansidar® (Hoffmann La Roche, Basel, Switzerland) as reference sample and some representative SP test samples which failed dissolution test: SP-Interchem Moshi Tanzania, Sulphadar® Shellys Pharmaceutical industry, Dar Es salaam Tanzania.

In total, 10/33 (30%) samples *i.e.* 8/33 (24%) SP and 2/33 (6%) AQ could not meet the USP specifications for dissolution.

Fansidar® and Camoquine® being innovator products for SP and AQ respectively were used as comparators in which all AQ and few SP representatives of the failed samples are illustrated. Identity tests were performed as described above and all the collected samples qualified.

4.3.2 Bioequivalence investigation

4.3.2.1 Demographic characteristics of the volunteers

Of the seventeen volunteers (7 males and 10 females) who were initially recruited, 2 of them were not healthy as assessed by clinical and laboratory screening so they were excluded from the study. Two others (male) whose blood samples were found to contain SDX prior drug administration were also excluded. One female subject did

not turn up after a wash out period of the first drug administration, so she was recorded as drop out.

The remaining 12 subjects who fully participated, (4 males and 8 females) had a mean age of 40.5 years (range 29-45yrs), mean body weight of 71.8 kg range (59-94 kg) and mean height of 163.6 cm (range 155-174 cm). The mean body mass index was 20.4 kg/m^2, range (17.8-24.2 kg/m^2) and normal range 18-25 kg/m^2.

Three volunteers (2 female and 1 male) reported headache 5 hours after intake of A and B. Two of them also experienced tachycardia on the second day after drug intake. The male volunteer further experienced skin itching and developed skin rashes on the second day. No danger sign associated with drug intake was reported. Two volunteers who got malaria during the study were treated with amodiaquine.

There were no clinically significant alterations in blood pressure, heart rate and respiratory rates.

4.3.2.2 Pharmacokinetics

The pharmacokinetic parameters of the two SP tablet samples are shown in Table 4-5, Figure 4-3 & Figure 4-4.

The mean blood concentration versus time profiles of SDX and PYR revealed that the concentrations of the test formulations were significantly lower than those of the reference formulation.

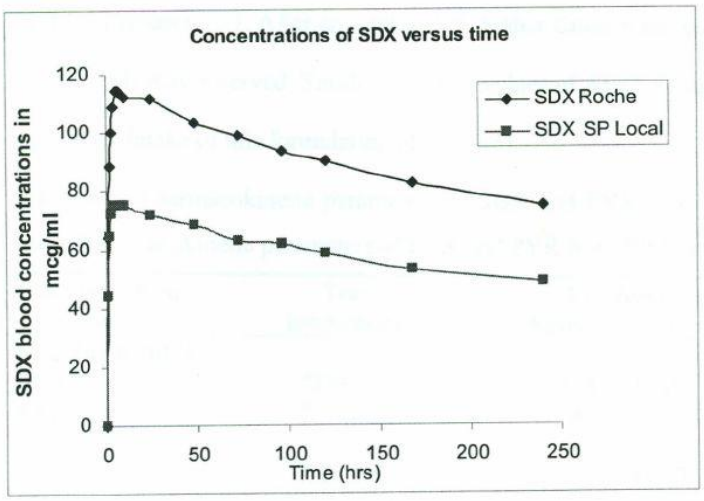


Figure 4-3 Pharmacokinetic profiles of SDX after a single oral intake of 3 tablets of SP (500mg SDX and 25 mg PYR) of SP Interchem, Moshi® and SP Fansidar® by healthy subjects, (mean, n=12).

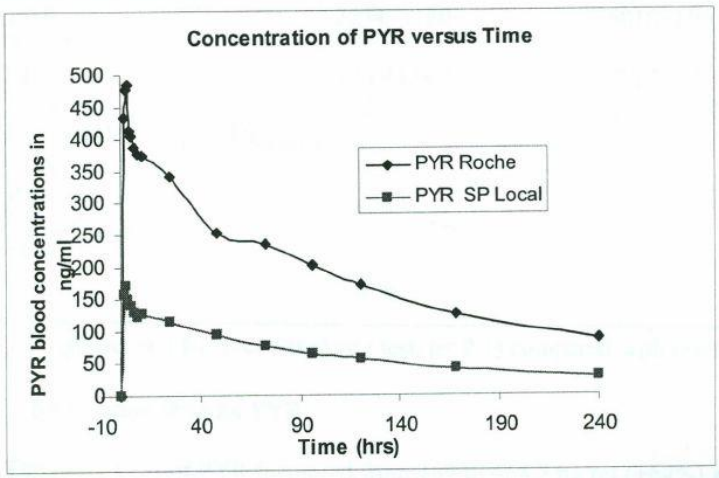


Figure 4-4 Pharmacokinetic profiles of PYR after a single oral intake of 3 tablets of SP (500mg SDX and 25 mg PYR) of SP Interchem, Moshi® and SP Fansidar® by healthy subjects, (mean, n=12).

Lower C_{max}, AUC and longer T_{max} were observed in A for both PYR and SDX as compared to B. The relative bioavailabilities (F_{rel}) of SDX and PYR in formulation A in relation to formulation B using AUC up to last sampling time (240 h) were 0.59

and 0.33 respectively. After an intake of B, higher Cmax with respect to SDX (114.6 *vs*75µg/ml) was observed. Similarly, higher values of AUC_{0-240} and $AUC_{0-infinity}$ were seen after intake of this formulation (Table 4-5).

