THE BIOCHEMICAL PHARMACOLOGY OF PRIMAQUINE

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

STEPHEN ANDREW WARD BSc (ASTON)

February 1985

ACKNOWLEDGEMENTS

I would like to acknowledge the financial support provided by the Mersey Regional Health Authority. I would like to thank Professor A. M. Breckenridge for his continued interest and support throughout this research project. I am also grateful to Doctors I.G. Edwards and G.W. Mihaly and Professor M L'E Orme for their valued guidance and helpful scientific comments. I would also like to thank Ms D D Nicholl for her excellent technical assistance and Miss L J Watson and Mr D E Kelly for typing the initial drafts of this thesis. Finally I would like to thank my parents for their enthusiastic support and understanding over the past three years.

THE BIOCHEMICAL PHARMACOLOGY OF PRIMAQUINE

S. A. WARD 1985

ABSTRACT

The 8-aminoquinoline primaquine is the drug of choice in the treatment of the tissue stages of human malaria. Despite the extensive use of this drug over the last 50 years very little is known of its disposition in both experimental animals and man. This lack of information has been due to the absence of suitable methods for the determination of primaquine in biological fluids.

In order to investigate fully the pharmacokinetics of primaquine it was essential to develop a number of selective and sensitive analytical methods. HPLC techniques have been established for the measurement of primaquine , the carboxylic acid metabolite of primaquine and the microbial metabolite N-acetyl primaquine, in plasma and urine.

The potential of racemic primaquine and the (+) and (-) isomers of primaquine to influence drug metabolism has been studied using the elimination of antipyrine from the isolated perfused rat liver preparation (IPRL) as an experimental model. Racemic primaquine was found to inhibit drug metabolism,the degree of inhibition being dependent on primaquine dose. The isomers of primaquine were equipotent as the racemate in inhibiting drug metabolism in this experimental model. The N-acetyl and 5-hydroxy derivatives of primaquine were shown to inhibit aminopyrine N-demethylase activity in hepatic microsomal enzyme preparations to a comparable extent to the parent drug.

The disposition of [^{14}C] racemic primaquine and the (+) and (-) isomers of primaquine has also been investigated in the IPRL. Racemic primaquine exhibited dose-dependent pharmacokinetics, metabolism and biliary excretion in this experimental model at doses of 0.5-5.0mg primaquine diphosphate, increasing dose being associated with significant reductions in the biliary excretion of [^{14}C] radioactivity and clearance of primaquine. Significant increases were observed in the the volume of distribution and elimination half-life of primaquine and in the formation of the carboxylic acid metabolite.. The isomers of primaquine exhibited stereoselective disposition in the IPRL after a 2.5 mg dose, the (-) isomer being eliminated more rapidly than the (+) isomer. This stereoselective difference was a consequence of a significantly lower value for clearance for the (+) isomer compared to the (-) isomer.

The pharmacokinetics of primaquine has been investigated in man. The drug was found to be rapidly and completely absorbed in man with a bioavailability of 1. The drug is a low clearance compound in man (Cl=20-30l/h) and the low plasma concentrations achieved after oral dosing is due to extensive tissue uptake (Vd= 200-3001). The carboxylic acid metabolite is the principal plasma metabolite. The pharmacokinetics of primaquine were found to be dose-independent in man at doses throughout the therapeutic range. Chronic administration had no effect on the mean pharmacokinetics of primaquine.

THE BIOCHEMICAL PHARMACOLOGY OF PRIMAQUINE

CONTENTS

CHAPTER 1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Malaria has existed since the dawn of civilisation. Fossil records have suggested that it originated in Africa at least 30 million years ago. Prior to World War II, approximately two thirds of the world's population were at risk from malaria. However, the development of antimalarial agents and the widespread use of insecticides such as dichloro-diphenyl-trichloroethane (DDT) were responsible for almost eliminating malaria and by the late 1950's it appeared that complete eradication of this disease was a possibility. Unfortunately, the last 10 years have seen a resurgence of malaria to a status close to its original level (Bruce-Chwatt 1979). The reasons for this resurgence are complex and numerous, the major contributing factors being the development of drug resistant parasites and insecticide resistant mosquitos, increased world travel and changes in socio-economic environments. The magnitude of the problem is such that in one year there may be as many as 300 million cases of malaria. In Africa alone more than one million malaria-related deaths occur annually (Lancet 1975). The world-wide concern over this problem has prompted a re-evaluation of the treatment of this disease and has stimulated research aimed at both the development of new antimalarial agents while making better use of drugs already available.

1.2 CHEMOTHERAPY OF MALARIA

1.2.1 THE MALARIA LIFE CYCLE

Malaria is a protozoan infection caused by various

species of plasmodia of which four are pathogenic to man: Plasmodium (P.) falciparum; P.vivax; P.ovale and P.malariae. The parasite has two independent life cycles, the first of which takes place in the female Anopholes mosquito. DDT and related insecticides, by virtue of their lethal effect on the mosquito, exert their effect at this stage of the parasite's development. In the second life cycle, in man (figure 1.1), the parasite goes through several stages of development and these stages are susceptible to attack by different chemotherapeutic agents.

The infective form of the parasite or sporozoite, resides in the salivary gland of the mosquito and is injected into the human host when the mosquito takes a blood meal. In order to survive, the parasites must invade the liver within one hour of entering the systemic circulation. This phase of the life cycle is termed the pre-erythrocytic stage. The parasites grow within the liver cell before undergoing schizogony (multiple fission of nuclei) forming multinucleate schizonts.

After a period of up to 15 days the tissue schizonts undergo cytoplasmic fission producing thousands of uninucleate merozoites. These merozoites rupture the liver cell and enter the general circulation terminating the pre-erythrocytic stage. For strains of plasmodia other than P.falciparum, a number of merozoites re-enter hepatocytes and begin the para-erythrocytic stage during which the parasite undergoes similar developments as described for the pre-erythrocytic stage. This process can continue over several years and is responsible for malaria relapse associated with

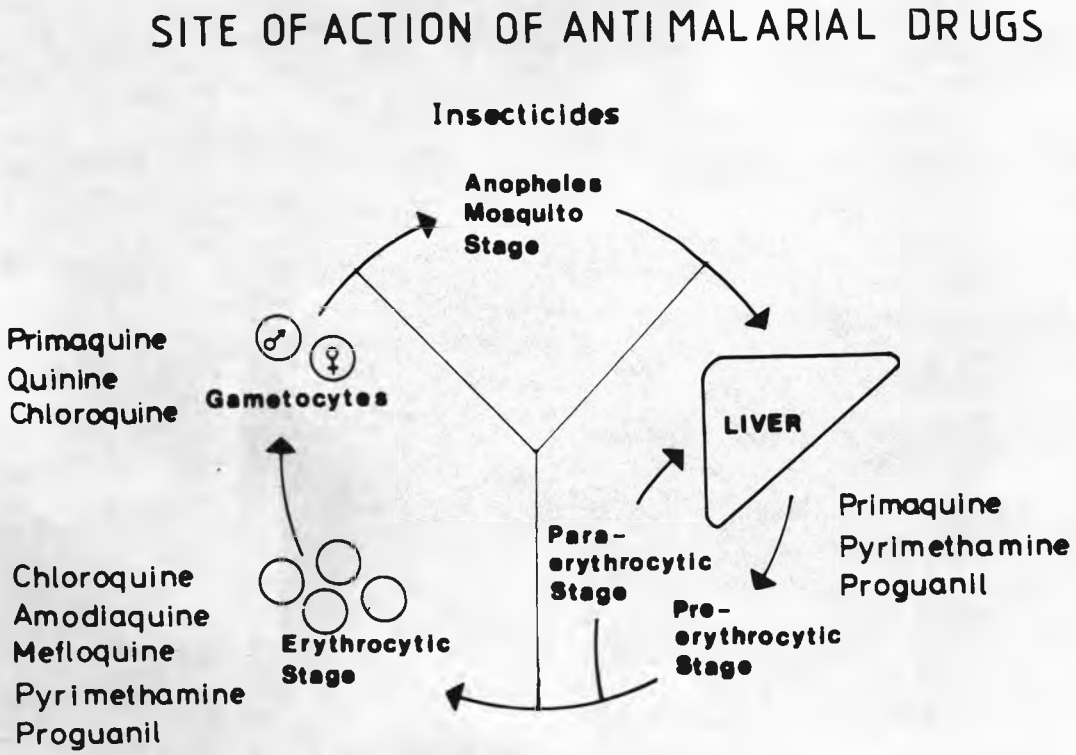


FIGURE 1.1 Schematic representation of the life cycle of a typical malarial parasite, indicating the site of action of commonly used drugs.

all forms of plasmodia other than P.falciparum.

On release into the general circulation the merozoites enter erythrocytes initiating the erythrocytic stage. Once in the erythrocyte the merozoite takes the form of a trophozoite which grows to fill the erythrocyte feeding on the protein fraction of haemoglobin. The parasite undergoes schizogony at this stage forming a multinucleate schizont.

These blood schizonts eventually divide into numerous uninucleate merozoites which burst the erythrocyte and enter the plasma prior to infecting other erythrocytes. It is this release of merozoites and associated pyrogens into the plasma which is responsible for the fevers associated with malaria.

Some merozoites undergo sexual differentiation into male or female gametocytes on entering an erythrocyte. These stages cannot undergo further differentiation until ingested by a feeding female Anopholes mosquito. After ingestion of gametocytes by the mosquito and the fusion of male and female gametes within the insects stomach wall,the parasite undergoes a number of developmental stages eventually producing several thousand merozoites which migrate to the salivary gland ready to be injected into the mosquito's next victim.

1.2.2 SITE OF ACTION OF ANTIMALARIAL AGENTS

Antimalarial drugs can be subdivided into classes depending on their principal site of action. The 8-aminoquinoline primaquine, the agent under study in this thesis, is the drug of choice for the treatment of tissue

schizonts. Primaquine exerts its major antimalarial activity against the pre-erythrocytic and para-erythrocytic stages of the life cycle (figure 1.1). Although the dihydrofolate reductase inhibitors pyrimethamine and proguanil also have antimalarial activity against these stages (figure 1.1), they are less widely used for this purpose in clinical practice.

A number of drugs are active against the erythrocytic stages of malaria. The first-choice blood schizonticide is the 4-aminoquinoline chloroquine (figure 1.1), which is thought to interfere with DNA replication processes within the parasite. The recent spread of resistance to this compound has prompted the use of other drugs against these blood stages, including another 4-aminoquinoline derivative, amodiaquine, the new quinolinemethanol, mefloquine and a number of dihydrofolate reductase inhibitors including pyrimethamine and proguanil (figure 1.1). However, parasite resistance to these compounds together with cross-resistance is now well documented (Bruce-Chwatt 1980). some sucess has been achieved by the use of drug combinations such as pyrimethamine plus a sulphonamide and in situations where all other drugs have failed, quinine has retained a role in the treatment of resistant infections.

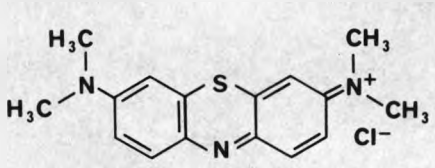
The gametocyte stages of malaria are potentially an ideal site at which to exert antimalarial activity. Primaquine, chloroquine and quinine are all effective to varying degrees against these stages and these drugs are now being prescribed, in certain areas, primarily for this purpose.

1.3 THE 8-AMINOQUINOLINE ANTIMALARIALS

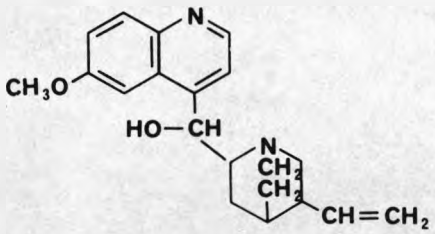
1.3.1 THE HISTORY OF THE 8-AMINOQUINOLINES

The chemotherapy of malaria was initiated late in the 19th century following the discovery that methylene blue (figure 1.2) possessed antimalarial activity when given to infected patients (Guttman and Ehrlich 1891). However, the first accepted drug for the treatment of malaria was quinine (figure 1.2), obtained from the bark of the cinchona tree. It was the short supply of quinine during World War I that initiated the search for alternative, synthetic antimalarials. It was found that alkylamino alkyl side chain substitution on to the N-atom in the aromatic ring of methylene blue increased antimalarial activity. Parallel studies were carried out using the quinoline moiety of quinine as a starting point. Modification of this structure gave rise to 8-aminoquinoline and 6-methoxy-8-aminoquinoline (figure 1.2) both of which possessed antimalarial activity. Schonhofer (1942) suggested that it was the 6 and 8 positions in the quinoline molecule which were responsible for the antimalarial activity.

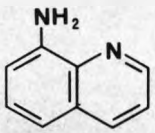
As a result of this research the 6-methoxy- 8-aminoquinoline derivative pamaquine (figure 1.3) was synthesised and introduced for the clinical treatment of malaria in 1926. This compound was found to be highly toxic, the toxicity being attributed to the 6-methoxy group. However, removal of this group resulted in a severe loss of antimalarial activity. Since the introduction of pamaquine,



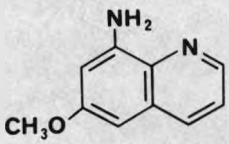
Methylene blue



Quinine

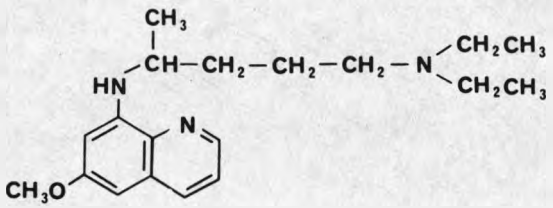


8-aminoquinoline

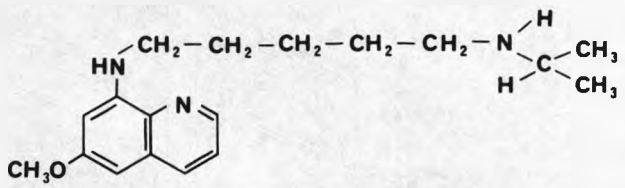


6-methoxy-
8-aminoquinoline

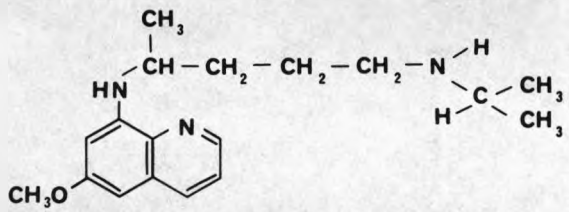
FIGURE 1.2 Chemical structures of compounds involved in the development of the 8-aminoquinoline antimalarials.



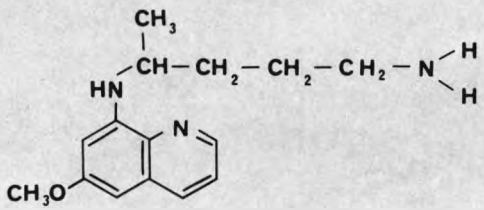
Pamaquine



Pentaquine



Isopentaquine



Primaquine

FIGURE 1.3 Chemical structures of important 8-aminoquinoline antimalarials

the search for high efficacy, low toxicity antimalarials has continued. During the Second World War a large scale co-operative antimalarial programme was initiated. Several hundred 8-aminoquinolines were synthesised and tested. Three of these compounds, pentaquine, isopentaquine and primaquine (figure 1.3) were selected for further study and it was primaquine which was shown to be the most suitable.

1.3.2 STRUCTURE-ACTIVITY RELATIONSHIPS OF 8-AMINOQUINOLINE ANTIMALARIALS

The antimalarial activity and toxicity of the 8-aminoquinolines is determined both by the functional group composition of the 8-amino side chain as well as the substitutions of the quinoline nucleus. It is generally assumed that the quinoline nucleus rather than other polyaromatic rings, is a pre-requisite for schizontocidal activity. Archer et al. (1980), having replaced the quinoline nucleus with naphthalene, produced several analogs of pamaquine and primaquine. Most of these compounds were either inactive or toxic, with the exception of 1,2-dimethoxy-4-4 - amino-1-ethylbutyl aminonaphthalene, which did possess antimalarial activity. This indicates that the quinoline nucleus is not an essential pre-requisite for antimalarial activity. In contrast, Carroll et al. (1976) have substituted the quinoline ring with various reduced quinoline ring analogues and none of the resulting compounds showed any antimalarial activity.

Extensive research has been carried out to investigate systematically the effects of substitution in the quinoline ring. Substitutions at the 2 position to form the benzoyloxy and ethyl derivatives of primaquine resulted in a reduction in both antimalarial activity and toxicity (Shefty 1978). Substitution at position 3 also produces a reduction in both activity and toxicity (Thompson and Werbel 1972). Substitution at position 4, to produce 4-methylprimaquine was reported to increase the activity relative to primaquine two-fold and at the same time reduce toxicity (Elderfield et al. 1953). Following on from this Lamontagne et al. (1977) and Carroll et al. (1979) synthesised a number of 4-substituted derivatives and found that methyl, ethyl and vinyl derivatives were less toxic and either as potent or slightly more potent as antimalarials than primaquine, but none of these derivatives have as yet become available for clinical use. Substitution at position 5 markedly reduces toxicity with a much smaller compromise of antimalarial activity (Chen 1977; Tanabe 1978). The 6 position on the quinoline ring is of primary importance to both the adverse and beneficial effects of the 8-aminoquinolines. Substitution at this position with an oxygen function, e.g. hydroxy or methoxy, greatly increases both toxicity and activity. Absence of these functional groups results in the loss of both of these responses. Primaquine and all 8-aminoquinolines presently under development retain this 6-methoxy group. Substitution at position 7 abolishes activity (Thompson and Werbel 1972) and multiposition substitution has yet failed to produce a compound with any advantage over primaquine (Carroll 1980).

The 8-amino side chain has been the most extensively studied structural feature of the 8-aminoquinolines. Optimal activity is obtained with an 8-amino side chain of between 2 and 6 methylene groups terminating with an amine group (an odd number of methylene groups being slightly more active than an even number). Although the terminal amine group may be primary, secondary or tertiary the 8-amino group must be secondary and not tertiary (Thompson and Werbel 1972). The 8-amino side chain of primaquine contains a chiral centre (figure 1.5).It has been shown that both racemic primaquine and its d (+) and l (-) isomers are equipotent antimalarials. The toxicity of the l (-) isomer was however, four to five times greater than that of the d (+) isomer in the rhesus monkey (Schmidt et al. 1977). The reverse order of toxicity was observed in rodents.

Structure-activity data for the 8-aminoquinolines has been obtained from studies which have tested the compounds against blood schizonts, although the major use of this group of drugs is as tissue schizonticides. In addition, it is widely believed that the 8-aminoquinolines themselves are inactive, requiring biotransformation to active metabolites (Greenberg et al. 1951). Therefore, analysis of structure activity relationships among parent compounds is unlikely to prove very useful in elucidating the mechanism of action and toxicity of this group of drugs. The investigation of metabolite structure-activity relationships is likely to prove most useful.

1.4 THE PHARMACOLOGY OF PRIMAQUINE

1.4.1 MECHANISM OF ACTION

Both primaquine and primaquine derivatives have been shown experimentally to influence the normal biochemical processes undertaken by the malaria parasite. Although all of these actions may contribute to the overall antimalarial effect, they are at present discrete observations and further investigation is required before the mode of action of this drug can confidently be described.

Primaquine has been shown to interfere with various aspects of parasite biochemistry including phospholipid metabolism (Wittels 1970; MIller 1976) and protein synthesis (Whichard 1968; Morris et al. 1970). The binding of primaquine to parasite DNA and the resultant inhibition of DNA function is presumed to be related in part to its antimalarial action. Primaquine produces morphological changes to the exo-erythrocytic stages of P.berghei and P.fallax parasites; these organisms being experimental models of malaria (Howells et al. 1970). Lesions of parasite mitochondria in cultured exo-erythrocytic stages of P.fallax has been associated with the presence of [^{3} H] substances after incubation with [^{3} H] primaquine (Aikawa and Beaudoin 1970). However, it is uncertain whether mitochondrial respiration is of importance for parasite survival in the tissue stages of mammalian plasmodia (Grewal 1981; Homewood 1977).

In spite of the observations described above, primaquine

is believed to be inactive *per se*, with both toxicity and antimalarial activity being attributed to one or more of its metabolites. This conclusion has been derived from experience gained from other 8-aminoquinolines, namely pamaquine and pentaquine, little information being available concerning the relative activity of primaquine *in vivo* versus *in vitro*. Indeed the evidence resulting in this theory is in many ways unsatisfactory as it is based on differences in methaemoglobinaemic, haemolytic or antimalarial properties of the parent 8-aminoquinoline when compared to either synthetically derived putative metabolites of the drug (Allahyari *et al* 1984) or to unidentified compounds extracted from biological fluids of animals dosed with the parent drug (Brodie and Udenfriend 1950; Josephson *et al* 1951a; Strother *et al.* 1981). Additional support for the role of metabolites as the active products of 8-aminoquinoline therapy comes from the observation that drug dose but not plasma concentration of the parent drug, correlate with toxicity and antimalarial activity (Carson 1984).

A mechanism of action for the 8-aminoquinolines based on metabolites has been proposed. Josephson *et al.* (1951a) pointed to the possible existence of a quinoline 5,6-diquinone metabolite of primaquine. This compound could exist in oxidation reduction equilibrium with the 5,6 dihydroxy derivative . It has been suggested that this metabolite, if it exists *in vivo*, could interact competitively with the naturally occuring redox compound ubiquinone, which is linked via dihydroorotate dehydrogenase to pyrimidine synthesis.

and Coombes 1977).

Recent work has pointed to the sensitivity of malaria parasites to oxidative stress, brought about by highly reactive chemical species, such as free radicals (Allison and Eugui 1982). Indeed it has been hypothesised that immunity to malaria parasites is due to the local production of free radicals of endogenous substances by macrophage metabolism. Consistent with this hypothesis, Clark and Hunt (1983) showed a rapid reduction in parasitaemia after administration of the diabetogenic agent alloxan which is itself metabolised to free radicals. In fact, primaquine is metabolised to compounds, presumably free radical species, capable of exerting an oxidative stress (Cohen and Hochstein 1964) and it is believed that these substances are the cause of toxicity in glucose-6-phosphate dehydrogenase (G-6-PD) deficient erythrocytes. It is conceivable that both the activity and toxicity of primaquine may be due to the generation of reactive oxygen species during the drug's metabolic breakdown. However, the studies carried out by Clark and Hunt (1983) utilised erythrocytic parasites, whereas the potential susceptibility of the tissue parasites, which is primaquine's primary site of action, was not investigated. Also, the above hypothesis suggests that any compound capable of producing free radicals should have an antimalarial effect against blood stages of malaria and primaquine has little effect at this site.

It becomes apparent from this section that one or any number of mechanisms may contribute to primaquine's activity,

however a complete understanding of the drug's metabolism and the interaction between the exo-erythrocytic parasite and primaquine or its metabolites is required before any firm conclusions can be drawn.

1.4.2 PRIMAQUINE TOXICITY

The major complications of primaquine therapy are methaemoglobinaemia and in individuals deficient in glucose-6-phosphate dehydrogenase (G-6-PD), haemolysis. Other side effects include dose-dependent gastro-intestinal disorders and immunosuppression caused by inhibition of lymphocyte proliferation.

Methaemoglobin is formed by the oxidation of the iron in haem from the ferrous [Fe^{2+}] to the ferric [Fe^{3+}] state. Putative primaquine metabolites exert an oxidant stress (i.e. by forming highly reactive species) on the erythrocyte resulting in methaemoglobinaemia. Intracellular reduced glutathione (GSH) is responsible for protecting the cell against oxidant stress and it is noticeable that methaemoglobin formation is most severe in older cells which generally have reduced levels of GSH.

The haemolytic effects of 8-aminoquinolines have been extensively investigated and discussed (Beutler 1959; Tarlov et al. 1962). Haemolysis was found to occur more readily in negroes than Caucasians. Carson et al. (1956) showed that the basis for this haemolysis was a deficiency in the enzyme glucose-6-phosphate dehydrogenase. Two variants of this x-linked deficiency are known: African variant A and the

1=G-6P dehydrogenase 2= 6-PG dehydrogenase 3= glutathione reductase 4= glutathione peroxidase 5= catalase

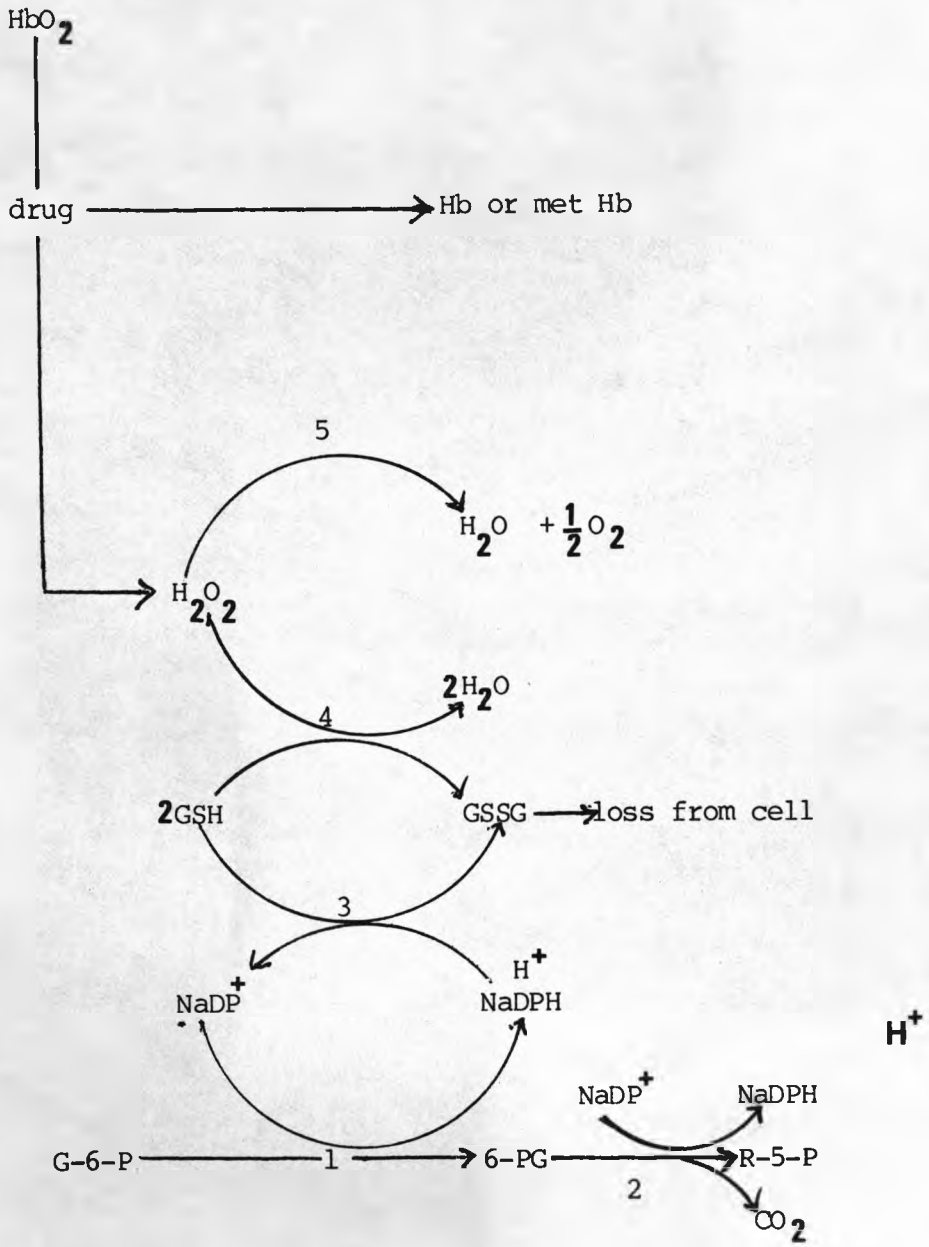


FIGURE 1.4 Glucose metabolism in the human red cell and the effect of oxidative stress.

Asian variant B. Haemolysis is most severe in individuals with type B deficiency. The severity of the haemolysis is directly related both to the dose of the drug and the degree of enzyme deficiency.

The biochemical basis for this haemolysis appears to be due to the role of G-6-PD in the pentose-phosphate pathway, (figure 1.4) which is responsible for 10% of cellular glycolysis. This pathway is involved in the generation of GSH via glutathione reductase with NADPH as a H^+ donor. GSH protects the cell against oxidant stress, detoxifying peroxides via glutathione peroxidase.

Erythrocytes deficient in G-6-PD are unable to detoxify oxidising compounds due to their reduced levels of GSH. These compounds are then able to react with vital cell components such as free sulphydryl groups in the cell wall. This is believed to cause a breakdown of cell walls resulting eventually in lysis.

It has been suggested that G-6-PD deficiency confers some degree of protection against malaria (Allison and Clyde 1961; Gilles et al. 1961). Luzzato et al. (1969) found a 2:80 ratio in parasite load between G-6-PD deficient and normal cells of females heterozygous for this deficiency. An explanation for selective advantage of G-6-PD deficient over normal individuals in resisting malaria infection has been put forward by Eaton et al (1976). They showed that the parasite itself exerts an oxidative stress on the red cell presumably by either directly producing free radicals or indirectly producing these species by an interaction with

endogenous compounds. Therefore, infection of G-6-PD deficient cells by the malaria parasite results in cell lysis, brought on by the combination of increased oxidant stress due to parasite derived free radicals and diminished capacity of deficient cells to neutralise this stress via reduced glutathione. This leads to destruction of the cell and the parasite within.

1.5 METABOLISM AND PHARMACOKINETICS OF THE 8-AMINOQUINOLINES

1.5.1 METABOLISM OF PRIMAQUINE RELATED 8-AMINOQUINOLINES

The metabolic fate of the 8-aminoquinolines has been under investigation since the 1950's with much of this work concentrated on the two compounds, pamaquine and pentaquine which are predecessors of the main therapeutic agent in this class, namely primaquine.

1.5.1.1 PAMAQUINE

In 1949 Zubrod showed that pamaquine was rapidly metabolised, with only 1% of the drug being excreted unchanged. Hughes and Schmidt (1950) found that the metabolism of various 8-aminoquinolines in the rhesus monkey was both fast and extensive. Two types of metabolites were formed; ethylene dichloride insoluble and ethylene dichloride soluble. Although exact structures could not be determined, both types of metabolite were isolated from all quinolines studied (including primaquine) irrespective of the route of administration. Brodie and Udenfriend (1950) identified two

metabolites from the urine and plasma of dogs receiving pamaquine. One metabolite was found to be stable to changes in pH, fluorescent at alkaline pH and non-toxic to erythrocytes in vitro. The other metabolite was stable only at acid pH and when incubated with erythrocytes caused methaemoglobin formation in lysed cells and haemolysis in normal cells. Both pamaquine and 8-aminoquinoline were shown to be non-toxic in in vitro incubations, but 5-hydroxy-8-aminoquinoline was. From these results they suggested that this pH sensitive metabolite could be a 5-hydroxy derivative. The therapeutic activity of the individual metabolites or the 5-hydroxy-8-aminoquinoline were not investigated.

Josephson et al. (1951 a and b) isolated a pamaquine metabolite from chicken droppings which was found to be 16 times more active against P.gallinaceum in culture than the parent compound. They showed the structure of this metabolite to be 8-(diethylamino-1 -methylbutylamino) -5,6-quinoline quinone. The identification of this metabolite was based on its conversion to a dipicrate salt and comparison with authentic diquinone similarly treated. However, this compound is produced when pamaquine is irradiated with UV light (Josephson et al 1951 a) and when pamaquine is incubated with chicken droppings (Greenberg et al. 1951). The existence of the quinoline quinone presumes the existence of one or more intermediates in which the methoxy group is demethylated and the 5 position oxidised.

Bami et al. (1960) suggested that pamaquine was metabolised via oxidative pathways eventually producing quinolinic acid from 5-6-quinoline quinone, nicotinic acid

quinolinic acid from 5-6 quinoline quinone, nicotinic acid and some smaller molecular weight compounds which he measured in urine. However, no evidence was given for the existence or structure of metabolic intermediates and the compounds he measured can be found in urine under normal conditions when the drug has not been administered.

1.5.1.2 PENTAQUINE

Elderfield and Smith (1953) studied the metabolism of [^{15}N] pentaquine (labelled in either the 8 position or at the terminal-N of the side chain) in rhesus monkeys. They showed metabolism to be rapid with initial cleavage of the side chain and further metabolism eventually releasing nitrogen.

The metabolism of [^{14}C] pentaquine labelled in either the 6-methoxy position or in the side chain was investigated by Smith (1956). He chromatographically characterised seven distinct radioactive peaks which he described as metabolites. The evidence presented suggested the first step in the metabolic pathway to be cleavage at the 6-methoxy position producing 6-desmethylpentaquine. The conversion of this metabolite to the 5-hydroxy-6-desmethyl derivative could possibly be the second step, this reaction having been shown to take place when 6-desmethylpentaquine is treated with molecular oxygen at room temperature and alkaline pH. These results contradict the findings of Brodie and Udenfriend that 5-hydroxylation is the first step in the metabolism of 8-aminoquinolines. The evidence of Smith is corroborated by the fact that a significant amount of the metabolites isolated react with diazotised sulphanilic acid which requires an

unsubstituted 5 position. Smith gave no evidence as to the structure of the radioactive peaks characterised.

Fraser and Vesell (1968) showed that hydroxylation increased the toxicity of 8-aminoquinolines. They found that 8 - amino -5,6- quinoline diol and 5- hydroxy-6 -desmethyl pentaquine were more active than the parent compound in increasing the mechanical fragility and methaemoglobin content of erythrocytes *in vitro* and these effects were greater in G-6-PD deficient cells than normals.

1.5.2 THE METABOLISM OF PRIMAQUINE

Baty *et al.* (1975) identified 6-methoxy 8-aminoquinoline as a primaquine metabolite, less than 4% of the dose being excreted as this metabolite and primaquine over 24 hrs. This compound was shown by Brodie and Udenfriend to have no toxicity *in vitro*, is thought to be a contaminant in some commercial preparations of primaquine and has eluded detection by other workers.

