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# **INTER-SUBJECT VARIABILITY IN THE ACTIVATION OF PROGUANIL TO THE ACTIVE CYCLOGUANIL IN GHANAIS**

A THESIS SUBMITTED BY

**WILLIAM KUDZI**



**IN PARTIAL FULFILMENT OF THE REQUIREMENT  
FOR A MASTER OF PHILOSOPHY DEGREE**

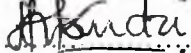
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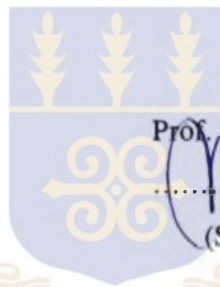
## **DECLARATION**

I hereby declare that this submission is my own work and that, to the best of my knowledge, it contains no material previously published or written by another person, except where due acknowledgment is made in the text. This work has not been accepted for the award of any other degree of a University or other institute of higher learning.

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### **ABSTRACT**

One hundred and thirty-two healthy Ghanaian volunteers were randomly selected and the activation of the anti malarial drug proguanil (PG) to the active metabolite cycloguanil (CG) was evaluated in them. These volunteers were made up of 16.7% females and 83.3% males. The volunteers were phenotyped either as extensive metabolisers (EMs) or poor metabolisers (PMs) by measuring the proguanil/cycloguanil ratio in their urine following a single dose of the pro-drug. One hundred and twenty-six subjects (97%) were EMs while 6 subjects (3%) were PMs.

The pharmacokinetic parameters of proguanil were not significantly different between 5 EM and 2 PM subjects, although the PM did show a higher plasma proguanil concentration after distribution phase and a longer elimination half life. Cycloguanil characteristics were not different between the groups as was expected. The prevalence of 3% of PM phenotype of proguanil metabolism in Ghanaians is comparable to results obtained in Caucasians 3-6%, but differ from what was obtained in Kenya (35%). This finding suggests a lesser variability of proguanil metabolism in Ghanaians. The maximum average plasma cycloguanil concentration of 93.4ng/ml obtained for the PMs in this study also showed that the metabolite concentrations achieved would be effective against many strains of *P. Falciparum*.

## **CHAPTER ONE**

### **INTRODUCTION AND LITERATURE REVIEW**

#### **1.1 GENERAL INTRODUCTION**

Many drugs are generally metabolised to reduce their pharmacological activity by forming an inactive product. In some cases, however, the biotransformation can lead to an increase in the therapeutic activity by forming an active metabolite.

Large differences often exist among individuals in drug metabolism and rates of plasma drug clearance, which may be due to genetic or environmental influences<sup>(1)</sup>.

For most drugs the variability in drug metabolism shows a normal (unimodal) distribution. However, for some drugs the distribution is bimodal or trimodal, indicating the existence of separate population of subjects capable of metabolising those drugs at discretely different rates.

The biotransformation of drugs into either active or inactive metabolites is controlled by one or more forms of cytochrome P450 enzymes. The inter-individual variability in the

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levels of expression of cytochrome P450 enzyme could determine the relative effectiveness of metabolism and efficacy of a particular drug. Recent interest has been focussed on the genetic regulation of the cytochrome P450 mixed function oxidases. This interest came from the observations that some of these isozymes exhibit genetic polymorphism.

There is a significant difference between the Caucasians and Orientals with reference to oxidative drug metabolism. These differences have been revealed using molecular genetics. The most common mutation of the CYP 2D6 gene (29B) is found in Caucasians but this is barely found among Chinese. Also, a high frequency of alleles (37%) was found to occur in the Chinese indicating that genes have been inserted and was reflected by Xba1 44Kb fragments. However, the situation is different among Caucasians; the fraction contains functional CYP 2D6 gene with mutations causing less amount of enzyme to be expressed<sup>(2)</sup>. Based on this result all CYP 2D6 substrates should be expected to be metabolised at a slower rate in Chinese than in Caucasians in a population kinetic perspective.

Pharmacogenetic studies carried out on Ghanaian population using debrisoquine and sparteine showed the prevalence of 2.1% of CYP2D6 poor metaboliser phenotype. Genomic DNA analysis showed 29kb, 44kb, and 11.5kb fragments which are comparable to those obtained in the Caucasian population (29kb, 44kb, 11.5kb and 16+9kb). The

44/11.5kb combination which was shown to be predictive of the PM genotype in the Caucasians were also found to be predictive of PMs in Ghanaian population. There was a 29B mutation in those individuals with 44/11.5kb and it was suspected to be derived from the XbaI 44kb allele <sup>(3)</sup>.

The ability of a drug to inhibit competitively the oxidation of one of the model compounds and vice versa in human liver microsome experiment has been used to implicate the corresponding isozyme in the metabolism of the candidate drug. By this method many clinically useful drugs have been shown to be metabolised by the debrisoquine path. These drugs include classical and novel anti depressants, neuroleptics, lipophilic  $\beta$ - blockers, antiarrhythmics and codeine.

Glucuronidation of codeine is also mediated through CYP2D6 enzyme, and it is slower in the Chinese than Caucasians. At the same time, the number of subjects that are unable to metabolize codeine to morphine is less among Chinese than among Swedes <sup>(4)</sup>. Similarly using mephenytoin as a probe drug for the enzyme CYP2C<sub>mp</sub> it was found that the number of slow metabolizers is 15% among Chinese and 3% among Swedes. Proguanil and omeprazole are two other drugs that have been found to be metabolised by the S-mephenytoin hydroxylase path using the microsome method <sup>(5,6)</sup>

Proguanil is an arylbiguanide which was introduced as an antimalarial agent in the 1940's. Its use was reduced with the introduction of chloroquine. Proguanil is relatively inactive, requiring metabolism to the active dihydrotriazine metabolite, cycloguanil <sup>(7)</sup>. This metabolic activation is dependent on S-mephenytoin hydroxylase. Cycloguanil is a potent inhibitor of the plasmodial enzyme, dihydrofolate reductase which is very important in the parasite viability. The efficacy of the drug is determined by the individual's level of expression of the cytochrome P450 isozyme belonging to the 2C subfamily. It has been established that large inter individual variability exists in the activation of proguanil to cycloguanil. This variation is about 2-6% in Caucasians, 18-20% in Japanese and 35% in the Kenyan populations <sup>(8,9,10)</sup>.

With the high incidence of *P. falciparum* resistance to chloroquine in Africa, the WHO now recommends a combination of chloroquine and proguanil for prophylaxis against malaria in West Africa. For this to be very effective, there is the need to know the incidence of this inter individual and inter-ethnic differences in the conversion of proguanil to the active metabolite cycloguanil. In a population with high percentage of PMs, it can be anticipated that the antimalarial effect of proguanil may be absent or greatly reduced. Subinhibitory concentrations of cycloguanil in plasma may also promote the development of parasite resistance to actions of dihydrofolate reductase inhibitors.

The aim of this study is to determine the inter-subject variability in the activation of proguanil to cycloguanil in Ghanaian population.

## 1.2 MALARIA

The term "malaria" derives from Latin, "Mala aria" meaning "bad air". The protozoan parasite that causes the disease malaria belongs to the genus Plasmodium and has four principal parasite species infective to humans. These are, Plasmodium falciparum (Pf), Plasmodium vivax (Pv), Plasmodium ovale (Po), and Plasmodium malariae (Pm).

Transmission of the infection occurs when the sexual parasite forms (male and female gametes) are ingested by the female mosquito of the genus Anopheles. The gametes mate and undergo sporogony in the mosquito mid-gut; a zygote is first formed which then develops into an ookinete and later becoming sporozoites. These migrate from the mid-gut into the salivary gland of the mosquito. The mosquito inoculates plasmodia sporozoites into the blood of a susceptible human host while feeding. The plasmodia then move through the blood stream to the liver where they invade the parenchymal liver cells. They go through a process of multiplication and several of them (now called merozoites) are released into the blood stream when the cells rupture after 5 to 20 days, according to the species. The merozoites so released into the blood stream invade the red blood cells. This marks the beginning of usual illness and symptoms associated with malaria. The

merozoites go through another phase of multiplication in the red blood cells and in about 28 to 72 hours after invasion, the red blood cells involved rupture, releasing the organisms which can invade other red blood cells and repeat the erythrocytic cycle. Some of the parasites will then develop into sexual forms called the gametocytes which are infective to mosquito. Fig.1

### MALARIA PARASITE LIFE CYCLE

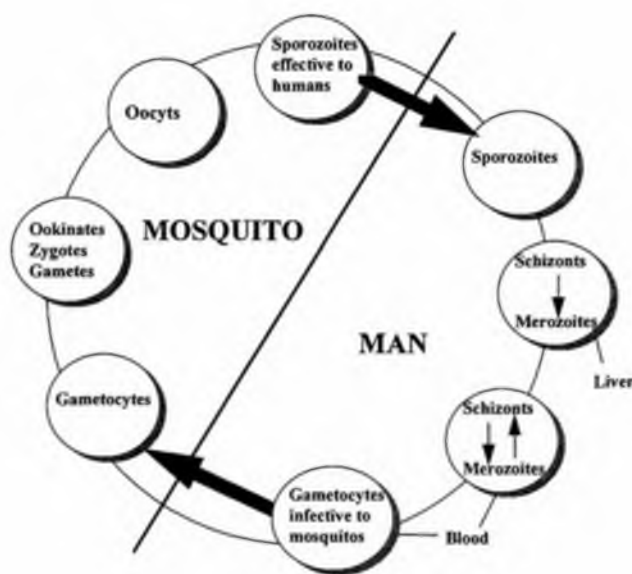


Figure 1

Clinical symptoms of malaria develop within 8 to 30 days of inoculation of infective sporozoites. They consist typically of sequential chills, fever and sweating which lasts for 20 to 30 minutes. This is followed by the "Hot Stage" in which the skin becomes very

warm and may last for up to eight hours. Then comes the "wet stage" where there is profuse sweating and leaves the patient weak and exhausted. Clinical response to infection depends both on the species of the parasite and immunological status of the patient. Non-immune travelers to malarious areas risk severe attacks. Acute malaria also occurs where exposure is limited or seasonal and where the collective immunity is relatively low. Acute falciparum malaria is a potentially fatal disease causing prolonged irregular high fever, intense headache and vomiting. Severe infection associated with intense parasitaemia, frequently gives rise to hyperpyrexia, convulsions, stupor collapse, copious vomiting and diarrhoea, haemolytic anaemia, and jaundice. Complications include cerebral malaria, hypoglycemia, pulmonary oedema, acute renal failure and massive haemolysis. Chronic or repeated infection often leads to splenomegaly and progressive anaemia. Splenic rupture is a dangerous complication of vivax malaria, and P. malariae infection occasionally gives rise to a fatal nephrotic syndrome.

Malaria is endemic in 102 countries of the world, although only 61% report epidemiological data to the World Health Organisation (WHO). About 2,700 million people, i.e. 56% of the world's population, live in areas endemic for malaria; 2,266 million in countries where malaria control has been or is still being practiced; 398 million in countries where no specific measures are being or have been taken against the disease.