Table 4-5: Pharmacokinetic parameters of SDX and PYR from SP formulations A and BPharmacokinetic parameters of SDX and PYR from SP formulations A and B

Pharmacokinetic Parameter	Test formulation A	Reference formulation B
C_{max} (µg✱.ml^{-1})		
SDX	[α]75±17	114.6±11.8
PYR	[α] 169±25	468.7±28.9
T_{max} (h)		
SDX	[α] 10.1±2.3	4.9±1.7
PYR	2.4±0.8	2.5±0.6
AUC_{0-240} (µg✱.h.ml^{-1})		
SDX	[α] 8838± 150	15077 ±148
PYR	[α] 18389± 200	55363 ±205
$AUC_{0-infinity}$ (µg✱.h.ml^{-1})		
SDX	[α] 13546± 209	28206±199
PYR	[α] 25341±180	76018±100
$T_{1/2}$ (h)		
SDX	158.433± 19	170.2±22
PYR	122.933± 28	125.621±20
Frel =$AUC_{0-240\ A}$ / $AUC_{0-240\ B}$		
SDX	0.59	
PYR	0.33	
$^{f}C_{maxA}$ / C_{maxB}		
SDX	0.66	
PYR	0.36	

[α] Significant difference (Student *t* test, p< 0.1) compared with reference formulation ✱ng for PYR

The mean C_{max} of PYR increased from 169 to 468.7 ng/ml (+64%) and the AUC_{0-240} from 18389 to 55363 ng.h.ml^{-1} (+67 %) while the $AUC_{0-infinity}$ increased from 25341 to 76018 ng.h.ml^{-1} (+67 %) after the administration of B formulation. No significant differences were observed in the $t_{1/2}$ values and the T_{max} of PYR between the two formulations. The concentrations of SDX and PYR three days after drug administration of B were respectively 98.9µg/ml and 236 ng/ml while in A , the

concentrations of both compounds were much lower (SDX-63 μg/ml and PYR-75.5 ng/ml).

Statistical evaluation of the pharmacokinetic data using C_{max}, AUC and T_{max} for SDX ($p< 0.1$), showed that the two formulatons were bioenequivalent. The same differences were observed in PYR with no significant difference in the T_{maxes} (cf 2.4±0.8 versus 2.5±0.6 hrs).

4.4 DISCUSSION

This study has confirmed low *in vivo* performance characteristics of a locally manufactured Sulfadoxine/pyrimethamine (SP) tablet formulation as compared to fansidar®, the original SP brand. The observed differences in C_{max}, T_{max} and AUC suggest that the locally produced SP formulation (formulation A) could not meet the criteria for bioequivalence with Fansidar® (Formulation B), an original SP brand. These findings are in agreement with the earlier predictions made after dissolution rate tests performed by Minzi et al (Minzi et al, 2003).

For a tablet formulation to qualify an assay test, USP requires that AQ tablet should contain an amount of amodiaquine hydrochloride ≥93% and not more than 107% of the label claim whereas an SP tablet should contain ≥ 90 % and not more than 110% of the label claim of SDX and PYR. For a tablet formulation to pass a dissolution test, USP requires ≥75% of the label claim of AQ and ≥60% of the labeled amount of SDX and PYR to be relesed in a dissolution medium in 30 minutes.

Based on these criteria, dissolution failures were found in 13% and 44% of the collected AQ and SP samples respectively. Malaridox® from India, Sulphadar® from Shellys Pharmaceutical industry of Dar Es salaam and SP-Interchem from Moshi failed dissolution test with the later exhibiting lowest dissolution properties for both SDX and PYR.

So far the dissolution test is the only in vitro quality control test that can predict the capability of a formulation to release the drug in vivo (Dressman et al 1998). Failure of a drug formulation to meet the USP dissolution specifications could be an indication of potential bioavailability problems. The formulation should be able to release the drug into solution otherwise the drug may pass through the gastrointestinal tract unabsorbed. Alterations of the rate and extent of absorption could be clinically relevant for agents such as antimalarials when a prompt and effective response is desired.

Further bioequivalence study was carried out to ascertain *the in vivo* release characteristics of one SP formulation manufactured in Moshi Tanzania (SP Interchem), after high dissolution failure rate of 44% (8/18) of the collected SP brands. This SP brand (as test sample A) was compared to Fansidar®, (reference sample B) an innovator SP product from Hoffmann La Roche, Basel, Switzerland. The former failed for both SDX and PYR in the dissolution test, while the later performed excellently. The SP tablets used as test and reference samples for bioequivalence study, were of the same batches to those used in the *in vitro* assays.

The two formulations were found to be bioinequivalent in terms of C_{max}, T_{max} and AUC as stated by FDA guidelines (FDA, 1992). The pharmacokinetics of the two products after oral administration showed significant differences ($P< 0.1$). The C_{max} and AUC (plus T_{max} for SDX) values of the test formulation were significantly lower than the respective values of the reference formulation (Figure 4-3 & Figure 4-4). The relative bioavailability for SDX and PYR for two formulations were 57 and 36% respectively, at 90% CI being completely below the predefined range of 80-125% according to international guidelines (FDA, 1992).

The observed differences in the pharmacokinetics were in agreement with the outcomes of the dissolution test of the two tablet formulations. Similarly, pharmacokinetic differences between the 2 formulations were observed for PYR as depicted in Figure 4-4.

The pharmacokinetic differences in C_{max}, T_{max} and AUC are consistent with lower extent of absorption of SDX and PYR in the gastrointestinal tract after administration of A and can be quantitatively related to substantially lower and slower release of these substances from A formulation in comparison with reference formulation B as observed in the dissolution rate test.

The high dissolution failure rate and consequently low bioavailability of marketed SP formulations as a first line drug in the treatment of malaria in the country, has serious therapeutic implications.

Falciparum malaria in sub-Saharan Africa, particularly in young children is life threatening and prompt achievement of adequate concentrations of an effective drug is essential. Reliable and adequate drug absorption after oral administration has to be ensured in order to achieve prompt and adequate systemic exposure to a drug.