Based on the evidence of earlier workers with pentaquine and pamaquine, Fletcher *et al.* (1977) suggested the existence of 8-amino-5-hydroxy-6-desmethyl quinoline as a primaquine metabolite though no direct experimental evidence for the existence of this compound was provided. Nonetheless, investigations *in vitro* showed this compound to have some of the toxic effects that are associated with primaquine therapy. The existence and action of such a metabolite *in vivo* remains to be proved. They also pointed to the work of Alving (1962) and speculated that such a diol may be further

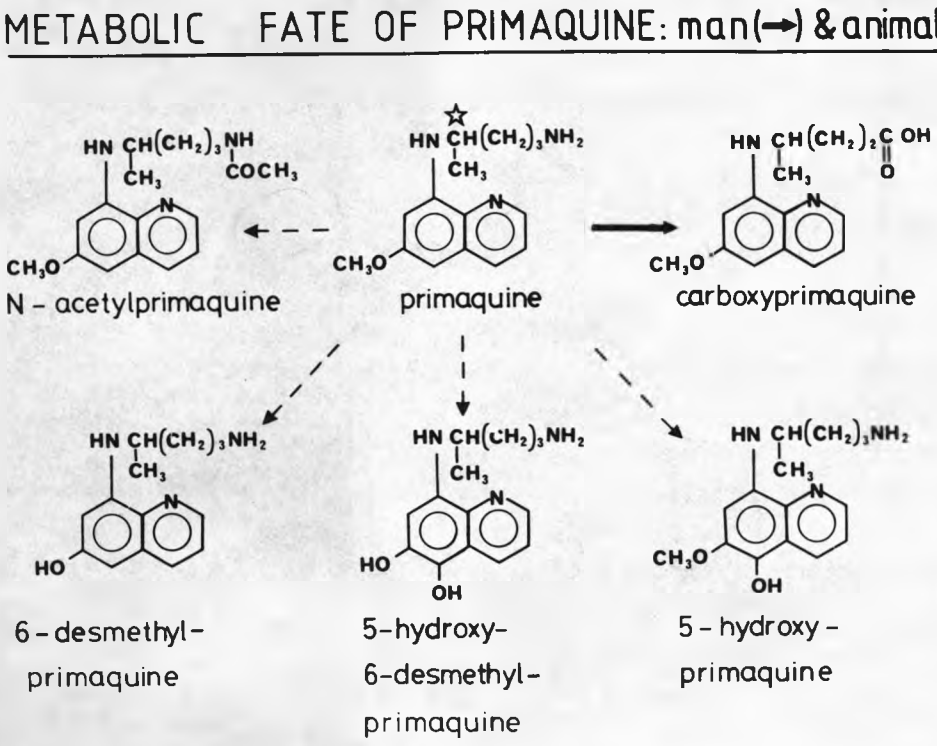


FIGURE 1.5 Chemical structure of primaquine, showing the position of the asymmetric C atom and the chemical structures of primaquine derivatives.

metabolised to form "a highly reactive resonating type of quinone structure", with a greater toxicity.

Holbrook et al. (1981) investigated the tissue distribution of tritiated primaquine in the rat (labelled in the 6 methoxy group). Tissue concentrations at each time interval were in the order:-

lung > liver > kidneys > spleen > heart > brain > arterial blood

Peak concentrations in all tissues except brain occurred at 15 mins. and at 3 hours 90% of the [^{3}H] label was in the form of a breakdown product. The [^{3}H] measured by Holbrook was mainly in the form of water and formaldehyde due to the rapid O-demethylation at the 6 position in the quinoline ring as shown by Smith (1956). Holbrook was unable to demonstrate any [^{3} H] metabolites containing the quinoline moiety in either tissue extracts or from [^{3} H] primaquine incubated with microsomal enzymes.

Carson et al. (1981) used high performance liquid chromatography to investigate the metabolism of primaquine. Volunteers were given a supra-pharmacological dose of primaquine (90-120mg). He reported the existence of two metabolites in plasma and a third in the urine. These metabolites could not be characterised and their therapeutic or toxic effects are unknown.

Strother et al. (1981) studied the metabolism of tritiated primaquine labelled in the quinoline ring. Metabolism in vivo was studied in the dog and metabolism in

vitro studied using microsomal liver extracts from mice. Only 16% of administered radioactivity was excreted over 8 hours in urine. Strother showed the existence of at least five metabolic products of primaquine using a variety of TLC systems and known standards. 5-hydroxyprimaquine was suggested as a major metabolite with some 6-desmethyl derivatives and only small amounts of N-dealkylated compounds. The 5-hydroxy and to a lesser extent the 6-desmethyl derivatives were able to promote methaemoglobin formation in vitro.

Strother suggested that the existence of both the 5-hydroxy and the 6-desmethyl metabolites could point to the existence of the 5-hydroxy-6-desmethyl derivative (figure 1.5) suggested by Brodie and Udenfried's earlier work on pamaquine (1950). This could then be converted to the 5,6 diquinone as implied by Josephson et al (1951a). Strother also suggested that the 5-hydroxy derivative could be converted to the powerfully oxidising quinoneimine.

It is apparent from the review presented here concerning primaquine that there is a dearth of definitive information on the metabolic fate of this compound. Furthermore, very little interest has been directed towards possible biotransformation products arising from metabolism at the alkylamino side chain. More recently, Clark et al. (1981) and Hufford et al. (1983) isolated two side chain metabolites of primaquine, but only from microbial cultures. These are the acetylated metabolite 8-(4-acetamido -1-methylbutylamino) -6-methoxyquinoline (figure 1.5) and the carboxylic acid

derivative, 8-(3-carboxyl-1-methylpropylamino)-6-methoxyquinoline (figure 1.5). Neither compounds have as yet been identified in man although the carboxylic acid metabolite has been identified in the plasma of rats treated with primaquine (Baker et al. 1982). The toxicity and antimalarial activities of these compounds are yet to be evaluated.

The metabolism of the 8-aminoquinoline class of compounds is poorly understood. The available evidence suggests that these compounds are rapidly and extensively metabolised. The metabolites are believed to have greater therapeutic and toxic activity than the parent drug, with the 5 and 6 positions of the quinoline moiety being of primary importance. In the case of primaquine it has been proposed that the compound undergoes metabolism to the 5-hydroxy-6-desmethyl derivative which can then be converted to the quinonimine. Primaquine has also been shown to undergo side chain metabolism to an acetylated metabolite in microbial cultures and to a carboxylic acid derivative in microbial cultures and the rat.

It is unknown whether both the toxic and therapeutic activity of primaquine can be attributed to the same metabolite. Schmidt et al (1977)investigated the sub-acute toxicities of racemic primaquine and its d (+) and l (-) isomers in the rhesus monkey. They found all three compounds to be equipotent antimalarials but the d isomer was some four times less toxic than the l isomer with the racemate toxicity falling between that of the two isomers (this trend was

reversed when acute toxicity was studied in mice). It is yet to be determined whether this effect is due to differences in metabolism of the isomers, intrinsic differences in the dynamic activity of the isomers or if this difference in toxicity is seen at therapeutic levels and with human strains of malaria.

1.5.3 DRUG INTERACTIONS AND PRIMAQUINE

In addition to undergoing metabolism itself, primaquine has recently been shown to have a marked effect on the metabolism of other drugs given concomitantly. Primaquine has been shown to inhibit mixed function oxidase activity in the rat *in vivo*, increasing pentobarbitone sleeping time and zoxazolamine paralysis time as well as decreasing antipyrine elimination (Back *et al.* 1983a). In addition primaquine also decreased ethoxyresorufin O-deethylase activity and aminopyrine N-demethylase activity in *in vitro* microsomes. These studies have since been extended to man (Back *et al.* 1983b) where primaquine was shown to significantly inhibit the clearance of antipyrine to its three major metabolites.

1.5.4 THE PHARMACOKINETICS OF PRIMAQUINE

The pharmacokinetics of primaquine are poorly understood and this is, in part, due to the lack of suitably selective and sensitive methods for its determination in biological fluids (2.1.1.1). Greaves *et al.* (1980) studied the pharmacokinetics of primaquine after the administration of a single 45mg dose of the drug orally in Caucasians (n=6), G-

6-PD normal Thais (n=6) and G-6-PD deficient Thais (n=5). In addition, a preliminary study of the pharmacokinetics of primaquine after chronic dosing (15mg per day for 5 days) was undertaken in three Thai volunteers.

The results of their single-dose study showed that the drug was rapidly removed from the plasma possibly due to extensive metabolism and/or tissue localisation. Less than 1% of the administered dose was recovered unchanged in the urine over 24 hours, and less than 2% of the dose was present in the plasma at peak concentrations. These low levels were attributed to either degradation in the stomach, incomplete absorption, gut wall metabolism or a first-pass effect. The elimination half-life ($t_{\frac{1}{2}}$) for primaquine ranged from 3.7 and 6.3 hrs. for normal Thais and normal Caucasians. The estimated systemic clearance (Cl) and volume of distribution (Vd) for the drug were approximately 25l/h and 200l respectively, assuming a bioavailability of 1.

Greaves et al. (1980) found no significant difference in the pharmacokinetics of primaquine after a single 45mg dose when compared with results obtained after multiple dosing (15mg for 5 days). In addition, they found no difference in primaquine disposition between Caucasians, normal Thais and G-6-PD deficient Thais. Pre-treatment of volunteers with the 4-aminoquinoline chloroquine produced no significant alteration in the pharmacokinetics of primaquine.

The only other study to have investigated the pharmacokinetics of primaquine in man was described by Nora et al. (1984). The drug was administered to healthy

volunteers in doses ranging from 30 to 120mg and plasma primaquine levels were determined by HPLC. Elimination half-life is the only pharmacokinetic parameter reported by the authors and is quoted as 6.7h in one volunteer, which is in accordance with the values obtained by Greaves et al. (1980) for this parameter.

A number of investigations carried out in animals have reported some limited pharmacokinetic data (Clark et al. 1984; Baker et al. 1982 and 1984). However, these studies were designed primarily to investigate the tissue distribution and metabolism of primaquine and not to characterise the pharmacokinetics of the drug.

The studies which will be described in this thesis will attempt to provide more complete information concerning the pharmacokinetics and metabolism of primaquine in both experimental models and man. Particular emphasis will be directed towards explaining the low systemic concentrations of primaquine achieved after oral dosage. A measure of the drug's bioavailability in man will be determined after simultaneous administration of a standard oral dose of primaquine (45mg) together with an intravenous tracer dose of [^{14}C] labelled drug. This technique minimises the time-dependent variability which often results when drugs are given on separate occasions to the same individual. Knowledge of the bioavailability of primaquine will allow accurate determination of both the volume of distribution and systemic clearance of this drug. This data will indicate which factors contribute to the low plasma levels achieved after oral

dosing.

The effect of increasing dose size on the pharmacokinetics and metabolism of primaquine will be studied using the isolated perfused rat liver preparation, dose ranging studies (across the therapeutic range) in man will also be performed. The isolated perfused rat liver preparation will be used to investigate the disposition of the (+) and (-) isomers of primaquine when compared with racemate in order to establish if primaquine exhibits stereoselective pharmacokinetics.

A detailed evaluation of the effect of chronic administration on the disposition of primaquine will be carried out. The pharmacokinetics and metabolism of the drug will be studied in healthy Thai volunteers following the initial and final doses of the standard fourteen day regimen. These subjects represent a population at risk from malaria. Comparison between the pharmacokinetics of primaquine in this population with Caucasian volunteers will be undertaken, in orderto assess the importance of ethnic differences in this drugs disposition.

Finally, factors which contribute to the propensity of primaquine to alter drug metabolism will be studied, using antipyrine elimination from the isolated perfused rat liver as a model system. The factors to be examined will be dose size and isomer form. In addition the potential of putative metabolites to influence drug metabolism will also be investigated in in vitro microsomes.

1.6 GENERAL PRINCIPLES OF PHARMACOKINETICS AND DRUG METABOLISM

1.6.1 INTRODUCTION

The work to be described in this thesis is directed towards the improvement of our understanding of the pharmacokinetics of primaquine. Pharmacokinetics represents a quantitative description of the processes of absorption, distribution, metabolism and excretion of drugs. The effect of a number of factors which might be expected to influence the absorption and disposition (distribution and elimination) of primaquine will also be examined. In addition, pharmacokinetic aspects of drug interactions involving primaquine will also be investigated. In view of this, it is pertinent to review the concepts and principles which will be applied throughout this thesis.

1.6.2 DRUG ABSORPTION

All drugs which are administered extravascularly and are required to act systemically must first be absorbed. This involves the transfer of drug in solution from the site of administration to the site of drug measurement (usually blood or plasma), across the separating biological membranes. The rate and extent of the absorption process can have marked effects on the time of onset, intensity and duration of a pharmacological response produced by a drug. Generally this transfer process results from the passive diffusion of drug molecules from a region of high concentration to a region of

low concentration, although for a number of compounds absorption is an active process requiring the expenditure of energy.

A number of factors can influence the absorption of a drug, they include: dissolution characteristics, molecular weight, the pka of the drug, lipophilicity, environmental pH, blood flow and gastric motility. A major determinant of the extent of drug absorption is the extent of pre-systemic elimination which refers to the loss of drug due to metabolism prior to entering the systemic circulation (Pond and Tozer 1984; George and Shand 1982). The term bioavailability or systemic availability is used to describe the fraction of a drug which is transported from its site of administration to the site of measurement, usually the systemic circulation (1.6.6.6).

1.6.3 DRUG DISTRIBUTION

Having entered the systemic circulation a drug can distribute throughout the body. Distribution is a reversible transfer process. The rate and extent of drug distribution is dependent uponthe physico-chemical properties of the drug, blood flow to various tissues and the binding of the drug to various tissue components and plasma proteins. Once distribution is complete drug concentrations throughout the body are at equilibrium. Alterations in plasma drug concentrations will then reflect similar changes in tissue concentrations throughout the body.

1.6.4 DRUG ELIMINATION

Drug elimination refers to the irreversible loss of drug from the body. Drug elimination is brought about by the processes of biotransformation and excretion. Hydrophilic compounds can readily be excreted, primarily by renal mechanisms. However, the majority of drugs are non-polar lipophilic compounds and as such require conversion to more polar species prior to excretion. Although drugs are often metabolised to polar, pharmacologically inactive compounds there are occasions when metabolism can result in the formation of either toxic or active metabolites of pronounced clinical relevance.

Many tissues of the body are capable of metabolising drugs including: lung, kidney, muscle and plasma, quantitatively, however the most important organ with respect to drug metabolism is the liver. Drug metabolism can generally be divided into phase I reactions which include drug oxidation, reduction and hydrolysis and phase II reactions which involve the conjugation of drug or drug metabolites formed by a phase I reaction with an endogenous molecule, such as glucuronic acid or glutathione. Drugs frequently undergo metabolism simultaneously by several pathways and the amount of metabolite formed is dependent upon the relative rates of metabolism by each route.

A large number of drug biotransformations have been shown to be catalysed by enzymes located in the endoplasmic reticulum of hepatocytes. These enzymes have been termed microsomal, as they can be isolated from microsomes which are

small vesicles formed after mechanical disruption of the endoplasmic reticulum. In particular, a group of enzymes classified as cytochrome P_{450} mixed function oxidase have been identified which are capable of metabolising a large number of drug substrates. The observation that these enzymes can metabolise a wide variety of structurally unrelated compounds has led to the assumption that they show a wide and overlapping substrate specificity (Timbrell 1982; Bentley and Oesh 1982; Lake and Gangolli 1981; Trager 1981). Although microsomal enzymes appear to be of primary importance with respect to drug metabolism, drug metabolising enzymes have been isolated from many other sources such as mitochondria, lysosomes, nuclei and cytosol.

Excretion is the process whereby both drug and drug metabolites are removed from the body. Renal and biliary mechanisms are the most important excretory processes, although excretion can take place through sweat, saliva or expired air. Drugs and metabolites are excreted into the urine by glomerular filtration, active secretion and diffusion. Certain compounds may, because of their physico-chemical properties, be subject to tubular reabsorption.

Excretion into bile can be a major route of elimination for some compounds. There is some evidence to suggest that the processes of renal and biliary excretion may be complementary elimination processes (Hirom et al. 1976). In general compounds secreted into bile have a larger molecular weight (>300 in the rat) than compounds excreted into urine; they also tend to be polar, ionised molecules (Rollins 1979). Drug conjugation results in an increased

molecular weight which can make the drug-conjugate complex an ideal candidate for biliary secretion. Drugs and drug metabolites which are secreted into bile can undergo reabsorption from the intestine back into the systemic circulation which can have both pharmacokinetic and pharmacodynamic consequences. This process is termed entero-hepatic recirculation (Klaassen and Watkins 1984).

1.6.5 DRUG INTERACTIONS

Any drug which has the potential to alter the processes of drug absorption, distribution or elimination may have a profound effect on the pharmacokinetcs of concomitantly administered drugs (Kristensen 1976). However, this brief review will be restricted to drug interactions which affect drug metabolism. As it is this aspect of drug interactions which will be investigated in this thesis in order to assess the potential of primaquine to influence the metabolism of other drugs (Chapter 3).

Enzyme induction is the term used to describe the increased functional capacity of drug metabolising systems after exposure to a variety of chemical substances. Hepatic enzyme induction can have profound clinical implications with respect to drugs that are extensively metabolised by the induced enzyme species. Induction can produce an enhancement of drug metabolism and therefore a reduction in drug levels within the body and an associated increase in metabolite formation (Park and Breckenridge 1981). Enzyme induction can

result from either increased enzyme synthesis and/or reduced enzyme degradation.

Inhibition of drug metabolism results in increased systemic concentrations of drugs that are metabolised by the inhibited enzyme. Studies have indicated that there are a large number of drug metabolising pathways which are potential targets for inhibitors, (Park and Breckenridge 1981). Testa and Jenner (1981) have characterised enzyme inhibitors as either reversible (non-destructive) or irreversible (destructive) and each of these mechanisms can be subdivided to include agents which are either directly acting or indirectly acting (i.e. those which require biotransformation,to form products which inhibit the enzyme). However, these categories are not all-inclusive since inhibitors can conceivably operate by either decreasing enzyme biosynthesis or enhancing enzyme degradation as opposed to a direct action against the enzyme itself.

1.6.6 THE PHARMACOKINETIC PARAMETERS

1.6.6.1 INTRODUCTION

Pharmacokinetics describes the processes of absorption and disposition in mathematical terms based on the time related changes in drug concentrations within the body. Classical pharmacokinetics has considered drug disposition in terms of compartmental models (Gibaldi and Perrier 1982; Riegelman et al. 1968). However, due to the difficulties which have been encountered in fitting plasma concentration/time data to specific models (Wagner 1976) and

the disparity which exists between compartments and true physiological or anatomical entities, there has been a movement towards the use of model-independent methods of pharmacokinetic analysis (Branch et al. 1973; Rowland et al. 1973; Wilkinson and Shand 1975).

Pharmacokinetic parameters are generally derived from time related drug and metabolite concentration changes in plasma or whole blood and the concentrations of drug and metabolites in urine. All pharmacokinetic evaluations presented within this thesis will be based upon the analysis of plasma and blood concentration data, using model-independent formulae, therefore it is these methods which will be described here (for pharmacokinetic evaluation from urine analysis see Gibaldi and Perrier 1982; Rowland and Tozer 1980).

Drug concentration/time data are generally presented graphically on a semi-logarithmic plot, that is time is plotted on the abcissa using a linear scale while concentration is plotted on the ordinate using a logarithmic scale (figure 1.6). This approach clearly discriminates between alterations in the rate of change in drug concentrations with time and also presents changes in drug concentration which usually occur exponentially, as linear processes (figure 1.6).

For all drugs that are administered by the extravascular route, the semi-logarithmic plot of drug concentration versus time will exhibit at least three phases. However, some of these processes may be so rapid that they

Typical semi logarithmic plot of plasma concentration versus time after oral drug administration

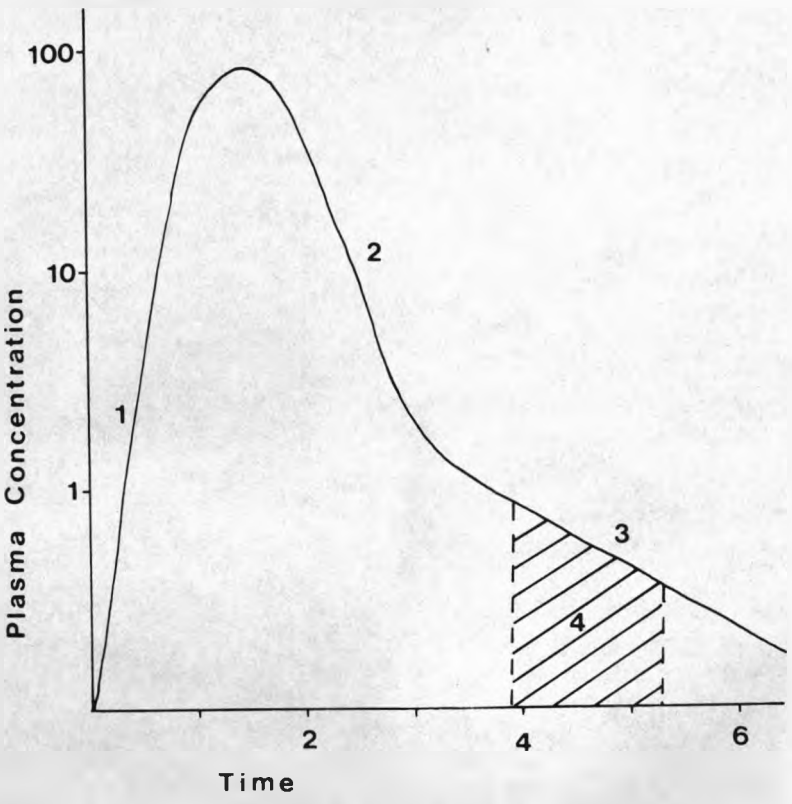


FIGURE 1.6 Plasma concentration/time profile

1=absorptive phase 2=distributive phase 3=elimination phase

4=trapezoid

cannot be determined experimentally. Initially drug concentrations rise due to absorption from the site of administration i.e. absorption phase. This is followed by one or more distributive phases where drug concentrations decline due to distribution throughout the body and finally there is an elimination phase, when drug concentrations decline due to irreversible loss of drug from the body.

In general, the rates of the above processes obey first-order kinetics and as such the rate of each process is proportional to the concentration of drug in the body. However, there are exceptions and a number of compounds exhibit non-linear or zero-order kinetics which can be described by the Michaelis-Menten equation (Gibaldi and Perrier 1982). The methods described here to determine pharmacokinetic parameters apply only to drugs which obey linear, first-order kinetics.

The pharmacokinetic parameters to be used throughout this thesis to describe the absorption and disposition of a drug may be divided into two categories: primary pharmacokinetic parameters i.e. clearance and volume of distribution and secondary or derived parameters including elimination half-life, area under the drug concentration/time curve and bioavailability. Alterations in the value of primary parameters can be reflected by a change in the values derived for the secondary parameters.

1.6.6.2 ELIMINATION HALF-LIFE

Elimination half-life is the time taken for plasma drug concentration and therefore the amount of drug in the

body to fall by one-half after the attainment of distribution equilibrium. This value is a constant for a drug obeying first-order elimination kinetics and is independent of the amount of drug in the body. Therefore the amount of drug eliminated decreases in successive half-lives. Theoretically then, the drug can never be completely removed from the body, however, in practical terms elimination is accepted as being complete after five half-lives have elapsed when 97% of the drug has been removed.

The elimination half-life can be determined from the semi-logarithmic plot of drug concentration versus time. The elimination phase is characterised by the linear equation:

$$\log Cp = \log Cp_{\emptyset} - Ks.t \qquad (1.1)$$

where Cp = drug concentration at time t, $Cp_{\emptyset}$ is the concentration at time Ø and Ks is the slope of the line (figure 1.6). Converting to natural logarithms produces:

$$Ln\ Cp = Ln\ Cp_{\emptyset} - Kt \qquad (1.2)$$

where K is the elimination rate constant equal to Ks X 2.303 (2.303 being the factor required to transpose values of log to Ln). The half-life is the time required for drug concentration to fall by one-half, therefore, if $Cp=Cp_{\emptyset}/2$ and $t = t_{\frac{1}{2}}$, substitution into equation 1.2 allows us to calculate $t_{\frac{1}{2}}$

$$K.t_{\frac{1}{2}} = Ln\ 2 = \emptyset.693 \text{ or } t_{\frac{1}{2}} = \emptyset.693/K \qquad (1.3)$$

1.6.6.3 CLEARANCE

Drug clearance describes the relationship between the rate of drug elimination and drug concentration. This is the most useful parameter for the evaluation of an elimination mechanism. Clearance is the volume of a biological fluid from which drug is removed per unit time. The value of clearance is dependent upon the site of measurement but independent of concentration for drugs which obey first-order elimination kinetics (i.e. clearance is constant). Total systemic clearance (Cl) is the sum of all metabolic and excretory clearance processes which is the sum of all organ clearances (1.6.6.4). There is an inter-relationship between the clearance of a drug, apparent volume of distribution and elimination half-life as follows

At equilibrium:

$$\textbf{Rate of elimination} = Cl \times Cp \qquad (1.4)$$

(where Cl = clearance and Cp = plasma concentration)

also $$\textbf{Rate of elimination} = K \times Ab \qquad (1.5)$$

(where K is the elimination rate constant and Ab is the amount of drug in the body i.e the drug concentration X volume of distribution or Cp X Vd)

therefore, combining 1.4 and 1.5

$$Cl \times Cp = K \times Cp \times Vd \quad \text{or} \quad Cl = K \times Vd \qquad (1.6)$$

since

$$t_{\frac{1}{2}} = 0.693/K \quad \text{then} \quad Cl = (0.693 \times Vd)/t_{\frac{1}{2}} \qquad (1.7)$$

From this relationship (1.7) it is apparent that changes in either drug clearance, volume of distribution or both can produce alterations in a drug's elimination half-life. However, calculation of drug clearance from this relationship relies on a knowledge of the drug's apparent volume of distribution which is only rarely available. An alternative method for calculating drug clearance from drug concentration versus time data is obtained as follows:-

At equilibrium:

$$\frac{dAb}{dt} = Cl \times Cp \qquad (1.8)$$

where dAb = the rate of drug elimination i.e. the rate of change in the amount of drug in the body with time. Therefore during a small interval of time dt. The amount of drug eliminated = Cl X Cp X dt.

Integration of equation 1.8 between time Ø to infinity produces the following:

$$\int_{\infty}^{0} dAb = Cl \times \int_{\infty}^{0} Cp.\ dt. \qquad (1.9)$$

$$\int_{\infty}^{0} dAb = \text{drug dose} \qquad (1.10)$$

$\int_{\infty}^{0} Cp.\ dt$ = the overall change in drug concentration from time Ø to infinity. (1.11) which is equal to the area under the plasma concentration/time curve from time Ø to infinity. therefore 1.9 can be rewritten as

$$\text{dose} = Cl \times \text{area} \qquad (1.12)$$

or $$Cl = \text{dose/area} \qquad (1.13)$$

The area under the drug concentration/time curve (AUC) is a measure of the amount of drug which has entered the systemic circulation. This area is measured by the trapezoidal rule (Gibaldi and Perrier 1982). The area under the curve from time =Ø to the time of the last data point t is divided into trapezoids (figure 1.6).The area of each trapezoid is calculated and the sum of these areas is equal to the area under the curve from time =Ø to time =t. The additional area from time = t to infinity is calculated from the ratio C_t /K, where C_t = drug concentration at time t and K = elimination rate constant, provided sufficient data points are available to characterise K.

$$AUC = AUC_{Ø-t} + C_t / K \qquad (1.15)$$

(ideally sampling schedules should be such that C_t /K constitutes a small percentage of total AUC).

1.6.6.4 PHYSIOLOGICAL APPROACH TO DRUG CLEARANCE (ORGAN CLEARANCE)

Clearance is the parameter which relates drug concentration to the rate of drug elimination. Systemic clearance (calculated as described in 1.6.6.3) is the sum of both metabolic and excretory clearance processes which is the sum of all organ clearances. The clearance of a drug across

an organ can be described in physiological terms which is useful in predicting the effects of changes in blood flow, plasma protein binding, enzyme activity or secretory activity on the clearance of a drug (Rowland and Tozer 1980; Wilkinson and Shand 1975; Wilkinson 1982; Nies et al. 1976; Tucker 1981). In this instance organ clearance is the product of organ blood flow (i.e. delivery of drug to the organ) and the extraction ratio for the drug by the organ i.e.

$$Cl = Q \times \frac{(CA - CV)}{CA} \qquad (1.16)$$

where Q = organ blood flow; CA = arterial drug concentration i.e. input; CV = venous drug concentration i.e. output. Or:

$$Cl = Q \times E \qquad (1.17)$$

where E = extraction ratio.

Drugs with an extraction ratio approaching unity are said to be high clearance compounds while drugs with an extraction ratio less than 0.2 are considered to be low clearance compounds. Alterations in blood flow, protein binding and intrinsic clearance (metabolising activity of the organ) can produce marked effects on a drug's pharmacokinetics and the degree and direction of these changes are dependent upon the drug's extraction ratio.

If we consider drugs which are primarily eliminated by the liver:

For high clearance drugs such as propranolol and lignocaine, it is assumed that the enzyme metabolising capacity of the liver (or the intrinsic clearance, which equals the theoretical maximal clearance of unbound drug at infinite hepatic blood flow)is more than sufficient to clear nearly all the drug delivered to the liver. In this case the value for hepatic clearance approaches the limiting value of liver blood flow and is sensitive to changes in liver blood flow,ie clearance is "flow dependent".Because of the excess of metabolising enzyme available for these high clearance processes, enzyme induction or inhibition should have little or no effect on the elimination of such compounds. The extraction process is so avid that protein bound as well as unbound drug will be metabolised. Under these circumstances clearance is termed "non-restrictive".

For low clearance drugs such as antipyrine, the primary determinant of hepatic clearance is intrinsic clearance. These compounds are insensitive to changes in blood flow and sensitive to changes in enzyme activity (ie induction or inhibition) and protein binding. If protein binding is high clearance will be "restrictive" as only free drug can be metabolised.

1.6.6.5 APPARENT VOLUME OF DISTRIBUTION

This parameter relates the measured drug concentration to

the total amount of drug in the body when distribution equilibrium has been achieved:

$$\text{i.e.} \qquad Vd = \frac{Ab}{Cp} \qquad (1.18)$$

where Vd = volume of distribution; Ab = the amount of drug in the body and Cp = the measured drug concentration. The value of this parameter rarely corresponds to a real physiological volume and is largely dependent upon the plasma and tissue binding of the drug, in addition to the physico-chemical properties of the drug which determine the extent to which it will partition into biological membranes. The volume of distribution can be calculated from plasma concentration versus time data as follows:-

combining 1.13 and 1.17,

$$Vd = \frac{dose}{area \times K} \qquad (1.19)$$

From the graphical plot of log drug concentration versus time the elimination rate constant and elimination half-life can be determined. After intravenous drug administration, both primary and secondary pharmacokinetic parameters can be derived from plasma drug concentration time data,as drug dose is known and AUC can be calculated.

1.6.6.6 BIOAVAILABILITY

Calculation of clearance and volume of distribution as described above can be carried out only when the dose of drug reaching the site of measurement is known, which, after intravascular dosage is equal to the dose administered. However, for drugs administered extravascularly, the amount of drug that reaches the site of measurement can be influenced by such factors as incomplete absorption, degradation in the stomach and intestine, metabolism by the gut flora, gut wall or liver and entero-hepatic recirculation. Under these circumstances the actual dose of a drug reaching the site of measurement is clearly different from the dose administered.

The term bioavailability is used to describe the completeness of absorption and is the fraction of the administered dose which reaches the site of measurement intact. The value of bioavailability (F) can be calculated following intravenous and oral administration of the drug.

$$F = \frac{dose_{IV}}{dose_{oral}} \bullet \frac{AUC_{oral}}{AUC_{IV}} \qquad (1.20)$$

This method for determining bioavailability assumes that the systemic clearance is the same following drug administration by either route at the drug concentration chosen (George and Shand 1983).

Therefore in order to determine the clearance and volume of distribution for a drug which is incompletely

bioavailable, the value for dose in equations 1.13 and 1.19 must be replaced by the value for F X dose.

The calculation of pharmacokinetic parameters is essential in order to predict alterations in drug concentrations as a function of time, dosage and route of administration. In addition, these parameters can be used in order to evaluate the effect of such factors as age, sex, disease, other drugs and environmental compounds on a drug's disposition. The pharmacokinetics of primaquine will be calculated using the equations described in this section after administration of the drug at a variety of dose sizes and by both the intravenous and oral routes. In addition, the pharmacokinetics of antipyrine in the IPRL will be calculated using the above formulae and the effect of primaquine on the value of these parameters will be assessed.

CHAPTER 2 METHODS

2.1 ANALYTICAL METHODS

2.1.1 DETERMINATION OF PRIMAQUINE IN BIOLOGICAL FLUIDS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.1.1.1 INTRODUCTION

Our understanding of the disposition of primaquine in animals and man has been severely limited due to the lack of suitably selective and sensitive methods available for the determination of the drug and its metabolites in biological fluids. Irvin and Irvin (1949) used fluorescence spectrophotometry to measure pamaquine concentrations. The fluorescence of pamaquine was attributed to the proton acceptor properties of the 6-methoxy group. This method was later modified for the determination of primaquine (Fletcher and Canning,personal communication) in concentrations greater than 1µg/ml. However, due to poor sensitivity, the necessity for large sample volumes (1-10ml), high blank values and interference from metabolites which retain the 6-methoxy group, this technique is unsuitable for the determination of primaquine after administration of therapeutic doses.

Other techniques which have proved unsuitable for the measurement of primaquine, again due to lack of sensivity and high blank values, include the spectrophotometric field test developed by Furanannu and Werrback (1965) and the thin layer chromatographic assay of Fusari et al. (1975). These methods had sensitivity limits of 3µg/ml and 13mg on plate

respectively.

A number of more sensitive and selective methods for the determination of primaquine in biological samples have recently been described. Rajagopolan et al. (1981) measured primaquine by electron capture gas chromatography (sensitivity limit 10 ng/ml) and Greaves et al (1979) developed a gas chromatographic-mass spectrometric procedure (sensitivity limit 30 ng/ml). However both these methods require derivatization of the parent drug. Additionally, they are unsuitable for the analysis of certain postulated primaquine metabolites which are thermally labile.

A high performance liquid chromatographic (HPLC) technique was developed by Carson et al. (1981). However this method could not detect primaquine at concentrations less than 50ng/ml. In addition they did not use an internal standard, which is essential for the accurate determination of drug levels. Baker et al. (1982) and Nora et al. (1984) have used HPLC to measure primaquine levels but again these procedures did not utilise an internal standard.

The HPLC method for primaquine determination described in this chapter is simple, sensitive, selective and overcomes many of the problems encountered by previous investigators.

2.1.1.2 CHROMATOGRAPHY

The method was developed on a Spectra-Physics liquid chromatograph. The SP 8700 solvent delivery system and SP 8750 organiser module equipped with a Rheodyne valve injection system was coupled to an SP 8300 fixed wavelength

UV absorbance detector, fitted with a mercury light-source emitting at 254nm. In the studies described in chapter 6 the separation was carried out on a Partisil 10 ODS III (10um particle size) reversed-phase column (20 cm X 0.6 cm O.D.). Reversed-phase chromatography was selected as it provides a higher degree of versatility than some other systems. In addition,metabolites (which are generally more polar than the parent drug) elute prior to the parent compound. Preliminary studies indicated that primaquine was strongly retained on columns which possessed active sites on the solid support, resulting in excessively long retention times and poor peak shape. Therefore the column which was eventually selected was termed "fully capped", that is the maximum number of active sites on the solid support had been reacted with the ODS stationary phase. The mobile phase consisted of water-acetonitrile-methanol (60:30:10) containing octanesulphonic acid (5×10^{-5} M) as an ion-pairing reagent, buffered to pH 3.5 with orthophosphoric acid and flowing at 1.5 ml/min. Originally a mobile phase of water-methanol (60:40) was used. However peak shape was improved by replacement of methanol (30%) with acetonitrile. The decrease in peak tailing is due to a reduction in non-specific hydrogen bonding produced by methanol. Octanesulphonic acid was used to produce an ion-pair with primaquine, resulting in a less polar, more lipid soluble complex with a greater affinity for the stationary phase and to prevent non-specific interactions between the ionised form of the drug and untreated sites on the solid support.

