THE PHARMACOLOGY AND TOXICOLOGY OF AMODIAQUINE

being a thesis submitted for the Degree of Doctor of Medicine,

by

PETER ANDREW WINSTANLEY MB ChB (HONS) MRCP (UK)

Department of Pharmacology and Therapeutics,
The University of Liverpool, L69 3BX.

July 1988.

TABLE OF CONTENTS

THE PHARMACOLOGY AND TOXICOLOGY OF AMODIAQUINE

P.A. Winstanley 1988

ABSTRACT

Amodiaquine, a 4-aminoquinoline antimalarial drug, is more effective, in vitro and in-vivo, than chloroquine against many chloroquine-resistant strains of Plasmodium falciparum. During the late 1970's and early 1980's, amodiaquine became increasingly popular for both treatment and prophylaxis but, at that time little was known of its pharmacology. The popularity of the drug for malaria prophylaxis was abruptly curtailed in 1986 when the unacceptably high prevalence of agranulocytosis and hepatitis, associated with its use, became obvious.

To investigate the clinical pharmacology of amodiaquine, high performance liquid chromatographic methods have been developed for the measurement of amodiaquine and desethylamodiaquine in plasma, blood, red cells and urine. These methods have been used to describe the pharmacokinetics of amodiaquine when administered orally to healthy subjects and to patients with acute malaria. The drug was found to be rapidly absorbed after oral administration to healthy subjects, but achieved low plasma concentrations, and was cleared rapidly with an apparent terminal half-life of 5h. Amodiaquine underwent rapid conversion to desethylamodiaquine, its major plasma metabolite, a compound with roughly equivalent antimalarial activity in-vitro. This metabolite was cleared more slowly than its parent drug. While amodiaquine did not appear to concentrate in the cellular components of blood, the whole blood:plasma concentration ratio of

desethylamodiaquine was 3:1. In further studies on healthy subjects, amodiaquine has been shown to exhibit linear [first-order] pharmacokinetics, over a clinically-relevant 3-fold dose range. In malaria patients, the disposition of amodiaquine in the plasma was found to be similar to that in healthy subjects. However the red cell concentrations of desethylamodiaquine, achieved in the presence of parasitaemia, were lower than those seen in unparasitised blood. Concentrations in the red cells were seen to rise as parasitaemia was cleared. These observations of changes in drug-disposition during parasitaemia are the reverse of those previously reported in the case of chloroquine, and further emphasise the differences between these two 4-aminoquinolines. To investigate the mechanism whereby amodiaquine causes its adverse effects, particularly agranulocytosis, the disposition of a radiolabelled form of the drug has been studied in laboratory animals, and the responses of normal human bone marrow cell cultures to amodiaquine and its derivatives have been observed. In rats, 96% of orally-administered radiolabel was found to be excreted, mainly in the faeces, 72h after dosing. Four % of the radiolabel was recovered from the tissues, the maximal sites of accumulation being kidney, liver, marrow and spleen. The effects of amodiaquine, desethylamodiaquine, amodiaquine-p-quinoneimine (a novel reactive derivative) and chloroquine on normal human granulocyte-monocyte colony-forming-units (GM-CFU) have been observed. All 4 compounds were found to be equally toxic, producing total or near-total inhibition of colony-formation at a concentration of 10 micromoles per litre.

DECLARATION AND ACKNOWLEDGEMENTS

The work for this thesis was undertaken between October 1984 and July 1988, whilst I was a research fellow, and subsequently a clinical lecturer in the Department of Pharmacology and Therapeutics, University of Liverpool. Except where otherwise indicated, all of the work presented here was performed by me. The two parts of the work for which I am indebted to others, are the clinical portions of the "malaria patient study", which were undertaken by Dr. J.M. Kofi-Ekue (Ndola, Zambia; chapter 4 of this thesis), and the "rat autoradiography" work, which was undertaken by Professor G.M. Powell and Dr. C.G. Curtis (Cardiff, UK; chapter 5 of this thesis). My thanks are due to many workers in the Department of Pharmacology, but most especially to Professors Alasdair Breckenridge and Michael Orme, and to Doctors Geoff Edwards and John Coleman for their continuous advice and encouragement. I would like to express my appreciation for the help given me by the Department of Haematology (University of Liverpool), particularly Doctor E. Rhodes, without whose advice the bone marrow work would have been very difficult. I would like to thank the many surgeons (especially Mr. John Monk) and theatre staff for their help in the "human marrow" work. For their financial assistance, I am indebted to Parke-Davis (UK), who enabled the purchase of radiolabelled amodiaquine, and to the World Health Organisation. Last, but not least, my thanks to Maria, Tom and Elizabeth for their tolerance over the course of the last three years.

PUBLISHED PORTIONS OF THE WORK

Winstanley PA, Edwards G, Orme ML'E, Breckenridge AM (1987); The disposition of amodiaquine in man after oral administration. Br J Clin Pharmac; 23: 1-7.

Winstanley PA, Edwards G, Orme ML'E, Breckenridge AM (1987); Effect of dose-size on amodiaquine pharmacokinetics after oral administration. Eur J Clin Pharmacol; 33: 331-3.

Winstanley PA, Edwards G, Curtis CG, Orme ML'E, Powell GM, Breckenridge AM (1988); Tissue distribution and excretion of amodiaquine in the rat. J Pharm Pharmacol; 40: 343-9.

LIST OF ABBREVIATIONS

AUC - Area under the curve

C - Degrees centrigrade

Ci - Curie

Cl - Clearance

DPM - Disintegrations per minute

h - Hours

HPLC - High performance liquid chromatography

IV - Intravenous

kg - Kilogrammes

λz - Elimination rate constant

L - Litre

M - Mole

ug - Microgrammes

mg - Milligrammes

ng - Nanogrammes

min - Minute

n - Number of observations

p - Level of significance

r - Correlation coefficient

RBC - Red blood cell

SD - Standard deviation

sg - Specific gravity

SEM - Standard error of the mean

VD - Volume of distribution

t - Time

$t_{\frac{1}{2}}$ - Half life

TLC - Thin layer chromatography

$\bar{x}$ - Mean

CHAPTER 1 Introduction and literature survey

1.1 HISTORICAL SURVEY

1.2 MALARIA

- 1.2.1 Advances in understanding of the disease
- 1.2.2 Malaria life-cycle
  - 1.2.1.1 Stages within the mosquito
  - 1.2.1.2 Stages within man
- 1.2.3 Geographical distribution
- 1.2.4 Clinical course of malaria
  - 1.2.4.1 Falciparum malaria
  - 1.2.4.2 Vivax, ovale and quartan malaria
- 1.2.5 Chemoprophylaxis
- 1.2.6 Treatment of acute malaria

1.3 ANTIMALARIAL DRUGS

- 1.3.1 Discovery of quinine
- 1.3.2 Development of synthetic antimalarial drugs
- 1.3.3 Drug resistance
- 1.3.4 Sites of drug action in the parasite life-cycle
- 1.3.5 The 4-aminoquinolines
  - 1.3.5.1 Development
  - 1.3.5.2 Structure-activity relationships
  - 1.3.5.3 Mode of action

1.4 CLINICAL PHARMACOLOGY OF AMODIAQUINE

- 1.4.1 Introduction
- 1.4.2 Absorption, excretion and tissue distribution
- 1.4.3 Metabolism
- 1.4.4 Antimalarial activity of amodiaquine and its metabolites
- 1.4.5 Pharmacokinetics
- 1.4.6 Toxicity
  - 1.5.6.1 In animals
  - 1.5.6.2 In humans

1.1 HISTORICAL SURVEY

Malaria has been a major cause of morbidity and mortality since prehistory. The great early civilizations of the Nile and Mesopotamia with their extensive systems of crop irrigation certainly had major problems with the disease, and the medical profession has grappled with malaria since its earliest days. Malaria was common in Classical Greece and the salient clinical and post-mortem features of falciparum malaria were concisely described by Hippocrates in Epidemics books I and III. Unfortunately, although Greek and Roman physicians described the disease accurately, their therapeutic armamentarium was limited to extracts of Hyoscyamus (Withington 1909) which can have been of little use. Roman engineering, rather than medical skills, probably kept the disease under some control through the drainage of the worst of the malarial swamps, but these drainage systems ceased to be maintained with the fall of Rome.