Successful SP therapy is dependent on the bioavailability characteristics of both SDX and PYR. Both components of SP drug combination are poorly soluble and each has a weak antifolate activity but when combined in such a fixed drug combination, this activity is enhanced by a synergistic mechanism of action. Low release of PYR from an SP tablet, has a serious clinical impact, since the compound is present in a very small quantity (25mg) as compared to SDX (500mg) in a fixed dose combination.

Plasma levels higher than 100 µg/ml for sulfadoxine and 175 ng/ml for pyrimethamine 72 hours after drug administrations are cut-off points associated with treatment success (Aubouy et al, 2001). With this respect, adequate SDX and PYR

blood concentrations of 98.9µg/ml and 236 ng/ml were achieved post dosing with reference formulation B but much lower levels of these substances were observed in A (SDX-63 µg/ml and PYR-75.5 ng/ml). This means individuals treated with the later formulation would suffer treatment failure.

Looking at individual values at day three after drug administration, all subjects in the test formulation group showed concentrations of between 36-53µg /ml for SDX and 51-154 ng/ml for PYR a range much below the therapeutic range and would also have been at the risk of treatment failure. SDX concentrations in reference formulation ranged between 90-117µg /ml while those of PYR were 151-287 ng/ml. No single subject in B group had SDX or PYR level significantly lower than 100 µg/ml and 175 ng/ml PYR respectively at day three after dosing with B formulation. SP-interchem batch which was earlier predicted to exhibit low bioavailability through dissolution rate failure both in terms of SDX and PYR indeed was proven to be so using healthy human subjects.

Low dissolution and consequently poor bioavailability of both SDX and PYR leads to sub-therapeutic concentrations resulting into only suppression of parasites rather than killing them and this may eventually render to emergence of resistant *Plasmodium falciparum* strains against SP (Kun et al, 1999, White, 1999). Currently, the treatment failure rate of SP in many areas in Tanzania is said to be > 25% a turning point for treatment policy changes.

AQ is a 4-aminoquinoline antimalarial drug resembling CQ in both structure and mechanism of action. This means, suboptimal treatment resulting from the uses of substandard drug formulations poses great risks of rendering AQ also ineffective.

The introduction of a free market economy in Tanzania in 1985, has witnessed an increase in number of different brands of antimalarials in the market. Private

provision of pharmaceuticals, particularly in developing countries has been associated with risks regarding availability, affordability, rational use and quality (Menkes, 1997).

The major reasons for existence of poor quality drugs in this area were not determined in this study, however it appears more likely that these preparations are substandard not because of fraudulent manufacturing, but as a result of bad manufacturing practice and poor storage conditions (Nazerali et al, 1998, Saville et al, 2001, Risha et al, 2003). A high dissolution failure rate of all SP samples from local pharmaceutical industries predicts lack of GMP. Use of raw material of low quality and application of different manufacturing procedure deviating from that of the innovator brand and consequently obtaining finished generic products with different physico-chemical properties may influence the absorption rate of the active substances from finished products. Use of micronised powder as raw material especially for drugs with poor solubility has been reported to improve both the *in vitro* and *in vivo* dissolution performance of the solid formulations (Scholz et al, 2003).

In Dar Es Salaam, where high humidity and temperatures are predominant, inappropriate packaging will affect the solubility characteristics of drug formulations (Rohrs et al, 1999, Risha et al, 2003). Drugs stored in high humidity and temperatures will undergo physical and chemical transformations (Murthy et al, 1993, Pandit et al, 1997, Saville 2001). Excipient-excipient and excipient-drug or drug-drug interactions may occur under the influence of such unfavourable storage conditions, consequently reducing the dissolution rate of a solid formulation (Rohrs et al, 1999). Under such conditions, the drug may undergo polymorphic or crystal changes that may decrease its inherent solubility (Rohrs et al, 1999). The

formulations may have contained disintegrants such as maize starch which could lose its capacity to swell on ageing or exposure to high humidity or temperature (Pandit et al, 1997).

The observed differences in the pharmacokinetics of the active components of SP may be clinically relevant because increased risk of treatment failure can be associated with low SP blood concentrations from formulation A.

In present study, it was observed that 75% (6/8) of SP samples which failed dissolution tests came from local pharmaceutical industries and were found in 75% of the wholesale pharmacies included in the study. Locally produced drugs are cheaper than imported brands therefore enabling the poor peasants in endemic areas to afford the costs of treatment. However, the predominance of low quality antimalarial drugs poses a risk to users in these developing countries.

Only 8 wholesale importers located in Dar Es Salaam were included in this study. Extrapolating the findings of this study to all pharmacies in the country suggest a higher proportion of substandard antimalarial drugs circulating on the market of Tanzania.

The finding of this study is in good agreement with the existence of poor quality antimalarials on the market of East African region reported by others (Mahamood et al, 1994, Rimoy et al, 1999, Risha et al, 2002). WHO recently compiled reports from selected African countries in which SP had high dissolution failure rates ranging between75%-100% especially with respect to pyrimethamine predicting poor bioavailability of the drug (World Health Organisation, 2003).

Poor bioavailability of SP will only lead to suppression of parasites and this eventually renders to emergence of resistant *Plasmodium falciparum* strains against SP (Petralanda, 1995, Kun et al, 1999).

Already such a problem has affected SP efficacy in many parts of Tanzania and currently, the Ministry of Health is under the preparation to move from SP to Artemisinin based combination therapy (ACT) after a high clinical failure of the former in many regions in the country. It is a pity losing SP since despite being cheap, the drug was easy to manage since it was administered as single dose and it could be given under direct observation at heath facilities in children so as to maximize adherence. Most of the proposed ACT are expensive and have complicated dosing regimen posing difficulty to maximize adherence

Local pharmaceutical manufacturers in developing countries should therefore invest more on producing quality drugs as their products are likely to be cheaper and can be delivered much faster to the target population than the imported products some of which are still under patent.