About 776 million people live in areas where malaria has been eliminated over the decades <sup>(11)</sup>.

In Africa, although no reliable statistical data exists on malaria morbidity and mortality, extrapolations from epidemiological studies suggest that the disease is probably responsible for no less than 500,000 to 1.2 million deaths annually. This high mortality is mainly in children below the age of five years. Worldwide, it is estimated that there are about 120 million clinical cases of malaria per year <sup>(12)</sup>. In Ghana the picture is not different, the disease is the most commonly reported case in the Out-Patients Departments (OPD) of the country's health institutions and accounted for 40-42% of all out-patient attendances from 1985 to 1987. It accounts for 7-8% of all certified deaths in the 0-4 year age group <sup>(13)</sup>.

Microscopic techniques are the main methods by which parasites are detected. The use of microscopes started towards the end of the nineteenth century. Although it is quite expensive and takes a bit of time to process and view blood films under the microscope, nevertheless, it has been used extensively in individual cases and for epidemiological surveys. In areas where microscopes are not available, clinical picture of the disease is used to do the diagnosis. This method is variable and can be misleading.

Several attempts to control or even eradicate this disease by the WHO have failed. Some of the ways by which WHO attempts to arrest the situation now is by controlling the vector, by chemoprophylaxis, personal protection, vaccines and chemotherapy.

### 1.3 ANTIMALARIAL DRUGS

Many comprehensive classification of antimalarial drugs have been proposed <sup>(14,15)</sup>. However, in practice, the choice of treatment is influenced not only by the intrinsic properties of the drug but also by the degree to which the locally occurring parasites have developed specific patterns of drug resistance. Antimalarial drugs can be classified as either tissue schizontocides or blood schizontocides. This classification is based on the stage of the parasite life cycle that the primary action of the of the compound is exerted.

#### 1.3.1 Tissue Schizontocides

Proguanil and chloproguanil are pro-drugs which are transformed in the liver into their active forms, cycloguanil and chlorcycloguanil. These are widely described as causal prophylactic agents since they are active against pre-erythrocytic intra-hepatic forms. Primaquine, unlike proguanil, is effective in eliminating the latent liver forms of P. ovale and P. vivax which persist after suppressive treatment with chloroquine, however, because of its toxicity and, in particular, the risk of haemolysis in patients with G6PD deficiency, it is not suitable for prophylaxis.

### 1.3.2 **Blood Schizontocides**

Drugs that are the mainstay of the treatment and prophylaxis of malaria infection. These include 4-amino quinolines (chloroquine), the related acylamino alcohols (mefloquine and quinine) and the phenanthrene methanol, (halofantrine). They suppress the disease by destroying the asexual blood forms of the parasite, thus they are not active against *P. vivax* and *P. ovale*, whose intra-hepatic forms can remain latent for months.

These schizonticidal properties are shared by various antimetabolites like pyrimethamine, sulphonamides and sulphones and also by some antibiotics particularly tetracycline. These generally act slowly and are not very useful when used alone. However, some antimetabolites act synergistically in combination: e.g. pyrimethamine in combination with a sulphonamide or sulphone. Tetracycline is used primary as adjunct to quinine where multiple-drug-resistance *P. falciparum* is prevalent.

### 1.4 **DRUG-RESISTANCE**

The WHO in 1973, defined drug resistance as "the ability of the parasite strains to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within limits of tolerance of the subject."

Based on this definition there are several responses of the asexual parasite to drugs. These responses range from mere survival at sub-patent level with subsequent recrudescence in the blood to active multiplication during treatment.

A system of grading the responses of asexual P. falciparum to normally recommended doses of chloroquine has been proposed as follows:

- a) S = Clearance of asexual parasitemia within 7 days of initiation of treatment, without subsequent recrudescence. (Parasite sensitive to drug)
- b) RI = Clearance of asexual parasitemia as in sensitivity (S) followed by recrudescence.
- c) RII = Marked reduction of asexual parasitemia but no clearance.
- d) RIII = No marked reduction of asexual parasitemia.<sup>(14)</sup>

The resistance of P. falciparum to chloroquine and almost all the available antimalarials poses a major obstacle to the control of malaria.

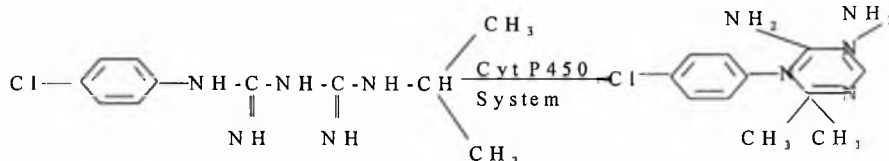
The resistance of P. falciparum to chloroquine has now spread to many areas of the world. Only few isolated areas with P. falciparum are exempted from this generalization. Resistance was established in most of South East Asia and South America in the 1960's and

1970's. In the 1980's, the trend spread to Africa. The Chloroquine Resistant P. falciparum (CRpf) is more common in South-East Asia than in South America and the frequencies of the resistant parasites in different areas still vary especially in Africa.

In vitro studies by Bjorkman and Philips-Howard showed that between 10% and 90% of P. falciparum were drug resistant <sup>(16)</sup>. Similar findings of drug failures in vivo were made, although in many areas partial protective immunity likely assists in the in vivo clearance of resistant parasites. Clinical efficacy therefore may be adequate in patients with partial immunity even when the parasites only partly respond to treatment. In Ghana, the first case of CRpf was reported in 1986 and by 1987 it was widespread in the country. After this initial report, there were many other reports on the failure of chloroquine to kill P. falciparum. The reduced response of Pf to chloroquine was reported from Nigeria and the Gambia around the same time <sup>(17,18,19,20)</sup>.

## 1.5 PROGUANIL

Proguanil (PG) is a white powder with a bitter taste and has the following structure:



It is a biguanide derivative which was introduced as an antimalarial agent in the early 1940's.

It is the pro-drug for the biologically active dihydrotriazine metabolite, cycloguanil (CG) <sup>(7)</sup>

Proguanil is administered at 200mg daily for adults including pregnant women for Children < 1 year, 25mg daily 1-4 years; 50mg daily, 5-8 years, 100mg daily; 9-14 years, 150mg daily. This regimen is generally effective even in areas where breakthrough resistance has been reported with previously recommended lower dosage regimens. The recommended treatment schedule must be followed meticulously and sustained until delivery in the case of pregnant women and for 4 weeks after the last risk of exposure to infected mosquitoes in the case of non-immune individuals. Proguanil is slowly but adequately absorbed from the gastrointestinal tract. After single oral dose, peak plasma concentrations of the drug are attained at 2 to 4 hours. The concentration then decreases to zero within 24 hours. Seventy-five percent of PG is bound to protein and the concentration of the drug in erythrocytes is six times that in the plasma. About 40% to 60% of PG absorbed is excreted into the intestinal

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tract and eliminated in urine and faeces as the unchanged form and the rest as the metabolite. Proguanil is extremely low in toxicity and relatively low in cost. But when proguanil is taken in large doses (1g daily) it may cause vomiting, abdominal pain, and diarrhoea. Excessive amount may also cause haematuria and transient appearance of epithelial cells and cast in the urine.

Proguanil is employed only in malaria. In both falciparum and vivax malaria, the compound adequately controls the overt clinical attack, but it is slower in abolishing fever and parasitemia than other antimalarial drugs. For this reason its use in the treatment of the acute attack of falciparum malaria is not recommended. In falciparum malaria, proguanil is a causal prophylactic, suppressive and radically curative agent if the plasmodia have not developed resistance to the drug.

In vivax malaria, proguanil controls the acute clinical attack but has no advantage over more rapidly acting 4-aminoquinoline antimalarial agent. It is effective in suppression of vivax malaria, but its use does not result in radical cure <sup>(21)</sup>.

Proguanil is lethal to actively developing pre-erythrocytic tissue forms of certain plasmodia and exerts a sterilizing action on gametocytes. It acts as good inhibitor of plasmodial

enzyme; dihydrofolate reductase which is essential for the parasites viability because it is plays an important role in the folate metabolic pathway.

### **Effect of Proguanil on Folate Metabolism**

Whereas the host depends upon preformed folic acid in diet, malaria parasite synthesizes the folate cofactors from pteridine, glutamate and para-aminobenzoic acid (PABA). Plasmodium derives glutamate and PABA from the host, while the pteridine molecule is synthesized from GTP which requires the enzyme guanosine triphosphate cyclohydrolase (GTP Case). The product of this enzyme activity then undergoes two further enzymatic transformations before being combined with PABA in the first stage of the folate synthesis.

The parasite can also salvage preformed folate from the host. Whether the folate is synthesized de novo or acquired exogenously, DNA synthesis requires that dihydrofolate (DHF) be converted to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR) and then to the methylene derivative which is used by the thymidylate synthase (TS) to convert dUMP to dTMP. The enzymes DHFR and thymidylate synthetase (TS) in P. falciparum are part of single bifunctional protein whose gene has been cloned and sequenced. Proguanil inhibits the enzyme DHFR and consequently the DNA synthesis.

Fig. 2.



regular use of proguanil by significant number of people. In another study, it was observed that whereas the activity of pyrimethamine was reduce 750-fold against resistant isolates, the corresponding reduction in cycloguanil was only 7.7-fold <sup>(24)</sup>. This observed difference has been shown to be due to differences in the mutation sites for pyrimethamine and proguanil resistant parasites <sup>(25,26)</sup>.

Chemoprophylaxis of malaria is still recommended for travellers from non-endemic areas, and as a short term measure for soldiers, police and labour forces serving in highly endemic areas. It is also desirable for pregnant women living in areas where the transmission is very intense and leads to high parasitemia, causing low birth weight and anaemia. For the mother and the fetus, it is beneficial to prevent malaria. The drugs that can be used for prophylaxis, taking cost, compliance and possible side effects into consideration are: chloroquine, mefloquine, proguanil and a combination of proguanil and pyrimethamine with some sulpha drugs.

Studies on the prophylatic combinations of proguanil and sulphonamide in South-East Asia showed that there was five to ten fold reduction of Pf incidence compared with proguanil alone <sup>(27,28)</sup>. A few other studies on this combination have also proved to be valuable in the treatment of *P. falciparum* infection <sup>(23,29)</sup>.

Proguanil is also extensively used in combination with chloroquine. In two consecutive studies, between 1985 and 1991, the efficacy and side effects of the combination of proguanil and chloroquine in malaria chemoprophylaxis in short term travellers to East Africa were assessed. It was deduced that the prophylactic effectiveness was 72% for chloroquine with proguanil as against 42% for chloroquine alone at various doses <sup>(30)</sup>.

In another study on British subjects returning from malarious areas, it was concluded after investigations in 1987 that chloroquine plus proguanil was a preferred chemoprophylactic regimen for *P. falciparum* infection in Africa <sup>(31)</sup>.