The discovery of the curative properties of Cinchona bark, in the seventeenth century, provided the first effective treatment for malaria, and by 1820 the active ingredients in extracts of bark had been isolated. This therapeutic advance was paralleled by advances in understanding the pathophysiology of transmission of the disease, both of which had been described by the end of the nineteenth century.

The first half of the twentieth century was dominated by two World Wars, and by the consequent need for secure supplies of effective antimalarial drugs for troops fighting in the tropics. Since quinine supplies might have been interrupted, the development of synthetic antimalarial agents became important. Many of these agents were toxic, but in the 1940s chloroquine, a safe, effective and cheap drug, was produced. Reliance was to be placed on

choroquine, both for malaria prevention and treatment, during subsequent decades. At much the same time, the availability of cheap and effective insecticides permitted the planning of mosquito-control programmes, which together with chloroquine, caused some to envisage the elimination of malaria on a world-wide scale, as was to be achieved with smallpox.

Unfortunately such large-scale attempts at vector control have failed, and resistance of Plasmodium falciparum to chloroquine (and other drugs) is becoming more prevalent. Furthermore visits to the tropics, by people lacking any immunity to malaria parasites, are increasingly popular. Consequently falciparum malaria in non-immune travellers is an increasing risk, and malaria remains as much of a scourge as ever to indigenous populations. Such indigenous peoples remain poor and often malnourished, malaria being just one of many environmental health problems.

During the 1980s attempts have been made to find effective alternatives to chloroquine. New drugs, including mefloquine, halofantrine and derivatives of artemisinine, have been under development but are not, even now, generally available. Therefore many existing drugs have been re-examined, in order to optimise their use. Amodiaquine, the subject of this thesis, is one such compound which, because of its effectiveness, enjoyed a brief increase in use, cut short when evidence of its unacceptable toxicity became known.

1.2 MALARIA

1.2.1 Advances in understanding of the disease

The 19th century saw the development of the disciplines of pathology and bacteriology and the establishment by workers such as Pasteur, Koch and Loeffler of the concept of specific pathogens

causing defined infections. In the 1840s post-mortem specimens of brain from patients who had died with cerebral malaria had been found by Meckel, Virchow and Shutz to contain microscopic, black-pigmented round bodies. However it was not until 1880 that identification of the malaria parasite was made by Laveran in Algeria. Laveran's findings were confirmed by Celli and Marchiafava in 1885 and the parasite was given the generic name Plasmodium. By 1891 staining methods had been developed by Romanovsky, and it was established that the patients' fevers corresponded to the release of parasite "spores" from the red cells.

However the mode of transmission of the disease was still not understood, even though it had been suspected since the early 18th century that mosquitos were responsible. Other tropical illnesses were shown to be transmitted by arthropods, including guinea worm and filariasis, and theories on arthropod transmission of malaria gained ground. The first demonstration of malarial parasites in mosquitos was made by Ross in 1897 which established Anopheles as the malaria vector. The life-cycle of P. falciparum was first described by Grassi, and thereafter the same was done for P. Vivax and P. malariae by Bastianelli and Bignami (Reviewed by Manson-Bahr 1963).

1.2.2 Malaria life-cycle

Malaria, a name originating in Italy and referring to 'bad air', is caused by Protozoal parasites of the family Plasmodiidae, within the order Coccidiida, sub-order Haemosporidiidea. There are nearly 100 species of Plasmodia infecting a wide variety of animals from reptiles through to man. The 4 species recognised to cause infection in man are P. falciparum, P. Vivax, P. malariae and P.

ovale. Within each species of parasite are morphological variations referred to as 'strains' which are defined as populations of common stock descended from a single ancestor (Bruce-Chwatt 1980). All of the species of human Plasmodia have similar life-cycles involving an exogenous sexual phase in female Anopheles mosquitos, and an endogenous asexual phase in man. Transmission of infection from insect to man and vice-versa results from ingestion of human blood by a female Anopheles.

1.2.2.1 Stages within the mosquito

When a female Anopheles feeds on an infected human, malaria parasites are released from red cells as they are digested. The asexual forms of the parasite too are digested, but the mature sex cells (gametocytes) develop further. The male gametocyte releases 4 to 8 flagella (male gametes), while the female gametocyte matures into a single female gamete. Male and female gametes unite to form a zygote within the mosquito stomach. Within 24 h of formation, the zygote becomes mobile and this oökinete forces its way between epithelial cells of the insect's stomach wall ending on the outer surface of the stomach where it remains. It becomes rounded into a small spherical cell known as an oöcyst. The oöcyst enlarges and the nucleus divides repeatedly forming great numbers of spindle shaped sporozoites ~ 15μ in length. The rupture of the oöcyst releases the sporozoites into the insect's coelom from whence they gain entry to the salivary glands. The insect is now infective, but not apparently perturbed by its parasite load.

1.2.1.2 Stages within man

Infected female Anopheles inoculate sporozoites into their victims with their saliva. The sporozoites rapidly clear from the blood, many gaining entry to hepatocytes where they multiply asexually (pre-erythrocytic schizogony). The nucleus of the parasite divides repeatedly forming a tissue schizont, and nuclear division is accompanied by cytoplasmic division so that latterly the schizont consists of many thousand uninucleate cells known as merozoites. This pre-erythrocytic stage lasts from 5½ to 16 days depending upon the species of Plasmodium.

Rupture of the cell membrane releases the merozoites into the circulation where many gain entry to erythrocytes. In the cases of P. vivax, P. ovale and P. malariae, merozoites were thought to re-enter hepatocytes to commence secondary exo-erythrocytic schizogony, however, the cause of late relapses seen with these infections are now thought more likely to be due to 'dormancy' of the primary exo-erythrocytic phase, or in the case of P. malariae persistance of erythrocytic forms for many years. P. falciparum does not cause such late relapses.

The interval between the date of innoculation and the first appearance of parasites in the blood is known as the pre-patent period. The released merozoites invade erythrocytes where they enlarge. Because there is a central vacuole within the parasite which displaces the nucleus to one side, this early erythrocytic stage is known as a ring-form. The enlarging parasite (now referred to as a trophozoite) ingests the haemoglobin of the red cell. The product of digestion is malaria pigment or haemozoin which remains within the trophozoite in the form of dark granules. After its period of growth the parasite nucleus and cytoplasm divide asexually to form a schizont which is composed of a large

number of new merozoites. When the schizont is mature, the red cell membrane ruptures releasing the merozoites into the plasma where they will attempt to invade further erythrocytes. This process of erythrocytic schizogony is repeated during the course of infection leading to a rapid increase in parasite numbers, until the process is either controlled by the development of host immunity, or results in the death of the host (as can occur with P. Falciparum infections).

The duration of erythrocytic schizogony varies between species of Plasmodia, being ~ 48h in the cases of P. falciparum, P. vivax and P.ovale and ~ 72h in the case of P. malariae.