4.5 CONCLUSION:

This work has found existence of poor quality antimalarial drugs on the market of Tanzania after malaria treatment policy changes.

CHAPTER 5: APPLICATION OF HPLC METHODS IN DETERMINING HOUSEHOLD ADOPTION OF THE NEW MALARIA TREATMENT POLICY IN RURAL COMMUNITIES IN TANZANIA USING KIBAHA DISTRICT.

5.1 INTRODUCTION

Malaria is a major threat to public health worldwide and one third of the world's population is exposed to the risk of malaria (Snow et al, 1999, Snow et al, 2001). Most deaths occur in sub-Saharan Africa and children under the age of five years are at highest risk.

In Tanzania, malaria is ranked as number one cause of morbidity and mortality with an estimated annual death rate of 0.1 million and the trend is increasing every year (Ministry of Health, Dar Es Salaam Tanzania, 1999). Because of rapidly spreading resistance against CQ, many Eastern and Southern African countries changed their malaria treatment policies to sulfadoxine/pyrimethamine (SP). Malawi was the first to change in 1993, followed by South Africa and Kenya (Shretta et al, 2000). Tanzania, faced with an average of CQ treatment failure rate of over 42%, in 1999 (Ministry of Heallth Tanzania, 2000), changed its national malaria treatment policy and switched to SP as first line treatment in August in 2001. Since this was affected CQ is officially not used. Widespread self-treatment with antimalarials in febrile illnesseses has been reported in several surveys where a high proportion of households were found to stock antimalarials (Wernsdorfer et al, 1991, Nsimba et al, 1999).These drugs were stocked without having proper knowledge on their doses. Irrational antimalarial drug uses through self-medication has been reported to increase the risk of toxicity and promote drug resistance (Mnyika et al, 1995).

High rate of self-medication with chloroquine, resulted in wide spread resistance in many Sub-Saharan countries (Schapira et al, 1993, Mnyika et al, 1995). Tanzania is among the known countries with high chloroquine consumption (Hellgren et al, 1994) and inadequate dosage regimens (Schapira et al, 1993, Mnyika et al, 1995, Ronn et al, 1996). However, considering that SP is not a new drug as it was a secondline to CQ and due to irrational drug exposure, its utility life span is in jeopardy and already there are reported high resistance rates in some parts of Tanzania which may necessitate replacement of this drug soon (EANMAT, 2003). Earlier, SP treatment failures were reported in mid 1990's in 12 villages in North-East Tanzania, indicating that clinical resistance would develop fast if appropriate measures were not introduced to protect SP against irrational use in the community (Trigg et al, 1997).

Combination therapy (CT), especially Artemisinin-based combinations (ACT), is currently the option for improving cure of *falciparum* malaria (EANMAT, 2003). Introducing this treatment strategy in low-income countries is a great challenge not only from a financial point of view, but also due to inadequacy in health systems in these countries. Compared with other antimalarials, SP is cheap and is administered as a single dose thus ensuring adherence.

This study aimed at investigating the extent of antimalarial drug uses, knowledge and acceptability of SP in a rural community following a change in malaria treatment policy. Verification of SP and CQ uses was done through analysis of blood for SDX and CQ respectively, using filter paper HPLC methods.

5.2 METHODOLOGY

5.2.1 Study area

The study was conducted in Kibaha, a rural district 40 km North-west of Dar Es Salaam, Tanzania. The district is holoendemic for malaria with a population of 132,443 people (Central Census Office National Bureau of Statistics; 2003 January 2003).

5.2.2 Study population

The administrative structure of the Tanzanian districts formed the basis for a three-stage stratified sampling procedure used to select wards, hamlets and households.

Of the 9 wards involved in the study at Kibaha district, 5 were rural. A list of the villages and hamlets in the chosen wards was obtained from the District Planning Officer. A total of 20 hamlets were randomly chosen. The required sample size was 730 households.

A total of 729 households from 20 hamlets were included in the study. In these households, only children at the age less than five years were targetted.

From each of the 20 hamlets 36 or 37 households were randomly selected upon arrival in the field by letting the flipping of a coin decide which direction to follow.

5.2.3 Data collection

A semi-structured questionnaire consisting of close- and open-ended questions was used. The questionnaire wanted to extract the following information:

- If the community has stopped using CQ
- If SP is used in the community
- The level of SP self-medication

- The mothers' accuracy on recall of an antimalarial and other drugs they gave their children during febrile episodes

The questions also addressed home stocking of antimalarial drugs, home-treatment practices of underfives with malaria by mothers or guardians, source and knowledge on the recent change of the national malaria treatment policy. The questionnaire was translated into Kiswahili and pre-tested (See appendix 1A & 1B). All heads of households were interviewed and in households with underfives, the mothers of the underfives were also interviewed. One part of the questionnaire concerned the last febrile episode of the child and was only addressed to mothers/caretakers of underfives using a recall period of four weeks. Knowledge of correct paediatric dosage and regimen was assessed using the exact recommendations for children 1-5 years of age as given by the National Malaria Control Program.

After obtaining informed consent from mothers/guardians of the the children, capillary blood samples were collected from one underfive in all the households with children. Blood samples of 100 μL were collected in duplicate, dried on filter paper and asssayed for chloroquine (CQ) and sulfadoxine (SDX), the later representing sulfadoxine-pyrimethamine (SP) drug combination. In households with more than one underfive (Table 5-1), blood samples were taken from the child who had the most recent episode of febrile illness. The household survey was performed from January to March 2002, about seven months after the policy change became officially effective.