One issue which needs to be investigated properly is the individual variability in the metabolism of proguanil into active cycloguanil. S-mephenytoin hydroxylase (CYP2 Cmp) enzyme is of critical importance in the polymorphic oxidation of proguanil to cycloguanil, whereas debrisoquine hydroxylase polymorphism appears not to influence it<sup>(5)</sup>. This inter-individual variation was reported by Watkins et al in 1987 <sup>(29)</sup>. Other studies have also confirmed the large inter-subject variability with the incidence of 2-3% in Caucasian population, 18-20% in Japanese subjects <sup>(32,8)</sup> and 35% in Kenyan adults<sup>(10)</sup> as poor metabolisers.

## 1.6 **PHARMACOKINETIC PROCESS**

Pharmacokinetics tries to understand how drugs cross membranes to enter the body, how they distribute in the blood and other fluids, how they are eliminated from the body. These processes can be expressed in mathematical terms and the information used to develop dosing regimes and not the process of try and error. The pharmacokinetic process comprises: (a) drug absorption and bioavailability (b) drug distribution (c) drug metabolism (d) drug excretion.

The extent of bioavailability of a drug depends on the extent of the absorption and the extent of metabolism. Plotting the curve of drug concentrations over a period of time following oral administration gives the opportunity to assess the bio availability. The peak height and the time taken for the drug to reach maximum concentration ( $t_{max}$ ) are measures of rate of availability. The total area under the curve (AUC) is a measure of the proportion of the drug which reaches the system circulation intact. These parameters gives you the fair idea of the right amount of drug that will give you the right effect for the right duration, with the minimum risk of unpleasantness and harm. For these reasons, it is important to do a pharmacokinetic studies when investigating inter -subject variability of proguanil.

## 1.7 METABOLISM OF DRUGS

Drugs are generally lipid soluble substances which ionise partially at physiological pH. This ionisation allows for the redistribution and excretion of drugs. Apart from these, drug metabolism is also a major mechanism by which drug action is terminated. Almost all drugs undergo metabolic transformation to form products (metabolites) which are more polar than the parent compound. Thus the rate of drug metabolism determines the duration and intensity of drug action.

One primary characteristic of biotransformation of drugs is generally the diminishing of their pharmacological activity by forming less active or inactive products. In a few cases, this biotransformation can lead to an increase in the pharmacological activity by producing an active metabolite. A typical example is proguanil, which in its native state is inactive but very active when metabolised into cycloguanil.

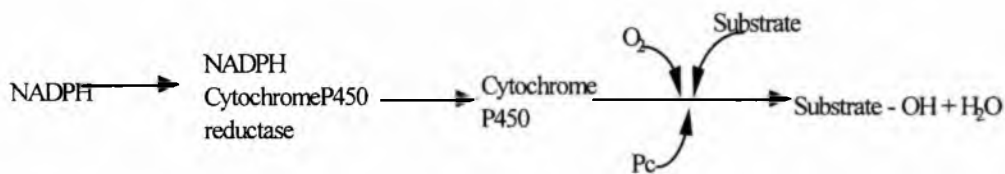
The rate at which a drug diminishes from the body is thus, a function of both its rate of biotransformation and rate of excretion by renal, biliary, pulmonary and other means. If the effects of the metabolism and the excretion results in first order kinetic rate of elimination of the drug, then the rate of concentration decrease can be described mathematically and called biological half-life,  $t_{1/2}$ . This  $t_{1/2}$  is the time required to reduce by half the quantity of drug present in a particular body compartment.

There are a number of enzymes that play a major role in the metabolism of carbohydrates, proteins, and lipids. But these enzymes are quite different from those enzymes that participate in biotransformation of foreign compounds which are chemically unrelated, mostly exogenous and largely lipid-soluble. These set of enzymes metabolise drugs, insecticides, herbicides, food additives (colouring agents and preservatives), industrial and automotive pollutants. One single organ which is rich in these microsomal enzymes is the liver. However other tissues, like the kidney, lung, intestinal mucosa and placenta also have significant amounts of these enzymes<sup>(33)</sup>. Chemical pathways by which drugs are metabolised are; oxidation, reduction, hydrolysis and conjugation with other metabolites.

**Oxidative Reactions:** These reactions are the most common ways by which drugs are metabolised. It is quite different from the oxidation - reduction reactions in intermediary metabolism because in the latter, there is a hydrogen transfer. The oxidation of foreign substances proceed with an insertion of one atom of oxygen molecule into the substrate and this process is catalysed by monooxygenases. The other oxygen atom is used to form water. The source of reducing equivalent is reduced nicotinamide adenine dinucleotide phosphate (NADPH). This process is frequently called microsomal hydroxylation because most of the oxidative reactions are, infact, hydroxylation reactions and the process occurs, generally, in the endoplasmic reticulum. The NADPH reduces NADPH-Cytochrome C reductase, which, in turn, reduces cytochrome P450. The reduced

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cytochrome P450 - substrate - O<sub>2</sub> ternary complex then rearranges to form the product. There are other oxidative reactions like, epoxidation, dealkylation, deamination, sulphoxidation, N-oxidation, oxidation of aliphatic alcohols. The monooxygenase system has obligatory requirements for NADPH and O<sub>2</sub> and is almost exclusively associated with the endoplasmic reticulum of intact cells. It is therefore closely associated with the phospholipid membranes of organelles. This monooxygenase system is composed of an electron transfer chain consisting of endogenous compounds which have been isolated, purified and studied in detail. These are flavoprotein known as NADPH - cytochrome P450 reductase, a hemoprotein called cytochrome P450 and a lipid component; phosphatidylcholine (Pc). The microsomal electron transfer chain can be schematically presented as follows:



**Reductive Reactions:** Reduction reactions also take place in drug metabolism. Unlike the oxidases which are present in the endoplasmic reticulum, the reductases occur both in the organelles and in the cytosol. In addition, anaerobic micro organisms present in the colon are rich in reductive enzymes and contribute to the metabolism of xenobiotics in vivo.<sup>(34)</sup>

**Hydrolytic Reactions:** The enzymes involved in these types of reaction are in contrast to oxidative and reductive enzymes in drug metabolism. They are found mostly outside the endoplasmic reticulum and virtually all tissues and organs. But the concentration is high in the liver, kidney and plasma. Substrates for these enzymes are compounds with ester linkages.

**Conjugated (Synthetic) Reactions:** After the metabolism of xenobiotics, enzymatically oxidised, reduced, or hydroxylated metabolites formed often contain a reactive chemical group such as hydroxyl, amino or carboxylic acid. The reactive group is either inserted directly into the drug or it may be unmasked by oxidative reactions. The reactive groups are able to combine with molecules provided by the body eg. glucuronate, sulphate, glycine and acetate. These reactions are frequently involved in the conversion of both drugs and endogenous substrates to compounds that are in general, pharmacologically much more water soluble for excretion. Some of the conjugation pathways are; glucuronide conjugation, sulphate conjugation, N-acetylation, methylation, glutathione conjugation and amino acid conjugation.

### 1.7.1 **Factors Affecting Drug Metabolism**

Drug metabolising enzyme activity is quite low under the age of six month, but increases during perinatal period and dramatically increases to adult levels at about puberty. In the elderly, however, the rate of biotransformation gradually decreases, thus elderly individuals become more and more sensitive to a given drug dose as their ability to metabolise foreign

compounds decreases. The elderly they may have a reduced renal function which can also contribute to a decrease in drug elimination.

**Nutrition and Diet:** The endoplasmic reticulum which contains the drug metabolising enzymes, Is composed of nearly equal parts of lipid (phospholipid, cholesterol and triglycerides) and protein. It also contains limited amounts of carbohydrates. This shows that diet influences rates of metabolism of drugs. Protein deficiency impairs drug metabolism. Vitamin A, riboflavin, ascorbic acid and Vitamin E deficiencies also impair drug metabolism.

**Hormonal Effects:**

**Cortic Steroids:** Hepatic drug metabolism appears to correlate universally with plasma glucocorticoid levels. And in some species, adrenalectomy impairs the metabolism of a variety of drugs. This effect can be reversed by the injection of cortisone, cortisol or prednisolone.

**Estrogens and Progesterone:** Some studies involving human subjects, suggests that drug metabolism is impaired in pregnant women. This may be due to estrogen and progesterone levels in the pregnant women. Contraceptives have also been found to influence drug metabolism <sup>(35)</sup>

**Disease:** Hepatic drug metabolism is impaired in patients with malaria and schistosomiasis. Also neoplastic disease which does not involve the liver, still inhibits drugs metabolism in the liver. This may be due to humoral factors. Biotransformation is also impaired by the presence of such pathological conditions as hepatitis, obstructive jaundice, and advanced cirrhosis.

**Species Difference:** The metabolism of drugs in different species of animals are commonly manifested either as differences in rates of metabolism or as differences in the metabolic products formed. An example is the deamination of amphetamine to inactive ketone in rabbit while the rat produces active substance called 4-hydroxyamphetamine <sup>(36)</sup>

**Sex Differences:** There are marked differences in the biotransformation of many, but not all drugs in male and female animals. The sex related differences in rates of drug metabolism are due to stimulatory effect of androgens and can be abolished by castration of the male rats. The administration of testosterone to females also increased the rate of metabolism. This sex differences in rates of drug metabolism have only been reported in rats and strains of mice and not in man <sup>(37)</sup>.

**Intestinal Microflora:** The anaerobic microflora present in the ileum and colon are rich in reductases. These may be responsible for a significant proportion of azo reductase activity

in humans. This is because the therapeutic agents in the gastrointestinal tract of human come into close contact with the intestinal microflora.

**Inhibition:** From kinetic studies it has been established that one substrate for the microsomal drug metabolising system can competitively inhibit the metabolism of a second substrate <sup>(38)</sup>. The effects of ethanol ingestion on hepatic drug metabolism will differ depending on whether the ingestion has been acute or chronic. Chronic ethanol ingestion increases the hepatic contact of drug metabolising enzymes and accelerates drug clearance from plasma. Acute ethanol ingestion, however, inhibits drug metabolism and prolongs and intensifies the effects of drugs. Studies performed in human volunteers have shown that acute ethanol intoxication results in, at least, a twofold increase in the plasma half-life of phenobarbital and meprobamate <sup>(27)</sup>.

### 1.7.2 **Pharmacogenetics**

The extent to which an individual metabolizes a drug is partly determined genetically. This fact emerged from studies on monozygotic (identical) and dizygotic (non-identical) twins. In identical twins, drug metabolism is similar while in fraternal twins the difference is seizable and approaches that of unrelated individual. These adult twins lived in different households, ate different diets and were exposed to different enviromental conditions, but they still exhibited similarities in plasma half lives. This study showed that large differences that exist

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among individuals in their rate metabolism and plasma clearance is controlled predominantly by genes. For most drugs in population studies, the variability in drug metabolism shows an individual normal distribution. However, for some drugs the distribution is bi-or tri-modal indicating the existence of separate populations of subjects capable of metabolizing those drugs and at discretely different rates <sup>(1)</sup>

Two important pathways of drug metabolism subject to pharmacogenetic variability are acetylation and hydroxylation.