The chromatography conditions for primaquine

determination, for all subsequent studies other than those described in chapter 6, were modified from those described above. Samples were chromatographed on a u Bondapak "Rad-Pak" phenyl column housed in a Z-module which was fitted with a CN-Guard pack. Primaquine was eluted with a mobile phase of methanol:water (50/50 v/v containing triethylamine phosphate 1%), buffered to pH 7 with orthophosphoric acid and flowing at 3 ml/min. These modifications resulted in more rapid chromatography and a reduction in peak tailing.

2.1.1.3 SAMPLE TREATMENT PROCEDURE

The effect of different solvents (table 2.1), pH and solvent volume were examined in order to determine optimum extraction efficiency and the resulting procedure is described below. The extraction was carried out in a 10ml capacity glass culture tube, pre-treated with dichlorodimethylsilane in toluene (5% v/v) in order to minimise non-specific absorbtion. To samples of plasma, perfusate or urine (0.5-2.0 ml) containing the internal standard (8-(3-amino-methylpropylamino) -6-methoxyquinoline 100 ng) was added ammonia solution (0.88 specific gravity, 2ml), followed by vortex mixing (30 secs.). This mixture was extracted twice by mechanical tumbling (10 mins.) with a combination of hexane and ethyl acetate (9:1 v/v ,5ml) . After centrifugation (1000g for 10min) and separation,the organic phases were combined, evaporated to dryness under a steady stream of nitrogen and reconstituted in methanol (50

µl). An aliquot of 5-25 µl was injected onto the column.

2.1.1.4 STANDARD CURVES

Standard curves were prepared by adding known quantities of primaquine (5-200ng) to a fixed concentration of internal standard (100ng) in drug-free plasma ,perfusate or urine. Samples were analysed as described above and the peak height ratio of drug to internal standard was plotted against the corresponding primaquine weight. Peak height ratios of unknown samples were similarly determined and concentrations calculated from the standard curve. The extraction efficiency of primaquine was calculated; a) by comparison of peak heights of extracted primaquine with that of directly injected stock solutions and b) from the recovery of [^{14}C]-radioactivity following extraction of plasma or urine spiked with [^{14} C] labelled primaquine (specific activity 1.55 mCi/mmol- 10,000 DPM/ml, labelled in the quinoline ring).

2.1.1.5 ASSAY SPECIFICATIONS

The extraction procedure resulted in simple sample preparation. Chromatograms of plasma extracts derived from a blood sample taken pre-dose and another obtained after a single 45mg oral dose of primaquine (Chapter 6) are shown in figure 2.1 (A and B). These separations were achieved using the initial chromatography conditions described. The plasma extract from the volunteer receiving primaquine showed a distinct peak with a retention time of 7.6 mins. corresponding to primaquine (120ng/ml). This peak was

completely resolved from that of the internal standard which had a retention time of 5.2 mins. The peak eluting prior to the internal standard was an impurity in the internal standard stock solution and was not observed in extracts of blank plasma. Chromatograms of blank urine and a urine specimen collected after a single oral dose of primaquine (chapter 6) are shown in figure 2.2 (A and B). Representative chromatograms of a blank perfusate extract and a perfusate extract after administration of 5.Ømg primaquine diphosphate to the isolated perfused rat liver preparation (Chapter 4) are shown in figure 2.3 (A and B). This separation was achieved using the modified conditions and the "Rad-Pak" phenyl column. In figure 2.3B there are two distinct peaks at retention times of 3.5mins. and 4.5mins. which are not present in the blank extract (figure 2.3A). These peaks correspond to the internal standard (2μg/ml) and primaquine (1μg/ml) respectively.

The acetylated and carboxylated metabolites of primaquine (figure 1.5) described by Baker et al. (1983) did not interfere with the assay. These metabolites appeared in chromatograms of aqueous stock solutions with retention times of 14 mins. and 17 mins. respectively under the initial assay conditions. However, they did not appear in any plasma or urine extracts due to poor analytical recoveries under the conditions of this extraction.

We found no interference with chromatographic separation from the commonly used antimalarial drugs chloroquine, amodiaquine and pyrimethamine, from endogenous

compounds in the plasma or from the 5-hydroxy, 6-desmethyl or 5-hydroxy-6-desmethyl derivatives of primaquine (figure 1.5). A chromatographically resolved component of the extract from plasma obtained from a normal subject receiving primaquine (retention time 9 mins.; figure 2.1B), did not appear in blank plasma (figure 2.1A). This may be a metabolite, as yet unidentified. Additionally, in a number of urine extracts a minor endogenous component was seen to elute with a retention time between that of the internal standard and primaquine (figure 2.2). This resulted in a marginally reduced level of sensitivity in these samples.

The extraction solvent, hexane-ethylacetate (9:1) gave optimal recovery of primaquine with minimal extraction of endogenous compounds (table 2.1). Calibration curves were linear in the range 0-200ng (r=0.99, figure 2.4) and analytical recovery of primaquine was 93 + 5%(n=6). The minimum detectable quantity of primaquine in plasma which gave a peak size three times baseline noise at the highest detector sensitivity (X 0.0025 AUFS) corresponded to a concentration of 1ng/ml primaquine base. The intra and inter assay variations were between 2 and 9% (table 2.2). Adoption of the alternative chromatography conditions (i.e. using the "Rad-Pak" phenyl column) did not alter any of the above assay specifications.

The advantages of this assay over earlier methods are that both sample treatment and chromatography are rapid and simple. Furthermore, the assay is selective and due to its high sensitivity it is capable of measuring primaquine levels after clinically relevant doses.

TABLE 2.1. Choice of extraction solvent. % extraction efficiencies for Primaquine using various solvents after basification with ammonia (2ml)

Solvent	Ethyl acetate	Diethyl ether	Dichloro-methane	Chloro-form	Aceto-nitrile	Trichloro-ethane	Hexane / Ethyl acetate (9 : 1)
% Recovery	80	40	47	46	90*	*	93**

* Interference from endogenous Cpds.?

** % recovery determined by extraction of [^{14}C]labelled Primaquine, other recoveries determined by comparison of extracted samples with directly injected stock solutions.

TABLE 2.2 ASSAY COEFFICIENTS OF VARIATION

PRIMAQUINE

CONCENTRATION	25 ng/ml	100ng/ml
INTRA-ASSAY VARIATION	8.7% n=8	4.2% n=10
INTER-ASSAY VARIATION	5.0% n=5	2.7% n=5

CARBOXYLIC ACID METABOLITE

CONCENTRATION	400ng/ml
INTRA-ASSAY VARIATION	2.9% n=6
INTER-ASSAY VARIATION	5.0% n=6

ANTIPYRINE

CONCENTRATION	10 ug/ml
INTRA-ASSAY VARIATION	5.2% n=6
INTER-ASSAY VARIATION	3.9% n=6

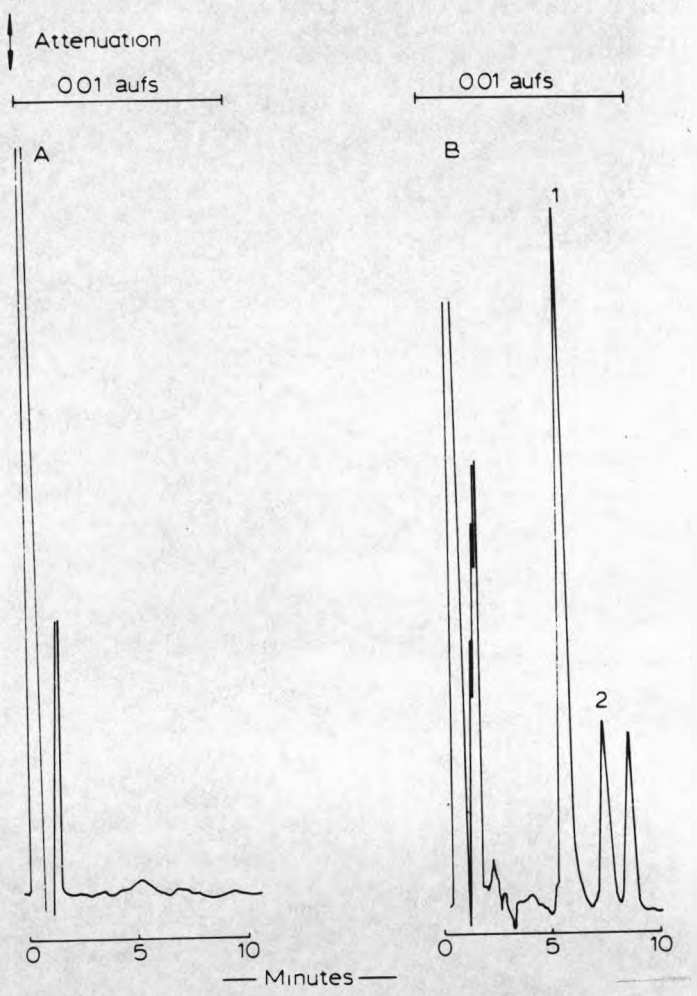


FIGURE 2.1 Chromatograms of (A) a blank plasma extract and (B) an extracted plasma sample obtained after administration of primaquine (45mg) to man (Chapter 6). 1= internal standard 2= primaquine (120ng/ml).

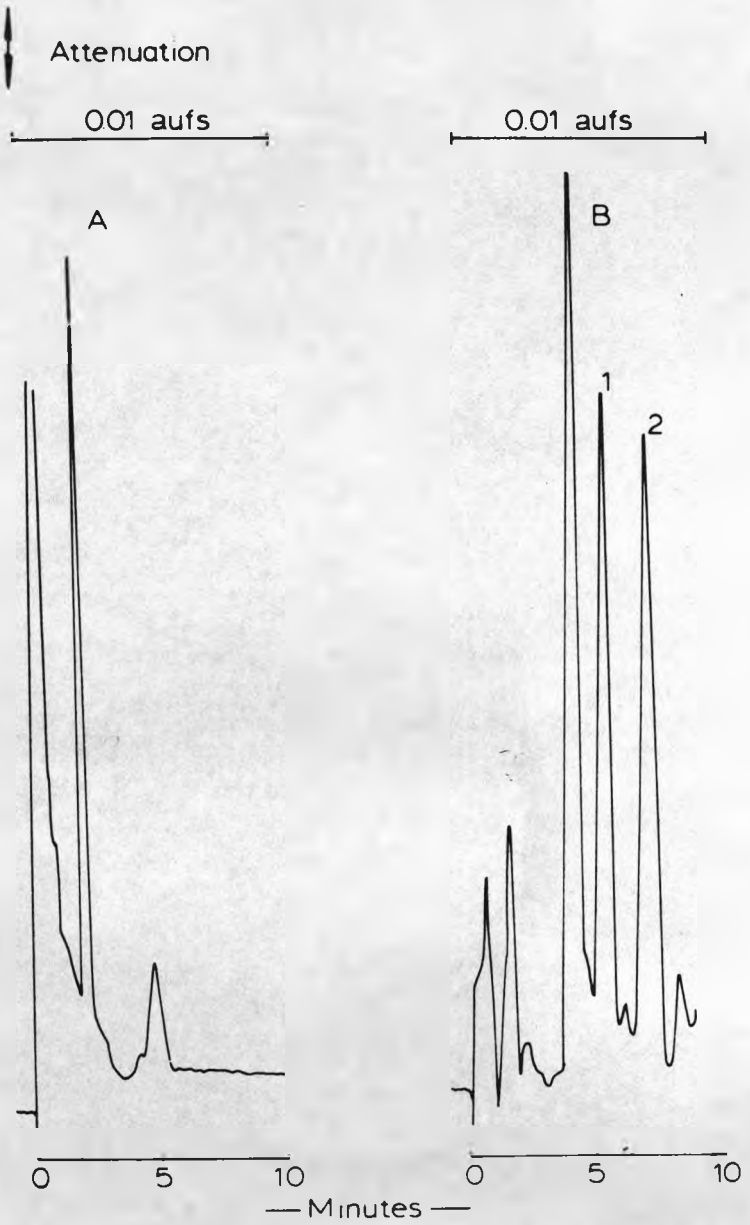


FIGURE 2.2 Chromatograms of (A) a blank urine extract and (B) a urine extract from a sample obtained after administration of primaquine (45mg) to man (Chapter 6). 1=internal standard 2= primaquine

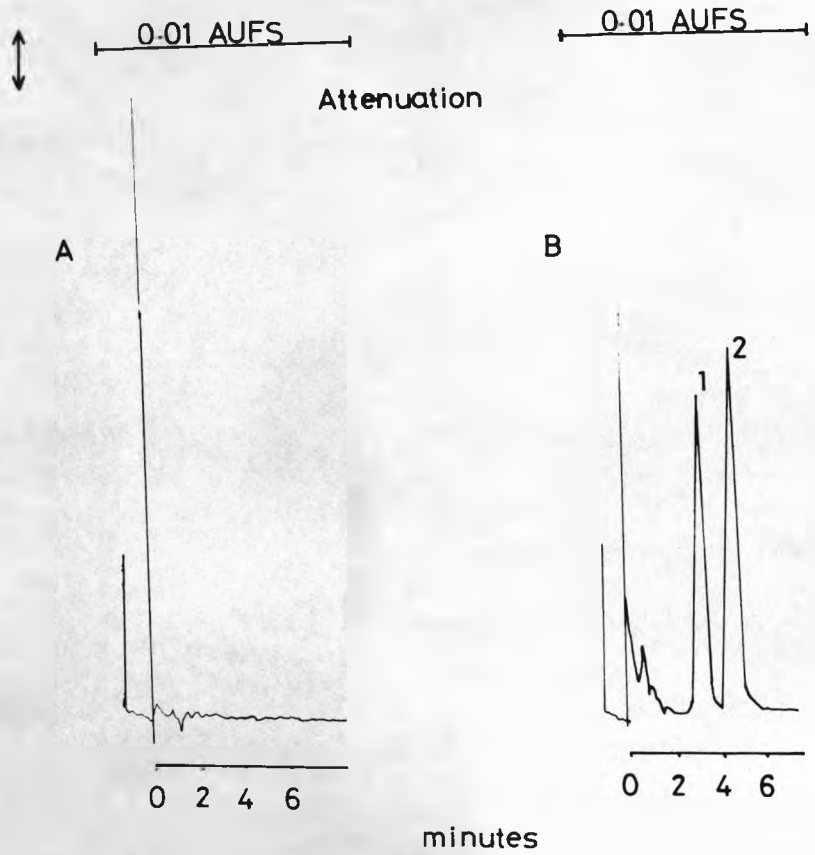


FIGURE 2.3 Chromatograms of (A) a blank perfusate extract and (B) a perfusate extract obtained after administration of primaquine to the IPRL (Chapter 4) 1=internal standard 2= primaquine (750ng/ml).

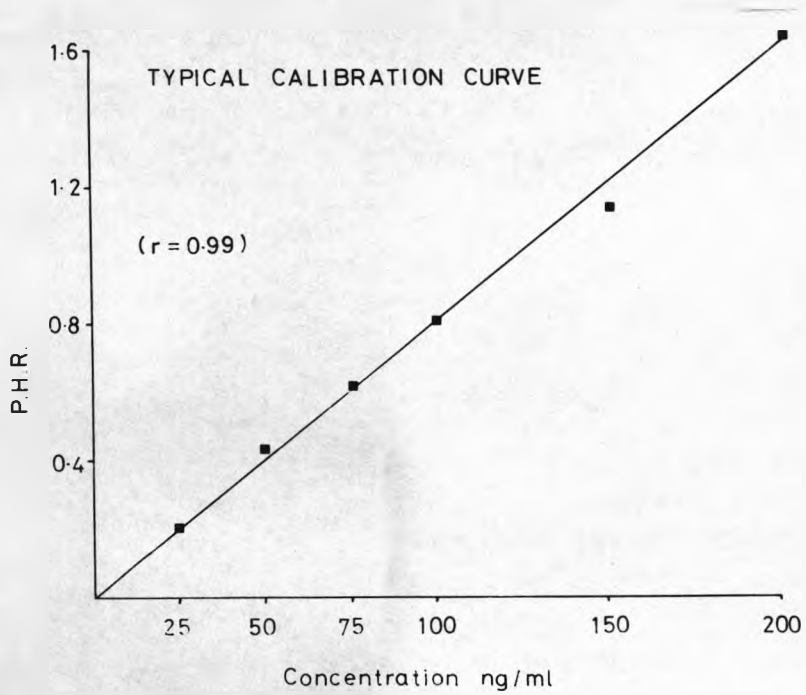


FIGURE 2.4 Typical calibration curve for primaquine

2.1.2 DETERMINATION OF THE CARBOXYLATED AND N-ACETYLATED DERIVATIVES OF PRIMAQUINE BY HPLC

2.1.2.1 INTRODUCTION

The carboxylic acid derivative of primaquine and N-acetylprimaquine have been isolated as primaquine metabolites following incubation of the drug with a variety of microbial cultures (Clark et al. 1981; Hufford et al. 1981). In addition, the carboxylic acid metabolite has been identified in the plasma of rats dosed with primaquine (Baker et al. 1982). HPLC assays are available for the quantitation of the carboxylic acid metabolite but not the N-acetyl derivative of primaquine (Baker et al 1982; Nora et al 1984). The method described here is capable of simultaneously measuring both of these compounds and the use of an internal standard increases the accuracy of quantitation. The development of this method is an essential prerequisite for investigations into the formation of these metabolites in both the isolated perfused rat liver preparation and in man.

2.1.2.2 CHROMATOGRAPHY

The liquid chromatograph used was as described in 2.1.1.2. Chromatographic separation of the carboxylic acid metabolite, N-acetylprimaquine and the internal standard indomethacin was carried out on a μ Bondapak "Rad-Pak" phenyl column housed in a Z-module which was fitted with a CN-Guard pak. The mobile phase consisted of water: methanol (65:35

v/v) containing triethylamine 1% (v/v) (buffered to pH 6.0 with orthophosphoric acid) and flowing at 3ml/min.

2.1.2.3 SAMPLE TREATMENT PROCEDURE

The extraction was carried out in a 10ml capacity glass culture tube as described in 2.1.1.3. To plasma, perfusate or urine samples (0.5ml) containing indomethacin (2.5 µg -25µl as an aqueous solution) was added an equal volume of phosphate buffer (NaH_2PO_4 ; 0.1M; pH 2.6) followed by vortex mixing (30 secs.). This mixture was extracted with a combination of hexane and ethylacetate (9:1 V/V; 5ml) by mechanical tumbling (10 mins.). After centrifugation (1000g for 10mins.) and separation, the organic phase was evaporated to dryness at 37°C under a steady stream of nitrogen. The residue was reconstituted in methanol (100µl) and 25µl was injected onto the chromatograph.

2.1.2.4 STANDARD CURVES

Standard curves were prepared as described in 2.1.1.4, with increasing quantities of the carboxylic acid metabolite (0.5-4 µg) or N-acetylprimaquine being added to a fixed quantity of indomethacin (5µg) in drug free plasma, perfusate or urine. Extraction efficiency was determined by comparison of the peak heights obtained from extracted samples with those from directly injected stock solutions of known concentration.

2.1.2.5 ASSAY SPECIFICATIONS

Sample treatment was rapid and simple. Chromatograms

of a blank plasma extract and a plasma extract obtained from blood of a volunteer who had received 45mg primaquine base orally (chapter 6) are shown in figure 2.6 (A and B). Chromatography of plasma extracts from both spiked samples and samples obtained from volunteers receiving primaquine consistently gave two peaks corresponding to the authentic carboxylic acid metabolite and indomethacin at retention times of 2.2 and 5.2 mins. respectively. N-acetylprimaquine was chromatographed with baseline separation at a retention time of 4.1 mins.

There was no chromatographic interference from either the commonly used antimalarials, chloroquine, amodiaquine and pyrimethamine, the postulated ring hydroxylated metabolites of primaquine, (figure 1.5) or from endogenous material. Furthermore, these basic compounds did not extract under the acidic conditions of the assay.

The assay has a limit of sensitivity of 75 ng/ml for the carboxylic acid metabolite and 1Ø ng/ml for N-acetylprimaquine as measured by a peak corresponding to four times baseline noise at Ø.Ø1 AUFS. Calibration curves were linear for the carboxylic acid derivative in the range Ø-4 ug/ml (r=Ø.99) and the analytical recovery was 45%. The intra and inter-assay variations for the carboxylic acid metabolite were 2.9% (n=6) and 5% (n=6) at a concentration of4ØØng/ml (table 2.2). Assay precision and variation for N-acetylprimaquine were not measured as it was never seen to exceed the minimum detectable concentration (1Øng/ml) in the plasma or urine of both human and animal studies.

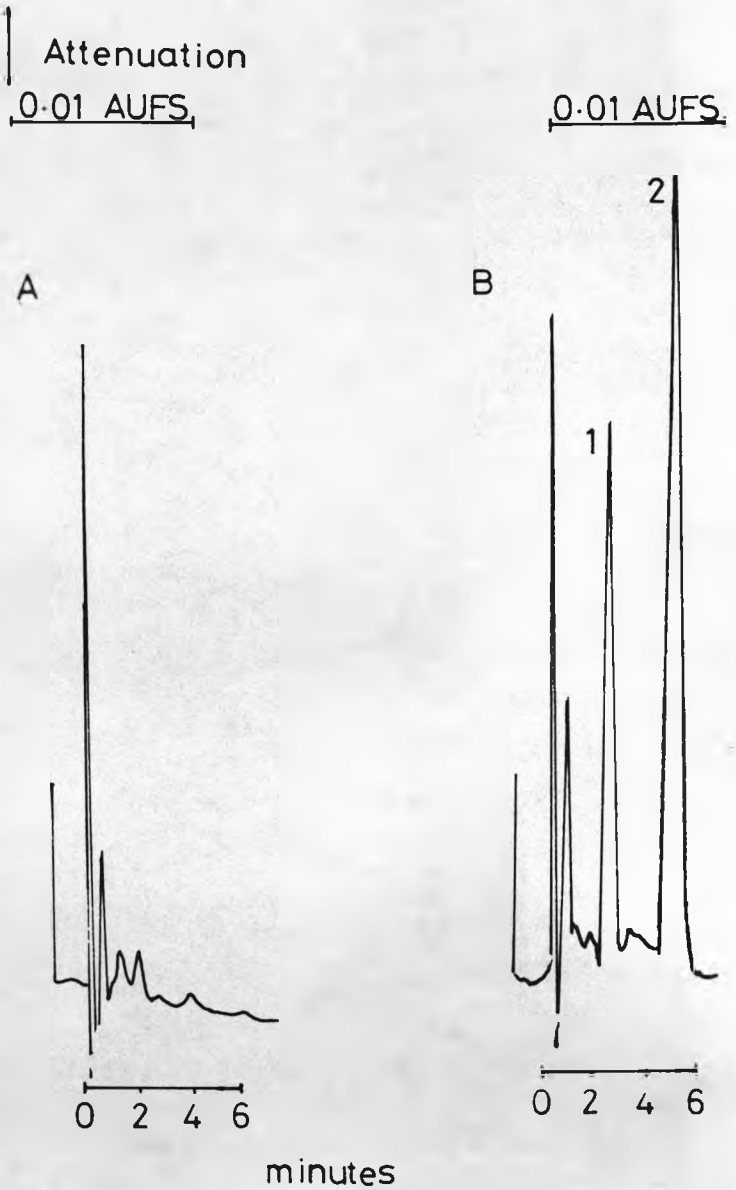


FIGURE 2.5 Chromatograms of (A) a blank plasma extract and (B) a plasma extract obtained after administration of primaquine 45mg to man (Chapter 6).1= the carboxylic acid derivative of primaquine 2= indomethacin.

2.1.3 DETERMINATION OF ANTIPYRINE CONCENTRATIONS BY HPLC

2.1.3.1 INTRODUCTION

Antipyrine is a low clearance drug which undergoes extensive metabolism. It is not significantly bound to plasma proteins and distributes within total body water. These characteristics make it a useful model substrate when investigating hepatic drug interactions, as antipyrine elimination is determined primarily by hepatic enzyme activity (Park 1982). This drug will be used as a model substrate to investigate the influence of primaquine on drug metabolising enzymes of the isolated perfused rat liver preparation. A number of methods are available for the determination of antipyrine and its three major metabolites and the method described here is a rapid and sensitive procedure based upon the methods described by previous workers.

2.1.3.2 CHROMATOGRAPHY

The liquid chromatograph used was as described in 2.1.1.2. Chromatographic separation of antipyrine, 3-hydroxymethylantipyrine, 4-hydroxyantipyrine, norantipyrine and phenacetin was carried out on a μ Bondapak C_{18} "Rad-Pak" column housed in a Z-module fitted with a CN-Guard Pak. The mobile phase consisted of methanol: Na_2 HPO_4 (50mM; pH 6.8) 45:55 (v/v) flowing at 3ml/min.

2.1.3.3 SAMPLE TREATMENT PROCEDURE

Antipyrine levels were determined by the method of Shargel et al. (1979) except that changes in chromatography conditions were made in order to avoid interference from the metabolites of antipyrine which have previously been described by Danhof et al. (1979). To perfusate plasma (200 µl), in a 1.5ml capacity microfuge tube, was added phenacetin as the internal standard (100 µg/ml, 20 µl). Perfusate proteins were then precipitated by the addition of 100µl each of $ZnSO_4$ (20%): methanol (50:50) and saturated $Ba(OH)_2$ solution. After vortex mixing (30 sec) and centrifugation (1000g, 1 min), 5 to 10µl of the supernatant was injected onto the chromatograph.

2.1.3.4 STANDARD CURVES

Standard curves were prepared as previously described 2.1.1.3, with varying quantities of antipyrine (5-30 µg/ml) being added to a fixed quantity of phenacetin (10 µg/ml) in drug-free perfusate. Extraction efficiency was determined by comparison of the peak heights of extracted samples with those of directly injected stock solutions of known concentrations.

2.1.3.5 ASSAY SPECIFICATIONS

Sample treatment was again rapid and simple. Chromatograms of a blank perfusate extract, a perfusate extract obtained from an isolated perfused rat liver experiment (chapter 4), and an injection of antipyrine, its three major metabolites and phenacetin stock solutions

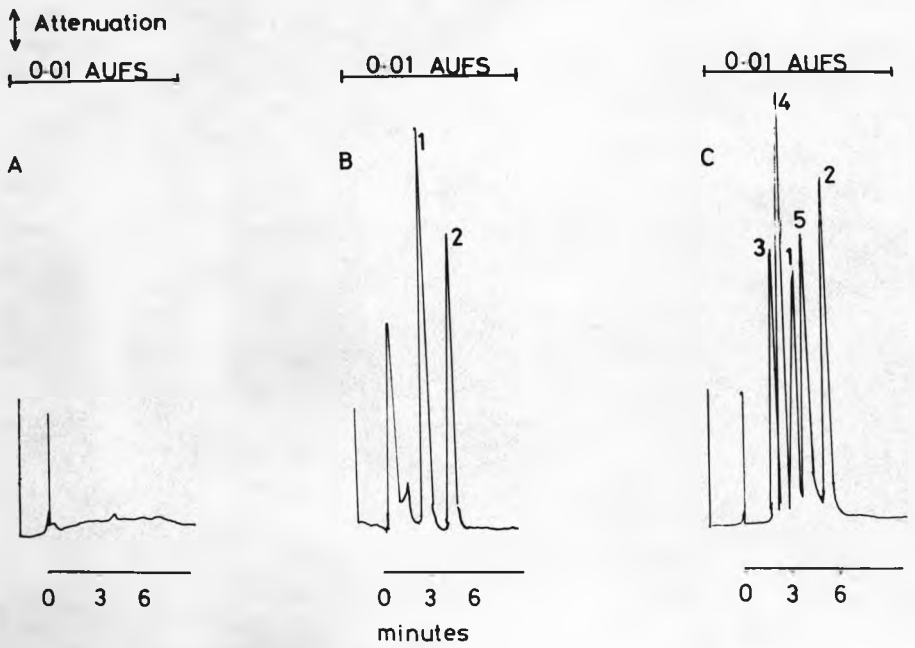


FIGURE 2.6 Chromatograms of(A) a blank perfusate extract (B) a perfusate extract obtained 1h after theadministration of antipyrine (2.5mg) and (C) injected stock solutions of antipyrine (1) phenacetin (2) 3-hydroxymethylantipyrine (3) norantipyrine (4) and 4-hydroxyantipyrine (5),250ng of each.

(250ng) are shown in figure 2.7 (A, B and C). Chromatograms of both spiked samples and a sample from the isolated perfused rat liver experiments gave peaks with retention times of 3.2 and 5.0 mins. corresponding to authentic antipyrine and phenacetin. 3-hydroxymethylantipyrine, norantipyrine and 4-hydroxyantipyrine were chromatographed with baseline resolution at retention times of 1.9, 2.4 and 3.5 mins. respectively.

The limit of sensitivity of this assay was 100ng/ml corresponding to a peak 4 X baseline noise at 0.01 AUFS. However, in all experiments in which antipyrine levels were assessed, the minimum concentration was in excess of 5 µg/ml. Calibration curves for antipyrine were linear in the range (0-30µg/ml) and analytical recovery was essentially complete. The intra and inter-assay variations were 5.2% and 3.9% respectively at a concentration of 10µg/ml (n=6, table 2.2).

2.1.4 DETERMINATION OF MICROSOMAL PROTEIN CONTENT

Microsomal protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as protein standard. BSA standard, 1ml (25-100 µg/ml) or microsomal protein accurately diluted (1:2500-1:3000) was incubated with 1ml of freshly prepared Lowry reagent ; (1% $CuSO_4$. $5H_2O$, 2% KNa tartrate, 10% Na_2CO_3 in 0.5M NaOH) for 15 mins. at room temperature. This was followed by the addition of Folin + Ciocalteau phenol reagent (3ml; 10% v/v in distilled water). After 30-45 mins., the absorbance of these samples was determined at 540nm using a

spectrophotometer. Protein concentration was calculated by comparison of the absorbance readings from unknown samples with the calibration curve (i.e. absorbance Vs. concentration) constructed using BSA standards.

2.1.5 DETERMINATION OF AMINOPYRINE N-DEMETHYLASE ACTIVITY

This assay was used to investigate the potential of a number of compounds to interfere with the ability of microsomal drug metabolising enzymes to N-demethylate aminopyrine via predominantly cytochrome P_{450} mediated processes(La Du 1971).

The reaction mixture consisted of aminopyrine (12.5mM), semicarbazide (9.37mM), test compound (0.10-0.18mM) and microsomes (0.5ml of a 4mg/ml suspension). After equilibration at 37°C in a shaking water bath, NADPH (12mM) was added to initiate metabolism. This mixture was incubated at 37°C for 10 mins. The reaction was stopped by the addition of $ZnSO_4$ (2ml; 15% w/v as an aqueous solution) and after a further 5 mins saturated $Ba(OH)_2$ (2ml) solution was added . The reaction mixture was then transferred into 20ml capacity "Sovirel" tubes and centrifuged (1000g X 15 mins.), 2ml of the supernatant was transferred to clean tubes (5ml capacity). Formaldehyde production (a by-product of aminopyrine N-demethylation) was measured following the addition of Nash reagent (0.8ml; Nash et al. 1953). After incubation (30 mins. at 60°C) the absorbance of the final solution was determined at 415 nm using a spectrophotometer. Aminopyrine N-demethylase activity in the absence of any other compound (i.e. control) was taken to be 100% activity

and a change in activity due to the presence of a test compound was expressed as the ratio of the absorbances of samples containing test compounds to controls.

2.1.6 DETERMINATION OF ETHOXYRESORUFIN O-DEETHYLASE ACTIVITY

Ethoxyresorufin O-deethylase activity was determined by the method of Burke and Meyer (1974). This assay was used to investigate the potential of a number of compounds to interfere with the function of microsomal drug metabolising enzymes to deethylate ethoxyresorufin (predominantly cytochrome P_{448} mediated processes).

Prior to the preparation of hepatic microsomes rats were pre-treated for three days with β-naphthoflavone (BNF 75mg/ml). This pre-treatment was necessary to induce the microsomal cytochrome P_{448} enzymes to a level of activity capable of deethylating ethoxyresorufin at the concentrations used.

The incubation mixture contained ethoxyresorufin (250 nM), test compounds (1 μM), microsomal protein (0.01 mg/ml) and NADPH (0.25mM) in 0.1M phosphate buffer (pH 7.8). The reaction was allowed to proceed in a cuvette,for 3 mins, at 30 °C, during which time the resorufin (a by-product of ethoxyresorufin O-deethylation) was monitored by the appearance of fluorescence (λ_{exc} =510nm;λ_{emm} =586nm). Ethoxyresorufin O-deethylase activity was measured as a percentage of the fluorescence of samples containing test compounds compared with the fluorescence of controls(i.e. 100% activity).

2.1.7 DETERMINATION OF CONJUGATES OF PRIMAQUINE AND PRIMAQUINE METABOLITES

Bile samples (chapter 4) and urine samples (chapter 6) were subjected to enzymic hydrolysis with H-1 hydrolase preparation obtained from H.Pomatia (100 units of enzyme activity per 100 ul of bile or urine, pH 5.0, 37 C, 3h). This hydrolysing enzyme preparation contains both arylsulphohydrolase and B-glucuronidase activity. Control incubations contained 0.1M sodium acetate buffer (pH 5.0).

2.1.8 DETERMINATION OF [^{14}C] RADIOACTIVITY

[^{14}C] radioactivity was measured by liquid scintillation spectrometry in an Intertechnique SL 30 counter, equipped with automatic quench correction. Biological samples 10-200 ul were mixed with a scintillation cocktail (5-10 ml; 10g/l 2,5-diphenyloxazole in toluene/Triton X 100; 2/1; V/V) and counted.

Tissue [^{14}C] radioactivity was determined following homogenisation of the tissue in saline (1:1 w/v) using a teflon in glass homogeniser. Duplicate samples (200ul) of homogenate were incubated overnight with NCS tissue solubiliser (200 ul) at room temperature. The digest was decolourised over two hours with hydrogen peroxide (200ul; 50 C) prior to the addition of the scintillation cocktail (5ml).

2.1.9 DETERMINATION OF THE OPTICAL PURITY OF (+) AND (-) PRIMAQUINE

The purity of the (+) and (-) isomers of primaquine was determined by measurement of the optical rotation of the individual isomers in aqueous solution using a polarimeter. The measured optical rotation was compared with the values previously reported by Carrol et al (1978) ie (+) primaquine $[\alpha]^{22}$+ 28.7° and (-) primaquine $[\alpha]^{22}$-27.8° .

2.2 EXPERIMENTAL METHODS

Experimental models were used to investigate certain aspects of the biochemical pharmacology of primaquine which could not be performed in vivo. For example it was not possible to investigate the hepatic component of primaquine disposition in vivo due to the myriad of factors such as tissue uptake, extrahepatic metabolism and urinary excretion which operate in the intact animal. In addition, ethical considerations and the short supply of compounds make it impossible to study the stereoselective disposition of primaquine or the potential of postulated primaquine metabolites to inhibit drug metabolism in man or whole animals. Two experimental models were utilised in order to overcome these problems. The first of these systems was the isolated perfused rat liver preparation, which is ideally suited to investigations into the hepatic handling of drugs in the intact organ, while avoiding considerations of the role of the other organs as is the case with whole animal

experiments. Secondly, hepatic microsomal enzyme preparations were used to provide a simple subcellular system for the investigation of certain drug metabolising processes. However it is not as physiologically realistic as the isolated perfused rat liver since the integrity and heterogeneity of the intact organ is lost after homogenisation of the liver.