5.2.4 Data analysis

The responses to the close-ended and the open-ended questions in the household interviews were all coded, data was entered, processed and analysed using the Statistica® software (StatSoft Inc, Tulsa, USA).

One blood sample collected from each child was assayed for CQ using an HPLC method developed by Minzi et al, (Minzi et al, 2003) and the second blood sample was assayed for SDX using another method (Bergqvist et al, 1987). The coefficients of variation (CV) were 5.3% and 8.9 for CQ and SDX methods respectively. The limit of determination was 100 nM for CQ and 25μM for sulfadoxine method. The data obtained was used to assess:

The proportion of children who took antimalarials during febrile episodes

Children whose mothers responded correctly on the drug used during fevers

Children who were given SP after visiting the health care facility (HCF)

5.2.5 Ethical Approval

Participation was voluntary and informed consent was obtained from all households. This study was done in collaboration with Karolinska Institute in Sweden, therefore, ethical approval was obtained from ethical committees of Muhimbili University College of Health Sciences and Karolinska Institute. Approval was also obtained from the Kibaha district, Coast Region administrative authorities, ward secretaries and leaders of the hamlets.

5.3 RESULTS

5.3.1 Socio-demographic characteristics

Table 5-1, shows the socio-demographic characteristics of the mothers of underfives and heads of the households who were interviewed. The mean age of the interviewees was 39.5 years. On average each household had a family size of 5 persons. In the 729 households, 443 heads of households and 336 mothers of underfives were interviewed, making a total of 779 interviewees. A total of 493

underfives were found in 336 households, but from each household only one child was included in the study ending up with an equal number of boys and girls.

Table 5-1 Socio-demographic characteristics of interviewed heads of households (n = 443 - fathers and mothers without under-five children) and mothers with underfives (n = 336) in 729 households.

Respondents (n = 779)*	Characteristics	Percentage (%)
Sex	Male	28
	Female	72
Education	No formal	30
	Incomplete primary education	8
	Completed Primary education**	54
	Secondary and higher education	5.5
	Adult***	2
Occupation	Farmers/Housewives	83
	Civil servants	5
	Private business	11
	Retired	0.1
	Others	2
Distribution of underfives (493 in a total of 729 households)	0	45
	1	43
	2	9
	3	2
	4	1

* In 50 households, both mothers of under-fives and heads of households were interviewed.

** Includes education in Muslim primary school (madrasa)

*** Adult education is a primary education for illiterate adults who never had formal education during childhood

5.3.2 Drug stocking at home and health seeking behaviour

Table 5-2 shows the health seeking behaviour outside homes in underfive children with reported febrile episode within 4 weeks prior to interview. Ten percent (n=33) of mothers could show antimalarials stocked at home. The most common were quinine, SP and CQ, but as many as 41% stated having previously stocked antimalarials, with no difference between the urban and rural wards. Most mothers who reported their children had febrile episodes (85% of 115, n=98) have sought care outside the home, mainly from public health facilities (78%, n=90), with no difference between the rural and urban wards (P< 0.001).

Table 5-2 Health seeking behaviour outside homes in underfive children (n=98 of 115 with reported febrile episode within 4 weeks prior to interview.

Type of care	CQ detected in blood %	SDX detected in blood %	SDX and CQ detected in blood %	Neither SDX nor CQ detected in blood %
Public HF (n=90)	2	32	5	61
Private HF* (n=8)	13	25	0	62
Total (n=98)	3	31	4	61

HF=health facility, CQ=chloroquine, SDX=sulfadoxine.

Includes private dispensaries, medical laboratory, Part II medical shop and ordinary shop (Figures add up in rows)

5.3.3 Community antimalarial uses in relation to blood drug concentrations.

Blood samples were taken from one underfive in 336 households. It was possible to assay 321 blood samples for Sulfadoxine (SDX). Eighteen percent (n=57) had detectable SDX levels and ranged between 7.7-332 μM, but 12% (n=38) were below 25 μM quantification (determination) limits (therapeutic range = *100-300 μM*)

(Aubouy et al, 2003). Among the 320 samples assayed for CQ, only 6% (n=18) had detectable levels of the drug and less than 2% (n=5) were in the range quantifiable by the method used (100 nM), the highest detected level being 1803 nM. In six (2%) of the children, both CQ and SDX could be detected.

There was low antimalarial dispensing at Home and HCF as only 18% (57/320) had detectable SDX 7.7-332 μM and only 32% who visited private and public HCF had SDX.

Among 115 children with febrile episodes within last 4 weeks, 32% were detected with SP and 6% with CQ.

Drug level analyses confirmed that the majority (61%) who received care for fever at HCF did not have SP in blood. The majority (69%) of mothers stated to have given only analgesics.

5.3.4 Relationships between SP and CQ blood concentrations and mothers' history.

Of the children who were reported to have had fever within the last 4 weeks, 32% (n=37) and 6% (n=8) had detectable levels of SDX and CQ, respectively (Table 5-2). One hundred fifteen (115) underfives were reported to have had fever episodes during the last 4-week period and 77% (n=88) of the mothers/guardians of these reported to have given them drugs at home with no difference between rural and urban wards ($P< 0.001$). The majority (69%, n=79) of mothers stated to have given analgesics and very few (5%, n=6) antimalarials at home. Among the 98 children who had a febrile episode during the last 4 weeks and where mothers stated to seek care outside the home, 35% (n=34) had detectable levels of SP in blood (Table 5-2).

5.3.5 Antimalarial policy change

In the visited households, 41% stated that the current first line treatment for malaria was SP and 32% explained that CQ was not used anymore because of drug resistance.

Of the 115 mothers whose underfives had a febrile episode during the last four weeks, 61% knew that SP was the first line antimalarial. Of 336 interviewed mothers/guardians only 12% (n=39) were able to mention the correct paediatric SP dosage and regimen as stated in the new national malaria treatment policy guideline.