**Acetylation:** Many drugs are acetylated by the hepatic enzyme N-acetyl transferase and the distribution of the rates of acetylation in population is bi-individual. The difference between fast and slow acetylation depends upon the amount of N-acetyl transferase, rather than a change in its inherited property. The fast to slow ratio of acetylators is 40:60 in Europe and 85:15 in Japan <sup>(39)</sup>.

**Hydroxylation:** Genetic variability in drug metabolism which stably exist at frequencies higher than 1% in a population is commonly referred to as polymorphism. Two independent polymorphisms have been clearly defined. One affects the 4-hydroxylation of debrisoquine and N-oxidation of sparteine and is controlled by cytochrome P450 2D6 (CYP 2D6) enzyme. The other affects 4-hydroxylation of 5-mephenytoin and mediated by cytochrome P450

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enzyme in the 2C subfamily (CYP2Cmp)<sup>(40,41,42)</sup>. The activities of these isozyme lead to the definition of distinct phenotypes that is, extensive metabolisers (EM) and poor metabolisers (PM). There are many drugs some with wide therapeutic uses such as novel antidepressants, neuroleptics, lopopluilic B-blockers which are mediated by this debrisouquine type of polymorphism. The percentage of phenotype is much less common among Orientals (< 1%) than among Caucasians (7%) and 2.1% in Ghana<sup>(43,3)</sup>. Drugs that are mediated by S-mephenytoin hydroxylase, the PMs were 13% Koreans, 23% Japanese, 17% Chinese, 11% Indonesian, 3-6% Caucasians. The metabolism of proguanil to cytoguanil is mediated by the S-mephenytoin hydroxylase enzyme. The inter-subject variation in the activation of proguanil has been reported by Watkins since 1987. A few other studies have also confirmed the inter-subject variability with the incidence of 2-3% in the Caucasian population, 18-20% in Japanese subjects and 35% in Kenyan adults as poor metabolisers<sup>(8,9,10)</sup>. In all these studies, proguanil to cycloguanil concentration ratio in a 0-8 hour urine collection was used to classify the PMs and EMs. Individuals with PG/CG ratio  $\leq 10$  were arbitrarily classified as EMs, while those with ratio  $> 10$  were classified as PMs.

Since drugs are administered on a fixed dose schedule, patients who are generally predisposed to metabolise and clear a particular drug quickly will be under medicated and will not receive the full therapeutic benefit, whereas, slow metabolisers may experience severe drug toxicity resulting from progressive drug accumulation.

### 1.7.3 Methods of Studying Drug Metabolism in Man

Interest in studying human drug metabolism has three interrelated objectives;

1. To investigate the enzymes involved in biotransformation of foreign compounds.
2. To examine the influence of genetic, racial and environmental factors as well as the consequences of ageing and disease on therapeutic agents.
3. To elucidate mechanisms of drug toxicity so as to improve the prediction and diagnosis of advance drug - drug reactions.

Three principal methods for studying drug metabolism in man have been developed.

- a) In vivo methods; allow overall routes of drug metabolism to be investigated.
- b) In vitro methods; permit detailed biochemical investigations to be performed with isolated tissues and subcellular fractions.
- c) The measurement of endogenous substances in biological fluids may offer a simple non-invasive approach to the assessment of drug metabolising activity.

The non invasion method of assessment of drug metabolism was used in this study

### **RESEARCH PROBLEM**

It has been recognised that individuals respond differently to drugs from other individuals. This individual variation in response to drugs may lead to adverse response to drugs or failure of therapy. The skill of using these drugs require that the variation in response be recognised and taken into account when prescribing. An understanding of the reasons for individual variation in response to drugs is therefore relevant to all prescribers.

### **AIM**

The aim of this study therefore is to determine the inter - subject variability in the activation of proguanil to cycloguanil among Ghanaians.

### **OBJECTIVES**

The study would involve the identification from population studies, "Poor" and "Extensive" metabolisers of proguanil and the determination of pharmacokinetics of the individual phenotypes that will be established in the population study.

## **CHAPTER TWO**

### **MATERIALS AND METHODS**

#### **2.1 MATERIALS**

##### **2.1.1 Study Population**

The study was carried out at Center for Tropical Pharmacology and Therapeutics, Ghana Medical School and at the Department of Biochemistry, University of Ghana, Legon. The study population comprised of apparently healthy adult volunteers of both sexes who were drawn from:

1. The paramedical schools i.e. Nursing Training School, the Laboratory School and School of Hygiene.
2. Staff of the Korle-Bu Teaching Hospital; and
3. Volunteers living in the municipality of Accra

##### **2.1.2 Reagents And Chemicals**

The test kits containing alamine aminotransferase (ALT, EC: 2.6.1.2), aspartate aminotransferase (AST, EC: 2.6.1.1), blood urea nitrogen (BUN) were purchased from sigma diagnostics. Nitrogen gas was purchased from Air liquid (GH) Ltd. Acetonitrile, methanol,

perchloric acid, and triethylamine hydrochloride were all HPLC grade and were bought from BDH laboratories, Poole BH15 1TD England Proguanil hydrochloride, cycloguanil, chlorcycloguanil and 4-chlorophenylbiguanide standards were supplied by Zeneca pharmaceuticals, Hurdsfied Industrial Estates, Macclesfield, England. Three hundred tablets of Palludrine each containing 100mg were purchased from Zeneca through their local agents, Ernest Chemists.

## 2.2 **METHOD**

### 2.2.1 **Inclusion/exclusion Criteria**

Volunteers who were included in the study fulfilled the following criteria:

- i) They were all above 14 years of age, since that was the minimal adult age set by the manufacturers of proguanil.
- ii) They were from both sexes; male and female
- iii) Those who had normal hepatic function and normal renal function were included in the study, while those with abnormal hepatic and renal function were excluded from the study. For their liver function the enzymes, alanine amino transferase (EC:2.6.1.2) and aspartate amino transferase (EC:2.6.1.1) levels were determined and for renal function, the blood urea nitrogen levels were determined.

- iv) Volunteers were also asked to avoid alcohol and cigarette at least 12 days before the beginning of the study.

In the determination of the hepatic and renal functions of each subject, 5mls of blood were taken from the forearm vein into plain tubes and left for 30minutes to clot. The clotted blood was centrifuged at 3000g for 10 minutes using Gallenkamp centrifuge. The serum was taken into another plain tube and stored at -25°C until they were analysed for alanine, aspartate aminotransferase activities and blood urea nitrogen. Samples of the serum were analysed as follows:

### **Liver Function Test**

The essay was based on alanine aminotransferase (ALT) catalysing the transfer of amino group from alanine to form glutamate and pyruvate. The pyruvate formed was then reduced to lactate in the presence of lactate dehydrogenase. This was accompanied by the oxidation of reduced nicotinamide adenine dinucleotide. The rate of decrease in absorbance at 340nm is directly proportional to ALT activity.

Sigma diagnostic test kit provided the detailed procedure. The reagent was reconstituted with 10mls of distilled water and mixed several times by gentle inversion. The wavelength of

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spectrophotometer (Pharmarcia LKB Ultraspec III) was set at 340nm and the absorbance reading set to zero using distilled water as reference sample.

One milliliter of ALT reagent was dispensed by pipetteman (Gibson's P1000) into 1ml cuvet labelled "Test". The cuvet was left on the bench for 30minutes at 25°C. Hundred microliters of serum were then added to the 1ml reagent and mixed immediately by gentle inversion 5times and left on the bench to incubate for 90 seconds. The absorbance (A) was read after the 90 seconds and the results recorded as "Initial A". The incubation was allowed to continue at 25°C for another 30 seconds and the reading noted. After another 30 seconds the final absorbance was taken as "Final A". The activity of ALT at 25°C was determined using the following formula:

$$\begin{aligned} \text{ALT } (\mu\text{/L}) &= \frac{\Delta A \times TV \times 1000}{6.22 \times LP \times SV} \\ &= \Delta A \times 1768 \end{aligned}$$

Where  $\Delta A$  = change in absorbance per minute at 340nm

TV = Total Volume (1.1ml)

SV= Sample Volume (0.1ml)

6.22= Millimolar absorptivity of NADPH at 340nm

LP = Light path (1cm)

1000= Conversion of units per ml to unit per litre

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Aspartate aminotransferase (AST) activity was similar to that of ALT activity. This assay was also based on AST catalysing the transfer of the amino group from aspartate to 2 - oxoglutarate to yield oxolactate and glutamate. The oxolactate was reduced to malate in the presence of malate dehydrogenase with the simultaneous oxidation of reduced nicotinamide adenine dinucleotide. The rate of decrease in absorbance at 340nm was directly proportional to AST activity. The procedure and calculation was the same as that of ALT, the only difference was the reagent used.

The activity of both ALT and AST were expressed as  $\mu\text{L}$  which was defined as the amount of enzyme (AST or ALT) which produces 1 $\mu\text{mol}$  of  $\text{NAD}^+$  per minute under the conditions of these assay procedures.

### **Kidney Function Test**

This assay procedure is based on urea being hydrolysed to ammonia and carbondioxide by catalytic action of urease. The ammonia serves to aminate 2 - oxoglutarate to glutamate with the concurrent oxidation of  $\text{NADH}$  to  $\text{NAD}^+$  in the reaction catalysed by glutamate dehydrogenase (GLDH). The rate of oxidation of  $\text{NADH}$  to  $\text{NAD}^+$  which is directly proportional to blood urea nitrogen (BUN) concentration in the serum sample was measured by spectrophotometer at 340nm. The BUN concentration in the sample was determined by comparing the sample reaction rate to that obtained with a BUN standard provided in the

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diagnostic kit from sigma that was used. The test kit also provided the detailed procedures as follows:

BUN reagent was reconstituted with 10mls of distilled water and mixed several times by inversion. The spectrophotometer wavelength setting was maintained at 340nm. The absorbance reading of zero was checked using distilled water as blank sample and electrothermal water bath was switched on with the temperature control set to give maximum temperature of 35°C. One milliliter of the BUN reagent was then pipetted in a cuvet and then placed in the water bath for 2minutes. Ten microliters of the serum sample was then added to the reagent in the cuvet and gently mixed by inversion 5 times. The mixture was then placed back in the water bath to incubate for 30 seconds. The absorbance reading was taken after this 30 seconds using spectrophotometer and results recorded as “Initial A”. The incubation was continued at 35°C and two other absorbance readings were taken at 30 seconds and 60 seconds. The absorbance reading at 60 seconds was recorded as “Final A”. The change in the absorbance was obtained by subtracting the “Initial A” from the “Final A”. These same procedure was used for the standards. Urea nitrogen concentration in serum were calculated based on the absorbance change per minute of both sample and standard. This was done using the following formula:

$$\text{BUN Concentration (mg/ml)} = \frac{\Delta A \text{ per min of serum} \times \text{Conc of Standard}}{\Delta A \text{ per min of standard}}$$

Where  $\Delta A$  per serum = (Final A - Initial A) of serum

$\Delta A$  per standard = (Final A - Initial A) of standard

## 2.2.2 **Determination of concentration of proguanil and cycloguanil in urine and plasma samples**

### **Sample Collection (Urine)**

Basic background information like weight, height, age, sex and tribe were obtained from 132 apparently healthy Ghanaian adults who satisfied the inclusion criteria and agreed to participate in the study. After an overnight fast, each subject was asked to empty his/her bladder in the morning at 7.00a.m, 2 Paludrine tablets, were then administered orally to each subject with about 100mls of water. They were allowed food after one hour following drug administration. This was to ensure that drug absorption was not affected by food. The subjects were provided with 2L plastic containers into which they emptied their bladder over the next eight hours (7:00 a.m. to 3:00 p.m.). The total volume of urine after the eight hours was recorded and 10mls aliquot was stored at -25°C until it was analysed for proguanil and cycloguanil content.