2.2.1 THE ISOLATED PERFUSED RAT LIVER PREPARATION

2.2.1.1 SURGICAL REMOVAL OF RAT LIVERS

The livers of male Wistar rats (200-250g) were isolated using standard techniques (Bartosek et al. 1981) following anaesthesia with sodium pentobarbitone (75mg/kg). After exposing the liver, the common bile duct was isolated and cannulated (polythene tubing 0.10 X 0.61 mm). The hepatic portal vein was cannulated using an Argyle Medicut cannula (size G) and the liver voided of blood by injection of 1 ml heparin/saline (5 units/ml). Flow through the liver was maintained by slow infusion of sodium lactate solution (B.P.) via the hepatic portal vein (1-3 ml/min). Cannulation of the inferior vena cava through the left atria (polythene tubing 0.02 X 0.42mm) provided the route for hepatic effluent. A water tight system was ensured by tying off the inferior vena cava above the level of the renal branch.

The livers were then removed, mounted on a flat glass support and introduced into the perfusion circuit (figure 2.8). The total time of this surgery, from initial incision to perfusate connection was 15-20 mins, with less than 10

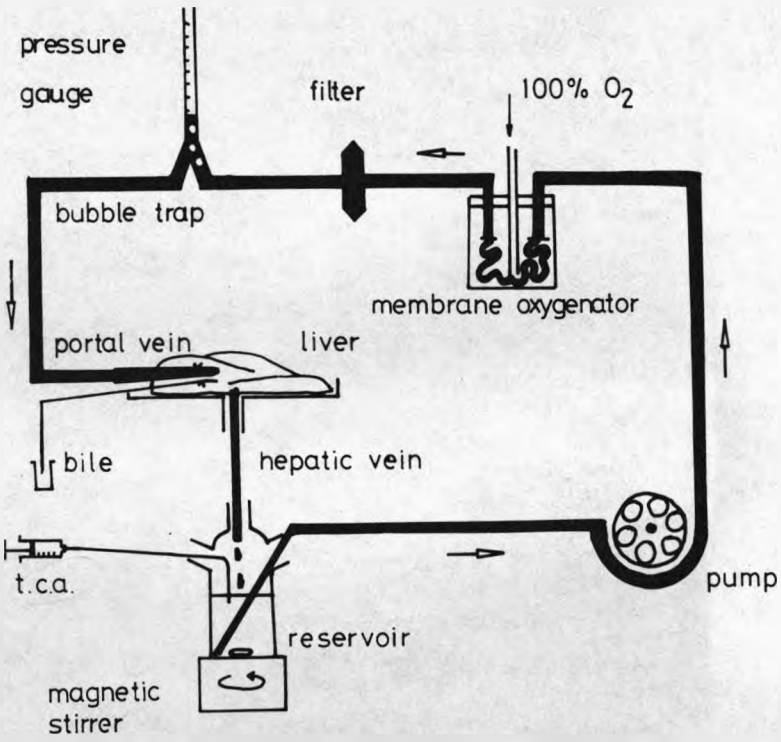


FIGURE 2.7 Schematic representation of the isolated perfused rat liver circuit.

minutes elapsing from the time portal vein flow was interrupted to the time the perfusate was connected.

2.2.1.2 THE PERFUSION CIRCUIT

Experiments were carried out in a humidified cabinet, thermostatically maintained at 37 C. Livers were perfused via the hepatic portal vein using a peristaltic pump (Gilson Minipuls 2) in a recycling system of 100ml capacity. A constant inflow to the liver of 15ml/min. was maintained at a portal pressure of 5 to 7 cm of water. The circuit included a silastic membrane oxygenator, filter,bubble-trap and pressure manometer (figure 2.7).

2.2.1.3 PERFUSATE COMPOSITION

The perfusate comprised 10% washed human red cells, 1% (w/v) bovine serum albumin and 0.1% (w/v) glucose in a standard electrolyte solution (Krone et al. 1974) equilibrated at pH 7.4 with 100% O_2 . Stable bile flow was maintained by constant infusion of sodium taurocholate (30 umole/hour; 0.5 ml/hour) into the perfusate reservoir.

2.2.1.4 INDICES OF LIVER VIABILITY

The principal indices of liver viability were steady oxygen consumption (1.5-2.0 umoles/g liver/min.), sustained bile production (0.4-0.6 ml/h.), consistent results for liver function tests and normal visual appearance.

2.2.2 PREPARATION OF HEPATIC MICROSOMES

2.2.2.1 PREPARATION OF 25% LIVER HOMOGENATE

Male Wistar rats (200-250g) were stunned and exsanguinated after severing the jugular and carotid vessels. The liver was rapidly exposed, excised and placed in a cold pre-weighed beaker. After calculation of the liver weight, KCl was added (1.15% in 1/15M phosphate buffer; 3 X liver weight). The liver was then cut into small fragments and transferred to a 55ml capacity homogenising tube. The liver was homogenised using a teflon in glass homogeniser.

2.2.2.2 PREPARATION OF POST-MITOCHONDRIAL SUPERNATANT

The liver homogenate was transferred into two 25ml MSE centrifuge tubes and balanced. Aluminium screw caps were fitted and the tubes placed in a pre-cooled (4°C) centrifuge head (MSE 8 X 25ml). The 25% homogenate was then centrifuged at 13,000g for 20 mins. at 4°C.

2.2.2.3 PREPARATION OF 105,000g MICROSOMAL PELLET

The post-mitochondrial supernatant was divided equally into four 10ml capacity centrifuge tubes. Aluminium screw caps were fitted and the tubes placed into a pre-cooled (4°C) centrifuge head (MSE 10 X 10ml). These tubes were centrifuged at 105,000g for 60 mins. at 4°C. The supernatant was discarded and the microsomal pellet covered with 1/15M

phosphate buffer (1.78g KH_2 PO_4 + 9.55g $NaHPO_4$ /1). Microsomal pellets were always used within 24 hours of preparation.

(NOTE for reagent and equipment sources refer to appendix 1 and 11)

CHAPTER 3 THE EFFECTS OF PRIMAQUINE STEREOISOMERS AND METABOLITES ON DRUG METABOLISM IN THE ISOLATED PERFUSED RAT LIVER AND IN VITRO RAT LIVER MICROSOMES.

3.1 INTRODUCTION

The antimalarial agent primaquine has been shown to inhibit drug metabolism both *in vivo* and *in vitro* in rats (Back *et al.* 1983a) and *in vivo* in man (Back *et al.* 1983b). These studies utilised racemic primaquine (±), which comprises of equal proportions of the (+) and (-) isomers. The toxicity but not the antimalarial activity of these two isomers has been shown to differ markedly (Schmidt *et al.* 1977). The potency of the (+) and (-) isomers in inhibiting drug metabolism when compared to increasing doses of racemic primaquine has not been examined.

In this chapter the clearance of antipyrine from the isolated perfused rat liver (IPRL) has been used as an index of mixed function oxidase activity. The results obtained from this experimental model, when applied to the study of hepatic drug-drug interactions for the H_2 -receptor antagonists (Mihaly *et al.* 1982) have agreed with findings from human studies (Henry *et al.* 1980). In addition, this model allows the direct evaluation of hepatic drug-drug metabolism interactions in a whole organ system.

In further studies the effects of the (+) and (-) isomers, racemic primaquine, the carboxylic acid metabolite of primaquine and several putative metabolites of primaquine on *in vitro* microsomal mixed function oxidase activity has been examined.

3.2 EXPERIMENTAL

3.2.1 ANTIPYRINE DISPOSITION STUDIES IN THE IPRL

The isolated perfused rat liver preparation was established as described in chapter 2 (2.2.1). The elimination of a 2.5mg bolus dose of antipyrine (250µl of a 10mg/ml aqueous solution added to the perfusate reservoir) was studied over four hours in the following groups: (a) control (without addition of a putative inhibitor; n=7); (b) (±) primaquine, (after bolus doses of 0.5, 2.5, or 5.0mg; n=4 in each set); (c) (+) primaquine, (after a bolus dose of 2.5mg; n=5); and (d) (-) primaquine (after a bolus dose of 2.5mg n=5). All drug solutions were added as aqueous solutions of the diphosphate salt of (±), (-) or (+) primaquine (10mg/ml), into the perfusate reservoir, thereby simulating systemic dosage. Prior to use, the optical rotation of the (+) and (-) isomers was found to correspond to that previously reported (Carrol *et al.* 1978, (+) $[\alpha]^{22}$ $+28.7^{\circ}$; (-) $[\alpha]^{22}$ -27.8°, chapter 2.1.9).Samples (1.5ml) were taken from the perfusate reservoir for antipyrine estimations,pre-dose and at 5, 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 minutes. After centrifugation (1000g for 1min) the separated perfusate plasma was removed and frozen at -20°C until assayed. Additional samples were taken hourly to measure primaquine concentrations. An equal volume of fresh perfusate was added to the reservoir to replace that removed by sampling. The total amount of antipyrine lost through sampling was less than 5% of the dose. Bile was collected into pre-weighed vials. Perfusate

blood gases were measured and liver function tests were performed before and after each experiment to ascertain the viability of the liver.

Perfusate antipyrine and primaquine levels were measured by the methods described in chapter 2 (2.1.1 and 2.1.3).

3.2.2 IN VITRO STUDIES

The potential of racemic primaquine to inhibit aminopyrine N-demethylation and ethoxyresorufin (ERR) deethylation, was compared with the degree of inhibition produced by the (+) and (-) isomers of this drug. As very limited quantities (< 5mg) were available of N-acetyl primaquine the carboxylic acid metabolite of primaquine (Hufford *et al.* 1983), 5-hydroxyprimaquine, 6-desmethylprimaquine and 5- hydroxy-6-desmethylprimaquine (Strother *et al.* 1981), these compounds were only tested for their potential to influence aminopyrine N-demethylation. The hydroxylated metabolites of primaquine were stabilised by the addition of ascorbic acid (1mM) to the reaction mixture.

Hepatic microsomes were prepared as described in chapter 2 (2.2.2) and the protein content of the microsomal pellet determined by the Lowry assay (2.1.4). Aminopyrine N-demethylase activity and ethoxyresorufin O-deethylase activity was determined as explained in chapter 2 (2.1.5 and 2.1.6 respectively).

3.2.3 PHARMACOKINETIC CALCULATIONS AND STATISTICAL ANALYSIS

Pharmacokinetic parameters (half-life, volume of distribution, clearance and AUC) were calculated using model-independent formulae (1.6.6). Statistical comparisons of antipyrine pharmacokinetic parameters were made by one-way analysis of variance and of % inhibition of substrate metabolism by comparison of sample means of unknown variance using the t-statistic (Chatfield 1978), both tests accepted $p < 0.05$ as significant. Data are presented graphically as mean $\pm$ SEM and are tabulated as mean $\pm$ SD.

3.3 RESULTS

The disappearance of antipyrine from the perfusate, in either the presence or absence of primaquine is shown in figures 3.1 and 3.2. Antipyrine concentrations in the perfusate declined monoexponentially in all experiments. Increasing doses of (±) primaquine were associated with slower rates of fall of antipyrine levels (figure 3.1). However, at doses of 2.5mg, the (±), (-) and (+) forms of primaquine resulted in no appreciable differences in the antipyrine perfusate concentration/time profile (figure3.2).

The resultant pharmacokinetic parameters for antipyrine are summarised in table 3.1 and illustrated in figure 3.3. Racemic (±) primaquine produced a dose and plasma level related reduction in antipyrine clearance (tables 3.1 and 3.2, figure 3.3) which at the highest dose of primaquine represented a decrease to 46% of the control values. The dose of (±) primaquine that produced 50% inhibition of antipyrine

clearance ($I.D._{50}$) was 7.0μmole (3.2mg primaquine diphosphate; figure 3.5).

Antipyrine clearance was reduced to a comparable extent by the (+) and (-) isomers and by the racemic mixture of primaquine (each at a dose of 2.5mg) with mean reductions of 45, 49 and 47% respectively. These changes in clearance were reflected by significant increases in elimination half-life compared to control (table 3.1, figure 3.3). The volume of distribution remained constant in all experiments and was comparable to the actual volume of the perfusion circuit (table 3.1, figure 3.3).

The mean perfusate concentrations of primaquine throughout the course of each set of experiments are listed in table 3.2, showing that primaquine underwent hepatic elimination in all groups (figures 3.4). There appeared to be some stereoselective differences in the disposition of (+) and (-) primaquine compared to the racemic drug at a dose of 2.5mg (figure 3.4), the (-) isomer being eliminated more rapidly than the (+) isomer .

Racemic primaquine and its (+) and (-) isomers were equipotent in inhibiting aminopyrine N-demethylase activity producing reductions of 56, 59 and 55% respectively (table 3.3). These three substances produced corresponding reductions of 73, 58 and 73% in ERR deethylase activity. Both the N-acetyl and 5-hydroxy derivatives of primaquine inhibited aminopyrine N-demethylase activity to a comparable extent to that seen for the parent drug. By contrast the

TABLE 3.1 Summary of Antipyrine pharmacokinetics (Mean ± S.D.) clearance (Cl); half-life ($t_{\frac{1}{2}}$); and volume of distribution (Vd).

Treatment Group	Cl (ml/min)	$t_{\frac{1}{2}}$ (min)	Vd (ml)	n
CONTROL	0.550 ± 0.053	165 ± 17	130 ± 16	7
(±) Primaquine 0.5mg	0.446 ± 0.044*	194 ± 20*	124 ± 5	4
(±) Primaquine 2.5mg	0.292 ± 0.074*	300 ± 121*	118 ± 10	4
(±) Primaquine 5.0mg	0.253 ± 0.083*	404 ± 143*	116 ± 28	4
(+) Primaquine 2.5mg	0.302 ± 0.057*	277 ± 51*	118 ± 11	5
(-) Primaquine 2.5mg	0.279 ± 0.035*	303 ± 45*	121 ± 9	5

* Significantly different from Control $P < 0.05$

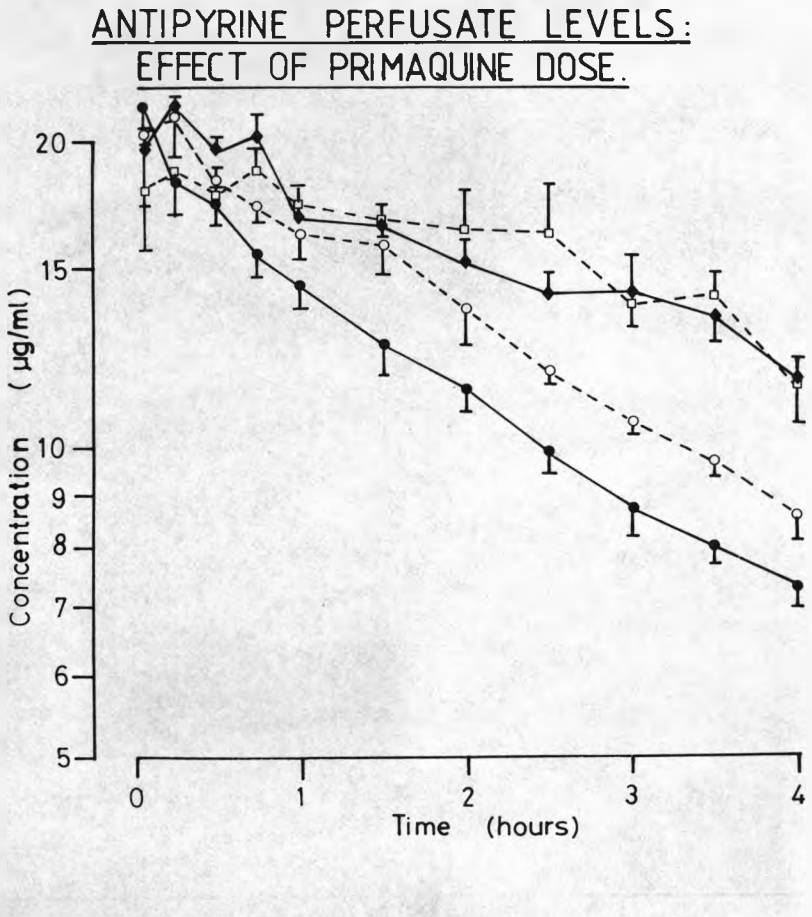


FIGURE 3.1. Perfusate concentrations of antipyrine (after a 2.5mg dose) in control experiments (●-●) and after administration of racemic primaquine at doses of 0.5 (○-○), 2.5 (◆-◆) and 5.0 (□-□) mg of the diphosphate salt. Increasing primaquine dose produced a gradual reduction in the rate of decline of antipyrine perfusate levels.

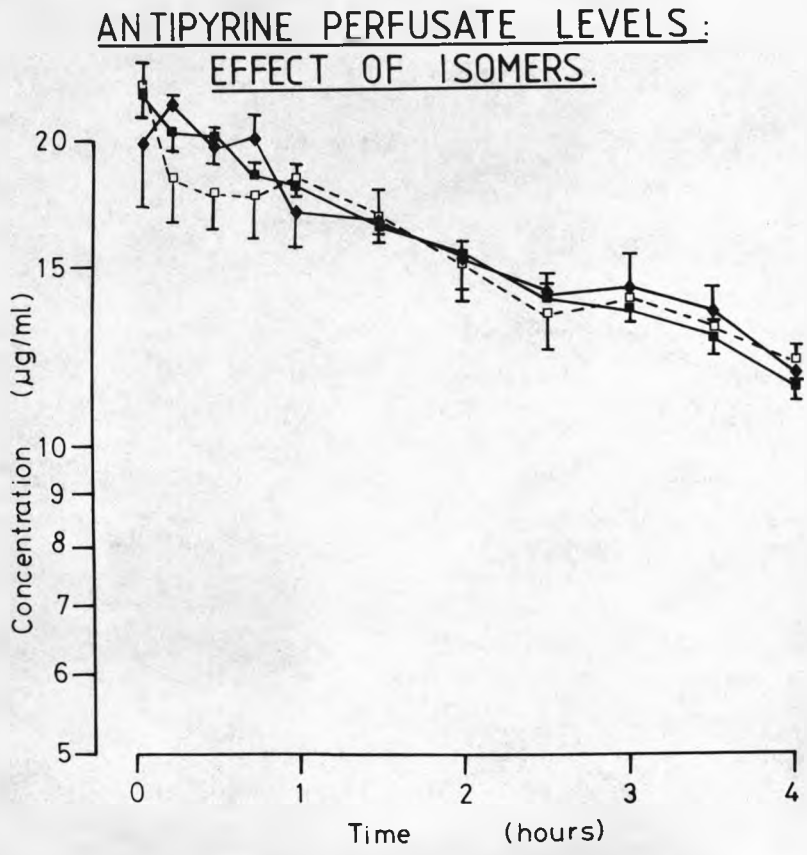


FIGURE 3.2. Perfusate concentrations of antipyrine (after a 2.5mg dose) after the administration of (±) - (■-■), (+) - (□-□) and (-) - (◆◆) primaquine diphosphate at a dose of 2.5mg.

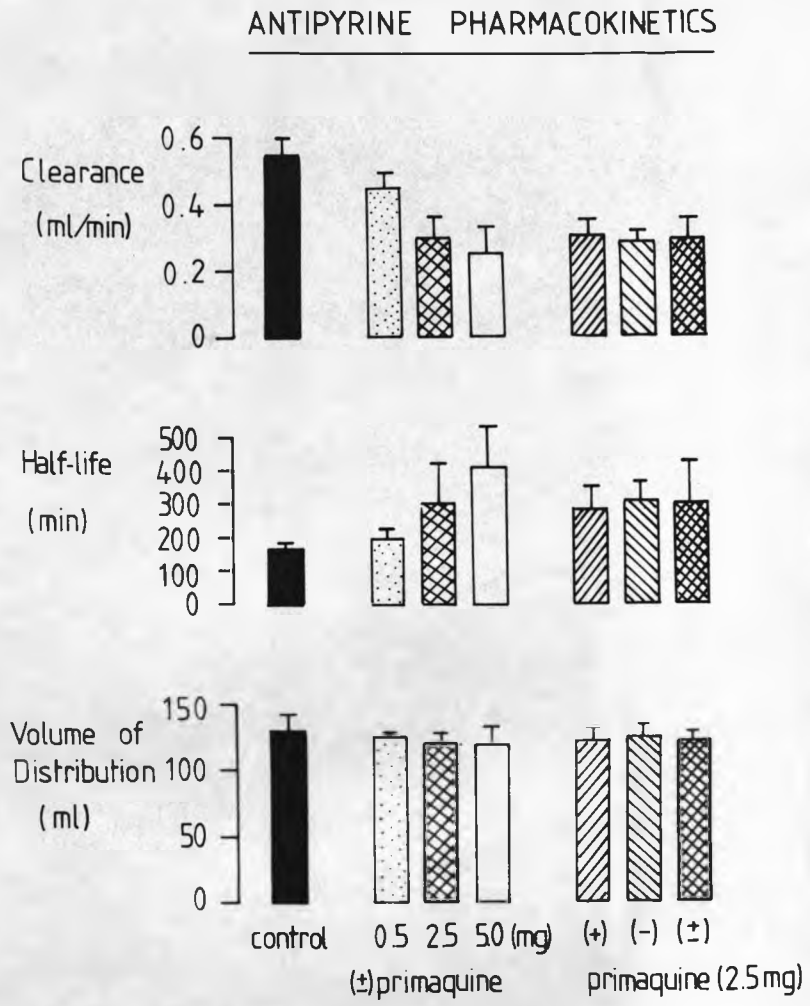


FIGURE 3.3. The effect of primaquine dose and isomer forms on the pharmacokinetics of antipyrine in the IPRL.

TABLE 3.2 Mean Perfusate primaquine concentration (ng/ml) (± S.D.)

Drug	Dose (mg)	Time (min) 60	120	180	240
(±) Primaquine	0.5	136 ± 19	99 ± 9	68 ± 19	46 ± 18
(±) Primaquine	2.5	682 ± 237	710 ± 183	624 ± 191	481 ± 199
(±) Primaquine	5.0	1985 ± 819	1337 ± 415	1028 ± 151	1287 ± 413
(+) Primaquine	2.5	995 ± 121	859 ± 62	767 ± 94	673 ± 209
(-) Primaquine	2.5	819 ± 181	564 ± 116	455 ± 74	383 ± 68

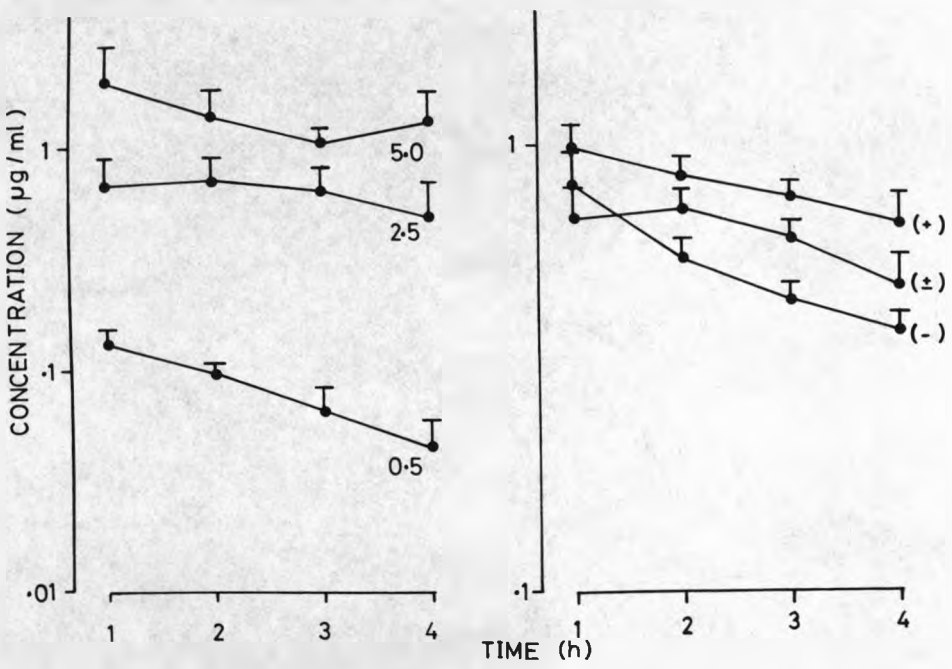


FIGURE 3.4 Primaquine perfusate concentrations after administration of A racemic primaquine Ø.5 (-),2.5 (-) and 5.Ø mg of the diphosphate salt and B (±)-(-), (+)-(-) and (-)-(-) primaquine diphosphate at a dose of 2.5mg.

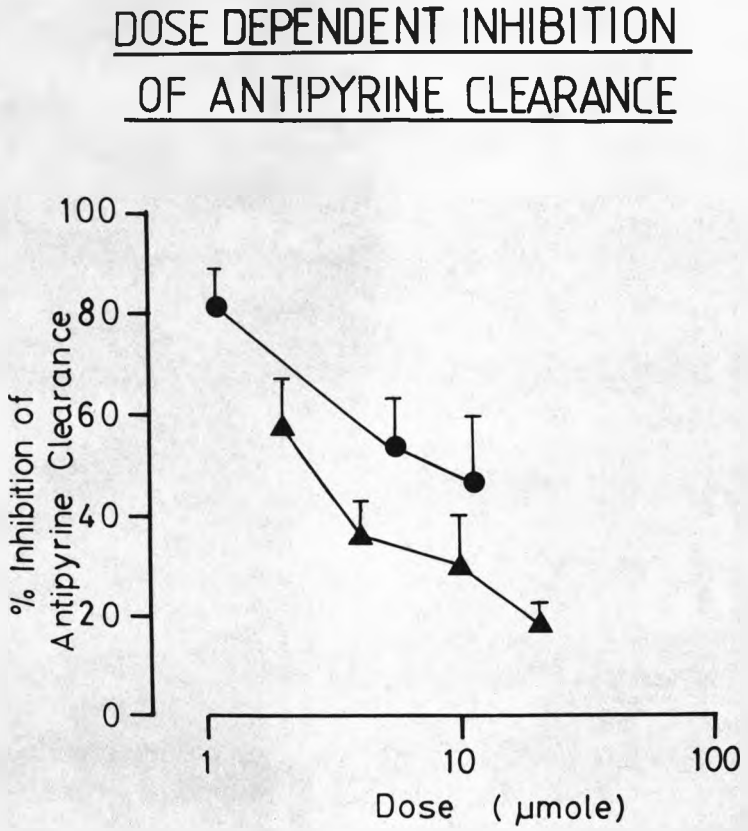


FIGURE 3.5 The effect of dose size on the inhibition of antipyrine clearance produced by racemic primaquine (●-●) and cimetidine (▲-▲) in the IPRL.

TABLE 3.3 % Inhibition of drug metabolism activity in-vitro, in liver microsomes - control (no drug) = 100% activity (mean ± S.D.).

Substance (concentration)	Aminopyrine Demethylation (%)	Ethoxyresorufin Deethylation (%)
Control	100 (n = 8)	100 (n = 5)
(±) Primaquine (0.11mM)	*56 ± 11 (n = 8)	*27 ± 4 (n = 5)
(+) Primaquine (0.11mM)	*59 ± 11 (n = 5)	*42 ± 6 (n = 5)
(-) Primaquine (0.11mM)	*55 ± 13 (n = 5)	*27 ± 3 (n = 5)
N-Acetylprimaquine (0.17mM)	*55 ± 12 (n = 4)	-
Carboxylic acid metabolite (0.18mM)	103 ± 7 (n = 3)	-
5-Hydroxy Primaquine (0.10mM)	*55 ± 7 (n = 4)	-
6-Desmethyl Primaquine (0.15mM)	95 ± 7 (n = 4)	-
5-Hydroxy-6-desmethyl primaquine (0.10mM)	91 ± 7 (n = 4)	-

* = level of significance compound to control $P < 0.005$

carboxylic acid metabolite of primaquine, 6-desmethylprimaquine and 5-hydroxy-6-desmethylprimaquine did not appreciably influence aminopyrine N-demethylase activity compared to control.

3.4 DISCUSSION

The disposition of antipyrine in the IPRL is a useful experimental model for the study of hepatic drug-drug metabolism interactions (Mihaly et al. 1982, Webster et al 1984). In the case of the H_2 - receptor antagonists cimetidine and ranitidine, the benzimidazole gastric acid secretion inhibitor omeprazole and in the present study primaquine, the qualitative findings from this experimental model have correlated with the conclusions reached from human studies (Back et al. 1983b; Henry et al. 1980; Webster et al. 1984). In addition, as a relatively simple and versatile experimental system, studies in the IPRL can readily examine the contribution of factors such as dose size, isomer form and drug metabolites to the hepatic component of drug metabolism interactions.

In the present study a dose and plasma level dependent inhibition of drug metabolism has been seen. This was shown by the progressive decrease in antipyrine clearance with increasing doses and associated higher plasma levels of (+) primaquine (tables 3.1 and 3.2, figure 3.3). This effect was virtually immediate suggesting a direct interaction between the inhibiting agent and the drug metabolising enzymes,

leading to a decrease in their activity. These changes in antipyrine clearance were reflected in prolonged half-life values. However, the values for volume of distribution were not significantly different within any of the treatment groups and were comparable in size to the actual volume of the perfusion circuit (100ml reservoir plus volume of the perfused liver), suggesting there was no perturbation of antipyrine distribution by primaquine.

The effects of various H_2- receptor antagonists on antipyrine clearance from the same IPRL model has previously been examined (Mihaly et al. 1982). They calculated the $I.D._{50}$ for cimetidine to be 2.8μmole which is two to three times more potent an inhibitor of drug metabolism than primaquine ($I.D._{50}$ =7.0μmole - figure 3.5). By contrast, in a recent study in the rat in vivo of the inhibition of the plasma disappearance of tolbutamide, primaquine was shown to be two to three fold more potent than cimetidine (Back et al. 1984). This reversal of the order of potency may be due either to dissimilarities in the experimental models used or to differences in the capacities of these inhibitor compounds to influence different substrate reactions.

In animal studies, the (+), (-) and racemic forms of primaquine have different therapeutic index values (i.e. ratios of toxic to effective doses). This disparity does not arise from differences in antimalarial activity, but is due to variations in the toxicity between the three substances (Schmidt et al. 1977). However, the (+), (-) and racemic forms of primaquine were equipotent in their capacity to

decrease antipyrine clearance in the IPRL (table 3.1, figure 3.2 and 3.3). These findings were further supported by the *in vitro* microsomal studies, in which aminopyrine N-demethylase activity was influenced by primaquine isomers and the racemate to a comparable extent (table 3.3).

In the case of ERR O-deethylase activity, a similar pattern of inhibition was evident for the three substances, however the (+) isomer appeared marginally less potent than the (-) isomer or racemate (table 3.3). Nonetheless, in this study the results of the *in vitro* substrate inhibition experiments , using racemic primaquine were in agreement with earlier reports (Back *et al.* 1983a).

In all experiments primaquine underwent hepatic elimination (table 3.2). However, throughout the study period and at all doses, drug concentrations persisted at levels equal to or greater than those encountered in human studies, where therapeutic doses of primaquine had been given (chapters 6, 7 and 8).

Despite any difference in the inhibitory potential of (±), (+) or (-) primaquine there appeared to be stereoselective differences in the disposition of these three compounds (figure 3.4). The (+) isomer of primaquine achieved higher perfusate concentrations and showed a marginally reduced elimination half-life when compared to the (-) isomer, with the racemic compound, as expected, falling between the two extremes. Stereoselective aspects of primaquine's disposition are discussed in chapter 5. The observation that primaquine shows stereoselective differences in its own disposition but no stereoselective differences in

its inhibitory capacity suggest that antipyrine disposition in the IPRL model may not be a good discriminator for highlighting subtle differences in inhibitory potency, or that inhibition of drug metabolism in this instance is due to a particular metabolite or metabolites which do not exhibit stereoselective differences in their extent or rate of formation.In addition, primaquine appeared to show dose dependent elimination,increasing doses of the racemate being associated with disproportionate changes in the AUC for primaquine (figure 3.4).

Using the limited quantities of identified and "postulated" metabolites of primaquine available, it was possible to evaluate the effects of these substances on microsomal aminopyrine N-demethylase activity (table 3.3). Only the carboxylic acid derivative of primaquine has been identified as a metabolite of this drug in man (chapter 6), however it did not effect aminopyrine N-demethylation. By contrast, N-acetylprimaquine, which has been detected as a primaquine metabolite in microbial culture studies (Hufford et al. 1983), but not in human plasma or urine (chapter 6), was as potent as racemic primaquine in inhibiting aminopyrine demethylation. As for the three hydroxylated derivatives (Strother et al. 1981, figure 1.5) only 5-hydroxyprimaquine inhibited drug metabolism, whereas 6-desmethylprimaquine and 5-hydroxy-6-desmethylprimaquine did not produce any appreciable inhibition (table 3.3).

The differences in potency of inhibition of drug metabolism between these primaquine derivatives implies that

subtle changes in functional group substitution of the parent molecule, but not optical activity, may have considerable bearing on the propensity of these compounds to influence drug metabolism. In this vein, it appears that the methylene group of the 6 position is necessary for inhibition of microsomal drug metabolism. This 6-methoxy substitution is present in all the compounds studied except 6-desmethylprimaquine and 5-hydroxy-6-desmethylprimaquine, neither of which produced any inhibitory effect. Although the carboxylic acid metabolite of primaquine does retain this methylene group, it failed to inhibit drug metabolism. This implies that the retention of the terminal amine group on the side chain may also be an essential molecular component for this inhibitory phenomenon.

Recently, interest has centred on the association of the 5-hydroxy primaquine compound with both antimalarial activity and toxicity (Strother et al. 1981; Fletcher et al. 1977). In addition the present study has implicated this substance in drug metabolism inhibition. However, in spite of its association with both therapeutic and adverse effects this compound has evaded detection in human studies probably due to the lack of suitably selective and sensitive analytical methods.

These studies have shown that in the IPRL, the inhibition of drug metabolism by racemic primaquine is rapid in onset and both dose and plasma level dependent. The results of further studies in the IPRL together with in vitro experiments in liver microsomes have shown the (+) and (-)

isomers to be equipotent to racemic primaquine in inhibiting drug metabolism. Separate *in vitro* studies have also shown the carboxylic acid metabolite of primaquine, which represents the major plasma metabolite in man, to have no influence on drug metabolism. In addition, although drug metabolism was inhibited by the N-acetyl and 5-hydroxy derivatives of primaquine, the significance of these observations awaits the complete elucidation of the metabolic fate of primaquine.

CHAPTER 4 THE EFFECT OF DOSE SIZE ON THE PHARMACOKINETICS, METABOLISM, BILIARY EXCRETION AND TISSUE LOCALISATION OF PRIMAQUINE IN THE ISOLATED PERFUSED RAT LIVER.