Once several cycles of erythrocytic schizogony have occurred, some of the invading merozoites give rise to sexually differentiated forms of the parasite known as gametocytes. These grow within the red cell, but the nucleus remains undivided. Further development of gametocytes is delayed until ingestion by a female Anopheles mosquito.

1.2.3 Geographical distribution

P. falciparum is generally confined to the tropics or subtropics, because its development within the mosquito at temperatures $<20^{\circ}C$ is retarded. P. falciparum is the principal organism in areas of endemic malaria in Africa. P. vivax is distributed throughout much of the temperate zone as well as in the tropics. However P. vivax is uncommon in sub-Saharan Africa. P. malariae is distributed throughout much of the tropics and subtropics, but its distribution tends to be patchy. P. ovale is less commonly reported but is found in tropical Africa, and the western Pacific.

1.2.4 Clinical course of malaria

1.2.4.1 Falciparum malaria

The incubation period of falciparum malaria varies between 9 and 14 days being most commonly around 12 days. The term 'incubation period' refers to the delay between inoculation and appearance of clinical symptoms (cf pre-patent period). The disease usually starts with non-specific symptoms such as myalgia, back pain, fatigue, anorexia, headache, diarrhoea and vomiting. There may be few parasites in the blood at this stage, especially if the patient has been taking suppressive drugs. It is easy to misdiagnose the disease at this stage, or to misinterpret its severity, especially if the patient is examined upon his return to temporate climes, and if his trip to the tropics is not reported to the physician.

Fever is irregular, and does not usually comply to the classical tertian periodicity. Examination may reveal profuse sweating even though the fever may be low. There may be slight jaundice, and mild hepatosplenomegaly. Dipstick urinalysis may reveal albuminuria, and granular casts may be seen upon microscopic examination of urinary sediment. The full blood count may reveal slight anaemia, and there may be a degree of leucopaenia with a relative monocytosis.

Recognition and treatment of the infection at this early stage may produce rapid improvement in the patient's condition. If the infection is neglected however, life-threatening complications may appear very suddenly, especially if the patient has no immunity to the organism, or has lost acquired immunity as can occur during pregnancy. The World Health Organization's definition of severe falciparum malaria involves the demonstration of asexual forms of

P. falciparum in a patient with a potentially fatal complication of malaria, in whom other diagnoses have been excluded. The WHO recommends that such patients require intensive management which should include parenteral chemotherapy (WHO 1986). These complications include the presence of > 5% peripheral blood parasitaemia, cerebral malaria, a haematocrit < 20%, renal failure, serum bilirubin > 50μmol l^{-1}, pulmonary oedema, shock, clotting disturbances, haemoglobinuria, hypoglycaemia, and complicating infections (such as aspiration pneumonia). Figures are not available indicating the overall mortality-risk of falciparum malaria, but it is estimated that the mortality rate for cases of malaria imported into the USA is ~ 0.5% (Sturchler, 1984).

1.2.4.2 Vivax, ovale and quartan malaria

The incubation period of P. vivax is usually between 12-17 days, that of P. ovale is between 16-18 days, and that of P. malariae is 18-40 days. These figures are approximate, and in the cases of all three species the incubation period can be much longer. The clinical picture resulting from infection with these species of malaria is similar excepting the periodicity of the fevers, which typically occur every 48h in the cases of P. vivax and P. ovale but every 72h in the case of P. malariae (quartan malaria). Complications of vivax, ovale and quartan malaria include anaemia and splenomegaly. P. malariae infection in semi-immune children is associated with nephrotic syndrome. Consequently although the risk of death is small from infection with these strains of Plasmodia, morbidity may be large; inadequately treated, such infection may relapse repeatedly.

1.2.5 Chemoprophylaxis

The prevention of malaria on an individual scale is largely confined to non-immune travellers exposed to malaria risk for a short time, and to gravid women whose partial immunity to local strains of parasite is generally perturbed during pregnancy (Bruce-Chwatt 1980). Measures designed to reduce exposure to mosquito bite are generally safe and should be strongly encouraged; these include the proper use of mosquito nets, adequate screening of houses, use of insecticides and insect repellent, and the avoidance of unecessary exposure after dusk. In contrast, prophylactic drugs especially if used for prolonged periods may not prove safe, and risk:benefit ratios for drug combinations need to be considered (Peto and Gilks 1986). The situation can otherwise arise where the traveller is more at risk from his chemotherapeutic agent than malaria infection, which has been a problem with Fansidar (MMWR 1985), Maloprim (Friman et al 1983) and most recently amodiaquine (Hatton et al 1986).

1.2.6 Treatment of acute malaria

The clinician needs to establish first whether the infection is due to P. falciparum and if so, whether the parasite is likely to be resistant to chloroquine. The severity of the patient's illness should be carefully assessed and the need for parenteral treatment deliberated. The drugs required in the treatment of acute malaria are rapidly-effective blood schizontocides (Bruce-Chwatt 1980), such as chloroquine, amodiaquine, quinine, quinidine, or mefloquine. If the patient is well and can take drugs by mouth then oral therapy is usually preferable. However if the patient is vomiting, or suffering from complicated falciparum malaria then

I.V. therapy is generally preferred. As important as the choice of drug and route of administration are careful supportive measures such as fluid replacement, blood transfusion, oxygen and termination of epileptic convulsions (White 1988).

While the clinical cure achieved by blood schizontocidal drugs is adequate for P. falciparum infections, relapsing malarias particularly infections with P. vivax and P. ovale require destruction of hepatic forms of the parasite. Consequently on or before the final day of chloroquine therapy (to which most types of P. vivax, P. ovale and P. malariae are still sensitive) treatment is commenced with primaquine. One practical limitation of the use of primaquine is its toxicity, especially in subjects deficient in the enzyme glucose-6-phosphate dehydrogenase.

1.3 ANTIMALARIAL DRUGS

Prior to the seventeeth century, there was no effective remedy for malaria, as was the case with other microorganism-induced diseases. However the therapeutics of malaria was greatly advanced by the transport to Europe of Cinchona bark, whose medicinal properties had long been known to the native Peruvian civilizations. The first European to be cured by an extract of the bark, in 1630, was Countess Francisca de Chinchon, wife of the Viceroy of Peru. The bark had been sent to her by Juan de Canizares, a regional governor, but was assessed and administered to the Countess by members of the Jesuit order, with whom the bark was to be long identified (Stephens 1937).

By 1632 Barba, the Professor of Medicine at Valladolid, had supplies of Cinchona bark in his possession, and by 1682 the Dauphin of France (the future Louis XIV) was cured of a fever by

its use (Stephens 1937). By 1656 Cinchona bark (held up initially by its Catholic associations) was in use in England, where seasonal malaria outbreaks were a problem. However due to lack of distinction between malaria and other 'agues' the bark was used indiscriminately, and its toxic effects were soon described by Harvey (1683) (Stephens 1937).

1.3.1 Discovery of quinine

The "fever tree" had been given the generic name Cinchona by Linnaeus (1749) in honour of the Countess of Chinchon (but unfortunately mis-spelling her name). Despite continued widespread use throughout the 18th century, the active ingredients of Cinchona bark were not discovered until 1820. Gomez in Portugal, and Kharkhow in Russia had independently obtained crystalline substances from the bark, but it was Pierre Pelletier and Joseph Caventou working in Paris who first isolated quinine.

Throughout the 19th century the European nations continued colonial expansion in the tropics, and demands for quinine both for the prophylaxis and treatment of malaria grew. The principal world supply of bark at that time came from the Dutch East Indies.

1.3.2 Development of synthetic antimalarials

The principal impetus for the development of synthetic antimalarial drugs was the fear, particularly by the German military, of future interruption of the supply of quinine. During the first World War quinine supplies had been adequate, but in the late 1930's future conflict seemed likely and the German government desired an alternative. Consequently intensive research was undertaken.