### **Sample Pre-treatment (Urine)**

Urine samples were thawed and 20  $\mu$ l of each sample was taken into a new test tube and 980  $\mu$ l of distilled water added. Three hundred microliters of 5  $\mu$ g/ml chlorcycloguanil was added as internal standard IS. One milliliter bond elut C<sub>8</sub> cartridge (supelco clean LC 8) was then preconditioned with 1ml of methanol, followed by 1ml of distilled water. Each sample with the IS was loaded on the cartridge, the sample was allowed to drain out of the cartridge and was washed with 1ml of distilled water, followed by 1ml of methanol. Proguanil, CG and IS were then eluted from the cartridge with 1% perchloric acid in methanol which was prepared as

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shown in appendix 2. The eluent was evaporated to dryness under gentle stream of nitrogen gas and the sample reconstituted in 100ml of mobile phase. Thirty microliters of the sample was then injected into the column through the rheodyne injector.

The concentrations of proguanil and cycloguanil in urine was estimated using standardised calibration curves. Stock and working solutions of pure proguanil and cycloguanil standards were prepared as shown in appendix 1.

a. **Proguanil:** Blank urine samples were spiked with proguanil working solution to produce 5 different concentrations in the range of 0 to 10 $\mu$ g/ml. These standards were 0, 2 $\mu$ g/ml, 4 $\mu$ g/ml, 6 $\mu$ g/ml, 8 $\mu$ g/ml, 10 $\mu$ g/ml and were arrived at by serial dilutions from a working concentration of 20 $\mu$ g/ml. The standard curve for the determination of proguanil concentration in plasma samples were similar to that of the urine samples. The curve ranged between 0 and 250 $\mu$ g/ml. Blank plasma samples were spiked with proguanil working solutions of 25 $\mu$ l, 50 $\mu$ l, 75 $\mu$ l, 100 $\mu$ l and 125 $\mu$ l to produce individual ranges of 50 $\mu$ g/ml, 100 $\mu$ g/ml, 150 $\mu$ g/ml, 200 $\mu$ g/ml and 250 $\mu$ g/ml. The same working solution of 2 $\mu$ g/ml prepared in appendix 1 was used.

b. **Cycloguanil:** The blank urine samples were again spiked with different volumes of 5 $\mu$ g/ml working solution to produce 5 different concentrations of the range 0 to 2 $\mu$ g/ml. The individual standards were 0, 0.4 $\mu$ g/ml, 0.8  $\mu$ g/ml, 1.2  $\mu$ g/ml, 1.6  $\mu$ g/ml and 2.0  $\mu$ g/ml. The blank plasma

samples were also spiked with 1  $\mu\text{g/ml}$  working solution to produce 5 different concentrations of the range of 0 to 125  $\mu\text{g/ml}$ . Using 25  $\mu\text{l}$ , 50  $\mu\text{l}$ , 75  $\mu\text{l}$ , 100  $\mu\text{l}$  and 125  $\mu\text{l}$  of the working solution, these individual standards were obtained; 25ng/ml, 50ng/ml, 75ng/ml, 100ng/ml and 125ng/ml. Several of these spiked urine and plasma samples were produced and stored at  $-25^{\circ}\text{C}$ . These were analysed together with the urine samples collected from the volunteers. Plotting the curves of peak height ratio of PG on IS and CG on IS against the known concentrations, calibration equations were obtained by linear regression. The unknown concentrations of the drug and its metabolite in the samples collected from volunteers were estimated from the standard curves. The peak heights were determined using high performance liquid chromatography technique.

### 2.2.3 **High Performance Liquid Chromatography (HPLC)**

HPLC is an analytical method used in the determination of proguanil and its metabolite in plasma and urine after single oral dose of 200mg of the parent drug. The chromatographic separation was based on the method of Taylor<sup>(44)</sup>. The HPLC set up comprised of pump (Water and Associates model 6000A solvent delivery system), Rheodyne sample injection system (with 100ml loop) Shimadzu UV-VIS. Spectrophotometer detector (SPD-6AV), Philips single pen recorder (model PM 8251) and Supelco column (Supelcosil LC-18, 15cm x 4.6mm ID, 5  $\mu\text{m}$  particle size) were all serially connected to each other. The flow rate on the pump was set at 1.5 ml/min and the wavelength for the detector was also set at 238nm. The HPLC set up was

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conditioned for the analysis of the samples by running mobile phase through it for two hours. The mobile phase was prepared as shown in appendix 3. Thirty microliters of the pre-treated sample was reconstituted and loaded on to the rheodyne injector using micro syringe. The sample was carried from the rheodyne sample loop on to the column where the separation of the parent drug, the metabolite and the Internal Standard (IS) occurred. Since these components have different retention times, they were detected on the spectrophotometric detector at different times. These different components were then identified as different peaks on the recorder with specific retention times.

The area under the peak height of PG, CG and IS gave an indication of their concentration level in the various samples. The concentrations of PG and CG of urine samples were estimated from standard calibration curves drawn from drug spiked urine samples. The ratio of the concentration of proguanil to cycloguanil in the urine samples collected from each individual was used as a measure of the drug metabolising activity of each subject. Based on the results obtained from this urine analysis, the population was classified as either a PM or an EM.

**Sample collection (Plasma)**

After the analysis of the urine samples in the population studies, seven of the healthy volunteers, previously designated as “Extensive” (n=5) and “Poor” (n=2) metabolisers of proguanil, were drafted in to this study having given their full and informed consent. Subjects were made to fast

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overnight and emptied their bladder in the morning at 7:00 a.m. Indwelling cannula was fixed into their forearm vein and 5mls of blood was taken just before the tablets were administered. This sample provided the baseline data. Two tablets were administered and 5mls blood samples were subsequently removed through the cannula at 0.5, 1, 2, 3, 4, 6, 8 hours. Syringe and needle was then used to take the 24 and 48 hours samples. All these samples were collected into heparinised tubes (sterilin, UK) to prevent clotting. The samples were centrifuged at 3000g for 15 minutes to produce about 2.5ml of plasma which was removed into another plastic tube and stored at -25°C until ready for analysis. Urine sample was also collected for these ten volunteers over 24 hours from the time the drug was administered. Ten milliliters aliquot was taken for each subject and stored in refrigerator at -25°C after the total volume was noted.

**Sample pretreatment (Plasma)**

The same sample treatment procedure was used for the plasma samples. The difference was with the dilution; 0.5ml of plasma sample was diluted with 0.5ml distilled water and 70  $\mu$ l of chlorcycloguanil (IS) was added to the diluted plasma sample. The process of passing the sample through the bond elut cartridge was the same as that of the urine sample. Samples and standards were subjected to the same pretreatment procedure. The concentrations of the drug and its metabolite in plasma samples at various time intervals were also estimated from 0 hours to 48 hours using standardised calibration curves obtained from drug spiked plasma samples.

### 2.2.5 **Data Analysis**

The concentration ratio of proguanil to cycloguanil in urine for each subject was presented as a frequency distribution table and was used as a measure of the drug metabolising activity of each subject. Plasma concentrations of proguanil and cycloguanil were presented graphically as means  $\pm$  standard deviation. The PG/CG concentrations were also tabulated from these concentration profiles, the peak concentration ( $C_{max}$ ) for proguanil and cycloguanil were determined by visual inspection. The terminal phase elimination rate constant (K) was also determined by the regression analysis of the post absorptive and distributive phase of plasma concentration-time data. The terminal phase elimination half life ( $t_{1/2}$ ) was also calculated from the ratio  $0.693/K$  for all the ten subjects. The area under the curve (AUC) from time = 0 to time  $T = x$  was also calculated by the trapezoidal rule. Oral clearance ( $CL_0$ ) was calculated from the ratio of Dose/AUC and apparent volume of distribution, which was expressed as a function of bioavailability (F), was calculated from the expression

$$\frac{V}{F} = \frac{CL_0 \times t^{(1/2)}}{0.693}$$

## **CHAPTER THREE**

### **RESULTS**

#### **3.1 POPULATION STUDIES**

One hundred and thirty-two apparently healthy volunteers took part in the study. These were made up of 22 females and 110 males. The females constituted 16.7% and the males 83.3% and were of different ethnic background.

The average age of the volunteers was 25 years with the minimum and maximum ages being 18 years and 61 years respectively. The age that occurred most in the population was 23 years. Their mean height was 167.7 cm with the minimum and the maximum heights being 152 cm and 186.0 cm. One hundred sixty five centimeters was the most frequent height that occurred. The volunteers had an average weight of 64.5 kg with 46kg and 94 kg being the minimum and maximum weights respectively.

All the 132 volunteers were found to have normal liver functions based on the serum ALT and AST activities for the subjects which were found to be within normal ranges. The mean value obtained was 16.8 $\mu$ /L with the minimum and maximum values occurring at 4.8  $\mu$ /L and 50.1  $\mu$ /L respectively. The most frequent activity that run through the results was 12.1  $\mu$ /L.

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The serum Aspartate amino transferase (AST) activity of the subjects were also found to be within normal ranges. The average AST value was  $22.9\mu\text{L}$  with the minimum and maximum values being  $4.8\mu\text{L}$  and  $42.3\mu\text{L}$  respectively. The enzyme activity of  $21.8\mu\text{L}$  was the enzyme activity that occurred most frequently during the analysis. All these have been illustrated in table 1.