4.1 INTRODUCTION

Studies carried out in micro-organisms and animals have suggested that primaquine is rapidly and extensively metabolised to a number of ring hydroxylated (Strother et al. 1981; and 1984) and side chain modified compounds (Baker et al. 1982; Hufford et al. 1983 chapter 1). In addition, the tissue distribution of primaquine and primaquine metabolites has been investigated after administration of radiolabelled drug to rats (Holbrook et al. 1981; Clark et al. 1984 chapter 1). These studies revealed high concentrations of unidentified radioactive material in well perfused tissues, particularly the liver, and substantial levels of radioactivity in faeces. However, the hepatic component of primaquine disposition and the identity of radioactive material described by previous workers has not been studied in detail.

In addition,preliminary evidence suggests that the disposition of primaquine in the isolated perfused rat liver preparation (IPRL) is dependent upon dose size, with disproportionate increases in primaquine perfusate concentrations being associated with increased dose (chapter 3) . This observation, coupled with the fact that primaquine is administered in doses ranging from 15 to 45mg in the radical treatment of malaria (Bruce-Chwatt 1980) has prompted the investigation of the hepatic disposition of primaquine and the influence of dose size on the disposition of this drug in the IPRL.

4.2 EXPERIMENTAL

4.2.1 STUDY DESIGN

The isolated perfused rat liver preparation was established as described in chapter 2 (2.2.1). The elimination of primaquine from the IPRL was studied over five hours in the following groups: (a) after a 0.5mg bolus dose of primaquine diphosphate (n=6) containing 0.42μCi of [^{14}C] labelled primaquine; (b) after a 1.5mg bolus dose of primaquine diphosphate (n=6) containing 0.31 μCi of [^{14}C] labelled primaquine and (c) after a 5.0mg bolus dose of primaquine diphosphate (n=6) containing 0.42μCi of [^{14}C] labelled primaquine. Primaquine was added as an aqueous solution (250 to 1000 μl) into the perfusate reservoir thereby simulating systemic dosage.

Samples (1.5ml) were taken from the perfusate reservoir pre-dose and at 5, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270 and 300 minutes. After centrifugation (1000g for 1 min.) the separated perfusate plasma was removed and frozen at -20°C until assayed for primaquine, the carboxylic acid metabolite of primaquine and [^{14}C] radioactivity. An equal volume of fresh perfusate was added to the reservoir to replace that removed by sampling. Bile was collected over 30 min. intervals into pre-weighed vials and bile volumes measured before freezing at -20°C.

4.2.2 ANALYTICAL PROCEDURES

Perfusate concentrations of primaquine and the carboxylic acid metabolite were determined by HPLC as described in chapter 2 (2.1.1 and 2.1.2). Perfusate and bile

concentrations of [^{14}C] radioactivity were determined directly by scintillation counting and the liver concentrations of [^{14}C] radioactivity were determined by scintillation counting after homogenisation and decolourisation of the tissue as detailed in chapter 2 (2.1.8). Pharmacokinetic parameters (half-life, volume of distribution, clearance and AUC) were calculated using model independent formulae (Chapter 1.6.6). Statistical analysis between three groups were made by one way analysis of variance and between two groups using the Student t statistic, $p > 0.05$ was accepted as significant. Data are tabulated as mean + SD and presented graphically as mean + SEM.

4.2.3 ANALYSIS OF BILIARY METABOLITES

Half-hourly bile samples from each IPRL preparation were combined and the total volume recorded. An aliquot (10µl) of this pooled bile obtained from each IPRL preparation was used to determine the biliary concentration of [^{14}C] radioactivity. A further aliquot (150µl) was removed and exposed to enzymic hydrolysis (pH 5.0, 37°C, 3h) with H-1 hydrolase preparation obtained from H. pomatia (100 units of enzyme activity per 100 µl of bile) (Chapter 2.1.7).This hydrolysing enzyme preparation contains both aryl-sulphohydrolase and β-glucuronidase activity. Control incubations contained 0.1M sodium acetate buffer (pH 5.0).

Primaquine and primaquine metabolites present in bile samples were separated by HPLC. Effluent fractions corresponding to the retention times of authentic standards

were collected and assayed for [^{14}C] radioactivity after the addition of scintillation fluid (20ml). Primaquine and the carboxylic acid metabolite were separated on a μ Bondapak 'Radpak' phenyl column and eluted with a mobile phase consisting of methanol:1% triethylamine phosphate (58:42), pH 3.5, flowing at 3ml/min. Elution times were 2min. 55sec. and 5min. 20sec. for primaquine and its carboxylic acid metabolite respectively. The ring hydroxylated metabolites: 6-desmethylprimaquine; 5-hydroxy-6-desmethylprimaquine and 5-hydroxyprimaquine were separated as described above, except that the mobile phase consisted of methanol:1% triethylamine phosphate (20:80) at pH 3. The retention times of 5-hydroxy-6-desmethylprimaquine and 6-desmethylprimaquine were 4 min. and 5 min. 35 sec. respectively. The 5-hydroxy derivative of primaquine was eluted with a retention time of 3 min. However, this compound was very unstable in aqueous solution and broke down to a number of compounds that eluted between 1.5 and 3.5 min. Nonetheless, an attempt to quantify this derivative has been made relying on the collection of this entire fraction (i.e. 1.5 to 3.5 min.), to provide an estimate of the levels of [^{14}C] radioactivity attributable to this metabolite.

Due to the complex composition of the liquid-chromatography mobile phase, the eluted fractions corresponding to each of the metabolites could not be subjected to mass-spectrometric characterisation. In the present study therefore identification of this metabolite has relied on comparison of the chromatographic retention times

of components in bile, with those of authentic standards of each compound.

4.3 RESULTS

4.3.1 PERFUSATE DISPOSITION OF PRIMAQUINE

Perfusate primaquine levels fell biexponentially in all dosage groups. However, the rate of decline of plasma levels in the terminal elimination phase became more gradual as the size of the primaquine dose was increased. This resulted in an increase in the elimination half-life from 33.2+10.7 min after 0.5mg primaquine to 175.3+84.5 min after 1.5mg and 413+239.3 min after 5.0mg (figure 4.1,table 4.1). This dose-dependent elimination of primaquine was reflected in a disproportionate increase in the area under the mean perfusate drug concentration/time curve (AUC) from 24.5+5.9μg.min./ml to 171.9+35.1μg.min./ml and 1128.6+575.7 μg.min./ml as the primaquine dose was increased (table 4.1). Furthermore, these changes in AUC were accompanied by significant decreases in the systemic clearance and significant increases in the apparent volume of distribution of primaquine (table 4.1).

Increasing doses of primaquine also produced alterations in the contribution of both the parent drug and its carboxylic acid metabolite, to the area under the [^{14}C] concentration time curve from time =0 to time =5h. (figure 4.2 A,B and C). When expressed as % dose.h/ml there was no change in the magnitude of the [^{14}C] AUC for the three doses (61.9+17.1, 72.3+16.8 and 60.9+18.8% dose.h/ml for the 0.5, 1.5 and 5.0mg doses respectively).

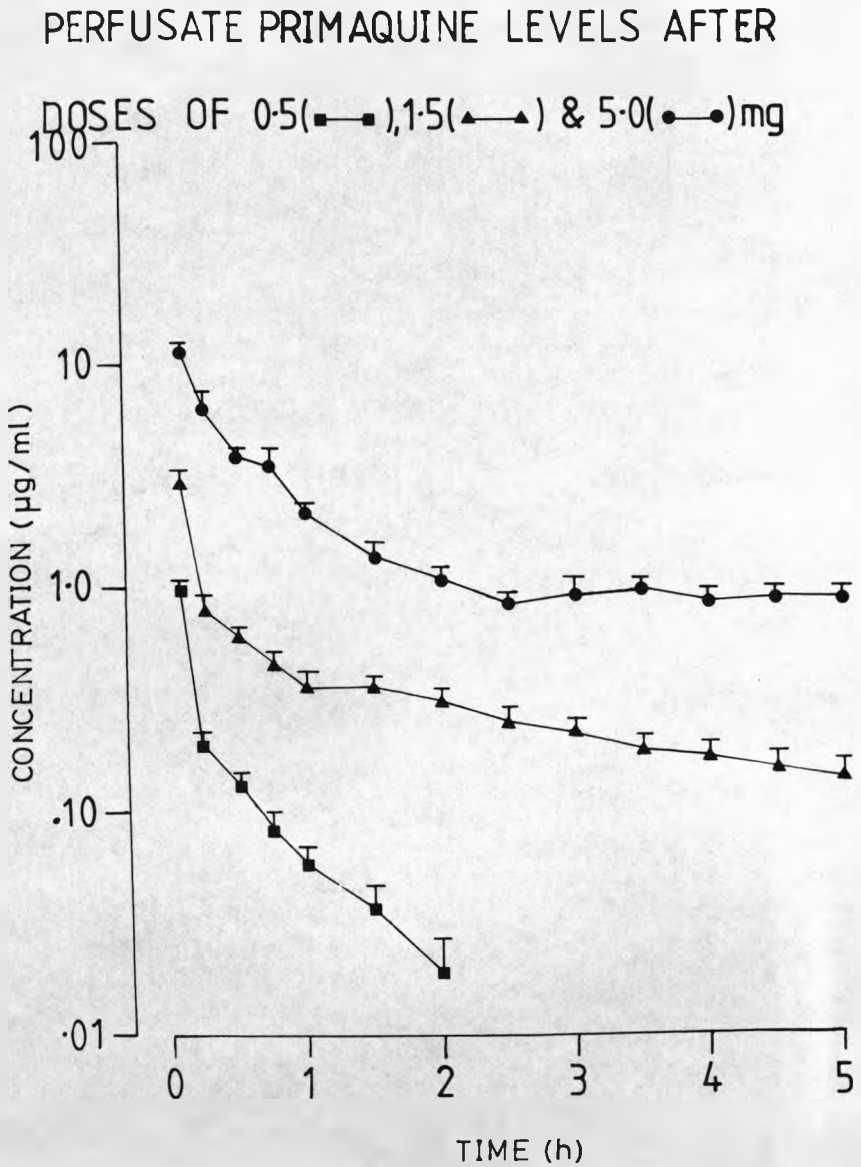


Figure 4.1. Perfusate concentrations (ug/ml) after administration of Ø.5 (■-■), 1.5 (▲-▲) and 5.Ømg (●-●) of radiolabelled primaquine diphosphate, demonstrating the dose-dependent pharmacokinetics of this drug.

TABLE 4.1 Primaquine pharmacokinetic parameters after systemic administration of 0.5,1.5 and 5.0mg. doses of the diphosphate salt. (results are expressed as mean± SD).

Dose (mg)	n	AUC (μg.min/ml)	Half-life (min)	Clearance (ml/min)	Volume of distribution (ml)
0.5	6	25.4 ± 5.9	33.2 ± 10.7	11.6 ± 2.5	547.7 ± 153.1
1.5	6	171.9 ± 35.1	175.3 ± 84.5	5.2 ± 1.0	1216.0 ± 359.0
5.0	6	1128.6 ± 575.7	413.0 ± 239.3	2.9 ± 1.0	1489.0 ± 249.0

However, the relative contribution of primaquine perfusate levels to this AUC increased from 16.9% after the Ø.5mg dose to 22.6% after 1.5mg and 35.1% after 5.Ømg. The carboxylic acid derivative of primaquine could not be detected in perfusate after the lowest dose of primaquine. Nonetheless, 3.6% and 4Ø.Ø% of the total [^{14}C] AUC were accounted for as this metabolite after 1.5 and 5.Ømg doses of primaquine respectively.

4.3.2 BILIARY EXCRETION OF PRIMAQUINE AND PRIMAQUINE METABOLITES

In addition to a dose dependency in primaquine perfusate pharmacokinetics, increasing drug dosage altered the extent of biliary excretion of [^{14}C] radioactivity from 64% of the dose after Ø.5mg primaquine to 34% after 5.Ømg (table 4.2). This decreased excretion was seen even though bile flow did not differ significantly between dosage groups (table 4.3).

The nature of the radioactivity excreted in bile was examined by HPLC analysis. HPLC fractions were collected corresponding to 5-hydroxyprimaquine, 5-hydroxy-6-desmethylprimaquine, 6-desmethylprimaquine , primaquine and the carboxylic acid metabolite of primaquine. Bile samples were analysed before and after enzymic hydrolysis with Hl-hydrolase and the results are summarised in table 4.4. Less than 4Ø% of the total radioactivity excreted in bile could be accounted for in these fractions at all of the dosage levels. Of the remaining radioactivity, 4-8% could be

recovered in the solvent front and the remaining 40-50% could be recovered in all cases by flushing the HPLC system with methanol (5ml) between injected samples (table 4.4). Even though the total radioactivity recovered in bile over 5h decreased significantly as the dose of primaquine increased, there was no overall difference in the composition of this radioactivity between doses. However, there was a small but significant increase in the excretion of both primaquine and the carboxylic acid metabolite at the two highest doses with a decrease in the excretion of 6-desmethylprimaquine (table 4.4). Primaquine, 5-hydroxyprimaquine and 5-hydroxy-6-desmethylprimaquine each accounted for 6 to 20% of the excreted [^{14}C] radioactivity. Only small amounts of radioactivity (2 to 4% of that excreted) could be accounted for as either 6-desmethylprimaquine or the carboxylic acid metabolite of primaquine (Table 4.4). As all the hydroxylated metabolites were highly unstable under the conditions of enzymic hydrolysis, the presence of conjugates of these metabolites could not be established. Deconjugation resulted in a small but significant increase from 4.1% to 4.9% and from 3.3% to 4.5% of the total radioactivity recovered in bile as the carboxylic acid metabolite after the 1.5 and 5.0mg doses respectively. Similarly, a significant increase from 7.1% to 7.8% in the percentage of [^{14}C] radioactivity excreted as the parent drug was obtained at the highest dose level. This indicates that only minor proportions of these compounds were excreted as glucuronide and sulphate conjugates.

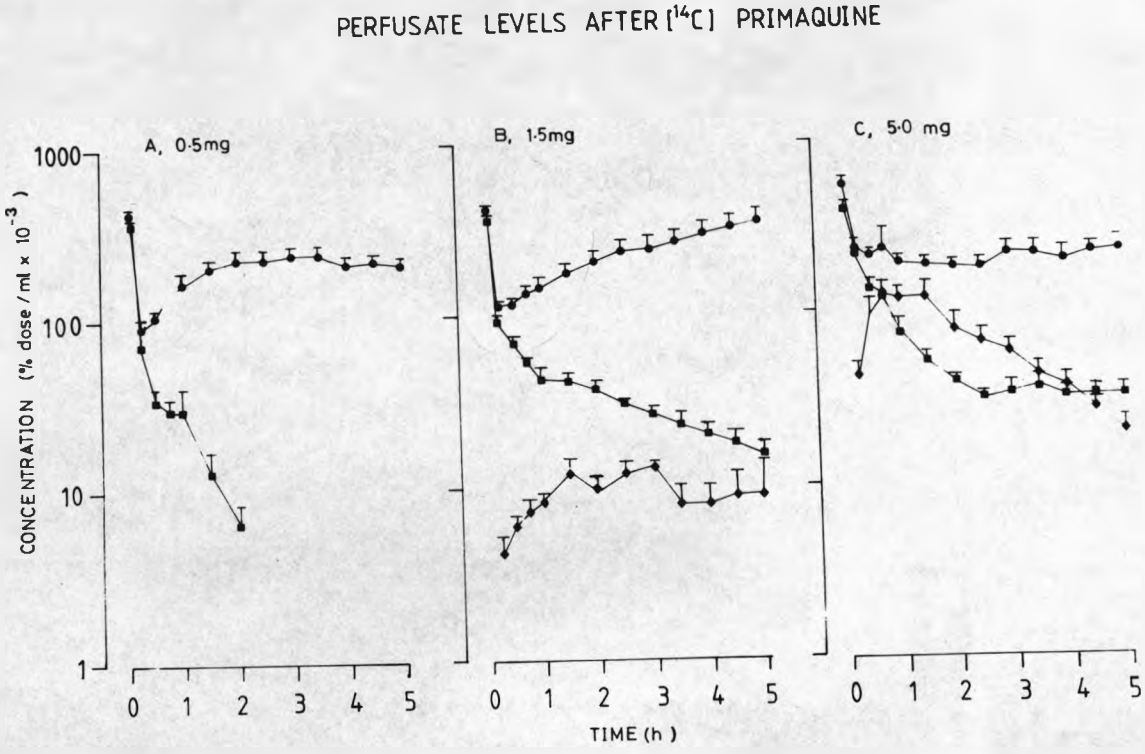


FIGURE 4.2 Perfusate concentrations (% dose/ml X $1\emptyset^{-3}$) of primaquine (■-■) [^{14}C] radioactivity (●-●) and the carboxylic acid derivative of primaquine (◆-◆) after the administration of radiolabelled primaquine (A) Ø.5mg ,(B) 1.5mg, and (C) 5.Ømg. Increasing dose size was associated with changes in the contribution of the AUC's for primaquine andthe carboxylic acid metabolite to the total [^{14}C] radioactivity AUC

TABLE 4.2 Mass Balance: The percent of the administered [^{14}C] radioactive dose recovered in bile over 5h and present in the liver and perfusate at 5h, after administration of primaquine (0.5 to 5.0mg) to the isolated perfused rat liver.

		% [^{14}C] Radioactive dose			
Dose (mg)	n	Liver	Bile	Perfusate	Total recovery
0.5	6	11.9 ± 1.6	63.8 ± 9.8	19.8 ± 4.1	95.5 ± 7.2
1.5	6	15.9 ± 3.4	40.5 ± 9.6	34.6 ± 9.9	91.0 ± 5.3
5.0	6	35.6 ± 7.2	33.8 ± 7.1	21.7 ± 11.0	90.2 ± 12.5

TABLE 4.3 Serial hourly bile flow after administration of primaquine (0.5, 1.5 and 5.0mg) to the isolated perfused rat liver preparation.

	Serial hourly bile flow (ml/h)				
Dosage	1	2	3	4	5
0.5	0.42 ± .06	0.31 ± .04	0.26 ± .02	0.24 ± .03	0.19 ± .04
1.5	0.47 ± .18	0.47 ± .04	0.37 ± .14	0.30 ± .15	0.21 ± .11
5.0	0.40 ± .03	0.33 ± .09	0.24 ± .03	0.22 ± .03	0.18 ± .05

4.3.3 MASS BALANCE

Table 4.2 shows the mass balance data for each dosage group at 5 hours. Although the amount of [^{14}C] radioactivity in the perfusate at 5h did not differ between dosage groups there was a significant dose-related reduction in the amount of radioactivity recovered in the bile. This reduction was translated into an increased localisation of [^{14}C] in the liver with higher doses. There was no significant difference in the total amount of radioactivity recovered collectively from bile, perfusate and liver at each dose level and total recovery approached 100% in all cases.

4.4 DISCUSSION

Evidence suggesting that primaquine exhibits dose-dependent elimination was obtained from a recent study investigating the effect of primaquine on the disposition of antipyrine in the isolated perfused rat liver preparation (IPRL) (chapter 3). Analysis of hourly perfusate samples from that study indicated that increasing primaquine dose resulted in a disproportionate increase in the perfusate primaquine concentrations. The present study has investigated the effect of increasing doses of [^{14}C] labelled primaquine (0.5, 1.5 and 5.0mg) on the pharmacokinetics and overall mass balance of the drug in the isolated perfused rat liver. The IPRL preparation enables experimental conditions to be rigorously controlled while allowing the hepatic component of drug elimination to be examined in a whole-organ system and without the influence of the other routes of elimination that

TABLE 4.4 Composition of [^{14}C] radioactivity recovered in bile from the isolated perfused rat liver preparation after administration of [^{14}C] labelled primaquine (0.5, 1.5 and 5.0mg).

Compound	Percent of total [^{14}C] radioactivity excreted in bile					
	0.5mg		1.5mg		5.0mg	
	f	f + c	f	f + c	f	f + c
5-hydroxyprimaquine	12.6 ± 1.7	-	12.4 ± 5.2	-	8.8 ± 5.2	-
5-hydroxy-6-desmethyl-primaquine	15.4 ± 4.9	-	7.1 ± 4.4	-	12.8 ± 3.2	-
6-desmethylprimaquine	3.4 ± 0.6	-	2.2 ± 0.4	-	2.1 ± 0.2	-
Primaquine	5.8 ± 0.9	5.9 ± 0.6	9.4 ± 1.6	8.6 ± 2.0	7.1 ± 1.0	7.8 ± 1.0*
Carboxylic acid derivative of primaquine	1.9 ± 0.6	2.5 ± 0.3	4.1 ± 0.7	4.9 ± 1.9*	3.3 ± 0.1	4.5 ± 0.8*

* represents a signficant increase in the [^{14}C] radioactivity present in the fraction after enzyme hydrolysis $p > 0.05$ (f = free compound c = conjugated compound)

are present in the intact animal.

Primaquine perfusate concentration/time profiles were biphasic at all three dosage levels. Initially perfusate drug concentrations fell rapidly, followed by a more gradual rate of decline (figure 4.1). Less than 50% of the dose could be accounted for as primaquine in the perfusate five minutes after drug administration. The initial rapid fall in perfusate drug concentrations is due to the avid hepatic uptake of primaquine. Perfusate primaquine levels continued to fall throughout the studies. By contrast the concentration of [^{14}C] radioactivity (expressed as % dose/ml) persisted at grossly elevated levels when compared to the corresponding primaquine levels, regardless of primaquine dose (figure4.2 A,B and C). This disparity between the concentrations of primaquine and total [^{14} C] radioactivity in the perfusate indicates the rapid formation of primaquine metabolites within the liver and their subsequent release into the perfusate.

The area under the perfusate primaquine concentration time/curves (AUC) were seen to increase, though disproportionately, with increments in dose size. A ten-fold increase in dose (0.5 to 5.0mg) produced a forty-five fold increase in the value for AUC (table 4.1). This was the combined result of a 75% decrease in clearance and a three-fold increase in volume of distribution. The reduction in elimination efficiency and increased hepatic uptake was reflected by a thirteen-fold increase in the elimination half-life of primaquine as dose was increased from 0.5 to 5.0mg.

At the Ø.5mg dose level, systemic clearance of primaquine was 11.6 ± 2.5 ml/min, representing 74% of liver blood flow (15 ml/min.) and indicating primaquine to be a high clearance compound in this experimental model. The capacity of the hepatic processes involved in the elimination of this drug were exceeded with increasing dose leading to a substantial fall in clearance, which represented only 19% of liver blood flow after the highest dose (5.Ømg). As primaquine dose was increased from Ø.5 to 5.Ømg the volume of distribution increased three-fold. As there is no selective uptake of this drug into erythrocytes (Chapter 7) and as there was a corresponding and equivalent three-fold increase in the amount of radioactivity within the liver at the conclusion of the experiment (table 4.1 and 4.2), this increased volume of distribution must reflect greater hepatic localisation of the drug. The increased hepatic uptake was associated with the higher perfusate concentrations that resulted from the considerably reduced clearance of primaquine at these higher doses (table 4.1).

Increasing dose size produced alterations in the pattern of primaquine metabolism, in addition to the pharmacokinetic changes discussed above (figure4.2A,B and C). The carboxylic acid derivative of primaquine, which has been identified as the principal plasma metabolite in man (chapter 6), was not detected in perfusate after a Ø.5mg dose of primaquine. After the 1.5 and 5.Ømg doses of primaquine the respective areas under the curves for this carboxylic acid

derivative were 3.6+1.4% and 40.0+17.5% of the corresponding areas under the [^{14}C] radioactivity concentration/time curves.

The increased AUC for this metabolite could conceivably be due to a reduced capacity of the liver to remove this metabolite after the 1.5 and 5.0mg doses of primaquine, similar to the limitation in elimination observed for the parent drug. Alternatively, the disproportionate increase in the AUC for this metabolite after higher doses of primaquine may be due to enhanced formation of the compound. However, the relative contribution of these processes could not be delineated in the present study, as it would be necessary to administer the metabolite, per se, to the IPRL.

Biliary excretion is a major route of elimination for primaquine in this experimental model with substantial levels of [^{14} C] radioactivity recovered in bile at all doses. However, this route of elimination was capacity limited with increasing dose size of primaquine being associated with a progressive reduction(from 60% of the dose after 0.5mg to 30% of the dose after 5.0mg) in the amount of the administered radioactivity recovered in bile. Increasing dose had no cholestatic or choleretic effects (table 4.3), and the actual composition of the [^{14}C] related material excreted in bile did not change (table 4.4). At all doses about 20% of the excreted radioactivity corresponded to 5-hydroxyprimaquine and a further 20% was associated with the remaining identified substances; 5-hydroxy-6-desmethylprimaquine, 6-desmethylprimaquine, primaquine and

the carboxylic acid metabolite of the drug (table 4.4). Over 50% of the radioactive material excreted in the bile was not identified although it was recovered by flushing the HPLC systems with methanol. Incubation of bile samples with deconjugating enzymes enabled the identification of small levels of conjugates (glucuronides and sulphates) of primaquine and its carboxylic acid derivative at the higher doses (table 4.4). Due to the unstable nature of the hydroxylated metabolites to the conditions of conjugate hydrolysis it was not possible to further examine the fate of these compounds in the present experiments.

At the conclusion of each experimental preparation (i.e. 5h) close to 100% of the administered radioactivity could be accounted for in the perfusate, bile and liver collectively. Although the amount of radioactivity present at 5h in the perfusate did not differ between the three dosage groups, increasing dosage produced a significant reduction in the amount of radioactivity recovered in bile with a significant increase in the amount remaining within the liver (table 4.2).

In conclusion, primaquine exhibits dose- dependent pharmacokinetics, metabolism and biliary excretion when administered to the isolated perfused rat liver preparation at doses ranging from 0.5 to 5.0mg of primaquine diphosphate. At the highest doses used, the carboxylic acid metabolite was the major metabolite in the perfusate, due either to enhanced formation or reduced elimination of this compound.

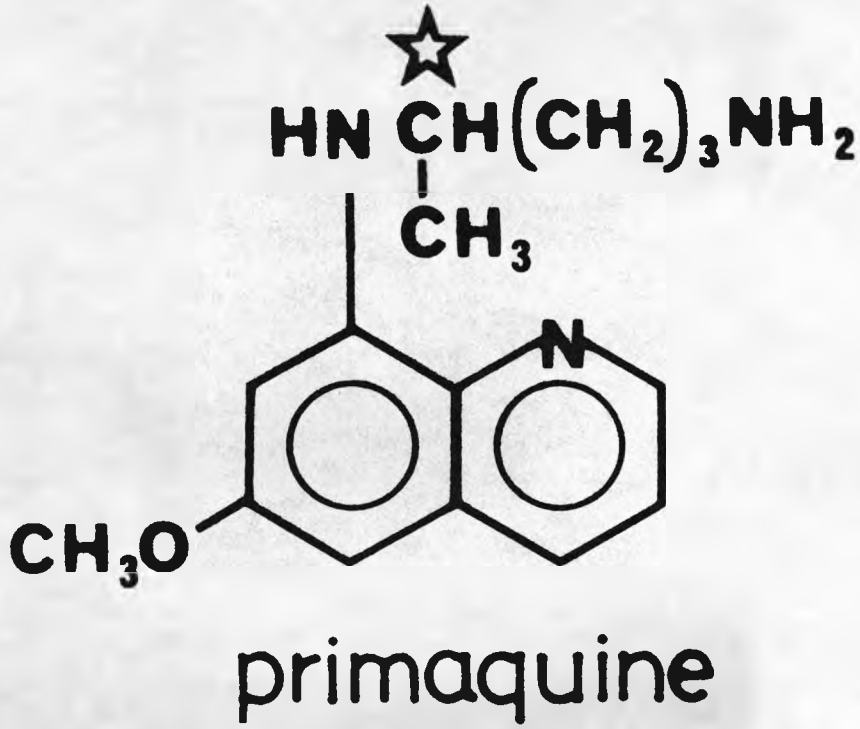
CHAPTER 5 THE PHARMACOKINETICS OF (+) AND (-) PRIMAQUINE IN THE ISOLATED PERFUSED RAT LIVER PREPARATION

5.1 INTRODUCTION

It is well documented that many compounds which exist in more than one isomeric form may exhibit stereoselective differences with respect to their pharmacological activity , metabolism and elimination e.g. warfarin (Breckenridge et al 1974, O'Reilly et al 1974) and propranolol (Sibler et al 1982). Primaquine possesses an asymmetric carbon atom in the alkyl amino side chain (figure 5.1). As a result of this chiral centre, the drug can exist as either (+) or (-) stereoisomers, although in clinical practice it is administered as the racemate .

A comparison of the curative and toxic activity of (+) and (-) primaquine with racemic primaquine showed that all three forms were equipotent antimalarial agents at doses of Ø.375-Ø.75mg/kg given daily over seven days(Schmidt et al 1977).The isomers however differed significantly with respect to toxicity (Schmidt et al 1977). Acute toxicity studies in the rat showed the (+) isomer to be three to four times more toxic than the (-) isomer, while subacute toxicity studies in the rhesus monkey showed the (-) isomer to be three times more toxic than (+) primaquine. The toxic dose of the racemate in both studies lay between the toxic doses of each of the isomers. These findings have not been further investigated, and it is unknown whether they are of a pharmacodynamic and/or a pharmacokinetic origin.

The potential of equivalent doses of (±), (-) and (+) primaquine to inhibit drug metabolism is described in this thesis (Chapter 3). Although all three compounds were



primaquine

FIGURE 5.1 Structural formula of primaquine, showing the position of the asymmetric carbon atom ☆.

equipotent inhibitors of drug metabolism, (-) primaquine was eliminated more rapidly than (+) primaquine, with the racemic compound having an elimination rate constant between those of the two isomers.

The purpose of the present study is to examine, in greater detail, the disposition of (+) and (-) primaquine, in the isolated perfused rat liver preparation. Earlier studies, in this experimental model, have shown that racemic primaquine exhibits dose-dependent pharmacokinetics (Chapter 4). Therefore, the disposition of the isomers of primaquine has been studied after a 0.5mg dose, where racemic primaquine behaves as a high clearance compound and after a 2.5mg dose, where the racemate behaves as a low clearance compound.

5.2 STUDY DESIGN

The optical purity of each stereoisomer was determined by measurement of the optical rotation of an aqueous solution of each isomer (1mg/ml ; 5ml) as previously described (2.1.9). The isolated perfused rat liver preparation (IPRL) was established as described earlier (2.2.2).

The elimination of primaquine isomers from the IPRL was studied, over three hours ,after administration of either the (+) or (-) isomer of primaquine as a 0.5 and 2.5 mg bolus dose of the diphosphate salt. In all groups n was equal to three. Primaquine isomers were added as aqueous solutions(50-250ul) directly into the perfusate reservoir thereby simulating systemic dosage.

Samples (1.5ml) were taken from the perfusate reservoir pre-dose and at 5, 10, 15, 30, 45, 60, 90, 120, 150, and 180 minutes. After centrifugation (1000g for 1 min) the separated perfusate plasma was removed and then frozen at -20°C, until it was assayed for primaquine. An equal volume of fresh perfusate was added to the reservoir to replace that removed by sampling. Bile was collected serially in 1 hour intervals into pre-weighed vials and the bile mass was measured. Perfusate blood gases and liver function tests were performed before and after each experiment to ascertain liver viability.

Perfusate plasma primaquine concentrations were determined by the previously reported selective and sensitive HPLC method (2.1.1).Pharmacokinetic parameters (clearance, volume of distribution, half-life and area under the perfusate plasma concentration/time curve) were calculated as described in chapter 1 (1.6.6).Statistical comparisons were made using Student's t-test. Data are tabulated as mean ± SD and illustrated graphically as mean ± SEM. Statistical significance was accepted when $p < 0.05$.

5.3 RESULTS

Perfusate plasma primaquine concentrations declined biexponentially following the administration of (+) or (-) primaquine at doses of 0.5 and 2.5mg (figure 5.2). Perfusate drug concentrations fell rapidly over the first 15 minutes after drug administration, followed by a more gradual rate of decline to the end of each experiment (3h). There were no

significant differences in the elimination half-lives clearance, volume of distribution or area under the curve for the (+) and (-) isomers of primaquine respectively after the Ø.5mg dose (table 5.1).

Inspection of the mean log perfusate drug concentration/time curves obtained for the (+) and (-) isomers of primaquine after a 2.5mg bolus dose, revealed a marked disparity in the two profiles (figure 5.2) , with the perfusate levels of the (-) isomer falling more rapidly than those of the (+) isomer . At this dose size there was no significant difference in the volume of distribution of the (-) and (+) isomers of primaquine (522 ± 115ml and 475 ± 207ml respectively).In contrast, the clearance of the (-) isomer (4.7 ± 1.1ml/min) was significantly greater than the clearance of the (+) isomer (1.4 ± Ø.6ml/min).This difference in hepatic elimination efficiency was translated into a significantly shorter half-life (81.6 ± 37.8min) and smaller area under the curve (31Ø ± 84µg.ml/min)for (-) primaquine when compared with the half-life (245 ± 122min) and area under the curve (11Ø5 + 4ØØµg.ml/min) for the (+) isomer (table 5.1).

5.4 DISCUSSION

The disparity in the toxicity of the two isomers of primaquine observed by Schmidt et al (1977) may be due to either stereoselective differences in the

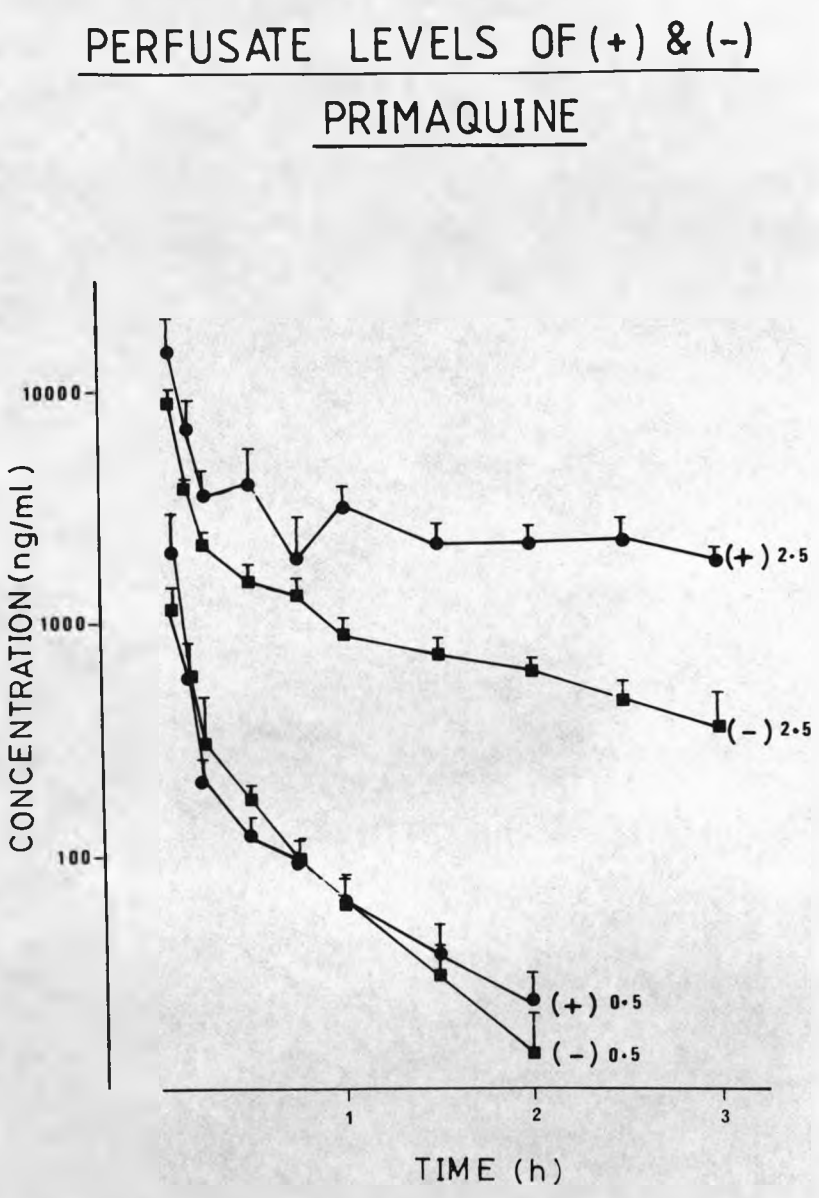


FIGURE 5.2 Perfusate primaquine concentration/time curves for primaquine after administration of the (+) or (-) isomer at a dose of 0.5 and 2.5mg of the diphosphate salt.