Much had already been achieved. Guttman & Ehrlich (1891) noted that methylene blue had chemotherapeutic effects in malaria patients. Substitution of a dialkylaminoalkyl chain for one of the methyl groups in methylene blue was noted by Schulemann et al (1930) to increase both efficacy and toxicity. It was later demonstrated that 8-aminoquinoline had schizontocidal activity in infected canaries, and the combination of a basic side chain with 8 aminoquinoline led to the first synthetic antimalarial agent, pamaquine (Schonhofer, 1942). Unfortunately pamaquine proved too toxic for routine use. This same basic side chain was introduced to a variety of heterocyclic systems largely without success, but its addition to the acridine nucleus led to the discovery of mepacrine which proved extremely valuable to both sides during World War II. Mepacrine is however, a relatively toxic compound and consequently research towards a better synthetic drug continued.

Early during World War II, the Allies discovered that a potent new antimalarial agent, free from serious toxicity, was under study by the Germans in N. Africa. This was the 4-aminoquinoline sontoquine, a sample of which passed into Allied hands and which will be considered in more detail in section 1.3.5.

At about the same time (the late 1940's) the biguanide drug proguanil was under development in Britain as a chemoprophylactic agent, and was noted to bear a structural relationship to certain pyrimidine inhibitors of dihydrofolate reductase. This led on to the demonstration by Falco and colleagues (1949) that proguanil was an inhibitor of this enzyme, and to the demonstration by Carrington and colleagues (1951) that the triazine metabolite of proguanil, cycloguanil (which itself bears a closer structural relationship to

the pyrimidines), was considerably more active than its parent drug. Subsequently the pyrimidine drug pyrimethamine, also a dihydrofolate reductase inhibitor, was shown to have antimalarial activity. The sulphones and sulphonamides, which are antifolate agents through inhibition of protozoal dihydropteroate synthetase, were developed as antibacterial agents in the 1930's, but were also shown to possess antimalarial activity. These antifolate agents have generally been used in combination (e.g. primethamine - dapsone in Maloprim, and pyrimethamine - sulfadoxine in Fansidar), and have generally been reserved for malaria prophylaxis, since many consider their onset of action to be too slow to permit their use in acute infections. However Fansidar is a current second-line agent for the treatment of acute chloroquine-resistant falciparum malaria (White 1988), and the combination of dapsone with chlorproguanil (an analogue of proguanil) has recently been shown to be effective treatment for P. falciparum infections in Kenya (Watkins et al in press).

To complete this description of the development of currently-important antimalarial agents mention must be made of the 8-aminoquinoline primaquine, which remains the only drug capable of achieving a radical cure in P. vivax, P. malariae and P. ovale infections, through its action on tissue schizonts.

1.3.3 Drug resistance

It should not be surprising that so complicated an organism as Plasmodium should develop drug resistance, especially given the often poor standards of drug-usage resulting in suboptimal concentrations of drug in the blood, possibly persisting for long periods of time. Perhaps more surprising is that so far only

Plasmodium falciparum has developed widespread clinically-relevant drug resistance. This however is of major import since P. falciparum is the organism which can kill acutely and which is most prevalent in the areas of maximal malaria transmission such as sub-Saharan Africa.

Resistance to dyhydrofolate reductase inhibitors such as proguanil and pyrimethamine was noted nearly 30 years ago but this has not been a major problem - the drugs are not first-line agents in acute infection and furthermore, despite widespread resistance, they remain effective prophylactic agents in many parts of the world (WHO 1984). However, resistance to chloroquine, which throughout the post-war years had become the mainstay of malaria therapy due to effectiveness, low toxicity and low cost, has become a matter of grave concern. Chloroquine-resistant P. falciparum was first documented on a large scale in Colombia in 1960. Since then chloroquine resistance has spread to many parts of the tropics (WHO 1973), but its presence in east and central Africa is the most worrying because it is here that transmission rates of P. falciparum are among the highest in the world.

Widespread chloroquine resistance has come at a time when international travel has increased massively, and large-scale vector control programmes have failed. Great efforts have been made in the fields of new drug development and malaria immunology, and as a result several "new" antimalarials are being, investigated (including mefloquine, halofantrine and artemisinine), and the understanding of immune responses to malaria has progressed. However, such large endeavours are slow by their very nature; vaccines for malaria are still a long way off, and the roles of newer antimalarial drugs have yet to be fully assessed.

Consequently there is a need for existing antimalarial drugs to be re-assessed and used optimally. It was this which prompted a re-appraisal of amodiaquine especially against chloroquine-resistant P. falciparum. The demonstration of superiority of amodiaquine over chloroquine in the treatment of such infections (Watkins et al 1984) led to the drug's increasing use in malaria prophylaxis during the period 1984 to 1986, and unfortunately to the subsequent demonstration of its unacceptably frequent toxicity (Hatton et al 1986).

1.3.4 Site(s) of drug action in the parasite life-cycle

Antimalarial drugs have selective actions on the phases of the parasite life-cycle which determine their therapeutic roles. Amodiaquine like chloroquine, quinine, quinidine and mefloquine is primarily a blood schizontocide producing rapid reduction of parasitaemia and correspondingly rapid clinical improvement; this effect is seen against all four types of human malaria (Bruce-Chwatt, 1980). In addition these same drugs have activity against the gametocyte stage of the life cycles of P. vivax, P. malariae and P. ovale and are thus said to be gametocytocidal to these species. Primaquine, which is an 8-aminoquinoline, is gametocytocidal against all four species of parasite in man although it is not primarily used for this purpose.

Drugs which inhibit parasite development within the vector, producing interruption of sporogeny, are said to be sporontocidal. Primaquine, pyrimethamine and proguanil are sporontocidal against all species of human malaria parasite.

There is no drug with activity against sporozoites immediately after their innoculation into the host (true causal prophylaxis).

However some drugs have activity against the exo-erythrocytic (liver) stages of infection, most notably primaquine and, to a much lesser extent, proguanil and pyrimethamine. Such drugs are said to be primary tissue schizontocides.

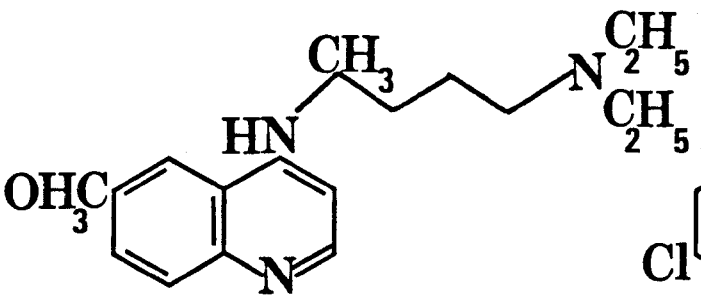
1.3.5 The 4-Aminoquinolines

1.3.5.1 Development

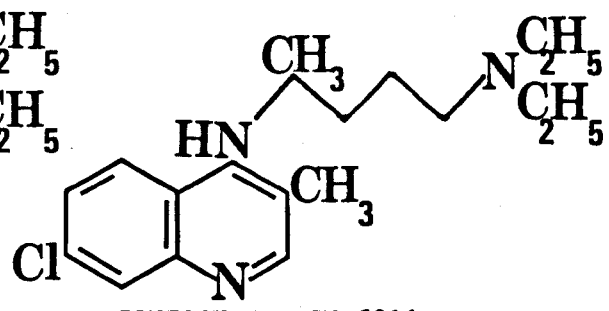
The 4-aminoquinolines were first synthesised and tested in the mid 1930's as part of the German programme to develop an antimalarial more effective but less toxic than mepacrine. Several agents including chloroquine were tested by H. Andersag (Coatney 1963), and of these sontoquine was considered to have the most favourable activity/toxicity relationship. In the early part of the second World War, a sample of sontoquine fell into Allied hands and was tested together with several other 4-aminoquinolines as part of the American antimalarial survey (Wiselogle 1946). The structures of some of the 4-aminoquinolines studied are given in figure 1.1. Chloroquine (Survey No. 7618), set aside by the Germans, was found to have the best activity/toxicity relationship, while amodiaquine (SN 10751) was felt worthy of further investigation; these two 4-aminoquinolines, together with their structural analogues hydroxychloroquine (Surrey & Hammer 1950) and amopyroquine (Thompson et al 1958), have remained in use for malaria chemotherapy and, to a lesser extent, the treatment of such auto-immune conditions as rheumatoid disease and systemic lupus erythematosus.