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**TABLE 1:** Statistical analysis of values for height, weight, age, PG, CG and Enzymes (AST, ALT,BUN) for volunteers

	Mean	STD DEV	MIN	25% ile	Median	76% ile	MAX	Mode
a. Height (cm)	167.7	8.2	152.0	161.0	168.0	174.0	186.0	165.0
b. Weight (kg)	64.5	8.6	46.0	58.3	64.0	69.0	94.0	65.0
c. Age (yrs)	25.0	6.3	18.0	21.0	23.0	27.0	61.0	23.0
d. AST ( $\mu$ /L)	16.8	9.9	8.2	17.0	21.0	26.7	42.3	21.8
e. ALT ( $\mu$ /L)	16.8	9.9	4.8	9.7	14.5	23.0	41.1	12.1
f. BUN(mmol/l)	3.2	0.9	2.1	2.5	3.1	3.8	5.1	3.8
g. PG ( $\mu$ g/ml)	87.5	62.0	10.6	42.8	70.7	112.2	384.9	53.9
h. CG ( $\mu$ g/ml)	25.3	17.2	2.5	11.7	20.3	36.0	77.1	11.7

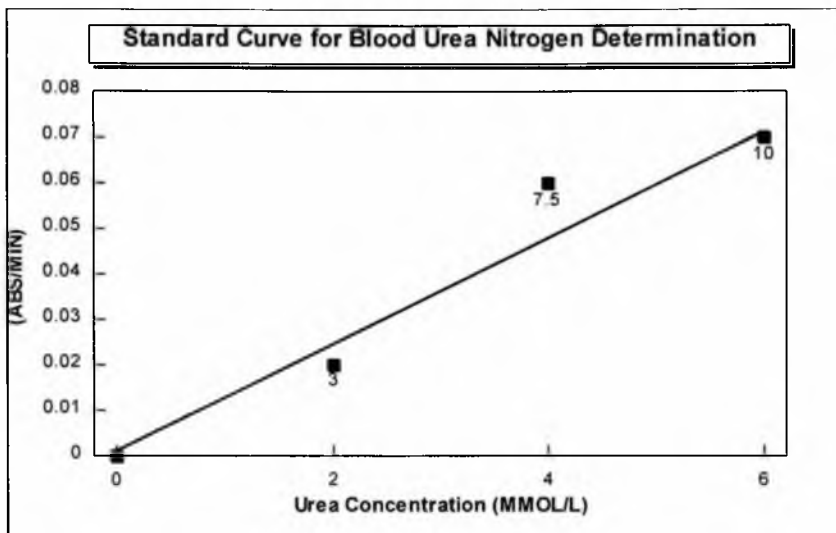


Figure 3

Standard curve for Blood Urea Nitrogen determination, showing the change in absorbancy per minute against standard concentration of urea

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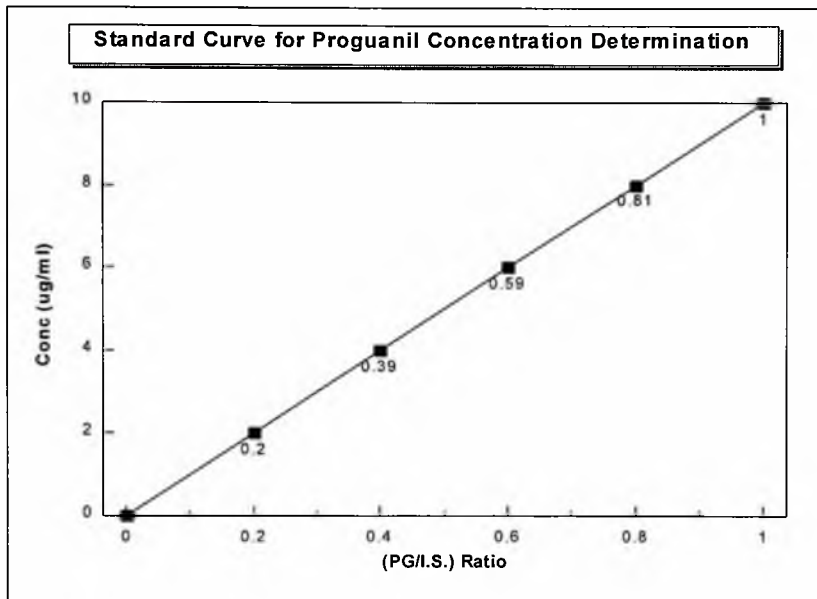
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The Kidney function of the individual subjects were also determined and found to be normal. The blood urea Nitrogen (BUN) levels were determined from figure 3 and found to be within normal ranges. The mean BUN level was found to be 3.2 mmol/L with the minimum and maximum levels being 2.1 mmol/L and 5.1 mmol/L. The most frequent level that was measured during the assay was 3.8 mmol/L

The level of the parent drug, proguanil and the metabolite, cycloguanil excreted in an eight hour urine sample was also measured for each subject from the standard curves shown in figure 4 and 5. The average amount of excreted proguanil measured was 87.5 $\mu$ g/ml. The minimum and maximum amount measured were 10.6 $\mu$ g/ml and 384.0 $\mu$ g/ml with the 53.9 $\mu$ g/ml occurring most frequently. For the excreted metabolite cycloguanil, the mean concentration was found to be 25.3 $\mu$ g/ml with the minimum and maximum concentrations as 2.5 $\mu$ g/ml and 77.1 $\mu$ g/ml respectively. The most frequent concentration that occurred was 11.7 $\mu$ g/ml. An example of the Chromatograph obtained during the assay is shown in figure 6 with retention times of 17 minutes, 2.5 minutes and 6 minutes for PG, CG, and IS respectively.

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**Figure 4**

Standard curve showing proguanil concentrations plotted against PG/IS ratio.

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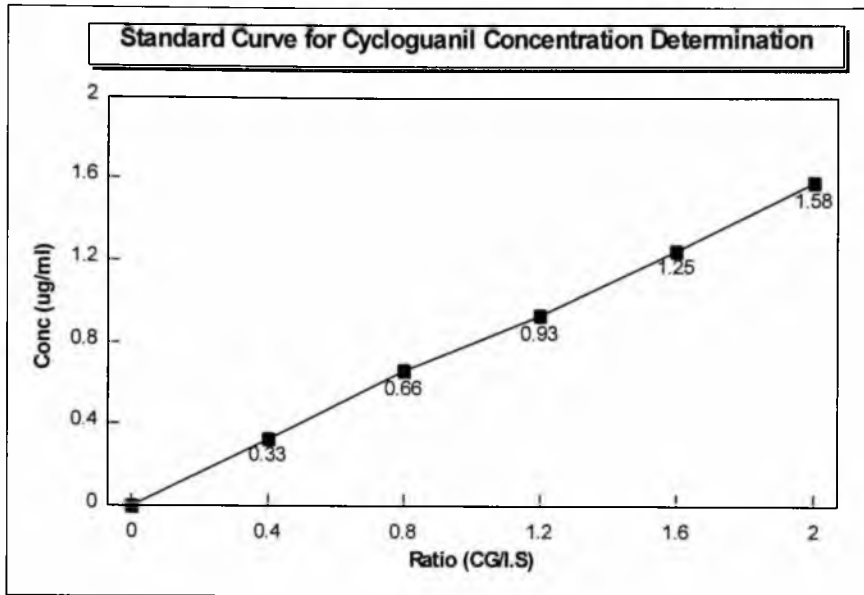
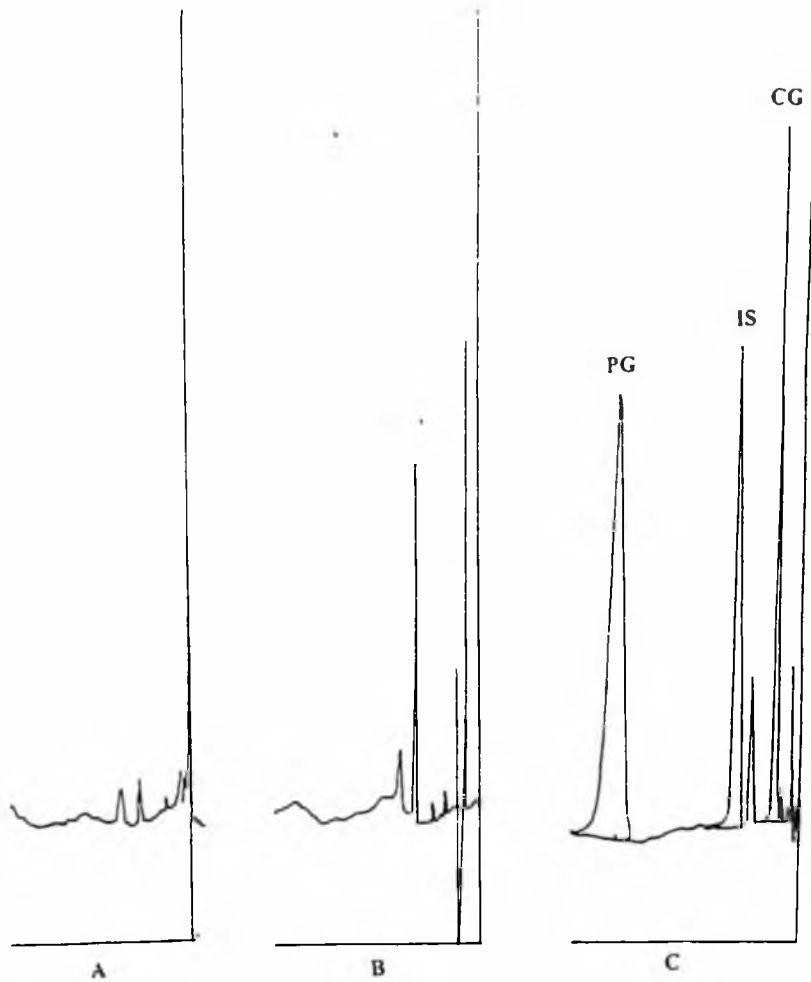


Figure 5

Standard curve showing cycloguanil concentrations plotted against CG/IS ratio.

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**Figure 6**

Representative Chromatogram obtained during Assay :(A) Mobile Phase; (B) Blank Urine Sample; (C)Urine sample spiked with PG,CG,IS.

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The frequency distribution profile drawn for the urinary PG/CG ratios in the one hundred and thirty two healthy volunteers is shown in figure 7. There was a non-normal distribution with PG/CG ratios ranging from 0.5 to 18.5. Individuals with proguanil to cycloguanil ratio  $\leq 12.5$  were arbitrarily classified as EMs while those with ratio  $> 12.5$  were classified as PMs. One hundred and twenty eight (97%) of the volunteers were tentatively identified as EMs because their PG/CG ratio was  $\leq 12.5$  and the other four subjects (3%) who had their ratio  $> 12.5$  were classified as PMs. The mean PG/CG ratio was 4.2 with a standard deviation of 2.8. The minimum and maximum ratios were 0.9 and 18.0 respectively.