5.3.6 Household experiences of SP

Interviews about SP experiences revealed 60% of the interviewees perceiving it to be a good drug that cures malaria, the rest reported that it increases fever (21%) or a bad drug giving body weakness and causing skin burns (18%). Only 2% had actually experienced skin reactions but none serious. Most newspapers and radio were reported to be folk media for a negative campaign against SP.

5.4 DISCUSSION

This study revealed low self-medication rates with antimalarial drugs at Kibaha district. Only 18% (57/321) children had SP in their blood as compared to the observations made in 1998 in which as many as 75% children had CQ in their blood prior visiting health facility (Nsimba et al, 1999).

In this study, only 32% of the children reported by mothers/guardians to have had fever within the last 4 weeks had detectable levels of SDX and CQ respectively, confirming much lower self treatment practice with antimalarials and less antimalarial drug dispensing at the health facilities (HCF) as most mothers visited the HCF during fever episodes. SP was detectable in about one third of the underfives,

yet 63% who according to the mothers had received care for fever at public HCF four weeks before interview, did not have detectable SP levels in their blood bearing in mind that SP has a wash out period of about 8 weeks after drug intake (Winstanley et al, 1992). The fact that SP was given at HCF under observation by a health worker and very few children were found with SP in blood can be explained by a staff diagnosing other febrile diseases and giving other treatments. In all these cases it seems that giving SP free of charge at the HCF under the supervision by a health worker was a positive move in reducing irrational dishing of this antimalarial drug. This is in line with recent findings in a study from rural Gambia (Clarke et al, 2003). Such a high number of children without measurable SP in this rural community is an indicator of low drug consumption, low self medication and rational dishing of the first line drug in this area.

However, low SP community uses in this area can also be explained by people's fear of the skin reactions caused by the drug. Skin reactions caused by intake of sulfonamides have been reported and range from skin rashes and itching to serious side effects (Zielinska et al, 1997, Gawronska-Szklarz et al, 1999). These reactions are associated with sulfonamide acetylation catalysed by polymorphic N-acetyltransferase-2 enzyme (NAT2) (Zielinska et al, 1997) and slow acetylators are prone to skin reactions (Zielinska et al, 1997, Gawronska-Szklarz et al, 1999). SDX, as a component of SP is acetylated to N4-acetylsulfadoxine by NAT2 and slow acetylators are likely to be victims of drug associated skin reactions. However there are sporadic undocumented cases of such reactions in patients treated with SP in Tanzania.The prevalence of skin reactions caused by the use of sulfonamides is low and complications such as Steven Johnson Syndrome and toxic epidermal necrolysis rarely occur.

Another concern is the validity of the mothers' history in relation to actual drug intake. In this study, most of the mothers' reponse to questions was in agreement with the actual drug intake.

Those children whose mothers denied to have given them any antimalarial drug during the febrile episode 4 weeks before the day of interview, indeed were confirmed to have neither measurable SDX nor CQ in the blood samples.

HPLC methods were used to determine the number of children who received SP or CQ and the levels of these drugs in the blood in relation to mothers' reporting.

Drug level analyses confirmed that the majority (61%) who received care for fever at HCF did not have SP in blood (Table 5-2). This is in agreement with mothers' reporting in which the majority (69%) of mothers stated to have given analgesics.

These are major achievements in the awareness of the mothers in this district as compared to the study reported by Nsimba et al in the same area in 1998, in which as many as 97% of the blood samples collected from children had already measurable CQ although their mothers stated to have not given CQ 4 weeks prior interview (Nsimba et al, 2002). Similar controversial findings were also reported in Malawi (Nwanyanwu et al, 1996).

Poor correlation between mothers reporting and the actual drug intake poses dangers of over-prescribing or under prescribing of an antimalarial drug. The validity of mothers' reporting is important in deciding the correct drug and dosage regimen for a child with malaria.

This study has confirmed through sensitive and selective HPLC method that the community has stopped using CQ since out of 320 children, only 6% had CQ ranging between 36-1803 nM and only 2% had CQ (n=5) within the permissible limit of quantification of the HPLC method used (Minzi et al 2003). Earlier, it was reported

that SP would be perceived negatively especially in the rural areas (Tarimo et al, 2001) where SP was less known and therefore many people would continue using CQ due to its antipyretic-like activity and its tolerable side effects as opposed to SP.

The study also revealed that only 12% of the mothers/guardians at Kibaha district were able to mention the correct paediatric SP dosage regimen as stated in the national policy guidelines. This finding is in agreement with observations in Kenya, Uganda and Malawi (Brooker et al, 2000, Nshakira et al, 2002, Slutsker et al, 1994), confirming that incorrect self-treatments at home remains one of the major problems in malaria case management in rural areas.

The fact that 88% of the mothers did not know the correct paediatric dose for SP in this rural community is evident that irrational use of SP through over-dosing or under-dosing is likely. Self-treatment and high antimalarial drug consumption have been associated with many negative outcomes including, poor adherence, under-dosing and over-dosing (Whyte et al, 2000).

However most of the public health facilities in malaria endemic areas suffer from insufficient supply of antimalarial drugs and lack of microscopes to correctly diagnose malaria (McCombie, 1996, McCombie, 2000, Forster, 2001, Kelly, 2001). Improving the performances of health facilities would automatically encourage people to visit them for seeking treatment and this in turn would dramatically reduce incidences of irrational antimalarial drug uses in the community.

In this study, it was revealed that, giving SP at health facilities free of charge to underfive children, dramatically reduced SP dispensing. Other strategies such as upgrading the knowledge of community health workers, drug sellers and mother co-ordinators on proper malaria case management in rural areas (Marsh et al, 1999, Yeboah-Antwi et al, 200, Deressa et al, 2003), pre-packaging of an antimalarial

drugs according to the age by drug-sellers have been shown to improve adherence and to curb down incidences of poor dosing of children at home (Ansah et al, 2001, Sirima et al, 2003).