1.3.5.2 Structure-activity relationships

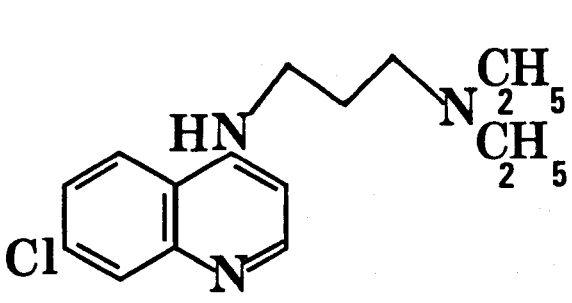
During the course of the wartime American antimalarial survey,



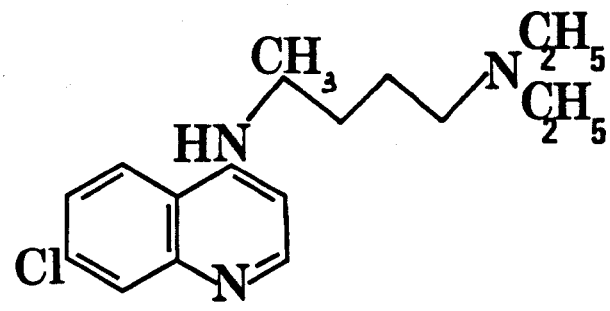
SN 3294*



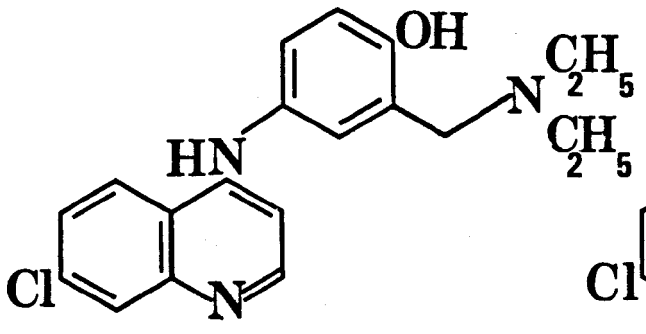
SONTOQUINE; SN 6911



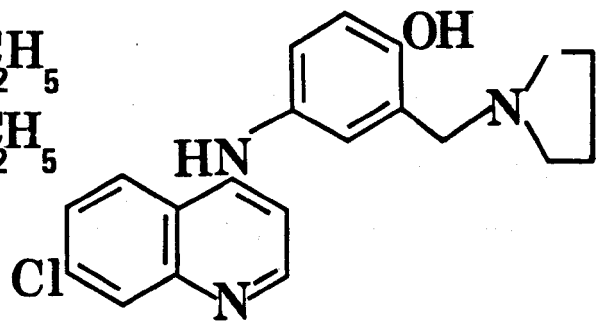
SN 9584



CHLOROQUINE; SN 7618



AMODIAQUINE; SN 10751



AMOPYROQUINE (NO SURVEY No.)

FIGURE 1.1 Chemical structures of some of the compounds important in the development of the 4-aminoquinolines

(* The survey No.'s are those used by Wiselogle 1946)

studies were undertaken on many hundreds of compounds including the 4-aminoquinolines (Wiselogle 1946). Substitutions on the quinoline nucleus and the 4-amino side chain were both found to be capable of changing antimalarial activity and toxicity. SN 3294 (figure 1.1) was a logical starting point for the programme, having an identical quinoline nucleus to that of quinine, together with a side chain identical to that of pamaquine but in the 4-amino position. SN 3294 proved less active and more toxic than mepacrine (Wiselogle 1946), but substitution of a 7-chloro group for the 6-methoxy group on the quinoline nucleus improved activity/toxicity relationships (and this agent SN 7618 was chloroquine). Methylation of the 7 chloro derivatives in the 2 or 3 positions (as in sontoquine; SN 6911) was detrimental, as was substitution of a 7-bromo group for the 7-chloro group (Coatney et al 1953). Substitutions of the 4-amino side chain included reduction in the number of carbon atoms between the 4-N atom and the tertiary amino group (as in SN 9584) and oxidation of the 4 amino side chain (as in SN 8137; oxychloroquine) but these proved detrimental to activity/toxicity relationships (Wiselogle 1946, Coatney et al 1953). The aromatic 4-amino side chains of SN 10751, amodiaquine (Wiselogle 1946, Coatney et al 1953), and amopyroquine (Thompson et al 1958) gave activity/ toxicity relationships equivalent to those of chloroquine, and like chloroquine their side chains possess 4 carbon atoms between the 4-N and the tertiary amino group.

1.3.5.3 Mode of action

Early theories of the mode of action of 4-aminoquinolines proposed that the drugs formed a complex with parasite DNA thus interfering with transcription (Ciak and Hahn 1966). However this is no longer thought to be correct; at present two distinct theories are

is no longer thought to be correct; at present two distinct theories are advanced. The first suggests that 4-aminoquinolines act by delaying the sequestration into malaria pigment, of ferriprotoporphyrin IX, by the parasite; the second suggests that the drugs enter parasite cytoplasm on a membrane carrier and subsequently interfere with food-vacuole activity.

Erythrocytic forms of malaria parasite absorb haemoglobin and leave as a product of their digestion a 'malaria pigment' haemozoin, which can be seen upon microscopic examination as dark granules within the parasite cytoplasm. Approximately 90% of the haemozoin is made up of ferriprotoporphyrin IX, a form of oxidised haem (Fitch et al 1986). Ferriprotoporphyrin IX either alone or in complex with chloroquine is toxic to malaria parasites, causing their lysis (Fitch et al 1982). Chloroquine and amodiaquine have a high affinity for ferriprotoporphyrin IX (Fitch 1973) and it is suggested by Fitch (1986) that 4-aminoquinolines exert their effect by binding to ferriprotoporphyrin IX thereby inhibiting its incorporation into haemozoin, and causing accummulation of the toxic complex within the cytoplasm of the parasite.

An alternative theory of the mode of action of 4-aminoquinolines (Warhurst 1986) proposes that the drugs are taken up into the parasites' cytoplasm by means of an active transport mechanism involving a membrane carrier (or permease). Warhurst (1986) suggests that an active mechanism is required because at the pH encountered within infected erythrocytes (~ 6.6-6.8) weak bases such as the 4-aminoquinolines would largely be in an ionised form and therefore hydrophilic/lipophobic (Friedman et al 1979; Mikkelsen et al 1982). It is suggested that the normal physiological function of the protease would be the active

transport of basic amino acids such as arginine (Warhurst 1986). Once within the parasite 4-aminoquinolines have rapid effects on the acidic digestive vacuoles causing a rise in their pH, and it is proposed that it is these effects which are responsible for parasite death.