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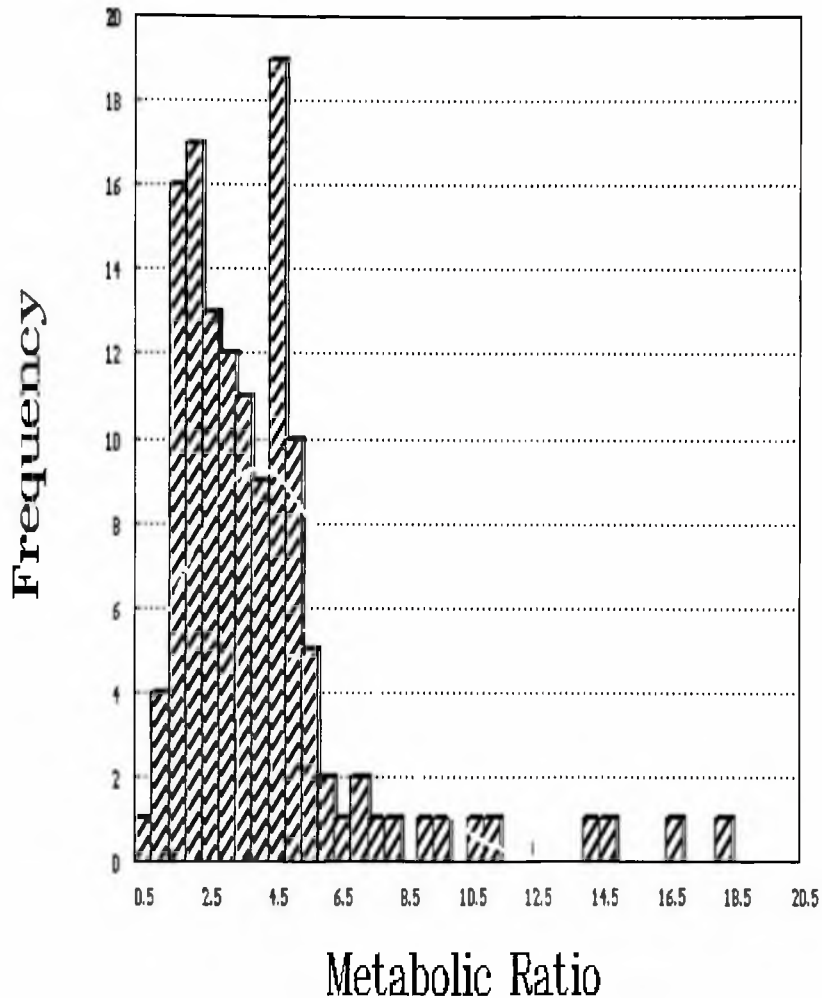


Figure 7

Frequency distribution of proguanil/cycloguanil ratio in 8 hours urine after an oral dose of 200mg of proguanil.

### 3.2 PHARMACOKINETICS STUDY

Seven volunteers (2 PMs and 5 EMs) again consented to take part in the pharmacokinetic studies which formed the second phase of the studies. The mean log plasma concentration-time profile for the five “Extensive metaboliser” of PG is shown in figure 8a. From this figure it was visually determined that proguanil appeared to be absorbed rapidly, achieving a mean peak plasma concentration ( $C_{max}$ ) of 126ng/ml ( $\pm 26$ ) within 3 hours ( $\pm 0.6$ ). Thereafter, the plasma concentrations fell throughout the study period with a terminal elimination half-life ( $t_{1/2}$ ) of 16.3( $\pm 6.1$ ) hours. The terminal phase elimination rate constant (K) was determined by the regression analysis of the post absorptive and distributive phase of plasma concentration-time data. The area under the curve (AUC) from  $t=0$  to time  $t=\infty$  was also calculated by the trapezoidal rule and was found to be 1890.3( $\pm 860$ ) ng, h, ml<sup>-1</sup>.

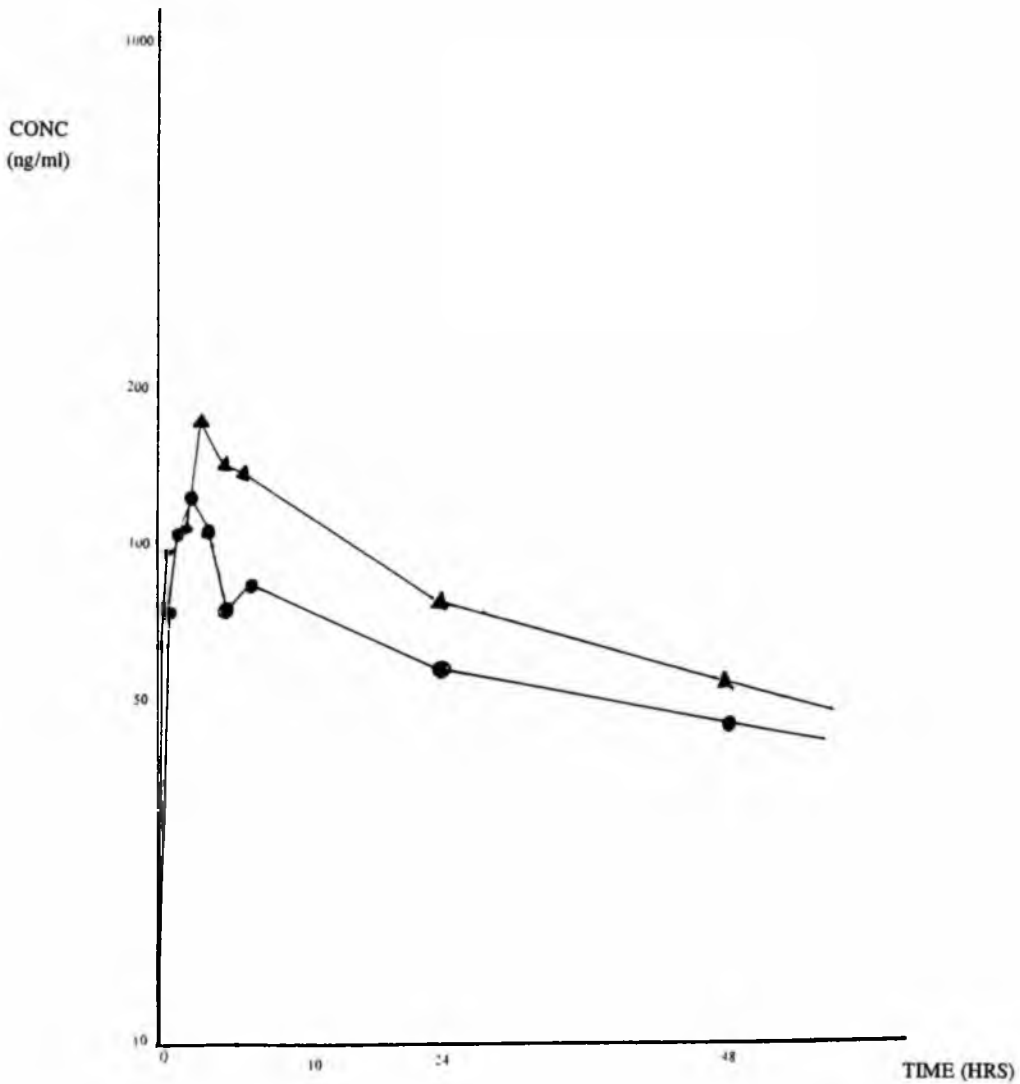
The mean log plasma concentration-time profile for the two PMs of proguanil is also shown in figure 8a. Proguanil, again, appeared to be absorbed rapidly and achieving peak concentration of 173.6ng/ml at about 4 hours. After achieving peak concentrations, plasma proguanil concentrations again fell gently. The maximum plasma proguanil concentration of 173.6ng/ml and consequently the AUC of 3039ng, h, ml<sup>-1</sup> in the PMs were higher than the mean values of  $C_{max}$  126.4ng/ml and AUC 1890.3ng, h, ml<sup>-1</sup> of EMs. The mean elimination half-life of the 19.0 hours of the PMs was also higher than that of the EMs which was 16.3 hours. This indicated that the parent drug remained in the PMs for much longer period and in a slightly higher concentration than the EMs.

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The mean log cycloguanil concentration-time profile was also drawn for the two phenotypes (EM and PM) as shown in figure 8b. Cycloguanil concentration in the EM subjects reached peak values of 88.7ng/ml in plasma at 7.2 hours while that of the PMs reached peak value of 93.4ng/ml at 5.5 hours. The elimination half-life and hence the AUC values were comparable for both the PMs and EMs. These pharmacokinetic parameters of PG and CG are shown in the table 2 and 3 respectively.

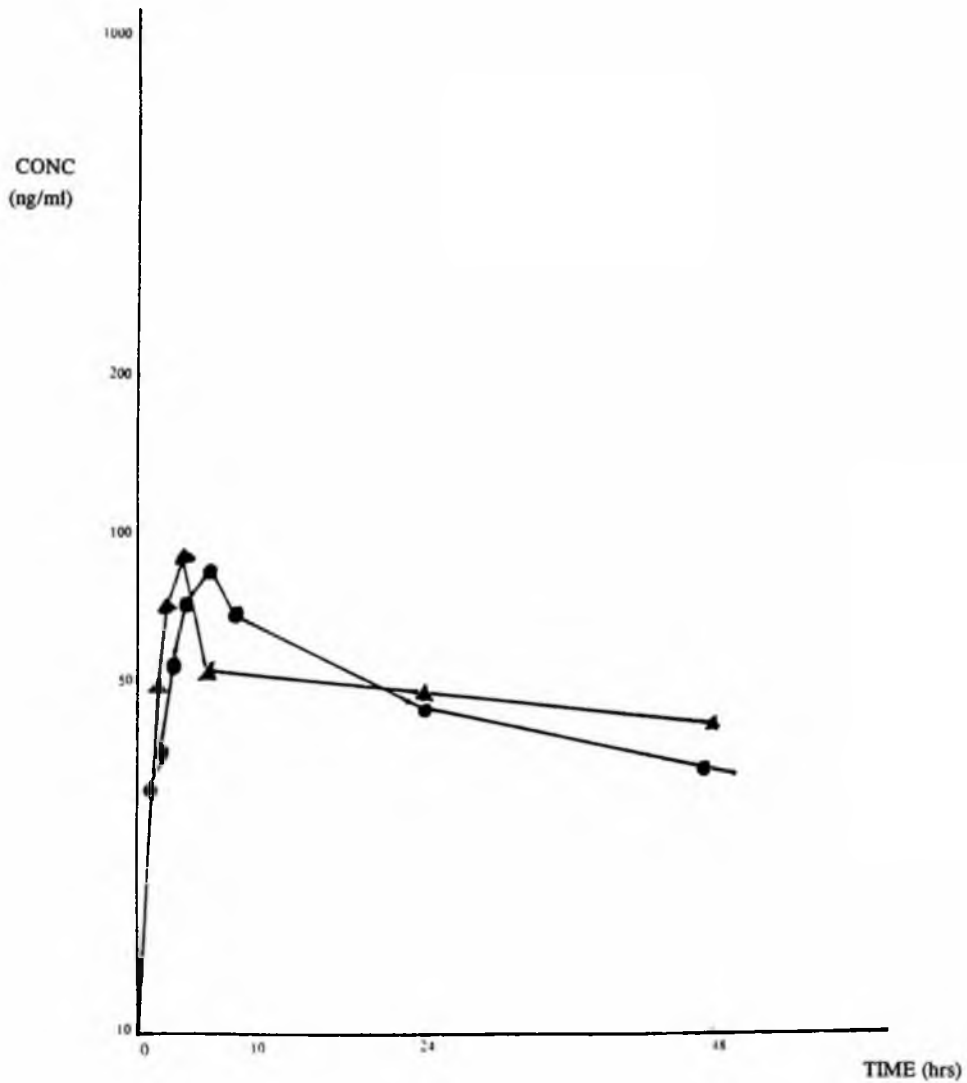
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**FIGURE 8a**

Proguanil concentration in plasma after a single oral dose of 200mg of proguanil hydrochloride in (A)extensive metaboliser (n=5;●) and (B) poor metabolisers ( n=2;▲)

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**FIGURE 8b**

Cycloguanil concentration after a single oral dose of 200mg proguanil hydrochloride in (A)extensive metabolisers (n=5;●) and(B) poor metabolisers (n=2;▲)

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**TABLE 2**

Pharmacokinetic parameters of EM (CB, RS, WK, AG, TT) and PM (CAK, ISS) for proguanil.