This study has confirmed through HPLC methods that, in spite of the negative perceptions against SP in the treatment of malaria in children in the same area (Tarimo et al, 2001) and an inclination for self-treatment, the policy seems to have diffused to the community. A probable reason could be lack of alternatives if CQ was perceived as less effective and made less available at the same time SP was sufficiently supplied to public HCF. The study has verified low SP overuse both at home and at HCF. By combining responses from interviews and drug level analysis it was possible to ascertain a decrease in self-treatment with antimalarials after malaria treatment policy change. It was also possible to acertain low SP drug pressure in the district and diffusion of the new malaria treatment policy (only 6% of 320 children had chloroquine in blood and only 1 child had quantifiable levels of chloroquine and desethylchloroquine).

5.5 CONCLUSION

This work has shown the exclusive importance of applying HPLC methods and drug level analysis as complement in epidemiological studies. The methods used in this work are field adapted for taking small volumes of blood samples in resource limited countries.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 GENERAL DISCUSSION

In this study, filter paper (FP) HPLC methods for determination of CQ and DCQ and PYR were developed. An HPLC method for determination of AQ and its principal metabolite DAQ, was also developed. The method applies small volume of blood samples (100µl). These methods were developed at the Unit of Tropical Pharmacology, Division of Clinical Pharmacology, Department of Medical Laboratory Sciences and Technology, Karolinska Institute, Huddinge Hospital Stockholm, in Sweden and transferred to an analytical laboratory established at MUCHS in the Department of Clinical Pharmacology, in Tanzania.

Sampling procedures which involve collection of small volumes (50-200 µl) of capillary blood from human volunteers are highly desirable in developing world particularly in Africa. Small volumes of blood samples can be dried as spots on filter paper and kept in plastic folders at room temperatues until assay. Such methods have been successfully applied to determine many antimalarial drugs (Lindström et al, 1985, Bergqvist et al, 1987, Kolawolem et al, 1995, Ericsson et al, 1996, Jansson et al, 2003).

FP methods have great advantages that they can be used in rural communities of endemic areas where huge number of samples can be easily collected and transported to a laboratory for drug analysis. Collection of small volumes of blood in pharmacokinetic and bioavailability studies is desirable to encourage volunteer participation. FP methods are therefore very useful in collecting samples for population pharmacokinetic studies.

The technique is more readily accepted by people in developing countries (low sample volume) than venous blood sampling. Using dried blood on filter papers reduces the need to provide facilities for separating and storing blood samples in the field. FP method does not require big volume of blood samples, making it easier to obtain consent from volunteers or mothers of children.

In Africa most studies are pursued in rural areas where storage and transportation of fresh blood can be cumbersome and impractical. FP sampling technique has advantage that it minimizes the amount of storage space needed which could be an issue in resource limited countries. There are also concerns in some laboratories about handling fresh blood samples due to the prevalence of HIV in sub-Saharan Africa.

This work has applied HPLC drug level analysis in conjuction with mothers' response to an interview in studying uses of antimalarial drugs at Kibaha rural community. Combining questionnaire and blood/plasma level analysis in epidemiological studies, drug efficacy or therapeutic drug studies give more reliable conlusions (Abouy et al, 2002, Khalil et al, 2002). Because of variations in extent of absorption, metabolism of drugs and parasite genotypes, assessment of *in vivo* outcomes of treatment with antimalarials in relation to drug plasma levels enables to make correct conclusions on drug efficacy (Khalil et al, 2002).

Treatment failure may be caused by inadequate plasma drug levels. Low plasma concentrations (bioavailability) may be due to, inadequate absorption caused by a patient experiencing vomiting or diarrhea.

In addition, low dissolution of the drug in the gastro-intestinal tract (GIT) due to poor quality of the formulation, low dose content of active components in a formulation, and poor adherence may be the reasons for sub-therapeutic plasma concentrations.

Ensuring that no drug is detected before drug administration and ascertaining the plasma levels of an antimalarial drug post administration through analytical measuresments by HPLC or by other analytical methods, enables the investigator to tell whether the absorption was adequate enough to exhibit parasiticidal activity *in vivo* (Abouy et al, 2002, Khalil et al, 2002).

To be able to obtain reliable data for a drug efficacy, the drug plasma level must suggest that inadequate absorption, abnormal drug metabolism or non compliance are not confounding factors, but strictly point to drug resistance (Khalil et al, 2002).

This work emphasises the importance of applications of drug level analysis as complement to questionaire or other methodologies in studying the utilisation of antimalarials in community. Lack of information on plasma levels in epidemiological or efficacy studies of antimalarial drugs creates difficulty in deciding whether treatment failure was due to drug resistance or suboptimal plasma drug concentrations (Macarthur et al, 2001, Ezedinachi et al, 1999, Aubouy et al, 2003).

Therefore, combining WHO methodology for clinical evaluation (WHO, 1996) with measurements of plasma drug levels would provide more reliable and robust data on antimalarial drug performances (Amukoye et al, 1997, Kun et al, 1999, Ezedinachi et al, 1999, Macarthur et al, 2001, Basco et al, 2002, Bwijo et al, 2003, Aubouy et al, 2003).

Relying entirely on interview response especially in rural communities in Africa where illiteracy rate is still high, may lead to wrong conclusions due to some respondents not remembering the drug used and therefore giving unreliable answers. Supplementing drug plasma levels in studies focusing on drug utilisation and efficacy assessment gives more reliable and workable data.