1.4 CLINICAL PHARMACOLOGY OF AMODIAQUINE

1.4.1 Introduction

Amodiaquine was first synthesised in the late 1940's (Burckhalter et al 1948) and was included by Wiselogle (1946) in the American wartime survey of antimalarial drugs (survey number 10751). Oral administration of the drug was subjected to field tests in Brazil (Penido et al 1947), Egypt (Halawani et al 1947), India (Simeons and Chkatre 1947) and the Philippines (Ejercito and Dugue 1948), and amodiaquine was tested by Payne and others (1949) by I.V. bolus administration. Amodiaquine proved free from major toxicity in these studies, the main problem encountered being nausea. Despite its equivalent potency to chloroquine and apparent lack of toxicity, amodiaquine was never used as widely as chloroquine possibly because it was more expensive. However the drug did remain in use, especially for administration to children, since unlike chloroquine, which has an unpleasant bitter taste, amodiaquine is virtually tasteless.

The in vitro testing of drug sensitivity of malaria parasites was developed in the early 1970's (Rieckmann 1971) in part as a tool in the global management of increasingly prevalent chloroquine-resistant P. falciparum. Using this technique Rieckmann (1971) was able to show that amodiaquine was more potent than chloroquine against the Vietnam (Marks) strain of P. falciparum (a

chloroquine-resistant strain), and these *in vitro* findings were supported by other workers (Schmidt *et al* 1977; Spencer *et al* 1983). Clinical support for the superiority of amodiaquine over chloroquine against certain strains of chloroquine-resistant *P. falciparum* was produced in studies from Kenya (Watkins *et al* 1984), and Thailand (Looareesuwan *et al* 1985). However, not all *P. falciparum* strains show this effect, some being less sensitive to amodiaquine (Watt *et al* 1987).

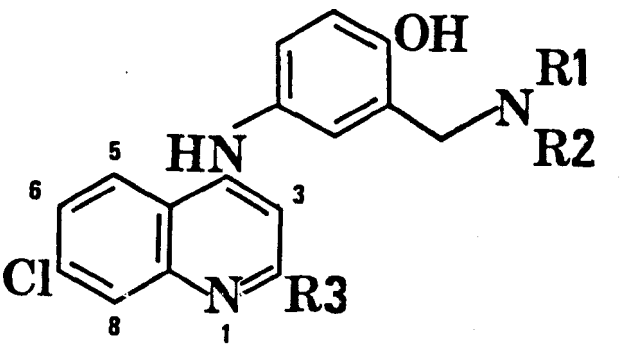
These data, largely showing advantages of amodiaquine over chloroquine, stimulated increasing use of the drug as part of chemoprophylactic regimens for travellers to areas where chloroquine-resistant *P. falciparum* was prevalent. Agranulocytosis had been recognised as a rare adverse effect of amodiaquine for some years (Glick 1957; Kennedy 1955). The widespread use of the drug for malaria prophylaxis involved a relatively large population subject to the scrutiny of adverse effect monitoring bodies, such as the Committee on Safety of Medicines in the UK, and a high prevalence of agranulocytosis was soon noticed in amodiaquine-recipients (Hatton *et al* 1986). Adverse effects have caused the abandonment of amodiaquine for malaria prophylaxis, although currently the drug remains in use for the treatment of acute infections.

1.4.2 Absorption, excretion and tissue distribution

In laboratory studies on amodiaquine using rats, Glazko and Wolf (1957) concluded that the drug was absorbed slowly after oral administration, and concentrated in the tissues particularly liver, kidney, spleen and lung. Urinary and faecal excretion of amodiaquine accounted for only a small percentage of the dose.

However, the fluorimetric assay method used (Brodie et al 1947) was neither specific for the drug, nor sensitive.

Barrow (1974) studied the excretion and, to a lesser extent, tissue distribution of ^{14}C-labelled amodiaquine in rodents (rats, mice and guinea pigs) following oral, I.V. or intraperitoneal administration. Following a single oral administration of ^{14}C-amodiaquine to rats < 50% of the administered ~~radiation~~ radioactivity was accounted for, ~ 10% in urine collections and ~ 38% in faeces collections (both over 216h); similar results were obtained after a single intraperitoneal administration of ^{14}C-amodiaquine with ~ 10% in urine collections and ~ 36% in faecal collections (both over 240h). That radiation was excreted in the bile following oral or I.V. administration of a single dose of ^{14}C-amodiaquine was confirmed by bile duct cannulation of intact rats (Barrow 1974). In contrast ^{14}C-labelled chloroquine was found to be excreted mainly in the urine of experimental animals whether given orally or parenterally (Barrow 1974). One possible explanation for the different routes of excretion of these two 4-aminoquinolines is the difference between their molecular weights; amodiaquine has a higher molecular weight than chloroquine and thus might be expected to be more likely to undergo biliary excretion. Barrow (1974) also examined the distribution of radioactivity in the tissues of mice following a single I.V. dose of ^{14}C-amodiaquine, and found that in an animal killed 48h after dosing, radioactivity was associated mainly with intestine, brown fat, liver, kidneys and lungs. Work will be presented in this thesis which quantitates the mass-fate of ^{14}C-amodiaquine in rats following oral and i.v. administration, and assesses the tissue distribution of the radiolabel.



KEY;	R1	R2	R3
Amodiaquine	C_2H_5	C_2H_5	H
Desethyl.	C_2H_5	H	H
Hydroxydesethyl.	C_2H_5	H	OH
Bisdesethyl.	H	H	H

FIGURE 1.2 Identified metabolites of amodiaquine (based on Churchill et al 1986)

1.4.3. Metabolism

Barrow (1974) subjected excreta from rats given oral ^{14}C-labelled amodiaquine to thin layer chromatographic (TLC) examination. At pH 9-10 40-66% of the radioactivity excreted in urine by 48h could be extracted into ether, while < 5% could be recovered by ether extraction at pH 2. This suggested that bases accounted for much of the urinary excretion products, and acids (such as carboxylic acid derivatives) accounted for little. TLC analyses of alkaline extracts revealed at least 6 radioactive compounds of which unchanged drug represented < 5%. The two major bands of radioactivity were subjected to further preparative TLC, and then to UV spectrum analysis. The UV spectra of both were similar to that of amodiaquine indicating that the quinoline nucleus was intact.

Analysis of bile by the same author, using paper chromatography, revealed that amodiaquine accounted for 20-40% of the excreted radioactivity in the first few hours after oral ^{14}C-amodiaquine, but that this fell to < 10% by 12h, and < 5% thereafter. Interestingly extraction of bile into ether at pH 9-10 recovered < 5% of the radioactivity present, but following acid hydrolysis a further 16-20% could be extracted, suggesting the presence of conjugates. The presence of such conjugates had also been proposed by Chambon and colleagues (1968) from experiments on dogs. Barrow (1974) was able to extract only 3-20% of the radioactivity voided in the faeces of rats by etheral extraction at pH 9-10. The same author examined methanolic extracts of faeces (presumably by TLC) and demonstrated the presence of unchanged amodiaquine plus the two principal metabolites previously demonstrated in the urine. Barrow (1974) felt that the two major

metabolites demonstrated in urine and faeces were likely to be desethylamodiaquine and bisdesethylamodiaquine (see figure 1.2). In contrast most of a dose of chloroquine, when administered to man, is excreted unchanged and only about 20% is in the form of desethylchloroquine (McChesney et al 1967); the metabolic fate of chloroquine is known to vary between species, but in general chloroquine clearance seems to depend less on hepatic biotransformation than that of amodiaquine.