<b>PROGUANIL</b>							
Volunteer	0-8hr (PG/CG)	C <sub>max</sub> (ng/ml)	max (hr)	t <sub>1/2</sub> (hr)	AUC <sub>∞</sub> (ng,h,ml <sup>-1</sup> )	$\frac{Cl_o}{f}$ (ml/min)	$\frac{V_d}{f}$ (L)
<b>CB</b>	2.3	158	3	14.7	1801.1	111	2354.5
<b>RS</b>	5.0	159.6	4	22.1	3465.6	57.7	1840.1
<b>WK</b>	1.8	105.8	3	24.8	1937.9	103.2	3693.2
<b>AG</b>	7.8	102	3	10.3	1244.6	160.7	2388.5
<b>TT</b>	4.1	106.7	2	9.6	1002.3	199.5	2763.6
<b>mean ± s.d</b>		126.4 ± 26	3 ± 0.6	16.3 ± 6.1	1890.3 ± 860	126 ± 49	2608 ± 617
<b>CAK</b>	11.3	183	4	19.7	3067.6	67.2	1910.3
<b>ISS</b>	18.0	164.5	6	18.3	3012	66.4	1753.4
<b>mean ± s.d</b>		173.6	5.0 ± 1	19.0 ± 0.7	3039.8 ± 27.8	66.8 ± 0.4	1831.9 ± 78.5

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**TABLE 3**

Pharmacokinetic parameters of EM (CB, RS, WK, AG, TT) and PM (CAK, ISS) for cycloguanil.

Volunteer	0-8hr (PG/CG)	CYCLOGUANIL					
		C <sub>max</sub> (ng/ml)	Max (hr)	t <sub>1/2</sub> (hr)	AUC <sub>∞</sub> (ng.h.ml <sup>-1</sup> )	<u>Clo</u> f (ml/min)	<u>Vd</u> f (L)
CB	2.3	64	8	21.5	1376.9	145.3	4498.6
RS	5.0	117	6	18.1	2490.2	80.3	1453.4
WK	1.8	124	6	20.7	2594.6	77.1	1596
AG	7.8	49	6	42.3	1753.6	114.1	4826.4
TT	4.1	64	8	26	1735.2	115.3	2997.8
mean± s.d		88.7±37	7.2±0.98	25.7±8.7	2786.5±1306	106.4±25	3074.4±1408
CAK	11.3	101.2	8	22.3	2313.7	86.4	2780.3
ISS	18.0	85.5	3	29.4	2068.3	96.7	4102.4
mean± s.d		93.4±7.9	5.5±2.5	25.9±3.6	2121.0±122.7	91.6±5.2	3441.4±661.1

## **CHAPTER FOUR**

### **DISCUSSIONS AND CONCLUSION**

#### **DISCUSSION:**

Risk/benefit analysis has lightened the importance of proguanil as the safest and most appropriate drug for malaria prophylaxis, when used in combination with chloroquine <sup>(45)</sup>. A previous Caucasian and Oriental population study showed inter-subject variability of 3-6 % and 18-20% respectively in the metabolism of proguanil <sup>(8,9)</sup>. This led to the observation that certain individuals have abnormally low circulating concentration of cycloguanil after standard dosage and raised a number of questions related not only to therapeutic failures associated with the use of this drug but also with respect to the promotion of parasite resistance to antifolate drugs. As a result of these reasons, it is important to determine the source of these inter-individual differences and to establish which factors contribute to the variability observed. The only data available on African population is from the population studies on black Kenya adults and the variation was 35% <sup>(10)</sup>.

This study has again confirmed the inter-subject variability associated with the activation of proguanil to cycloguanil in man. Based on PG/CG concentration ratio in 0-8 hours urine collection, 128 subjects (97%) of the population formed a distribution with ratios less than 12.5 while 4 individuals (3%) exhibited urinary ratios less than 12.5. These 4 individuals exhibited

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urinary ratios more than ten standard deviations away from the mean of the major group. There was the absence of subjects between the larger group, who had a ratio  $\leq 12.5$  and a smaller group whose ratio was  $> 12.5$ . The absence of subjects in between these two groups suggests that the smaller group of individual form a separate population. The incidence of 3% of volunteers with high urinary ratio is lower than the 35% reported in the Kenyan study, suggesting a lesser variability in the Ghanaian population. This implies that there are differences in the two population studied.

Differences in the mixed function oxidase system (cytochrome P450) has been found to account for both inter-species and intra-species variability of proguanil metabolism. A number of polymorphisms of oxidative drug metabolism have been demonstrated to have a genetic basis. These are debrisoquine/sparteine polymorphism <sup>(2)</sup> and the mephenytoin hydroxylase polymorphism <sup>(4)</sup> The S-mephenytoin hydroxylase which is an isozyme of cytochrome P450 and is in the 2C subfamily have been found to be responsible for the variation in the metabolism of proguanil in man. It will be worthwhile if the genotypic assay could be performed on both Ghanaians and Kenyans to determine the fragmentation patterns. It is possible that the genes causing the variation in the metabolism of proguanil in these two groups are different mutants. This will then explain the large differences that exists between the number of PMs found in Ghana and Kenya.

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This variability does not represent poor compliance as dosage administration of 2 tablets of paludrine was controlled and all individuals had measurable concentrations of PG and CG in their plasma and urine. Two groups of subjects were classified as EMs of proguanil if the urinary ratio of PG and CG were less than or equal to 12.5 and PM if this ratio is greater than 12.5. The plasma pharmacokinetics of proguanil and the formation of cycloguanil in EMs were similar in data reported in Caucasians and Kenyan studies. Although peak proguanil concentrations were not significantly different in the two PMs, their average did show a higher plasma proguanil concentrations in the post-distributive phase and a significantly longer elimination half-life. Additionally, cycloguanil concentration in both PMs and EMs fell parallel with the proguanil during the elimination phase.

In vivo sensitivity studies have indicated a mean 50% inhibition concentration ( $IC_{50}$ ) value for cycloguanil against *P. falciparum* of 5 to 150 ng/ml <sup>(46)</sup> The maximum average plasma cycloguanil concentration of 93.4 ng/ml was obtained for the PMs will be effective against many strains of *P. Falciparum* which is considered totally sensitive to biguanides.

Clinical consequences of polymorphic PG metabolism is two fold. First, the relative inability to form the active metabolite may result in prophylactic failures with this drug and secondly, sub-inhibitory concentrations of PG may promote the development of parasite resistance to the actions of dihydrofolate reductase inhibitors. In addition to the activity against erythrocytic

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stages of malaria. CG is thought to exert its primary anti-malaria activity at the exo-erythrocytic stages in the liver. The importance of polymorphic biguanide activation is dependent on the incidence of PM phenotype within populations. The incidence of PM phenotype of 3% in Ghana is low and PG can be put forward as a possible agent for treatment of malaria when combined with chloroquine.

## CONCLUSION

In summary, this study has shown that 3% of the population in Ghana cannot metabolise PG to CG. Proguanil/Cycloguanil ratio, in urine, 8 hours after proguanil dosage, is a quite stable individual characteristics and is possible to measure now with HPLC. The measurement should be incorporated into chemoprophylaxis trials to determine the extent of individual protection from the drug under trial.

**APPENDIX ONE****Preparation of stock and working Solutions of Standards:**

- a. **Proguanil:**
- i. **Stock Solution:** 5 mg of proguanil was weighed into a 100 ml volumetric flask. Distilled water was added to the 100ml mark and put in the sonicator to dissolve. This gives proguanil solution of 50 $\mu$ g/ml concentration.
- ii. **Working Solution:** The stock solution of 50 $\mu$ g/ml was diluted 1 in 2.5 to obtain 20 $\mu$ g/ml concentration for the working solution. The dilution involved 1 part of stock solution plus 2.5 parts of distilled water. 40mls of the stock solution was taken into 100 mls volumetric flask and distilled water was added to the mark.
- b. **Chlorcycloguanil (Internal Standard):**
- i. **Stock Solution:** 1mg of Chlorcycloguanil was weighed into 50 ml Volumetric flask. Distilled water was added to the 50ml mark and put in a sonicator to dissolve. The resultant - solution is Chlorcycloguanil stock solution of concentration of 20 $\mu$ g/ml.
- ii. **Working Solution:** The stock solution of 20 $\mu$ g/ml concentration was diluted with distilled water to obtain 5 $\mu$ g/ml working solution. The dilution involved 1 part of the stock Solution with 4 parts of distilled water. 25mls of the stock solution was taken into 100mls volumetric flask and distilled water added to the 100ml mark.

c. **Cycloguanil:**

- i. **Stock Solution:** 5 mg of Cycloguanil was weighed into a 100ml volumetric flask distilled water was added to the 100ml mark and put in the sonicator to dissolve. The resultant solution is cycloguanil stock solution of concentration of  $50\mu\text{g/ml}$ .
  
- ii. **Working Solution:** The stock solution of  $50\mu\text{g/ml}$  concentration was diluted with distilled water to obtain  $5\mu\text{g/ml}$  working solution. The dilution involved 1 part of the stock Solution with 10 parts of distilled water. 10mls of the stock solution was taken into 100mls volumetric flask and distilled water added to the 100ml mark. The resultant concentration is  $5\mu\text{g/ml}$  .

## APPENDIX TWO

### **Preparation of 1% Perchloric acid in Methanol:**

- i. 5mls of Perchloric acid was taken into 500mls volumetric flask.
  
- ii. 495 mls of methanol was added to get to the 500ml mark. This gives the 1% perchloric acid in methanol for sample elution.

**APPENDIX THREE****Preparation of Eluent System (Mobile Phase):**

- a. 1% Triethylamine phosphate solution at a pH 3.
- I. 5ml of Triethylamine solution was taken into a 500ml volumetric flask.
  - ii. Distilled water was added to the mark and mixed well by putting in a sonicator for 15 minutes.
  - iii. The pH meter was standardised using standard pH solutions of 4 and 9.
  - iv. The pH of the triethylamine solution was adjusted from pH of 8 to pH of 3 using phosphoric acid.
- b. For a 300ml volume of Mobile Phase of Composition: 1% triethylamine phosphate;

Acetonitrile: Methanol (82:8:1 v/v/v).

$$1\% \text{ Triethylamine Phosphate (pH3)} = \frac{82}{100} \times 300$$

$$= \underline{246 \text{ mls}}$$

$$\text{Acetonitrile} = \frac{8}{100} \times 300$$

$$= \underline{24 \text{ mls}}$$

$$\text{Methanol} = \frac{1}{100} \times 300$$

$$= \underline{3 \text{ mls}}$$

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246mls of 1% triethylamine phosphate buffer, 24 mls of Acetonitrile and 3 mls of methanol were put together as mobile phase for the assay. This mixture was filtered through Millipore filtering apparatus using an aqueous filter paper to remove particles and gas bubbles. It was latter degassed in a sonicator for 5 minutes.

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