The importance of drug level analysis in monitoring extent and pattern of antimalarial drug utilisation in the community is substantiated by a number of studies. A study conducted in schoolchildren in Tanzania and Nigeria, showed that, most of the children had malaria parasites despite high drug levels of CQ in their blood (Hellgren et al, 1994, Mockenhaupt, 1999).

In a study conducted in Malawi, a poor correlation was observed between mothers' reporting and actual drug intake where mothers/caregivers denied to have given CQ to the children two weeks before interview, although as many as 75% of the blood samples collected from these children had already high blood levels of CQ (Nwanyanwu et al, 1996).

This study has revealed persistence of substandard antimalarial drugs in Tanzania. The findings of this work are in agreement with other studies previously carried out in the region (Risha et al, 2002, Rimoy et al, 2002). Ogwal-Okeng made a systematic comparison of the quality of antimalarial drugs on Ugandan market between 1996 and 2002. He did not find any significant improvement in quality of antimalarials between the two years (Ogwal-Okeng et al, 1998, Ogwal-Okeng et al, 2003).

The commonly reported problem in most of these quality assessment studies is low dissolution rate of many brands especially those manufactured by pharmaceutical industries in resource limited countries. Dissolution rate is a predictor of drug bioavalability and for a drug to be effective; an adequate amount must be available for absorption to reach therapeutic plasma concentration.

Tanzania Food, Drug and Cosmetics Authority (TFDA) in collaboration with Malaria project at MUCHS are planning to start conducting bioequivalence testing of different antimalarial products as one of the conditions for product registration in the country.

Cost-effective methodologies aimed at cutting costs of bioequivalence tests should be identified and adopted. The traditional methodology of collecting blood samples up to 4-5 half-lives of a drug after drug intake is not cost effective in terms of time, compliance and analysis cost.

Cost-effective methodologies such as those involving the use of truncated AUCs instead of AUC up to infinity for drugs with long half-lives (Sathe et al, 1999, Gaudreault et al, 1999, Marzo et al, 1999) or 72 h truncation of AUC for drugs that have low intra-subject variability and long half-lives (FDA, 2000) and that one involving blood sampling up to 2h after Tmax, (except controlled release), (Niazi et al, 1997, Jackson et al, 2001) can be tested and adopted in resource limited settings. A limited sampling technique in which samples are collected using 2-3 time points after drug administration (Suarez-Kurtzet al, 1999, Suarez-Kurtz et al, 2001, Mahmood I et al, 1997-2000) has also been reported.

All these techniques aim at shortening the sampling period and reducing the number of collected blood samples and consequently reducing analysis costs.

An exclusive achievement of this work is the establishment of an analytical laboratory as a research tool at MUCHS. The laboratory is currently equipped with three HPLC systems. The laboratory can be used in therapeutic drug monitoring as well as studying drug-drug interactions in patients. It can also be used in investigating metabolic pathways of many drugs used in the tropics including antiretroviral drugs for management of HIV-AIDS. In addition the laboratory will provide support to TFDA in carrying out bioequivalence studies of generic products before they are recognized for registration.

6.2 CONCLUSION

This work has developed field adapted HPLC methods which use small volume of blood and apply UV detection and reversed phase systems. The methods could be applied in confirming diffusion of new malaria treatment policy and low SP uses in a rural Tanzanian community as well as persistence of poor quality antimalarial drugs on the market of Tanzania. The HPLC methods were useful in confirming an improved skill of mothers' reporting on the actual antimalarial drug they gave to their underfives when they had fever episodes.

The major findings in this work include ability of the developed analytical methods to apply small volume of blood samples making them suitable for field studies, in resource limited settings. By applying these analytical methods, it was possible to confirm persistence of low quality antimalarial drugs on the Tanzanian market and by combining mothers' responses from interviews with drug level analysis, it was possible to confirm diffusion of new malaria treatment policy and low SP uses in a rural Tanzanian community.

It can be concluded that HPLC methods applying small volume of blood samples as well as blood spots dried on filter paper give reliable data when complemented to questionnaires in epidemiological studies. HPLC methods are very useful tools in quality assessment of drugs.

6.3 RECOMMENDATIONS

This work has developed HPLC methods which are field adapted for taking small volumes of blood samples in resource limited settings.This study therefore recommends to carry out further investigation on field adapted analytical method which will ensure stability of AQ and DAQ in whole blood for more than a month and which applies simple liquid-liquid extraction procedure.

This study has provided evidence that there are low quality antimalarial drugs existing on the Tanzanian market and efforts should be made towards obtaining a permanent solution to this problem. Poor quality antimalarial drugs constantly available on the Tanzanian market and self-medication with lower doses of antimalarial drugs are key reasons of treatment failure and emergence of resistant strains of *P. falciparum*.

The study recommends to The Drug Regulatory Authority to test all generic antimalarials for bioequivalence with innovator brands before the products are registered. Prompt actions like recall of poor quality batches from the market and informing the public on poor quality products in circulation should be undertaken immediately when such cases are discovered.

To reduce analysis costs for bioequivalence studies, the study recommends to pharmaceutical and academic institutions to test the applicability of different sampling procedures aimed at shortening the period required to collect blood samples and consequently analysis time.

The study recommends the use of drug level analysis as complementary in epidemiological and quality assessment studies. Involvement of blood drug level analysis as a complement to other methodologies in studying drug resistance, efficacy, extent and pattern of drug utilisation in the community is exclusively important.

Currently many countries are reviewing their malaria treatment policies and are planning to introduce artemisinin combination therapy (ACT) as an alternative to SP. Very little is know about the pharmacokinetics of the ACTs in African population and there are no reliable, field-adapted analytical methods for determination of drug concentrations of the artemisinine derivatives.

It is recommended that the research instutions should work on field adapted analytical methods which will enable to carry out different studies on ACT in humans.

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