Isolation and identification of the major metabolites of amodiaquine following its oral administration to man has been undertaken recently by Churchill and colleagues (1985 and 1986). Following oral amodiaquine, the urine of a healthy volunteer was collected and extracted into organic solvent. Following preparative TLC (Churchill et al 1985) or HPLC (Churchill et al 1986) purified metabolites were examined by nuclear magnetic resonance and electron impact mass spectrometry. The two major metabolites thus identified were desethylamodiaquine and 2'-hydroxydesethylamodiaquine (figure 1.2). A third metabolite bisdesethylamodiaquine has been demonstrated in the urine (Churchill et al 1986) by HPLC comparison with authentic compound. Churchill and colleagues (1986) speculate that a further 2 metabolites of amodiaquine may be formed in man by hydroxylation of the parent drug and bisdesethylamodiaquine to give 2'-hydroxyamodiaquine and 2'-hydroxybisdesethylamodiaquine respectively. Quantitative data on the metabolic fate of amodiaquine in man, such as are available for chloroquine (Kuroda 1962, McChesney et al 1966 and 1967), have not been determined.

Like paracetamol, amodiaquine contains a p-hydroxyanilino moiety and might be expected to be subject to cytochrome P-450-mediated oxidation to a semiquinone or quinoneimine. Such

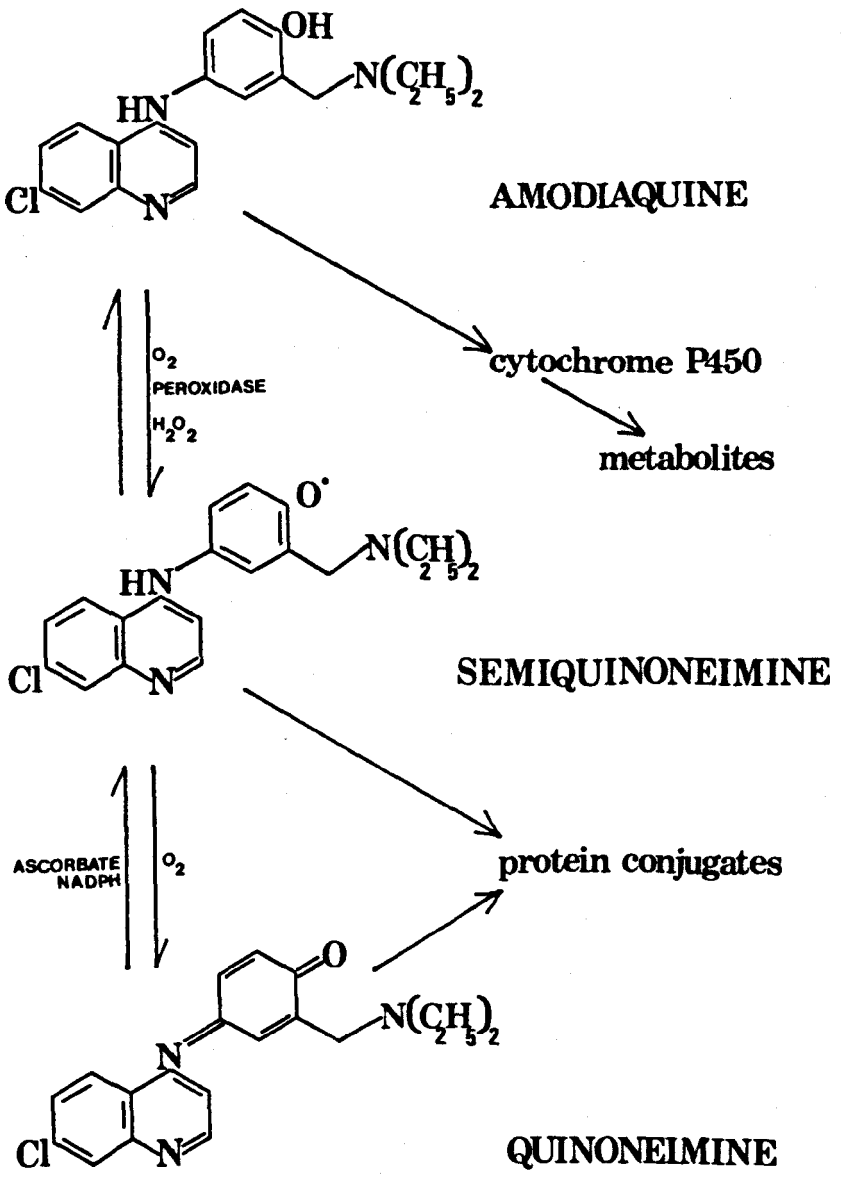


FIGURE 1.3 Autoxidative breakdown products of amodiaquine (after Maggs et al 1987)

compounds are highly reactive, and in the case of paracetamol, the p-quinoneimine produced by enzymic oxidation is thought to be the ultimate toxic intermediate of the drug (Holme *et al* 1984), probably producing its toxicity by arylation of vital intracellular proteins (Jollow *et al* 1973). The possibility that amodiaquine too might undergo enzymic oxidation yielding reactive intermediates has been studied by Maggs and colleagues (1987 *a,b* and 1988) (figure 1.3). These workers found that amodiaquine is an unstable molecule, undergoing autoxidation in aqueous solution at physiological pH. Evidence has been presented by the same workers which suggests that both a semiquinone and a quinoneimine may be produced by such autoxidation, and that the rate of this process could be increased by peroxidase activity (Maggs *et al* 1988). They also speculate that desethylamodiaquine too could be expected to be subject to autoxidation under similar conditions (Maggs *et al* 1988). In addition to its susceptibility to autoxidation, Maggs and colleagues (1988) have shown that amodiaquine may undergo N-chlorination, when exposed to chlorine solutions, which may also generate a potentially toxic reactive intermediate. These *in vitro* observations on the ability of amodiaquine to generate reactive compounds offer the possibility of an understanding of the toxicity of amodiaquine at a molecular level. Work will be presented in this thesis which investigates the effects of amodiaquine-p-quinoneimine on the *in vitro* proliferation of granulocyte-monocyte colony forming units (GM-CFU) obtained from the bone marrow of normal volunteers.

1.4.4 Antimalarial activity of amodiaquine and its metabolites

The relative antimalarial activity of the metabolites of

amodiaquine have been determined by Mount and co-workers (1987). The P. falciparum strains examined were NF54, Indochina 1, and HB3. The inhibitory concentrations of chloroquine and amodiaquine were similar for NF54 and HB3 which are both chloroquine-sensitive, being 0.08 and 0.1 μM respectively; however for Indochina 1 (chloroquine-resistant) the inhibitory concentrations were 0.6 and 0.1μM for chloroquine and amodiaquine respectively showing the higher activity of the latter. Of the metabolites, desethyl-amodiaquine and bisdesethylamodiaquine had similar inhibitory concentrations at 0.3 to 0.1μM for all 3 strains. The 2'hydroxy-desethyl metabolite was significantly less active, needing to be present at between 3-10mM in order to inhibit growth of the organisms. Pussard and colleagues (1987) have also determined the in vitro antimalarial activity of amodiaquine, desethylamodiaquine, and bisdesethylamodiaquine. Like Mount and colleagues (1987), these workers found comparable antimalarial activity between the parent drug, and the monodesethyl metabolite; but in contrast to the findings of Mount and colleagues (1987) found the activity of the bisdesethyl metabolite to be much lower than either of the other two compounds.