TABLE 5.1 The pharmacokinetics of the (-) and (+) isomers of primaquine after administration of each isomer at doses of 0.5 and 2.5mg to the isolated perfused rat liver preparation.

Dose	Cl (ml/min)	Vd (ml)	$t_{\frac{1}{2}}$ (min)	AUC (μg.ml/min)
(-) 0.5mg	11.9 ± 1.6	358 ± 64	21.2 ± 6.0	24.1 ± 3.5
(+) 0.5mg	11.6 ± 3.7	499 ± 106	32.9 ± 14.1	26.2 ± 9.2
Sig. diff.	NS	NS	NS	NS
(-) 2.5mg	4.7 ± 1.1	522 ± 115	81.6 ± 37.8	310 ± 84
(+) 2.5mg	1.4 ± 0.6	475 ± 207	245 ± 122	1105 ± 400
Sig diff.	P>.05*	NS	P>.05*	P>.05*

pharmacodynamics and/or pharmacokinetics of the two forms. Preliminary observations have suggested that there is a stereoselective difference in the disposition of (+) and (-) primaquine in the IPRL (Chapter 3), such that the (-) isomer appeared to be eliminated more rapidly than an equivalent dose of the (+) isomer.

In the present study the disposition of primaquine isomers in the IPRL preparation has been investigated in greater detail. Earlier studies have shown that the disposition of primaquine in this experimental model exhibits dose-dependent pharmacokinetics, behaving as a high clearance compound at low doses (< 0.5mg) and a low clearance compound at high doses (> 0.5mg) (Chapter 4). Therefore, in order to study the disposition of (+) and (-) primaquine at both extremes (i.e. when behaving as a high clearance and when behaving as a low clearance compound) the (+) and (-) isomers have been administered at doses of 0.5 and 2.5 mg.

There was no significant difference in the area under the perfusate plasma concentration/time curve, elimination half-life, volume of distribution or clearance derived for the (+) and (-) isomers of primaquine at the lower dose (i.e. 0.5mg,table 5.1). The value for half-life and area under the curve for each isomer following a dose of 0.5mg in this study was similar to that obtained after the administration of the racemate at an equivalent dose (Chapter 4). In addition the observed value for clearance indicated that both (+) and (-) primaquine behave as high clearance compounds at this low dose since the clearance of each isomer was more than 60% of

liver blood flow, (15ml/min) as was shown to be the case for the racemate (Chapter 4). As was also shown to be the case for the racemate (Chapter 4), the volumes of distribution for both the (+) and (-) isomers of primaquine were considerably greater than the physical volume of the system (i.e. 100ml perfusate + liver volume) suggesting extensive tissue uptake of both isomers.

There was a marked difference in the drug concentration /time profiles for (+) and (-) primaquine following the 2.5mg dosage(figure 5.2), a dose at which racemic primaquine behaves as a low clearance compound. Perfusate plasma concentrations of the (-) isomer fell more rapidly than the corresponding (+) isomer levels. This disparity could conceivably result from differences in either the clearance and/or the distributional characteristics of the two isomers at this dose . There was no significant difference in the volume of distribution of the two isomers at this dosage and the value for this parameter was again significantly greater than the physical volume of the system (table 5.1). In contrast,the clearance of (+) primaquine was only 25% of the clearance of the (-) isomer, although the clearance of both isomers was representative of low clearance compounds . This difference in the ability of the liver to eliminate (+) primaquine was reflected in a threefold greater elimination half-life and area under the curve for this isomer compared to (-) primaquine (table 5.1). The observation of Schmidt et al (1977) who showed (+) primaquine to be three to four times more toxic than (-) primaquine, may be a consequence of

the threefold difference in elimination half-life and area under the curve for the two isomers, as determined in the present study.

In summary, the isomers of primaquine show stereoselective disposition after administration of higher doses, where the isomers behave as low clearance compounds. This difference in the elimination of the two isomers is likely to be due to a difference in the affinity of metabolising enzymes for the two substrates, as shown by the differences in the values derived for clearance. At the lower dose both isomers behave as high clearance compounds.As such the elimination of the isomers is dependent primarily on liver blood flow. Under these conditions differences in intrinsic clearance (or the affinity of metabolising enzymes for the two isomer substrates) will not be reflected by measurable stereoselective differences in drug elimination. An alternative explanation for the lack of any difference in the elimination of the two isomers after the Ø.5mg dose could be because different metabolising pathways operate at the two dose levels used in this study. Such that at low doses high affinity, low capacity processes are responsible for metabolising the isomers of primaquine,in which case there may be no difference in affinity. However, at the higher dose this low capacity process becomes saturated and high capacity low affinity metabolising pathways take over and these pathways may show a difference in affinity for the two isomers.

The relevance of these findings to man, where

primaquine behaves as a low clearance compound at doses throughout the therapeutic range (Chapter 7), is currently under investigation as are studies to investigate the toxicity and antimalarial activity of the two isomers at therapeutic concentrations.

CHAPTER 6 THE PHARMACOKINETICS OF PRIMAQUINE IN MAN AFTER SINGLE ORAL DOSAGE AND THE IDENTIFICATION OF THE CARBOXYLIC ACID DERIVATIVE AS THE PRINCIPAL PLASMA METABOLITE.

6.1 INTRODUCTION

The pharmacokinetics and metabolic fate of primaquine is largely unknown. Earlier work in animal models with other 8-aminoquinoline compounds structurally similar to primaquine (ie. pamaquine and pentaquine), indicated that these compounds were rapidly removed from the body and extensively metabolised (Zubrod et al. 1948; Elderfield et al. 1953; Smith 1956; Bami et al. 196Ø) more recently an investigation in man (Greaves et al 198Ø) found that primaquine too was rapidly eliminated and extensively metabolised to unidentified products (Chapter 1.5).

The therapeutic and toxicological effects of primaquine therapy have been attributed to one or more of its metabolites (Chapter 1.5). Hence there has been considerable interest in the identification and quantitation of these compounds. Studies have generally focused on aryl-hydroxylated structures (Strother et al 1981;Chapter 1.5), and although a number of compounds have been postulated as metabolites none have been identified from studies in man.

By contrast, little attention has been paid to primaquine related compounds produced by metabolism of the 8-amino side chain. Two such metabolites have recently been isolated by Hufford et al. (1983) following the incubation of primaquine with microbial cultures. One of these compounds possessed acidic properties and was produced by deamination of the terminal primary amine group followed by oxidation to a carboxylic acid derivative (figure 6.1). The other was identified as N-acetylprimaquine (figure 6.1). The carboxylic

acid metabolite but not N-acetylprimaquine was also identified in the plasma of rats treated with primaquine (Baker et al. 1982; Chapter 1.5).

This chapter describes the pharmacokinetics of primaquine in healthy male volunteers after a standard oral dose (45mg free base) and examines the metabolism of this drug to the proposed N-acetyl and carboxylic acid derivatives.

6.2 STUDY DESIGN

Five healthy male volunteers (age 24-46 years) who were taking no other drugs each received primaquine (6X 7.5 mg tablets = 45mg as primaquine base) orally after an overnight fast. Venous blood samples were taken pre-dose and at Ø.5, 1, 2, 3, 4, 6, 8, 12, and 24h. Blood was centrifuged (1ØØØg for 15 min) and the separated plasma stored at -2Ø°C until time of analysis. Urine was collectedfor 1h pre-dose and from Ø-24h,urine volume and pH were recorded and an aliquot frozen at -2Ø°C.

In addition to the standard 45mg dose, one volunteer (RB) received an oral tracer dose of ring labelled [^{14}C] primaquine (8.2μCi; 2.4mg). In this volunteer blood was collected over 12Øh and urine collections were continued for 6 days. Plasma and urine samples were analysed for primaquine, N-acetylprimaquine and the carboxylic acid metabolite of primaquine by HPLC and for [^{14}C] radioactivity by liquid scintillation counting as described in chapter2 (2.1.1,2.1.2, and 2.1.8).Urine samples were incubated with hydrolysing enzymes as described (2.1.7) in order, to determine the concentrations of sulphate and glucuronide

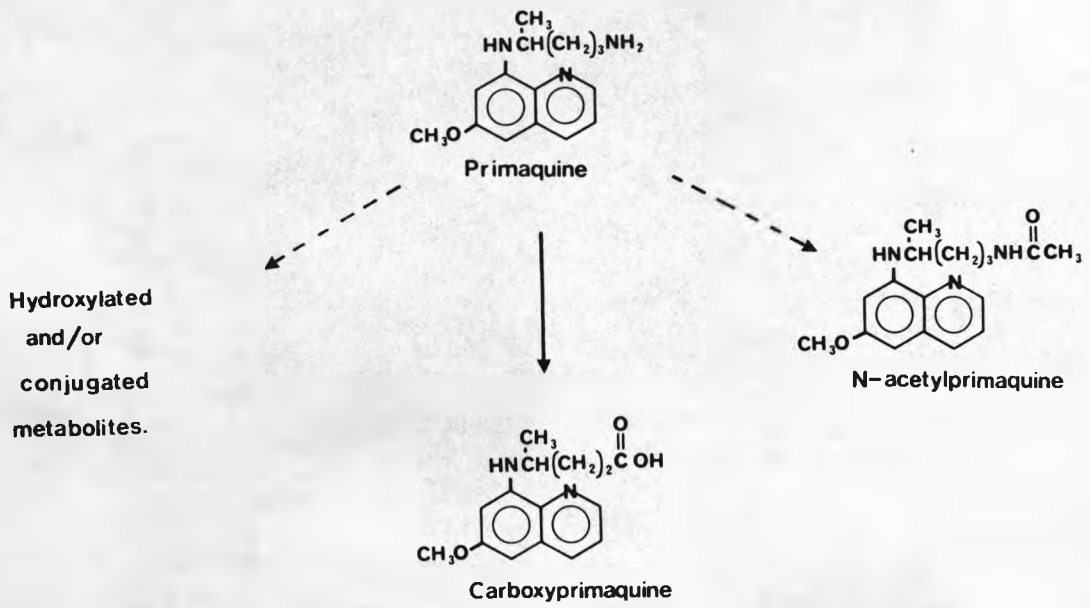


FIGURE 6.1 Primaquine conversion to identified metabolites (——) and postulated metabolites (- - - -).

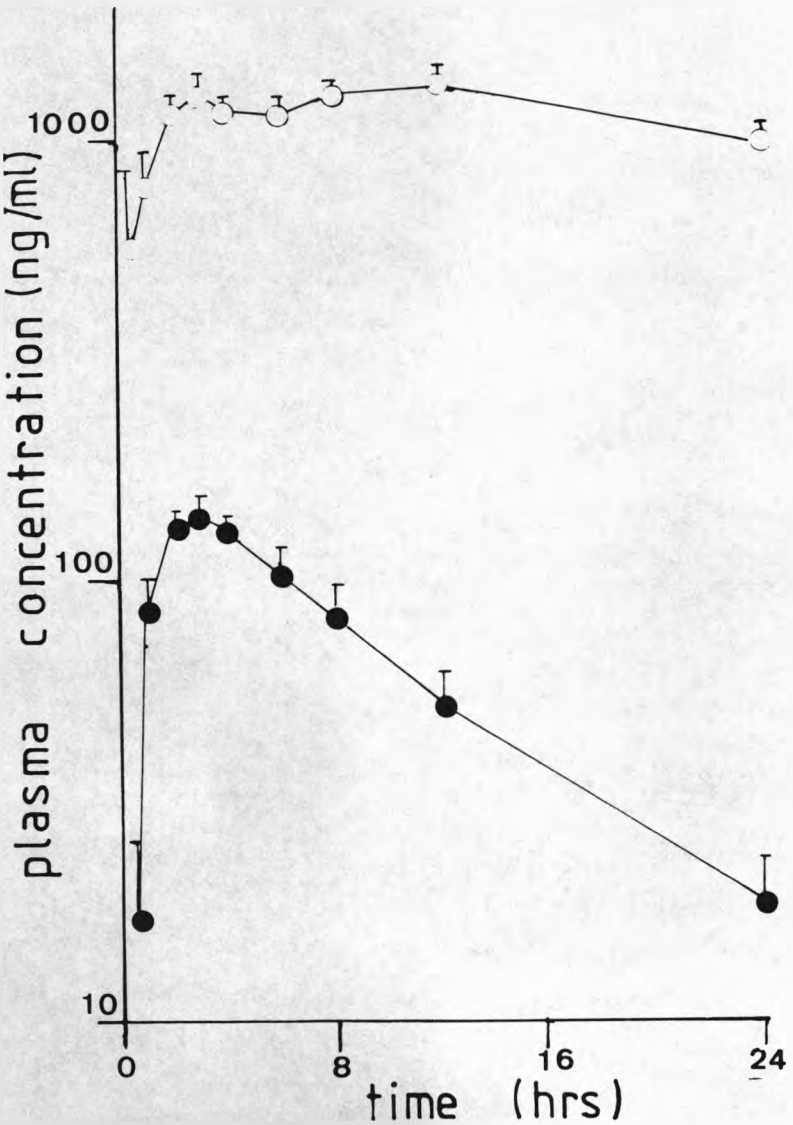


FIGURE 6.2 Plasma concentration/time profiles for primaquine (●—●) and the carboxylated metabolite of primaquine (○—○) after administration of primaquine (45mg free base) to man.

TABLE 6.1. Summary of pharmacokinetic parameters for Primaquine and its carboxy metabolite after oral dosage with Primaquine (45mg). Time to peak plasma concentration (T_{pk}); peak plasma concentration (C_{pk}); terminal elimination half-life ($t_{\frac{1}{2}}$); apparent oral clearance (Cl_o); and amount excreted in the urine to 24 hours (X_u).

Pharmacokinetic Parameter	Subjects SW	GE	AB	MO	RB	Mean ± SD
A. PRIMAQUINE (Primaquine)						
T_{pk} (h)	3.0	2.0	3.0	2.0	3.0	3 ± 1
C_{pk} (ng/ml)	131.2	132.8	180.4	146.0	176.0	153.3 ± 23.5
$t_{\frac{1}{2}}$ (h)	5.8	6.5	7.8	5.9	9.6	7.1 ± 1.6
Cl_o (l/h)	41.6	28.6	18.5	33.4	20.3	28.5 ± 9.5
X_u (% of dose)	1.4	0.5	0.4	1.9	2.4	1.3 ± 0.9
B. CARBOXYPRIMAQUINE ($PQCO_2H$)						
T_{pk} (h)	8.0	2.0	8.0	12.0	3.0	6.6 ± 4.1
C_{pk} (ng/ml)	1078.0	1386.0	1199.0	1808.0	1666.0	1427.4 ± 307.3
X_u (% of dose)	0.0	0.0	0.0	0.0	0.0	0.0

conjugates of both primaquine and the carboxylic acid derivative of primaquine. Pharmacokinetic parameters were derived using model independent formulae (Chapter 1.6.6). Data are tabulated as mean ± SD and presented graphically as mean ± SEM.

6.3 RESULTS

The mean plasma concentration/time profiles for primaquine and the carboxylic acid metabolite of primaquine are shown in figure 6.2 and the pertinent pharmacokinetic estimates are listed in table 6.1. Following oral administration primaquine was rapidly absorbed reaching peak plasma concentrations of 153.3 ± 23.5ng/ml between 2 and 3h. Thereafter, drug levels declined rapidly and monoexponentially, with a half-life of 7.1 ± 1.6h. Oral clearance was 28.5 ± 9.5 l/h. Renal clearance of unchanged primaquine represented a minor route of elimination since only 1.3 ± 0.9% of the dose was recovered in urine over 24h.

Primaquine undergoes rapid side chain metabolism to the carboxylic acid metabolite, involving initial deamination followed by oxidation. Peak levels of this metabolite of 1427 ± 307ng/ml were reached between 3 and 12h post-dose. These metabolite levels were maintained in excess of 1000ng/ml throughout the study period. However, the carboxylic acid metabolite was not detected in urine, suggesting that this compound is subjected to further metabolism prior to excretion. Furthermore, concentrations of N-acetylprimaquine, identified in microbial cultures by Hufford *et al.* (1983),

rarely exceeded the minimum detectable level of 10ng/ml in this study.

In the subject (RB) where a tracer dose of [^{14}C] primaquine was co-administered with the oral dose, the pharmacokinetics of primaquine were comparable with the other four volunteers. However, in this case the plasma concentration/time profile of [^{14}C] radioactivity differed markedly from that for primaquine (figure 6.3). Peak levels of [^{14}C] were attained rapidly (2-12h) but exceeded primaquine levels by ten to twenty fold. Only 1.9% of the total area under the plasma radioactivity concentration/time curve was accounted for by primaquine, whereas the carboxylic acid metabolite contributed 55% of the area (figure 6.3). This metabolite could not be detected by 72-96h, at which time 1.3 10^{-3}% of the dose was present per ml of plasma as [^{14}C] radioactivity, indicating the persistance of other metabolic products of primaquine.

Urinary recovery of [^{14}C] radioactivity over 6 days accounted for 64% of the dose (figure 6.4) and was essentially complete within 5 days (table 6.2). This radioactivity was mainly in the form of metabolites other than N-acetylprimaquine or the carboxylic acid metabolite, with only 3.6% of the dose recovered as the parent compound (figure 6.4;table 6.2). Incubation with hydrolysing enzymes did not result in an increase in the concentrations of primaquine or the carboxylic acid metabolite.

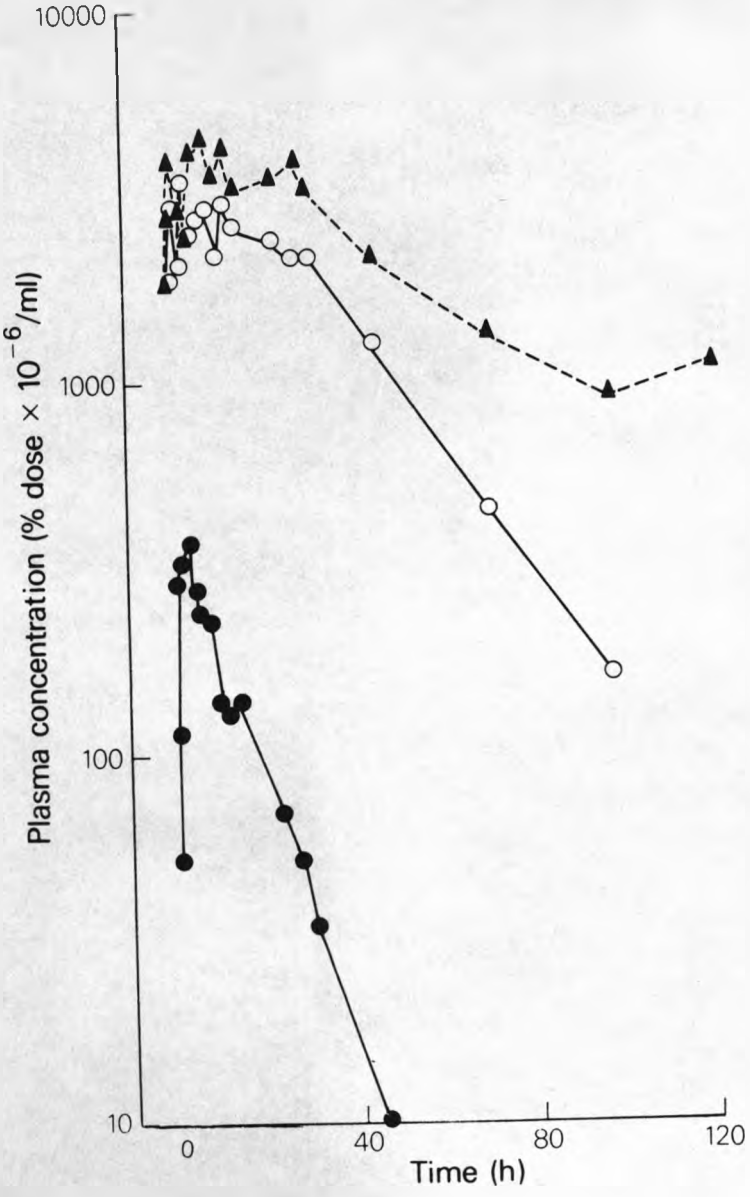


FIGURE 6.3 Plasma concentration/time profiles for primaquine (●—●) the carboxylic acid metabolite of primaqine (O—O) and [^{14}C] radioactivity (▲—▲), after administration of [^{14}C] labelled primaquine (8.25μCi) to volunteer RB.

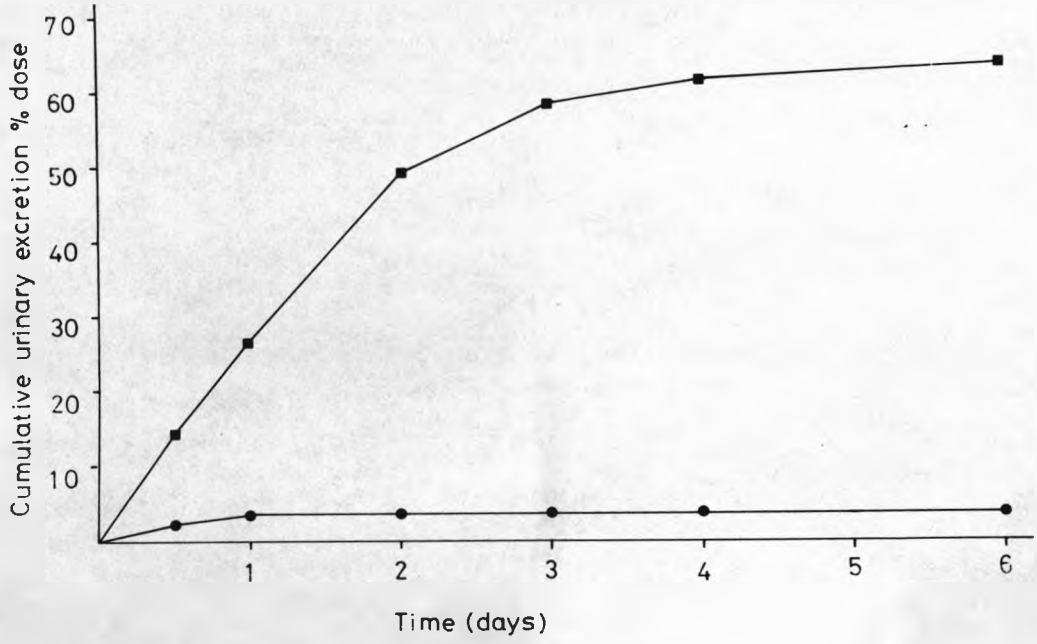


FIGURE 6.4 Cumulative urinary excretion curves for primaquine (PQ)●and [^{14} C]■radioactivity over 6 days in volunteer RB.

TABLE 6.2 Cumulative urinary excretion data for Primaquine and ^{14}C Radioactivity in one subject RB. The carboxylic acid metabolite was not detected in urine.

Serial Collection (h)	Cumulative % of Dose	
	Primaquine	^{14}C Radioactivity
0 - 12.0	2.4	14.6
12 - 24.0	3.5	26.8
24 - 48.0	3.6	49.6
48 - 72.0	3.6	58.7
72 - 96.0	3.6	61.4
96 - 144	3.6	63.8

6.4 DISCUSSION

The report of Greaves et al (1980) is the only other published investigation of the pharmacokinetics of primaquine in man.In that study primaquine reached peak plasma levels of 150ng/ml within 1-3h. Plasma clearance was 25l/h and the volume of distribution was estimated to be 200 l. The calculation of these parameters assumed a bioavailability of 1. Greaves et al.(1980) showed the renal elimination of unchanged primaquine to account for less than 1% of the dose administered.The findings of the present study on the pharmacokinetics of primaquine (figure 6.2; table 6.1) are in broad agreement with those of Greaves et al.(1980).However, without a reliable measure of primaquine's bioavailability, systemic clearance and apparent volume of distribution could not be calculated in the present study.

The study described here has shown the carboxylic acid metabolite of primaquine to be the principal metabolite in human plasma. Primaquine was rapidly converted to this metabolite which persisted at elevated levels in plasma for the duration of the 24h study period. At 24h the carboxylic acid metabolite levels in plasma were fifty-fold higher than the corresponding levels of the parent drug. The area under the plasma concentration/time curve for the metabolite was considerably greater than that for primaquine. An indication of the clearance of the carboxylic acid metabolite relative to primaquine can be obtained by substituting the values for area under the plasma concentration/time curve (AUC) into the following equation

(Rowland and Tozer 1980):

$$\frac{\text{AREA(met)}}{\text{AREA(drug)}} = Fm. \frac{\text{CLEARANCE OF DRUG}}{\text{CLEARANCE OF METABOLITE}} \qquad (6.1)$$

Where AREA(met)=area under metabolite concentration/time curve; AREA(drug)=area under drug concentration/time curve and Fm=the fraction of the drug converted to the metabolite.

The fraction of the drug converted to the metabolite cannot exceed the available dose of the drug (ie. it has a limiting value of unity).In the present study the ratio of AREA(met)/AREA(drug) is approximately 25. Consequently the ratio of drug clearance to metabolite clearance must be greater than unity irrespective of the value of Fm which as stated cannot exceed 1. Hence the clearance of this carboxylic acid metabolite must be considerably less than the clearance of primaquine although the difference cannot be quantitated without knowing the value for Fm.

In contrast to primaquine the more polar carboxylic acid metabolite would be expected to be restricted to plasma water to a much greater extent and have a much smaller volume of distribution than the lipid soluble parent drug. It is therefore conceivable, that this metabolite represents a smaller fraction of the dose administered than does primaquine. A more detailed pharmacokinetic study of this metabolite *per se* could not be undertaken as the compound is continuously being formed and eliminated over the 24h study period. In order to obtain further information concerning the pharmacokinetics of this compound it would be necessary to administer pure metabolite.As this metabolite was not

identified in urine, it must undergo further metabolism prior to excretion.

An analogous process of metabolism has been reported for the β blocking agent propranolol where side chain deamination and oxidation of the terminal carbon atom to the carboxylic acid produces naphthoxylactic acid (Walle et al. 1979). This compound is present in plasma at considerably higher levels than propranolol. However, unlike the carboxylic acid metabolite of primaquine, naphthoxylactic acid is eliminated from plasma at a comparable rate to propranolol and about 14% of the dose appears as this metabolite in urine.

The HPLC assay used for the determination of the carboxylic acid metabolite also enabled the simultaneous determination of N-acetylprimaquine (Chapter 2.1.2) previously identified in microbial metabolism studies (Hufford et al. 1983). In the present study the N-acetylated compound was never seen to exceed the minimum detectable plasma concentration of 10ng/ml and is therefore unlikely to be of importance with respect to the metabolism of primaquine in man.

Following [^{14}C] primaquine administration to subject (RB) the area under the plasma concentration/time curve for the carboxylic acid metabolite constituted 55% of the total area due to plasma radioactivity. This observation suggests that this compound is the principal metabolite in plasma. This is particularly apparent over the first 24h, during which metabolite levels closely follow [^{14} C] radioactivity levels (figure 6.3). Thereafter [^{14} C] levels in plasma

persisted, despite falling levels of this carboxylic acid metabolite, again suggesting further biotransformation of this metabolite. Over 6 days 3.6% of the dose was recovered inthe urine as primaquine and none as the carboxylic acid metabolite although 64% of the [^{14}C] radioactivity was recovered over the same period. In an attempt to identify the material excreted in urine, urine samples were incubated with H1-hydrolase obtained from H.pomatia(Chapter2.1.7). This enzyme preparation has both arylsulphohydrolase activity and β -glucuronidase activity. As such this preparation is capable of breaking down glucuronide and sulphate conjugates. This procedure failed to increase the urinary recovery of either primaquine or the carboxylic acid metabolite of primaquine. Therefore, neither glucuronide nor sulphate conjugation appear to be major processes of elimination for these two compounds.

The study described in this chapter has identified the carboxylic acid derivative as the principal plasma metabolite of primaquine in man. The association of this metabolite with the toxicological and pharmacodynamic effects of primaquine has yet to be examined.

CHAPTER7. STUDIES OF THE EFFECT OF DOSE SIZE ON THE PHARMACOKINETICS AND METABOLISM OF PRIMAQUINE AND A MEASURE OF THE DRUG'S ABSOLUTE BIOAVAILABILITY.

7.1 INTRODUCTION

Primaquine is used in doses ranging from 15mg to 45mg per day.Although the pharmacokinetics of this drug have been studied after a single 45mg dose (Chapter 6) the disposition of smaller oral doses is unknown.Interestingly, experimental evidence obtained in the isolated perfused rat liver preparation (Chapter 4) has shown primaquine to exhibit dose-dependency with respect to its pharmacokinetics and its metabolic fate.These observations have in part prompted the present investigation.

The absolute bioavailability of primaquine is unknown although it is believed to be less than one (Greaves et al 1980).To study the absolute bioavailability of primaquine, requires that the drug be administered by the intravenous route. It has been proposed that primaquine is subject to extensive first-pass hepatic extraction (Greaves et al 1980). This implies that i.v. dosage of this drug would be associated with substantially higher plasma levels than that seen with oral dosage and this could lead to greater pharmacological and possibly toxic effects after i.v. administration.

Therefore, the purpose of this study was firstly to evaluate the absolute bioavailability of primaquine,which was done by the simultaneous administration of a tracer dose of [^{14}C] primaquine i.v. (7.5 uCi; 1,55mCi/mmole) and a standard 45mg oral dose, thus eliminating the possibility of intra-subject day to day variation in disposition. Furthermore, the

use of an i.v.tracer dose circumvents suprapharmacological effects and toxicity that may result from i.v. doses of larger mass.The second aim of this study was to examine the pharmacokinetics of primaquine over the therapeutic dosage range (15-45mg).

7.2 STUDY DESIGN

7.2.1 PROTOCOL

Five healthy volunteers (24-46 years) who were taking no other drugs, participated in this study. On separate occasions (at least one week apart)in randomised order and after an overnight fast each subject received primaquine (15, 30 and 45mg as the base). The 45mg oral dose was accompanied by an intravenous dose of [^{14}C] primaquine (7.5uCi; equivalent to 4.5mg primaquine base in 5ml solution), administered over eight minutes.Venous blood samples (10ml), for plasma drug assays, were collected pre-dose and again at 0.5, 1, 2, 3, 4, 6, 8, 10, 14 and 24 hours post-dose and also at 10 and 17 minutes after the start of the intravenous dose. Blood was centrifuged (1000g for 15 min) and the separated plasma stored at -20^{o}C until analysis. In the simultaneous i.v./oral dose study, additional samples (5ml) were taken for whole blood assays at 2 and 6 hours post-dose and stored as for plasma.

7.2.2 ASSAY METHODS

(a) Primaquine and the carboxylic acid metabolite of primaquine: plasma and whole blood levels of the carboxylic acid metabolite of primaquine and primaquine were determined by selective and sensitive HPLC methods (2.1.2 and 2.1.1)

(b) [^{14}C] primaquine and [^{14}C] carboxylic acid metabolite of primaquine: plasma and whole blood levels of [^{14}C] primaquine and the [^{14}C] carboxylic acid metabolite of primaquine, derived from the simultaneous i.v./oral dose study were determined as follows. After HPLC analysis, the eluted fractions corresponding to primaquine and the carboxylic acid metabolite of primaquine were collected and the radioactive content determined by liquid scintillation counting. The absolute concentrations of [^{14}C] radioactivity in each sample were adjusted in order to account for differences in extraction efficiency between samples. Extraction efficiency in a sample of concentration C, was calculated as follows:

$$\text{Extraction efficiency (\%)} = \frac{C(\text{ng/ml}) \times Y(\text{mm/ng})}{P(\text{mm/ml})} \times 100$$

Were Y is the magnitude of the recorder deflection equal to 1ng of compound and P is the peak-height of compound obtained per ml, from that extracted sample. The absolute [^{14} C] concentration associated with each compound, per ml of sample, was then calculated as follows:

$$\text{Absolute } [^{14}C] \text{ concentration/ml} = \frac{\text{Observed } [^{14}C]/\text{ml}}{\text{Extraction efficiency}}$$

(C) $[^{14}C]$ radioactivity: plasma levels of $[^{14}C]$ radioactivity were determined by liquid scintillation counting using an Intertechnique SL33 liquid scintillation counter (2.1.9).

Pharmacokinetic parameters (clearance, volume of distribution, half-life, area under the curve and bioavailability) were determined as described in chapter 1 (1.6.6). Statistical comparisons between two groups were made by Student paired t test. When comparisons between more than two groups (of paired data) were made, two factor analysis of variance was used, $p < 0.05$ was accepted as significant.

7.3 RESULTS

After simultaneous i.v. and oral administration of primaquine, there was no significant difference between the values for the half-life, AUC, clearance, and volume of distribution (figure 7.1, table 7.1). From this data, the absorption of orally administered primaquine can be shown to be virtually complete as the calculated value for bioavailability approached unity in every subject (table 7.2).

Plasma total $[^{14}C]$ concentrations of primaquine fell sharply but only transiently after i.v. administration of $[^{14}C]$ primaquine (figure 7.2). However after 30 min, plasma $[^{14}C]$ radioactivity rose, until at 6h the levels of

radioactivity plateaued at 0.04% of the dose per ml of plasma. Over the 24h period of study, 38% of the plasma [^{14}C] radioactivity AUC was accounted for by plasma concentrations of the [^{14}C] carboxylic acid metabolite, whereas less than 5% was due to the parent drug.

The whole blood to plasma distribution (B/P) ratios of primaquine and the carboxylic acid metabolite of primaquine were determined at 2 and 6h after the simultaneous i.v./oral dose study. There were no significant differences in B/P in each individual, between the 2 and 6h samples for primaquine (B/P at 2h=0.81 ± 0.14 and at 6h=0.80 ± 0.14) and the carboxylic acid metabolite of primaquine (B/P at 2h=0.84 ± 0.37 and at 6h=0.83 ± 0.40).

After a single oral 15mg dose of primaquine, drug absorption was rapid, with peak plasma levels attained within 3h (figure 7.3). Thereafter plasma levels fell rapidly and monoexponentially with a mean elimination half life of 5.9 ± 2.1h. The mean AUC was 0.5 ± 0.1 μg.h/ml , oral clearance was 31.2 ± 7.0 l/h and apparent volume of distribution was 269.2 ± 120.9 l. The administration of 30 and 45mg oral doses of primaquine was associated with proportional increases in AUC (1.2 ± 0.2 μg.h/ml and 1.7 ± 0.4 μg.h/ml respectively). This linear rise in AUC ($r=0.99$; $p<0.01$; table 7.1) was not accompanied by any significant alteration in the values for elimination half-life, clearance, or volume of distribution for primaquine (table 7.1).

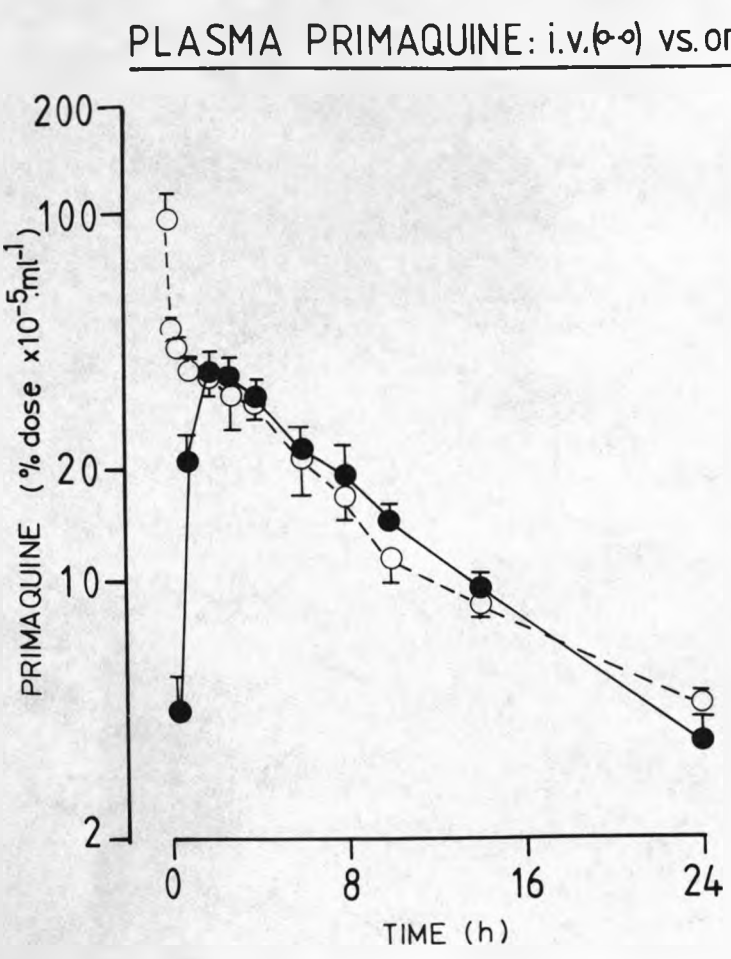


FIGURE 7.1 Semilogarithmic plot of plasma primaquine concentrations against time after simultaneous intravenous (O ;7.5μCi,4.5mg) and oral (● ;45mg) dosage.

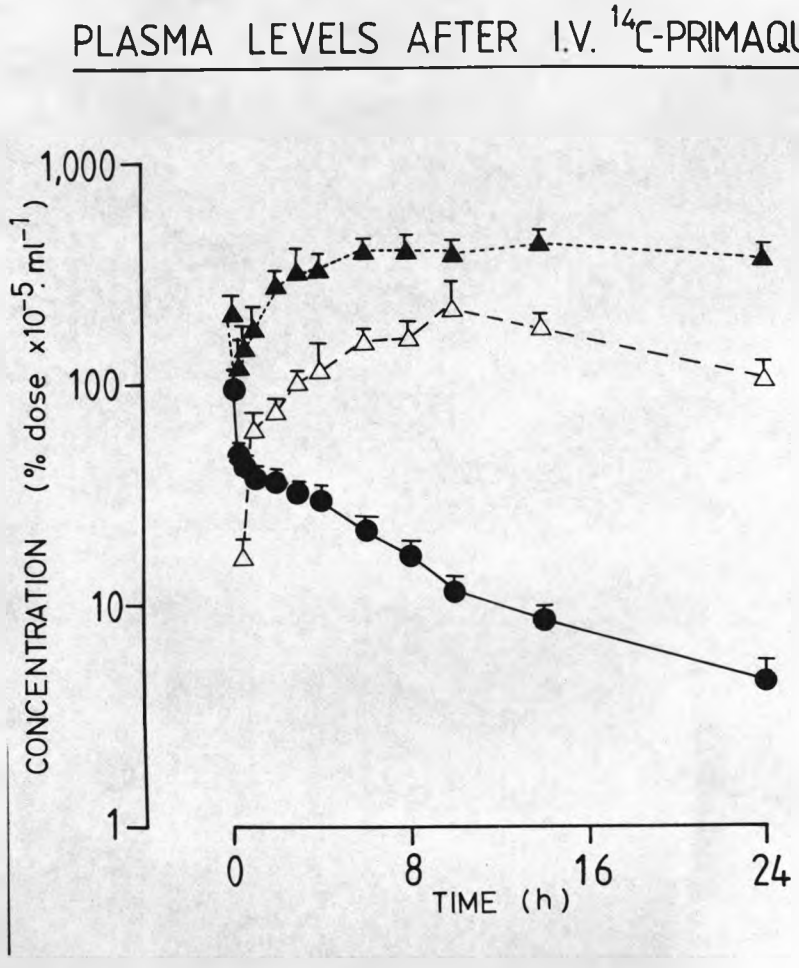


FIGURE 7.2 Semilogarithmic plot of total [^{14}C] radioactivity (▲),[^{14}C] primaquine(●) and [^{14} C] carboxylic acid metabolite levels(Δ) obtained after intravenous dosage with [^{14}C] primaquine (7.5uCi, 4.5mg).

TABLE 7.1 Summary of pharmacokinetic parameters for primaquine after oral primaquine (15 and 30mg) and simultaneous intravenous [^{14}C]-primaquine (7.5μCi; 1.55mCi/mmole; 4.5mg) and oral primaquine (45mg) to 5 healthy subjects. The parameters are; maximum plasma concentration(C_{max}), time to reach maximum levels (t_{max}), oral clearance (CL_o), systemic clearance (CL), elimination half-life ($t_{\frac{1}{2}}$), volume of distribution (Vd), area under the curve from time = 0 to 24h $AUC_{(o-24)}$ and area under the curve from time = 0 to infinity (AUC). Also shown are the values for $AUC_{(o-24)}$ for the carboxylic acid metabolite of primaquine ($PQCO_2H$).

Dose (mg)	Route	C_{max} (ng/ml)	t_{max} (h)	CL_o (l/h)	CL (l/h)	$t_{\frac{1}{2}}$ (h)	Vd (l)	$AUC_{(o-24)}$ (μg.h/ml)	AUC (μg.h/ml)	$AUC_{(o-24)}$ ($PQCO_2H$) (μg.h/ml)
15	oral	53 ± 25	2 ± 1	31.2 ± 7.0	-	5.9 ± 2.1	269 ± 120	0.5 ± 0.1	0.5 ± 0.1	15.1 ± 2.6
30	oral	104 ± 25	3 ± 1	27.3 ± 5.3	-	7.4 ± 2.5	281 ± 69	1.0 ± 0.2	1.2 ± 0.2	33.4 ± 4.2
45	oral	176 ± 43	2 ± 1	27.1 ± 5.5	-	6.7 ± 0.5	263 ± 57	1.6 ± 0.4	1.7 ± 0.4	39.9 ± 6.1
4.5	i.v.	-	-	-	24.2 ± 7.4	7.1 ± 1.3	242 ± 69	0.2 ± 0.1	0.2 ± 0.1	4.1 ± 0.8

TABLES 7.2 Individual areas under the plasma concentration time curve (AUC) for primaquine after simultaneous i.v. and oral administration. Bioavailability (F) was determined from the ratio of AUC's after oral and i.v. dosage.

Subject	AUC (% dose.h/ml x 10^{-5})		F
	oral	i.v.	
AB	313.6	330.7	0.95
GM	561.0	589.1	0.95
GE	336.4	302.2	1.10
MS	370.6	417.4	0.89
SW	528.4	573.6	0.92
Mean	422.0	442.6	0.96
± S.D.	114.4	133.7	0.08

The carboxylic acid metabolite of primaquine, previously identified as the major plasma metabolite in man (Chapter 6) was detected in plasma within 30 min of dosing. By 4h in each study , plasma levels of this compound were in excess of ten fold greater than those attained by primaquine. Despite falling primaquine levels, the concentrations of the carboxylic acid metabolite were maintained at their elevated level, throughout the remainder of each study (figure 7.3) The proportion of the primaquine dose converted to this acidic metabolite was unaffected by dose size, as illustrated by the linear increase in the AUC_{0-24} for this metabolite, with increasing dose size of the parent drug ($r=0.99; p< 0.01$; table 7.1). Consequently, the ratio of AUC_{0-24} metabolite to AUC primaquine was similarly unaffected by dose size and was approximately 30:1 in each case (table 7.1).

7.4 DISCUSSION

The therapeutic regimens for primaquine used in the radical cure of malaria infections, utilise doses ranging from 15 to 45mg p.o. daily (Rollo 1980). Pharmacokinetic studies have to date only examined the disposition of a single dose of primaquine after 45mg p.o. (Greaves et al 1980, Chapter 6).In the earlier of these studies, the finding that primaquine achieved only low plasma levels, was attributed to extensive first-pass metabolism or tissue

distribution of primaquine (Greaves et al 1980). Experimental evidence has demonstrated capacity limited hepatic disposition of primaquine in the isolated perfused rat liver preparation (Chapter 4). However, detailed studies in man of the absolute bioavailability of primaquine and the pharmacokinetics of this drug across the clinically used dosage range, have not been undertaken.

The extent of absorption of primaquine was shown to be virtually complete in the present study, as illustrated by the near unity values for the absolute bioavailability of this drug (table 7.2). As the i.v. and oral doses were administered simultaneously in this part of the study, the variance in the estimates for bioavailability are low. This advantage is achieved because intra-subject day to day variation in drug disposition is avoided by concurrent administration of the i.v. and oral doses. This method for the determination of bioavailability assumes that the clearance of the drug by either route of administration is the same at the doses used (Rowland and Tozer 1980, George and Shand 1982). The systemic clearance of primaquine was determined from the i.v. dose study (Cl= 24.2 ± 7.4 l/h), confirming that this drug is a low to intermediate clearance compound in man, (in contrast to the situation in the IPRL where primaquine clearance was seen to approach liver blood flow after the administration of a 0.5mg dose of the diphosphate salt, Chapter 4). Therefore, the low systemic drug levels obtained after oral dosage are due to the rapid and extensive tissue distribution of the drug (Vd=242.9 ±

59.5 l) and not due to avid hepatic elimination as previously suggested.

Following the intravenous administration of [^{14}C] primaquine plasma levels of both [^{14}C] primaquine and total [^{14}C] radioactivity declined sharply (figures 7.1 and 7.2). However, although [^{14}C] primaquine levels continued to fall, the levels of total radioactivity from 30 min post-dose climbed steadily before reaching a plateau at 6h, at a level which was maintained for the remainder of the 24h sampling period.Although this implies indefinite persistance of radioactivity, it has been previously noted that radioactivity declines from 24h onwards and returns to background levels after several days (Chapter 6).

Approximately 40% of the total [^{14}C] radioactivity AUC in plasma was accounted for by the AUC_{0-24} for the [^{14}C] carboxylic acid metabolite. Furthermore, the ratio of AUC_{0-24} for this metabolite to the AUC for primaquine was 25:1, which is similar to the ratios obtained after oral administration. These results confirm that the carboxylic acid metabolite is the principal plasma metabolite of primaquine in man (as has been reported in Chapter 6) and that the extent of formation of this metabolite is unaffected by the route of administration.

Some antimalarial agents, in particular the 4-aminoquinoline derivative chloroquine, have been shown to selectively concentrate in white and red blood cells

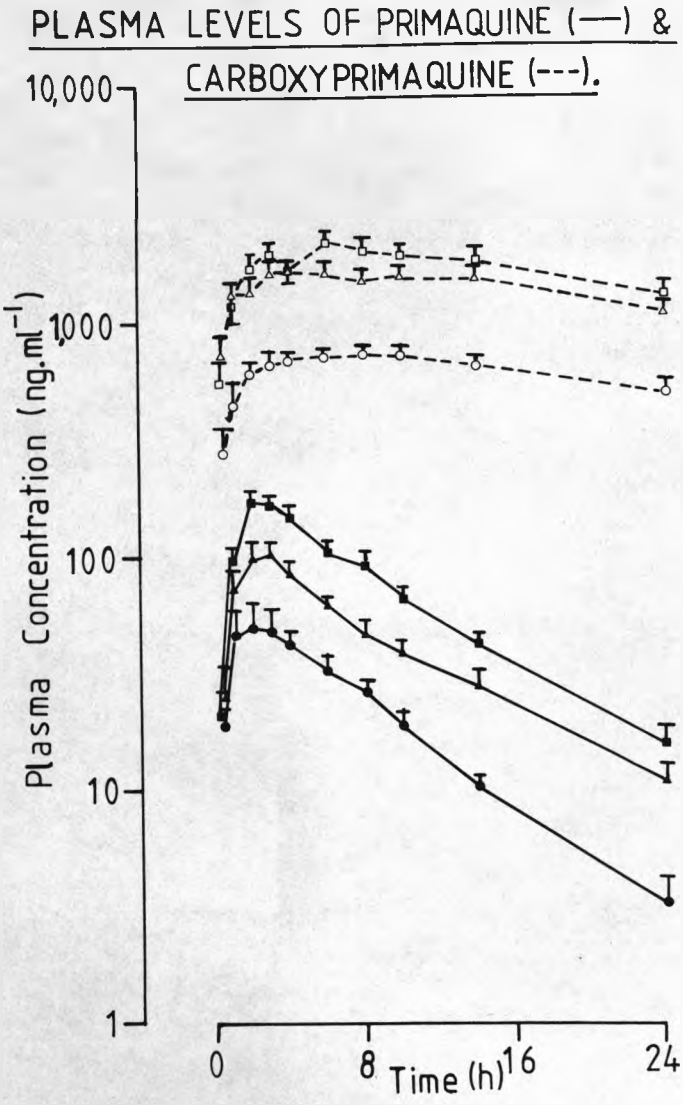


FIGURE 7.3 Semilogarithmic plot of plasma primaquine concentration against time after oral primaquine; 15mg(●), 30mg(▲) and 45mg(■)and of the corresponding plasma levels of the carboxylic acid metabolite after 15mg(○), 30mg(△) and 45mg(□) doses of primaquine.

resulting in whole blood :plasma concentration ratios of 10:1 (Bergqvist et al 1983). This study provides no evidence for any accumulation of primaquine or the carboxylic acid metabolite in blood cells. This is in keeping with the exo-erythrocytic mode of action of this antimalarial agent (Bruce-Chwatt 1979). In fact, as the whole blood:plasma ratio was less than unity, there appears to be some degree of exclusion of these compounds from blood cell components. A detailed evaluation of the distributional characteristics of primaquine into specific blood cell components was not addressed.

The results of the dose ranging study demonstrate that the pharmacokinetics of primaquine, after oral administration, are unaffected by dose size, within the clinically used dosage range (figure 7.3,table 7.1). The extent of formation of the carboxylic acid metabolite of primaquine, as measured by the ratios of AUC's for this metabolite and the parent drug, was also unaffected by dose size (figure 7.3,table 7.1). These results are in contrast to the primaquine disposition studies described in chapter 4. In those studies, primaquine doses in the range of 0.5 to 5.0mg produced marked differences in the perfusate pharmacokinetics, metabolic fate and extent of biliary excretion of the drug. This discordance in the results between man and rat may in part be due to differences in the mass of drug delivered to the liver of the human subjects compared to the isolated rat liver or to intrinsic differences between species in the disposition of the drug.

These studies show that the pharmacokinetics of primaquine and its conversion to the carboxylic acid metabolite are independent of both dose size and route of administration. Also neither the drug nor its metabolites undergo accumulation into blood cells. It has been demonstrated that primaquine is completely absorbed into the systemic circulation after oral administration and in contrast to earlier suggestions, this drug is a low clearance compound which is not subject to extensive first-pass metabolism .

CHAPTER 8. THE PHARMACOKINETICS OF PRIMAQUINE IN MAN: COMPARISON OF ACUTE VERSUS CHRONIC DOSAGE

8.1 INTRODUCTION

The two previous chapters have examined in detail the effect of both dose size and route of administration on the single dose pharmacokinetics of primaquine in man (Chapters 6 and 7). However, this drug is routinely adminstered in a regimen of 15mg/day for 14 days in the radical cure of malaria (Bruce-Chwatt 1980). The effect of chronic therapy on the pharmacokinetics and metabolism of primaquine has not been fully investigated. In a limited study by Greaves et al (1980) the pharmacokinetics of primaquine on day five of this regimen were examined in three subjects. However, inadequacies in study design and the absence of acute dose data in the subjects compromised the value of this data .

As described in chapter 3 of this thesis, primaquine is a potent inhibitor of mixed function oxidase (MFO) activity in both animals and man. Lignocaine has been shown to inhibit MFO activity and when administered repeatedly this drug was found to inhibit its own metabolism (Bauer et al 1982). In contrast, other drugs have been shown to induce their own metabolism after chronic administration, for example, carbamazapine and rifampicin (Eichelbaum et al 1975; Acocella et al 1979).

As a drug with a low therapeutic index (Bruce-Chwatt 1980), factors which affect the disposition of primaquine may alter therapeutic efficacy or produce toxicity. This chapter will examine the potential of primaquine to alter its own

disposition during chronic therapy. The study was carried out in healthy Thai volunteers, who, as inhabitants of an area where malaria is endemic, are clearly at risk of contracting the disease.

8.2 STUDY DESIGN

The study was carried out in the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand. Five healthy male Thai volunteers (25-46 years,45-57kg) who were taking no other medication took part in the study. Each subject received initially, an oral dose of primaquine (15mg). After a period of not less than one week each subject began chronic dosage with primaquine ie 15mg each morning for fourteen days. In all cases primaquine dosage followed an overnight fast. Venous blood samples (10ml), for plasma drug assays were taken pre-dose and at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24h after acute dosage and similarly on day fourteen of the chronic dosage schedule. In addition subjects presented for several blood samples to be collected between day 1 and day 13 of chronic dosing, three hours after the morning dosage. Drug level measurements in these samples were used for the assessment of compliance with the prescribed dosage schedule.

Plasma levels of primaquine and the carboxylic acid metabolite of primaquine were determined by selective and sensitive HPLC methods (2.1.1 and 2.1.2).Pharmacokinetic parameters (half-life, and area under the curve) were derived as explained in chapter 1 (1.6.6). Oral clearance (Clo) was calculated from the equation

Clo=dose/area. Statistical analysis was by paired Student t test accepting $p < 0.05$ as significant.Data are tabulated as mean ± SD and presented graphically as mean ± SEM .

8.3 RESULTS

Primaquine was rapidly absorbed after the acute administration of drug (15mg p.o.) to the five Thai volunteers. The mean peak plasma concentration of primaquine (65 ng/ml) was achieved by 2h (figure 8.1A, table 8.1). Thereafter plasma primaquine levels fell rapidly and monoexponentially with a mean terminal phase elimination half-life of 4.4 ± 1.4h. The mean value for oral clearance was 37.6 ± 15.5 l/h (table 8.1).

After chronic dosage with primaquine (15mg daily for 14 days) no appreciable changes were obtained in the mean plasma concentration/time profile for the drug, compared with that seen following acute administration (figure 8.1A). This is reflected in the almost identical values obtained for the mean pharmacokinetic parameters calculated forprimaquine after both acute and chronic dose studies (table 8.1).

Compliance to the 14 day dosage regimen was evaluated in each subject by measuring primaquine and metabolite levels, three hours post-dose, on several occasions during repeated dosage. The resultant plasma levels ranged from 35.7 to 107.0 ng/ml for primaquine and from 559 to 1361 ng/ml for the metabolite. This indicates that subjects had adhered to their dosage schedules ,since otherwise, at least in the case of primaquine, the drug levels would have been below the

sensitivity of the assay (1ng/ml).

Despite the close parity in mean pharmacokinetic parameters for primaquine, there was a high degree of intersubject variability in drug disposition at both the acute and chronic stages of therapy. This was reflected in the wide variance in plasma levels and calculated pharmacokinetic parameters (figure 8.1; table 8.1). Furthermore, each individual subject varied in the nature of the response to chronic therapy, yielding substantial increases or decreases in the values of the calculated pharmacokinetic parameters (figure 8.2). This disparity between the subjects is illustrated by the values obtained for oral clearance, which after chronic dosage increased markedly in subjects 2,4 and 5, (by 100,50 and 20% respectively), did not alter in subject 1 and showed a fall of 60% in subject 3 (figure 8.2).Inconsistent patterns of change were also obtained for the AUC for primaquine (figure 8.2).

The carboxylic acid metabolite of primaquine appeared in plasma within 0.5h after the acute dose of parent drug (figure 8.1B) and maximum plasma concentrations of this metabolite reached 736 ± 236 ng/ml at 8 ± 2h (table 8.1). Thereafter, plasma levels of this metabolite declined slowly to 498 ± 237 ng/ml at 24h. Chronic administration of primaquine resulted in significant accumulation of this

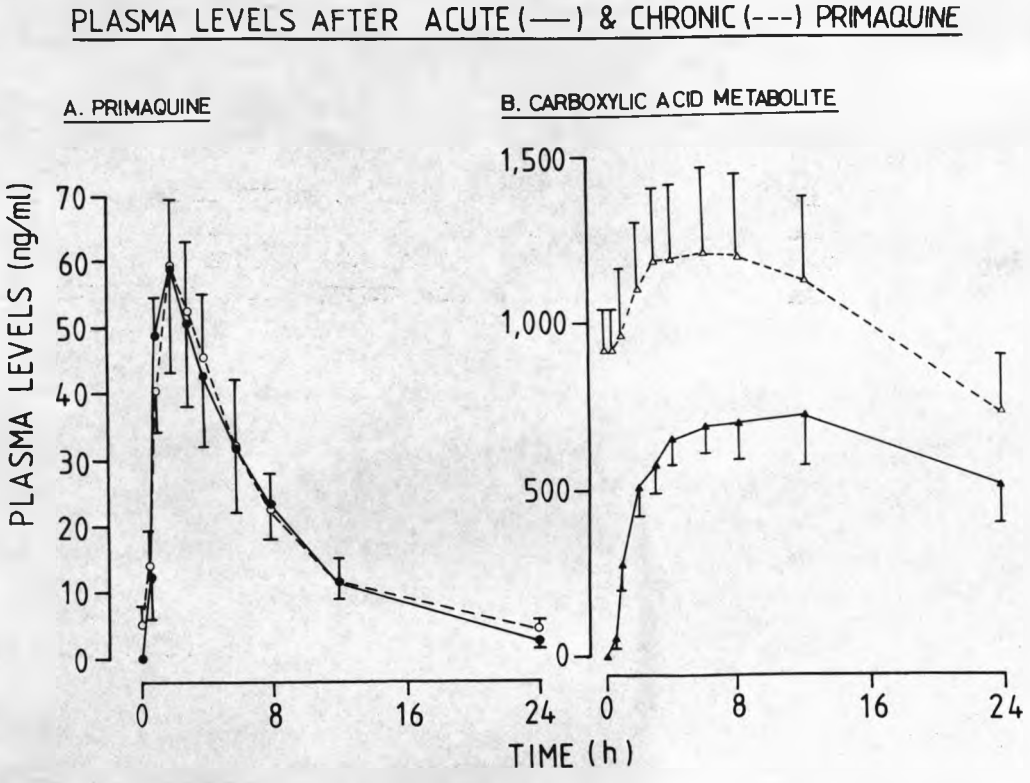


FIGURE 8.1 log plasma concentration versus time curves for (A) primaquine after acute (●) and chronic (O) dosage with primaquine and (B) the carboxylic acid metabolite after acute (▲) and chronic (△) dosage with primaquine.

TABLE 8.1 Mean Pharmacokinetic parameters for primaquine and its carboxylic acid metabolite after acute and chronic dosage with primaquine.

Pharmacokinetic Parameter	Acute dose (Day 1)	Chronic dose (Day 14)
A. Primaquine		
t_{max}(h)	2 ± 1	2 ± 1
C_{max}(ng/ml)	65 ± 34	66 ± 27
$t_{\frac{1}{2}}$(h)	4.4 ± 1.4	4.3 ± 1.5
CL_o (l/h)	37.6 ± 15.5	41.2 ± 21.0
AUC (ng.h/ml)	468 ± 229	443 ± 223
B. Carboxylic acid metabolite of Primaquine		
t_{max}(h)	8 ± 2	5 ± 2*
C_{max}(ng/ml)	736 ± 236	1240 ± 568*
AUC(0,24h) (μg.h/ml)	14.2 ± 5.3	24.7 ± 12.0*

(*significant difference to acute dose study; $p < 0.05$)

carboxylic acid metabolite. This was shown by a 74% increase in the area under the mean plasma metabolite concentration/time curve (measured from t=Ø to t=24h) and a 68% elevation in the value for peak plasma concentration of this metabolite, which was achieved earlier (by 5 ± 2h) on the fourteenth day of chronic primaquine administration (figure 8.1B;table 8.1). Unlike the findings with the parent drug, the direction of change in the values for the pharmacokinetics of this metabolite after chronic administration of primaquine were consistent between subjects.

8.4 DISCUSSION

It is now apparent that for a number of drugs the pharmacokinetic consequence of chronic therapy may manifest itself as an auto-induction or an auto- inhibition. Such alterations can have profound effects on both drug toxicity and efficacy, particularly for compounds with a narrow therapeutic index (ie the ratio of therapeutic to toxic dose), such as primaquine (Bruce-Chwatt 198Ø). A definitive study of the effect of chronic dosage on the pharmacokinetics of primaquine has not been performed, although a limited study has been carried out by Greaves et al (198Ø), the results of which were compromised because plasma samples were taken on day five of chronic therapy and no acute dose data was presented for the purpose of comparison. In addition, the three volunteers studied had been pre-treated with the 4-aminoquinoline chloroquine, which is known to increase the antimalarial efficacy of primaquine

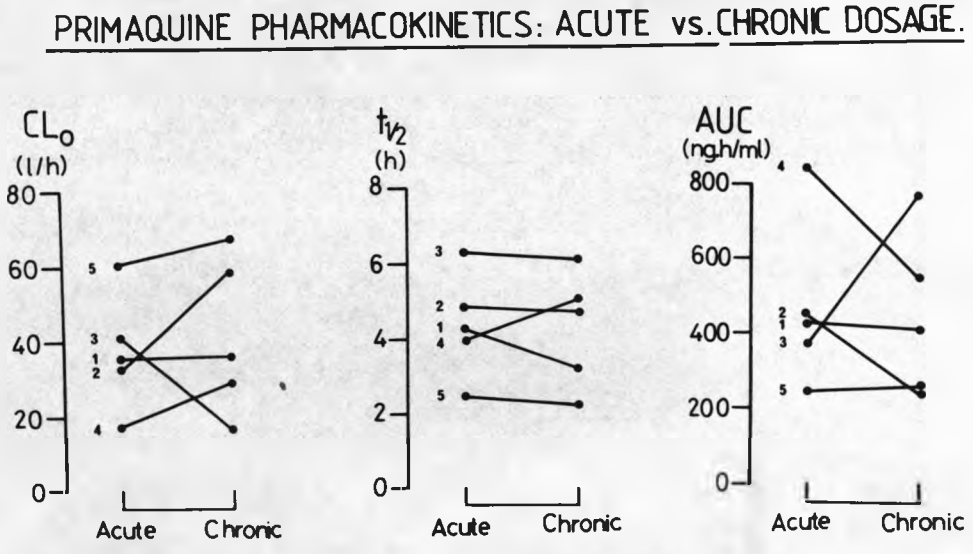


FIGURE 8.2 Tie diagram relating individual changes in oral clearance (Clo), half-life ($t_{\frac{1}{2}}$), and area under the plasma concentration/time curve (AUC) for primaquine after acute and chronic administration of the drug to five Thai volunteers (1-5)

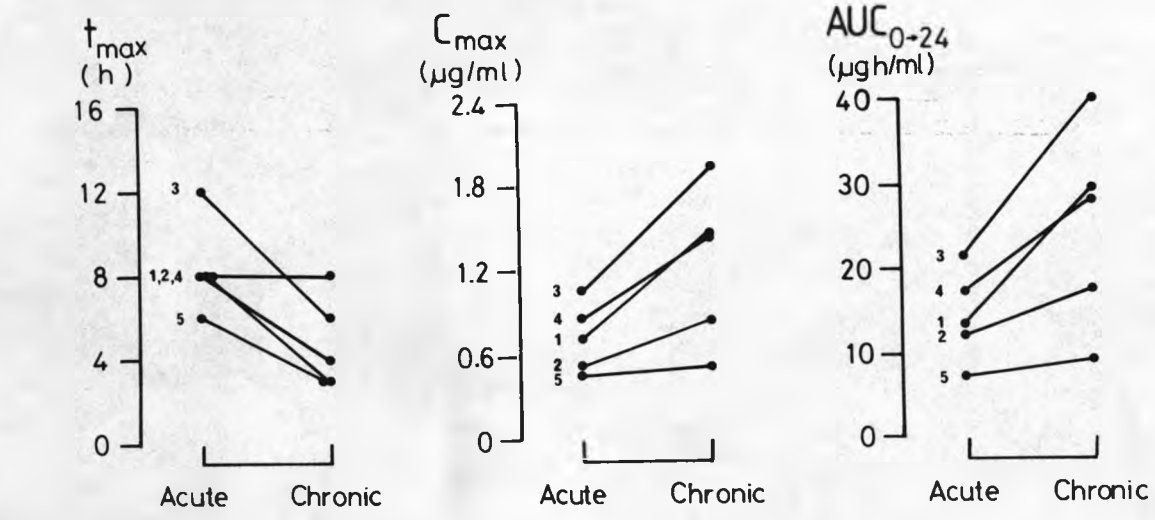


FIGURE 8.3 Tie digram relating individual changes in peak plasma concentration (C_{max}), time to reach peak plasma concentration (t_{max}) and area under the plasma concentration/time curve from t=Ø to t=24h ($AUC_{Ø-24}$)for the carboxylic acid metabolite of primaquine after acute and chronic administration to five Thai volunteers (1-5).

by an unknown mechanism (Carson 1984).

This study has shown that chronic dosage with primaquine has no effect on the mean pharmacokinetic parameters for the drug, when compared to those obtained following acute dosage (figure 8.1A, table 8.1). However,marked interindividual differences were seen in the magnitude and direction of change in the various parameters,after chronic therapy (figure 8.2). These changes in disposition may reflect in part changes in elimination efficiency(ie auto-induction or auto-inhibition inseparate individuals) or changes in distribution characteristics , but may also imply an alteration in the bioavailability of primaquine. Calculation of systemic clearance and apparent volume of distribution after oral dosage rely on an accurate measure of the drug's bioavailability. These parameters have not been calculated in this study due to possible inter and intra-individual differences in the value for this parameter in this population. The magnitude and direction of change in pharmacokinetic parameters could not be associated with the value of the parameter calculated after the acute dose study.Neither physical characteristics of the individual subjects, nor environmental factors (eg diet) could be related to the changes seen. It is unknown if these bidirectional changes in disposition characteristics could be translated into clinical relevance, namely toxicity or alternatively lack of efficacy.

It is of interest to note that there were no significant differences between themean pharmacokinetic parameters of primaquine determined in Thai subjects in this study (table

8.1) with those derived from Caucasians (Chapters 6 and 7). This implies that data from studies undertaken in European volunteers can be readily extrapolated to those individuals more at risk of contracting malaria. This is not to say however, that the same would apply to diseased patients in these endemic areas .

The carboxylic acid metabolite of primaquine was shown to accumulate with chronic administration with the drug as illustrated by the 74% increase in the AUC_{0-24} between days 1 and 14 of the study (figure 8.1B, table 8.1).However, as the pharmacology of this metabolite has yet to be investigated, it is premature to speculate as to the therapeutic significance of this accumulation.

In summary, this study has shown that there is considerable variation in the magnitude and direction of change within individuals in the pharmacokinetics of primaquine after chronic dosage. The carboxylic acid metabolite was seen to accumulate in every subject upon repeated dosage with the parent drug. The pharmacological activity of this metabolite and the clinical significance of its accumulation is unknown. There was no significant difference in the mean pharmacokinetic parameters for primaquine between the acute and chronic dose studies, although there was marked changes in the pharmacokinetics of primaquine for each individual . The possible correlation between these changes and the therapeutic efficacy or toxicity of antimalarial treatment with primaquine, in infected patients, awaits evaluation.

In this final chapter the experimental findings presented within this thesis will be collated and assessed. All aspects of the work carried out will be discussed together with those areas of research which merit further investigation.

The quality of all the experimental observations presented here,is dependent upon the selectivity, sensitivity and reproducibility of the analytical methods used (Chapter 2).The HPLC assays for primaquine, N-acetylprimaquine and the carboxylic acid metabolite of primaquine were developed and assessed as an integral part of this research project. In all cases these methods were found to be simple, selective , reproducible and capable of accurate quantitation of drug and metabolite concentrations at the levels encountered in both the human volunteer studies and isolated perfused rat liver experiments. All other analytical methods were adapted from previously reported procedures.

A large portion of the work presented within this thesis has used the isolated perfused rat liver as an experimental model (Chapters 3,4 and 5). The advantages of using this model in investigations of the hepatic component of drug disposition and in the investigation of hepatic drug interactions are discussed in chapter 2 (2.2.1).

This preparation has been used to assess the potential of racemic primaquine and the (+) and (-) isomers of primaquine to influence drug metabolism. Primaquine was found to inhibit the elimination of antipyrine in this preparation. The degree of inhibition was dependent upon the dose of

primaquine. It was shown that the (+) and (-) isomers of primaquine were equipotent inhibitors of drug metabolism (as assessed by a reduction in antipyrine clearance) when compared to the racemic drug at an equivalent dosage. Although the doses of primaquine used in these studies were greater than the doses used clinically , the findings using the racemate were in agreement with the results of studies carried out in the rat in vivo (Back et al 1983a) and with the results of drug inhibition studies undertaken in man after the administration of a therapeutic dose of primaquine (45mg; Back et al 1983b).

The potential of racemic primaquine,(+) primaquine,(-) primaquine and a number of identified and postulated metabolites of primaquine to inhibit drug metabolism was investigated using hepatic microsomal enzyme preparations. The (+) and (-) isomers of primaquine and the racemate were found to be equipotent inhibitors of both P_{450} and P_{448} mediated processes of metabolism. The carboxylic acid metabolite of primaquine, the only metabolite to be identified in man (Chapter 6,7 and 8) showed no potential to inhibit drug metabolism at the dosage used. This was also the case for the postulated metabolites, 6-desmethylprimaquine, and 5-hydroxy-6-desmethylprimaquine.The N-acetylated metabolite of primaquine, which is formed by various microbial cultures but not man (chapter 6) and the postulated metabolite 5-hydroxyprimaquine were equipotent with primaquine in inhibiting drug metabolism.

These findings suggest that the propensity of

primaquine to inhibit drug metabolism is governed not by stereochemistry but by functional group substitution. It would also appear that the methoxy group at the 6-position and the terminal amine group in the side chain are essential for inhibitory activity. Although the N-acetyl metabolite of primaquine did inhibit drug metabolism, this metabolite has never been identified in man and is therefore of no clinical relevance. The 5-hydroxy derivative of primaquine also possessed the capacity to inhibit drug metabolism . This has not yet been conclusively identified in man. If it is produced in man, the concentrations achieved by this metabolite and its rate of elimination need to be measured before any clinical significance can be discussed. Unfortunately, as only trace amounts of these compounds were available,the potential of these compounds to inhibit drug metabolism relied on single point determinations. Further investigations into the inhibitory potential of these compounds against drug metabolising enzymes need to be performed in which the concentration of the potential inhibitor is varied. Nonetheless, the study reported here has allowed the inhibitory potential of primaquine related compounds to be evaluated at similar concentrations.

Measurement of primaquine concentrations in hourly perfusate samples from the drug inhibition studies suggested that primaquine may exhibit dose-dependent elimination from the IPRL (Chapter 3). The effect of dose size on the disposition of primaquine in this experimental model was investigated in chapter 4. These studies revealed that

the pharmacokinetics of primaquine, particularly its metabolism to the carboxylic acid derivative and biliary excretion are dependent on dose in the IPRL at doses ranging from 0.5 to 5.0mg primaquine diphosphate. Pharmacokinetic analysis showed that both the clearance and the volume of distribution of primaquine were influenced by dose size. The systemic clearance of primaquine decreased as dose size was increased due to a limitation in the capacity of the liver to metabolise the drug. At the lowest dose (0.5mg) primaquine behaved as a high clearance compound while at the highest dose (5.0mg) the drug behaved as a low clearance compound. Increasing dosage (from 0.5-5.0mg) produced a three-fold increase in the volume of distribution, this is associated with a three-fold increase in the amount of [^{14}C] radioactivity in the liver at the end of each experiment. It is likely therefore that this increased volume of distribution may represent an increased hepatic uptake of primaquine as the dose is increased. A possible explanation for this change in the distributional characteristics of primaquine with increasing dose size comes from consideration of metabolite data. At all three doses used, primaquine was extensively metabolised. However, the contribution of the carboxylic acid metabolite to the total concentration of metabolites was found to increase with increased dose of primaquine. It is suggested therefore that at low doses, as yet unidentified metabolites of primaquine compete with the parent drug for binding sites in the liver, restricting the volume of distribution of primaquine. As the

dose of primaquine is increased, there is an alteration in the metabolic profile, resulting in greatly increased concentrations of the polar carboxylic acid metabolite. This compound is likely to be restricted to perfusate water.This change in metabolic pattern results in more binding sites in the liver becoming available for the parent drug, and an increase in the observed volume of distribution.

In addition to the dose-dependent pharmacokinetics and metabolism, primaquine also exhibited dose-dependency with respect to biliary excretion. Although this was a major route of elimination at all doses, increasing the dose (from 0.5 to 5.0mg) produced a significant reduction in the amount of [^{14}C] radioactivity excreted in bile (from 60%to 30%). Increasing the dose had no effect on bile flow. Although total [^{14}C] radioactivity excreted in bile decreased with increasing dose there was little or no change in the contribution of primaquine, the carboxylic acid metabolite of primaquine, 5-hydroxyprimaquine, 6-desmethylprimaquine or 5-hydroxy-6-desmethylprimaquine to the material excreted, as determined by HPLC. Even so 50% of the biliary radioactivity remained unidentified. The observation that this unidentified material could be recovered by flushing the HPLC system with methanol suggested that it represents either highly non-polar metabolites or compounds undergoing a very strong chemical interaction with the column support or the stationary phase, which would seem the more likely of the two explanations.

Evidence for stereoselectivity in the pharmacokinetics of primaquine has been investigated in detail (Chapter 5)

following the preliminary observations discussed in chapter 3. Stereoselective differences in the pharmacokinetics of primaquine were observed following the administration of (+) and (-) primaquine at a dose of 2.5mg to the IPRL.There was no significant difference in the elimination rates of the two isomers at lower doses where the racemic drug behaves asa high clearance (ie blood flow dependent) compound.Following the 2.5mg dose the (-) isomer was eliminated at a rate three times greater than the (+) isomer. This difference was due to a difference in the systemic clearance of the two isomers. At this dosage (2.5mg) primaquine behaves as a low clearance drug, therefore this difference in clearance must reflect differences in intrinsic clearance or the affinity of drug metabolising enzymes for the (+) and (-) isomer substrates. Earlier work (Schmidt et al 1977) showed that although the stereoisomers are equipotent antimalarial agents the (+) isomer was three to four times more toxic than the (-) isomer in rodent toxicity studies. These experiments utilised much larger doses than were used in the present study but it is possible that the differential toxicity of the stereoisomers of primaquine results from the difference in the elimination rates of the two isomers. It is not known whether this stereoselective difference in the elimination of (+) and(-) primaquine is a result of different metabolic pathways for each isomer or due to differences in the rates of reaction of the same pathways. This is an important point,since it is the metabolites of primaquine which are thought to be responsible for both the toxic and antimalarial effects of the drug

(Chapter 1).In addition earlier workers have suggested that it is the absolute dose of primaquine and not plasma concentrations that appear to correlate with toxicity and antimalarial activity (Carson 1984).

The observation that primaquine exhibits stereoselective disposition after administration of a 2.5mg dose to the IPRL raises a number of questions. This stereoselective difference was observed at a dose at which the racemic drug behaves as a low clearance compound. In man , at clinically relevant doses, racemic primaquine also behaves as a low clearance drug. Therefore, does primaquine show stereoselective differences in the disposition of its isomers at therapeutic doses in man ? If so,is it due to differences in the metabolic pathways available to each isomer or to differences in the rates of the same metabolic pathways ? Finally,can these differences in the elimination pharmacokinetics of (+) and (-) primaquine be translated into differences in the relative toxicity and antimalarial activity of the two isomers ?

Chapters 6 to 8 present a detailed evaluation of the pharmacokinetics of primaquine in healthy volunteers . After simultaneous intravenous and oral administration of primaquine to man bioavailability was calculated to be 1. In addition there was no difference in the pharmacokinetics of primaquine after intravenous or oral administration. Primaquine was shown to be rapidly and completely absorbed after oral administration. It is a low clearance drug (Cl 25-30 l/h) and the low plasma concentrations achieved after oral

administration are due to extensive tissue uptake (Vd 200-300l).Primaquine is rapidly metabolised to the carboxylic acid metabolite in man which achieves concentrations in plasma that are an order of magnitude greater than the corresponding concentrations of the parent drug. The formation of this metabolite appeared to be route of administration independent. Although this metabolite was the principal plasma metabolite in man, it could not be detected in urine and is thought to be further metabolised prior to excretion. After oral administration of [^{14}C] labelled primaquine more than 60% of the dose was recovered in the urine with less than 5% present as the unchanged drug. It is conceivable that some of the radioactivity unaccounted for may undergo biliary excretion and elimination in the faeces, biliary excretion having been shown to be a major route of elimination in the IPRL. The data obtained from the human studies, in addition to answering a number of questions pose many more. What is the toxic and therapeutic activity of the carboxylic acid metabolite and how is it ultimately removed from the body ? Nearly all of the [^{14}C] radioactivity excreted in urine was in the form of primaquine metabolites other than the carboxylic acid metabolite. what is the identity of these metabolites and do they contribute to the drugs pharmacological activities ?

Primaquine was shown to exhibit dose-dependent pharmacokinetics in the IPRL at a dose range of 0.5 to 5.0mg primaquine diphosphate. Therefore the effect of dose size on the pharmacokinetics and metabolism of primaquine in man was

investigated, using doses which covered the therapeutic range. These studies showed that dose size had no effect on the pharmacokinetics of primaquine or its conversion to the carboxylic acid metabolite in man.

The observed difference in the results obtained from the IPRL and man, with respect to increasing dose size, may be due to the difference in the mass of drug to which the liver is exposed in the two studies. However the Ø.5mg dose used in the IPRL experiments is equivalent to a 45mg base dose administered to man and even at these doses the disposition of primaquine in the two groups of experiments differed markedly. This difference in the disposition of primaquine in man and the IPRL may be due to the absence of extrahepatic elimination and distributional processes in the IPRL compared with the situation *in vivo*. Alternatively, high affinity, low capacity, drug metabolising pathways may be present in the rat liver which are not present in man.

The potential of primaquine to influence drug metabolism (Chapter 3) prompted an investigation into the effect of chronic dosage on the pharmacokinetics of primaquine. Chronic drug administration had no effect on the mean pharmacokinetic parameters derived for primaquine although the carboxylic acid metaboite was seen to accumulate. The pharmacology of this metabolite must be evaluated before the clinical relevance of this observation can be established.

There was no significant difference in the mean disposition parameters of primaquine after a single 15mg dose of the drug to healthy Caucasian or healthy Thai volunteers.

This suggested that it would be possible to extrapolate observations carried out in Caucasians to the Thai population. There was however greater inter-subject variation within the Thai subject group. When the results for each volunteer in the Thai study were analysed, chronic dosage was found to produce gross and unpredictable alterations in the pharmacokinetic parameters calculated for each subject. The toxicological and therapeutic consequences of these changes have not been investigated. The underlying mechanisms behind these observations are unknown but could be a result of changes in elimination efficiency, distribution or bioavailability of primaquine in these subjects.

The studies described in this thesis have added to our understanding of the biochemical pharmacology of primaquine and have posed many new questions. In addition all the work presented here has been carried out in healthy animal tissue or healthy human volunteers, it is therefore important to re-evaluate the observations described here in the clinical environment.

APPENDIX I REAGENT SOURCES

PRIMAQUINE - Aldrich Chemicals Ltd, Gillingham, Dorset, U.K.

PRIMAQUINE (tablets)- I.C.I. Alderley Edge, Manchester, U.K.

[^{14}C] PRIMAQUINE-(labelled in position 2 of the quinoline ring- radiochemical purity 99%) New England Nuclear, Boston, U.S.A.

(+) PRIMAQUINE, (-) PRIMAQUINE, 8-(3-AMINO-1-METHYLPROPYLAMINO)-6-METHOXY QUINOLINE , 5-HYDROXY, 5-HYDROXY-6-DESMETHYL AND 6-DESMETHYLPRIMAQUINE- Walter Reed Army Medical Research Centre, Washington DC, U.S.A.

N-ACETYL PRIMAQUINE AND THE CARBOXYLIC ACID METABOLITE- Professor J McChesney, School of Pharmacy, University of Mississippi, U.S.A.

CHLOROQUINE AND SAGITAL- May and Baker, Dagenham, U.K.

AMODIAQUINE- Warner Lambert, Eastleigh, U.K.

PYRIMETHAMINE- Burroughs Wellcome, Beckenham, U.K.

INDOMETHACIN- Merck Sharp and Dohme Research Laboratories, Hertfordshire, U.K.

ANTIPYRINE, PHENACETIN, AMINOPYRINE, NADPH, β-NAPHTHOFLAVONE, GLUCOSE, BOVINE SERUM ALBUMIN, SODIUM TAUROCHOLATE AND H1 HYDROLASE- Sigma Chemicals Ltd, Dorset, U.K.

3-HYDROXYMETHYLANTIPYRINE - Professor Breimer, Lieden, Netherlands.

4-HYDROXYANTIPYRINE AND NORANTIPYRINE- Dr McKillop, I.C.I. Alderley Edge, Manchester, U.K.

ETHOXYRESORUFIN- Pierce Chemicals Ltd, Chester, U.K.

RESORUFIN- Eastman Kodak Co, Rochester N.Y. U.S.A.

SODIUM LACTATE BP- Travenol Laboratories Ltd, Norfolk, U.K.

SOLVENTS(ALL HPLC GRADE)- Fisons, Loughborough, U.K.

ALL OTHER REAGENTS (ANALYTICAL GRADE)- British Drug Houses (BDH Chemicals Ltd),Dorset, U.K.

All clinical studies were carried out in healthy male volunteers. All aspects of these protocols were approved by the Mersey Regional Health Authority Ethics Committee and the Ethics committee of the World Health Organisation. Permission for the administration of [^{14}C]-primaquine was obtained from the D.H.S.S. Radioisotopes panel.

APPENDIX 11 EQUIPMENT SOURCES

HARDWARE

HPLC- Spectra Physics Ltd, St Albans, U.K.

UV SPECTROPHOTOMETER CE 373- Talbot Instruments, Manchester, U.K.

FLUORESCENCE SPECTROPHOTOMETER (PE 203)- Perkin Elmer, Beaconsfield, U.K.

INTERTECHNIQUE SL30 SCINTILLATION COUNTER- Kontron International, France.

GILSON MINIPULS 2 PERISTALTIC PUMP- Anachem Ltd, Luton, U.K.

THORN POLARIMETER- Thorn Automation, Nottingham, U.K.

Z-MODULE- (Waters) Millipore, Harrow, U.K.

COLUMNS

PARTISIL 10 ODS 111- Technicol, Stockport, U.K.

μ BONDAPAK RAD-PAK PHENYL AND C_{18} AND CN Guard PAK- (Waters) Millipore, Harrow, U.K.

REFERENCES

Acocella,G.,Nattinissi,R. and Seagre,G. (1979): Multicompartmental analysis of serum, urine and bile concentrations of rifampicin and desacetyl-rifampicin in subjects treated for one week. Pharmac. Res. Comm. 10 271-288.

Aikawa,M. and Beadoin,R.L. (1970): Morphological effects of 8-aminoquinolines on the exo-erythrocytic stages of P.fallax. Milit. med. 134 986-999.

Allahyari,R., Strother,A., Fraser,I.M. and Verbiscar,A.J. (1984): Synthesis of certain hydroxy analogues of the antimalarial drug primaquine and their in vitro methaemoglobin -producing and glutathione -depleting activity in human erythrocytes. J. Med. Chem. 27 407-410.

Allison, A.C.and Clyde, D.F. (1961): Malaria in African children with deficient glucose-6-phosphate dehydrogenase. Brit. Med. J. 1 1346-1349.

Allison, A.C. and Eugui, E.M. (1982): A radical interpretation of immunity to malaria parasites. Lancet. 2 1431-1434.

Alving, A.S., Powell, R.D., Brewer, G.J. and Arnold, J.D. (1962): Investigation of the haemolytic effect of primaquine and enhancement of its action against exo-erythrocytic forms of the chesson strain of P.vivax by intermittent regimen of drug administration. Bull. W.H.O. 22 621-631.

Archer, S., Osei-Gyimah, P. and Silbering, S. (1980): 4-[(aminoalkyl)amino]-1,2-dimethoxynaphthalenes as antimalarial agents. J. Med. Chem. 23 516-519.

Back, D.J., Purba, H.S., Staiger, C., Orme, M. L'E. and Breckenridge, A.M. (1983a): Inhibition of drug metabolism by the antimalarial drugs chloroquine and primaquine in the rat. Biochem. Pharmacol. 32 257-263.

Back, D.J., Purba, H.S., Park, B.K., Ward, S.A. and Orme, M.L'E. (1983b): effect of chloroquine and primaquine on antipyrine metabolism. Br. J. Clin. Pharmacol. 16 497-502.

Back, D.J., Sutcliffe, F. and Tjia, J.F. (1984): Tolbutamide is a good model drug for the study of enzyme induction and enzyme inhibition in the rat. Br. J. Pharmacol. 81 557-562.

Baker, J.K., McChesney, J.D., Hufford, C.D. and Clark, A.M. (1982): High performance liquid chromatographic analysis of the metabolism of primaquine and the identification of a new mammalian metabolite. J. Chromatogr. 230 69-77.

Baker, J.K., Bedford, J.A., Clark, A.M. and McChesney, J.D. (1984): Metabolism and distribution of primaquine in monkeys. Pharmaceut. Res. 2 98-100.

Bami, H.L., Dhatt, M.S., Ahulwalia, G.S. and Ray, A.P. (1960): Studies on the metaboilic degredation of pamaquine (plasmochin) in monkeys. 14 1-13.

Bartosek, I., Guaitani, A. and Miller, L. (1973): Isolated liver perfusion and its application. New York, Raven Press.

Baty, J.D., Price-evans, D.A. and Robinson, P.A. (1975): The identification of 6-methoxy-8-aminoquinoline as a metabolite of primaquine in man. Biomed. Mass. Spec. 2 304-306.

Bauer, L.A., Brown, T., Gibaldi, M., Hudson, L., Nelson, S., Raisys, V. and Shea, J.P. (1982): Influence of long term infusions on lidocaine "kinetics". Clin. Pharmac. Ther. 31 433-437

Bentley, P. and Oesch, F. (1982): Foreign compound metabolism in the liver. Prog. Liver. Dis. 7 157-178.

Bergqvist, Y. and Domeij-Nyberg, B. (1983): Distribution of

chloroquine and its metabolite desmethyl-chloroquine in human blood cells and its implication for the quantitative determination of these compounds in serum and plasma. J. Chromatogr. 272 137-148.

Beutler, E. (1959): Drug induced hemolytic anaemia. Pharmac. Rev. 21 73-103.

Branch, R.A., Nies, A.S. and Shand, D.G. (1973): The disposition of propranolol.8. General implications of the effects of liver blood flow on elimination from the perfused rat liver. Drug. Metab. Disp. 1 687-690.

Breckenridge, A.M., Orme, M., Wesseling, H., Lewis, R.G. and Gibbons, R. (1974): Pharmacokinetics and pharmacodynamics of the enantiomers of warfarin in man. Clin. Pharmacol. Ther. 15 424-430.

Brodie, B.B. and Udenfriend, S. (1950): Metabolites of pamaquine in urine. Proc. Soc. Exp. Biol. 74 845-848.

Bruce-Chwatt, L.J. (1979): Man against malaria: Conquest or defeat. Trans. R. Soc. Trop. Med. Hyg. 73 605-617.

Bruce-Chwatt, L.J. (1980): Essential malariology. William Heinemann medical books Ltd. London.

Burke, M.D. and Mayer, R.T. (1974): Ethoxyresorufin: Direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug. Metab. Disp. 2 583-588.

Carroll, F.I., Blackwell, J.T. and Philip, A. (1976): Reduced 8-aminoquinoline analogues as potential antimalarial agents. J. Med. Chem. 19 111-119.

Carroll, F.I., Berrang, B. and Linn, C.P. (1978): Resolution of antimalarial agents via complex formation with - (2,4,5,1 -tetranitro-9-fluoronylidene aminooxy) proprionic acid. J. Med. Chem. 21 326-330.

Carroll, F.I., Berrang, B.D. and Linn, C.P. (1979): Synthesis of 4 alkyl and 4-(beta-alkylvinyl) derivatives of primaquine as potential antimalarials. J. Med. Chem. 22 1363-1367.

Carroll, F.I., Berrang, G.D. and Linn, C.P. (1980): Synthesis of 2,4-disubstituted 6-methoxy-8-aminoquinoline analogues as potential antiparasitics. J. Med. Chem. 23 581-584.

Carson, P.E., Flanagan, C.L., Ickes, C.E. and **ALving, A.S.** (1956): Enzymatic deficiency in primaquine sensitive erythrocytes. Science N.Y. 124 484-485.

Carson, P.E., Hohl, R., Nora, M.V., Parkhurst, G.W., Ahmad, J., Scanlon, S, and **Frischer, H.** (1981): Toxicology of the 8-aminoquinolines and factors associated with their toxicity in man. Bull. WHO. 59 427-437.

Carson, P.E. (1984): 8-aminoquinolines:in antimalarial drugs II . Editors, W Peters and WHG Richards. Springer-verlag N.Y. pp 83-121.

Chatfield, C. (1978): Statistics for technology. 2nd edition, Chapman and Hall, London, pp 140-146.

Chen, E.H., Saggimo, A.J. and Tanabe, K. (1977): modifications of primaquine as antimalarials 1. 5-phenoxy derivatives of primaquine. J. Med. Chem. 20 1107-1109.

Clark, I.A., Virelizer, J.L., Carswell, E.A. and wood, P.R. (1981): Possible importance of macrophage-derived mediators in acute malaria. Infect. Immun. 23 1058-1066.

Clark, I.A. and Hunt, N.H. (1983): Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. Infect. Immun. 39 1-6.

Clark, A.M., Hufford, C.D. and McChesney, J.D. (1981): Primaquine: Metabolism by microorganisms and ^{13}C nuclear magnetic resonance assignments. Antimicrob. agents. chemother. 19 337-341.

Clark, A.M., Baker, J.K. and McChesney, J.D. (1984): Excretion, distribution and metabolism of primaquine in rats. J. Pharm. Sci. 73 502-506.

Cohen, G. and Hochstein, P. (1964): Generation of hydrogen peroxide in erythrocytes by hemolytic agents. Biochem. 3 895-900.

Danhof, M., Degroot-Van Der Vis, E. and Briemer, D.D. (1979): Assay of antipyrine and its primary metabolites in plasma and urine by HPLC and some preliminary results in man. Pharmacology. 18 210-223.

Dupovy-Comet, J., Verdy, E. Combrisson, A.and Lepierre, J. (1984): An additional argument for a protective effect of glucose-6-phosphate dehydrogenase against malaria. Presse. Medicale. 13 176.

Eaton, J.W., Eckman, J.R., Berger, E. and Jacobs, H.S. (1976): Suppresion of malaria infection by oxidant-sensitive host erythrocytes. Nature. 264 758-760.

Editorial (1975): Epitaph for global malaria eradication. Lancet 2 15-16.

Elderfield, R.C. and Smith, L.L. (1953): On the physiological disposition of pentaquine labelled in the side chain. J. Am. Chem. Soc. 75 1022-1024.

Eichelbaum, M., Ekbom, K., Bertilsson, L., Ringberger, W.A and Rane, A. (1975): Plasma pharmacokinetics of carbamazepine and its epoxide metabolite in man after single and multiple doses. Eur. J. Clin. Pharmacol. 8 337-341.

Fletcher, K.A., Canning, H.V. and Gilles, H.M. (1977): metabolites of primaquine and their effects on erythrocytes. Trans. Roy. Soc. Trop. Med. Hyg. 71 111-112.

Fraser, I.M. and Vessel, E.S. (1968): Effect of drugs and drug metabolites on erythrucytes from normal and glucose-6-phosphate dehydrogenase deficient individuals. Ann. N.Y. Acad. Sci. 777-794.

Fraser, I.M., Strother, A. and Tilton, B.E. (1976): Methaemoglobin formation in normal and G-6-PD deficient human erythrocytes by primaquine and a mouse liver microsomal system. Fed. Proc. 35 244.

Furranannu, Von G. und Werrbach, K. (1965): Zum nachweis von antimalarianuffel in korperflussigkeiken und ausscheidugen. s. mittelung. Ein feldtest zum quantitativen nachweis von primaquin im urin. Z. Tropenmed. Parasit. 16 397-400.

Fusari, S.A., Malcomb, I.J. and Leurs, R.B. (1975): assay of pharmaceuticals using thin layer-chromatography . J. chromatog. Sci. 13 589-592.

George, C.F. and Shand, D.G. (1982): Pre-systemic drug metabolism in the liver. in: pre-systemic drug elimination, clinical pharmacology and therapeutics. Edited by C.F.George and D.G.Shand. Butterworths (London U.K.).

Gibaldi, M. and Perrier, D (1982):Pharmacokinetics. 2nd edition. Marcell Dekker inc. N.Y.

Gilles, H.M. and Taylor, B.G. (1961): The existence of glucose-6-phosphate dehydrogenase deficiency trait in Nigeria and its clinical implications. Ann. Trop. Med. Parasitol 55 64.

Gollan, J., Hammaker, L. and Licko, V (1981): Bilirubin kinetics in intact rats and isolated perfused liver. J.Clin. Invest. 67 1003-1015.

Greaves, J., Price-Evans, D.A. and Gilles, H.M. (1979): A selective ion monitoring assay for primaquine in plasma and urine. Biomed. Mass. Spec. 6 109-112.

Greaves, D., Evans, D.A.P., Gilles, H.M., Fletcher, K.A., Bunnag, D. and Harinasutu, T. (1980): Plasma kinetics and urinary excretion of primaquine in man. Brit. J. Clin. Pharmac. 10 399-405.

Greenberg, J., Taylor, D.J. and Josephson, E.S. (1951): studies on plasmodium gallinaceum in vitro II. The effect of some 8-aminoquinolines against the erythrocytic parasites. J. Infect. Dis. 88 163-167.

Grewal, R.S. (1981): Pharmacology of 8-aminoquinolines. Bull WHO. 59 397-406.

Gutteridge, W.E. and Coombes, G.H. (1977): in: Biochemistry of parasite protozoa. Macmillan. London.

Guttman, and Erlich, (1891): Uber die wirkung des methyenblau bei malaria. Berl. Klin. Wochensclir 28 953-956.

Holbrook, D.J., Griffin, J.B., Fowler, L. and Gibson, B.R. (1981): Tissue distribution of primaquine in the rat. Pharmacol. 22 330-336.

Henry, D.A., Macdonald, I.A. and Kitchingman, G. (1980): Cimetidine and ranitidine-comparison of effects on hepatic drug metabolism. Br. Med. J. 281 775-777.

Hirom, P.C., Millburn, P. and Smith, R.L. (1976): Bile and urine as complementary pathways for the excretion of foreign organic compounds. Xenobiotica 6 55-64.

Homewood, C.A. (1977): Carbohydrate metabolism of malarial

parasites. Bull. WHO. 55 229-235.

Howells, R.E. and Fullard, F.J. (1970): Mitochondrial changes during the malarial life cycle. Ann. Trop. Med. Parasit 64 203-208.

Hufford, C., Clark, A., Quinones, I.,Baker, J.and McChesney, J. (1983): Microbial metabolism studies on the major microbial and mammalian metabolite of primaquine. J. Pharm. Sci. 72 92-94.

Hughes, H.B. and Schmidt, L.H. (1950): metabolism of various 8-aminoquinolines in rhesus monkeys. Proc. Soc. Exp. Biol. Med. 73 581-585.

Irvin, J.L. and Irvin, E.M. (1948): A fluorimetric method for the determination of pamaquine , SN-3276 and SN-3294. J. Biol. Chem. 174 589-586.

Josephson, E.S., Taylor, D.G., Greenberg, J. and Ray, A.P. (1951a): A metabolic intermediate of pamaquine from chickens. Proc. Soc. Exp. Biol. Med. 70 700-703.

Josephson, E.S., Greenberg, J., Taylor, D.J. and Bami, H.L. (1951b): A metabolite of pamaquine from chickens. J. Pharmac. Exp. Ther. 103 7-9.

Klaassen, C.D. and Watkins, J.B. (1984): Mechanisms of bile formation, hepatic uptake and biliary excretion. Pharmac. Rev. 36 1-67.

Kristensen, M.B. (1976): Drug interactions and clinical pharmacokinetics. Clin. Pharmacokin 1 351-372.

Krone, W., Huttner, W.B. and Kampf, S.C. (1974): Long term perfusion of the isolated rat liver, maintenance of its function state by use of a fluorocarbon emulsion. Biochem. Biophys. Acta. 372 55-71.

La Du, B.N., Mandel, H.G. and **Way, E.L.** (1971): Fundementals of drug metabolism and disposition. The Williams and Wilkins Co. Baltimore. U.S.A.

Lake, B.G. and Gangolli, S.D. (1981): Toxification and detoxification as a result of xenobiotic metabolism. in: Concepts in drug metabolism part B. Edited by B.Jenner and B.Testa. Marcel Dekker Inc. N.Y. pp 167-218.

LaMontagne, M.P., Markovac, A. and **Menke, J.R.** (1977): Antimalarials. 10. Synthesis of 4-substituted primaquine analogues as candidate antimalarials. J. Med. Chem 20 1122-1127.

Lowry, O.H., Rosebrough, J., Farr, A.L. and **Randall, R.J.** (1951): Protein measurement with Folin phenol reagent. J. Biol. Chem. 193 265-275.

Luzzato, L., Usanga, E.A. and **Reddy, S.** (1969): Glucose-6-phosphate dehydrogenase deficient red cells: Resistance to infection by malarial parasites. Sci N.Y. 164 839-842.

Mihaly, G.W., Smallwood, R.A., Anderson, J.D. Jones, D.B., Webster, L.K. and Vajda, F.J. (1982): H_2 receptor antagonists and hepatic drug disposition. Hepatology. 2 828-831.

Miller, A. and **Smith, H.C.** (1976): The in vitro effect of primaquine on red cell phospholipid metabolism. J. Lab. Clin. Med. 88 462-468.

Morris, C.R., Andrew, L.V., Whichard, L.P. and **Holbrook, D.J.** (1970): The binding of antimalarial aminoquinolines to nucleic acids and polynucleotides. Mol. Pharmacol. 6 240-250.

Nash, T. (1953): The colourimetric estimation of formaldehyde by means of the Hantzsch reaction. J. Biol. Chem. 55 412-416.

Nies, A.S., Shand, D.G. and **Wilkinson, G.R.** (1976): Altered hepatic blood flow and drug disposition. Clin. Pharmacokin. 1 135-155.

Nora, H.V., Parkhurst, G.W., Thomas, R.W. and **Carson, P.E.** (1984): High performance liquid chromatographic-electrochemical assay for primaquine in plasma and urine. J. Chromatogr. 307 451-456.

O'Reilly, R.A. (1974): Studies on the optical enantiomorphs of warfarin in man. Clin. Pharmac. Ther. 16 348-355.

Park, B.K. and Breckenridge, A.M. (1981): Clinical implications of enzyme induction and enzyme inhibition. Clin. Pharmacokin. 6 1-24.

Park B.K. (1982): Assessment of drug metabolism capacity of the liver. B. J. Clin. Pharmac. 14 631-651.

Pond, S.M. and **Tozer, T.N.** (1984): First-pass elimination Basic concepts and clinical implications. Clin. Pharmacokin. 9 1-25.

Rajagopolan, T.G., Anjaneyulu, B., Shanbag, V.D. and **Grewal, R.S.** (1981): Electron-capture gas chromatographic assay for primaquine in blood. J. Chromatogr. 224 265-273.

Riegelman, S., Loo, J.C.K. and Rowland, M. (1968): Shortcomings in pharmacokinetic analysis by conceiving the body to exhibit properties of a single compartment. J. Pharm. Sci. 57 117-123.

Rollins, D.E. and **Klaassen, C.D.** (1979): Biliary excretion of drugs in man. Clin. Pharmacokin. 4 368-379.

Rollo, I.M. (1980): Drugs in the chemotherapy of malaria. in: The pharmacological basis of therapeutics. Edited by A.Goodman and A.Gillman. Macmillan, pub, Co, N.Y. ppl051-

1054.

Roth. E.F., Raventossuarez, C., Rinaldi, A. and Nagel, R.L. (1983): Glucose-6-phosphate dehydrogenase deficiency inhibits in vitro growth of Plasmodium falciparum. Proc. Nat. Acad. Sci. -Biol. Sci. 80 298.

Rowland, M., Benet, L.Z. and Graham,G.G. (1973): Clearance concepts in pharmacokinetics. J. Pharmacokin. Biopharm. 1 123-135.

Rowland, M. and Tozer, T.N. (1980): Clinical pharmacokinetics concepts and applications. Lea and Febiger. Philadelphia. U.S.A.

Schmidt, L.H., Alexander, S., Allen, L. and Rasco, J. (1977): Comparison of the curative antimalarial activities and toxicities of primaquine and its d(-) and l(+) isomers. Antimicrob. Agents. Chemother. 12 51-60.

Schonhofer, F. (1942): Uber bedeutung der chinoiden binding in chinolinverbundungen fur die malariawirkung hoppeseyleiz. Z. F. Physiol. Chemie. 274 1-8.

Shargel, L., Cheung, W. and Yu, A.B. (1979): High performance liquid chromatographic analysis of antipyrine in small plasma samples. J. Pharm. Sci. 68 1052-1053.

Shefty, R.V. and Blanton, C.D. jnr. (1978): Synthesis of 2-substituted primaquine analogues as potential antimalarials. J. Med. Chem. 21 995-998.

Silber, B., Holford, N.H.G. and Riegelman, S. (1982): Stereoselective disposition and glucuronidation of propranolol in humans. J.Pharm.Sci. 71 699-701.

Smith, C.C. (1956): Metabolism of pentaquine in the rhesus monkey. J. Pharmacol. 116 67-76.

Strother, A., Fraser, I. Allahyari, R. and Tilton, B.E. (1981): The putative metabolism of 8-aminoquinoline antimalarial agents. Bull. WHO. 59 413-425.

Strother, A., Allahyari, R., Bucholz, J., Fraser, I.M. and Tilton, B.E. (1984): In vitro metabolism of the antimalarial agent primaquine by mouse liver enzymes and identification of a methaemoglobin-forming metabolite. Drug. metab. Disp. 12 35-44.

Tanabe, K., Chen, E.H., Verma, B.L., Saggimo, A.J. and Nodiff, E.A. (1978): Modifications of primaquine as antimalarials. 2. 5-phenylthio and 5-anilino derivatives of primaquine. J.Med. Chem. 21 133-136.

Tarlov, A.R., Brewer, G.J., Carson, P.E. and Alving, A.S. (1962): Primaquine sensitivity. Archs. Int. Med. 109 209-234.

Testa, B. and Jenner, P. (1981): Concepts in drug metabolism. Edited by P.Jenner and B.Testa. Marcel Dekker Inc. N.Y.

Thompson, P.E. and Werble, L.M. (1972): Antimalarial agents. Academic press , N.Y. pp100-122.

Timbrell, J.A. (1982): Principles of biochemical toxicology. Taylor and Francis Ltd. London.

Trager, W.F. (1982): Oxidative functionalisation reactions. in: Concepts in drug metabolism, Part A. Edited by P.Jenner and B.Testa. Marcel Dekker Inc. N.Y. pp 177-209.

Tucker, G.T. (1981): Measurement the of renal clearance of drugs. Br. J. Clin. Pharmac. 12 761-770.

Wagner, J.G. (1976): Model-independent linear pharmacokinetics. Drug. Intell. and Clin. Pharm. 10 179-180.

Walle, J., Conradi, E.C., Walle, K., Fagan, T. and Gaffney, T. (1979): Naphthoxylactic acid after single long term doses of propranolol. Clin. Pharmacol. Ther. 26 548-554.

Webster, L.K., Jones, D.B., Mihaly, G.W. and Smallwood, R.A. (1984): Effect of omeprazole and polyethylene glycol 400 on antipyrine elimination in the isolated perfused rat liver. J. Pharm. Pharmacol. 36 470-476.

Wilkinson, G.R. and Shand, D.G. (1975): A physiological approach to hepatic drug clearance. Clin. Pharmac. Ther. 18 377-390.

Wilkinson, G.R. (1982): Physiological factors affecting hepatic first-pass metabolism. in: Pre-systemic drug elimination, clinical pharmacology and therapeutics. Edited by C.F.George and D.G.Shand. pp 78-.95

Wittels, B. (1970): Modification of phospholipid metabolism in human red cells by primaquine. A possible mechanism in drug induced hemolysis. Biochem. Biophys. Acta. 210 74-85.

Whichard, L.P., Morris, C.R., Smith, J.H. and Holbrook, D.J. (1968): The binding of primaquine, pentaquine, pamaquine and plasmocid on deoxyribosnucleic acid. Mol. Pharmacol. 4 630-639.

Zubrod, C.G., Kennedy, T.J. and Shannon, J.A. (1949): Studies on the chemotherapy of human malaria. Vlll. The physiological disposition of pamaquine. J. Clin. Invest. 27 114-120.