1.4.5 Pharmacokinetics

Prior to the development of HPLC methods for the determination of amodiaquine and metabolites in biological fluids, reliance was placed on extraction of the drug into organic solvent followed by a method to increase the native fluorescence of the drug, and subsequent fluorimetric detection. Such methods included those of Brodie and others (1947), McChesney and others (1956) and Trenholme and others (1974); all suffered from a lack of sensitivity (lower

limits of detectability no lower than 50 ng ml^{-1} of plasma) but more importantly none was able to distinguish between the parent drug and its metabolites. Consequently although elaborate pharmacokinetic calculations were made (Ritschel et al 1978) the results were of dubious value. Ritschel and colleagues (1978) for example calculated the following values for amodiaquine:- half life ($t_{\frac{1}{2}}$) 49.5h, bioavailability 1.0 (for the meaning of these terms, see section 1.6 of this thesis). These values, which were used to give recommendations on dose schedules, suggested that the pharmacokinetics of amodiaquine closely resembled those of chloroquine; data reported in this thesis, and by others elsewhere, have shown that this is largely untrue.

It was evident however that more detailed investigation of the pharmacokinetics of amodiaquine were necessary to optimise dose schedules, and this became particularly important when the drug began to be used frequently for malaria prophylaxis in the early 1980's. The HPLC method for the measurement of amodiaquine in plasma developed by Mihaly and colleagues (1985) was the first to be published. These workers applied the method to plasma samples drawn from two Thai patients who had received amodiaquine 10mg kg^{-1} in a 4h I.V. infusion. In distinction to the long $t_{\frac{1}{2}}$ value for amodiaquine value given by Ritschel and others (1978) Mihaly and colleagues (1985) calculated amodiaquine terminal $t_{\frac{1}{2}}$ values of 4.3 and 9.7h for the two patients. This same method was applied to samples of plasma drawn from both healthy subjects and Thai patients who had received amodiaquine by mouth (Looareesuwan et al 1985) but the unchanged drug was detectable, close to the lower limit of detectability, for only a very short time after administration. Further development of the HPLC method of Mihaly

and colleagues (1985) to allow simultaneous determination of amodiaquine and desethylamodiaquine forms a part of this thesis. HPLC methods were reported by others (Churchill et al 1985, Pussard et al 1985 and 1986, Mount et al 1987) during the course of the work, and the pharmacokinetic data produced by these methods will be considered in Chapter 3.

1.4.6 Toxicity

1.4.6.1 In Animals

The wartime antimalarial survey, as well as investigating the activity of compounds, investigated their short and long-term toxicity in animals, using a simple set of protocols (Wiselogle 1946). Data were usually given in terms of the toxicity of quinine. Thus chloroquine (SN 7618) had oral toxicity 'values' of 5-10 in different animals, while amodiaquine's toxicity 'values' ranged from 3-5. Work undertaken by the manufacturer of the drug (Grühzit et al 1961) gave acute toxicity figures in more detail; for mice the mean lethal doses (LD 50) were 548 mg kg^{-1} (oral), 100mg kg^{-1} (intraperitoneal) and 17 mg kg^{-1} (I.V.), while for rats LD 50 values were 379 mg kg^{-1} (oral) and 490 mg kg^{-1} (subcutaneous).

1.4.6.2 In Humans

Few of the early reports on the pharmacology of amodiaquine mention toxicity, but Berliner and colleagues (1947) reported lassitude, insomnia, epigastric discomfort, and nausea, as main adverse effects seen in 16 healthy males who received 7.6g of the drug over 5 weeks. Symptoms were severe enough to warrant withdrawal of one subject. White cell counts do not appear to have been measured. More recently 12 out of 20 patients receiving oral

amodiaquine for the treatment of onchocerciasis (as part of a trial into its potential efficacy for this indication) suffered side effects (Kale 1982); seven had gastrointestinal upset, 12 had lassitude, and in 3 falls in peripheral granulocyte counts (ranging 18-27% of the pretreatment value, and lasting 7-14 days) were noted, but were not accompanied by clinical problems. Like chloroquine, amodiaquine could be expected to cause a dose-dependent retinopathy (Hobbs, 1959) but this seems to have been reported less frequently largely due to less frequent use of the drug. Other reported adverse effects to amodiaquine include peripheral neuropathy, and skin pigmentation, both of which occur principally in subjects taking the drug for prolonged periods (Martindale 1982).

The two most frequent life-threatening adverse effects of amodiaquine are agranulocytosis and hepatitis, and the exact mechanisms of neither are known. Hepatitis was reported as an adverse effect of the drug quite early in its history (Bepler et al 1959; Pomeroy et al 1959), but as with agranulocytosis, because amodiaquine was used largely in the tropics and therefore outside the scrutiny of drug monitoring bodies, the prevalence of hepatitis due to the drug was not appreciated. Thirteen cases of amodiaquine-induced hepatitis are to be found in the recent literature (Neftel et al 1986; Larrey et al 1986; Amourretti et al 1986) of which 3 ended fatally (one of these patients also had agranulocytosis). There are insufficient data to allow detailed speculation on the mechanism(s) involved, but Neftel and colleagues (1986) favoured a direct toxic effect, while Larrey and colleagues (1986), based upon the recurrence of hepatitis following rechallenge of 2 patients with amodiaquine, speculated that an

immune reaction was implicated. The mass of drug consumed ranged 1.2 to 9.6g, taken over 5-15 weeks for malaria prophylaxis; the interval from commencement of amodiaquine to the onset of symptoms ranged 4 - 11.5 weeks, and the duration of illness ranged 5-28 days. Jaundice was the commonest presenting symptom, and elevation of plasma bilirubin and alkaline phosphatase were the commonest laboratory finding. Deranged hepatic transaminase levels (alanine amino transferase was most often measured) was less common, as was perturbation of gamma glutamyltransferase. Post mortem studies were carried out in the 3 fatal cases reported (Neftel et al 1986; Amourretti et al 1986); the first showed a moderate mononuclear cell infiltrate into portal tracts, together with centrilobular cholestasis. Both the remaining cases had sub-total hepatic necrosis containing clusters of mononucleocytes or lymphocytes.

1.5 DRUG-INDUCED AGRANULOCYTOSIS

1.5.1 Introduction

Drug-induced agranulocytosis is a clinical condition characterised by a tendency to infection due to a reduction in the circulating numbers of neutrophils, often to levels $< 0.2 \times 10^9 L^{-1}$, as a result of an idiosyncratic response to a drug (Young and Vincent 1980). Other forms of neutropaenia (ie peripheral neutrophil count $< 2.0 \times 10^9 L^{-1}$), eg those predictably caused by anticancer agents, non drug-related neutropaenia and blood dyscrasias in which other cell types are affected, are therefore excluded from this definition. The important clinical result of agranulocytosis is susceptibility to infection (particularly from bacterial pathogens) which may result in death. The mechanisms of

agranulocytosis due to drug therapy are rarely fully understood. By their nature, such reactions are idiosyncratic and it is often not clear why a given patient should have developed agranulocytosis while taking a drug, or why other patients taking the same drug under similar circumstances were unaffected. There are however certain mechanisms which have been proposed.

1.5.2 Mechanisms

1.5.2.1 Interindividual differences in drug disposition

Genetic factors are known to account for differences in drug handling between different ethnic populations; for example fast acetylator status predominates among the Japanese and Eskimoes, while slow acetylator status predominates in mediterranean Jews (Evans and White 1964). This 'genetic polymorphism' of drug metabolism has clinically important implications for certain drugs, most notably in this case isoniazid, procainamide and hydralazine, where accumulation of parent drug or synthesis of toxic metabolites may cause disease. Examples of drugs subject to such genetically-determined interindividual variations in drug metabolism, which may have been implicated in agranulocytosis, include phenylbutazone (Cunningham et al 1974). In patients recovered from phenylbutazone-induced agranulocytosis (but not subjects recovered from idiopathic agranulocytosis who were used as control subjects) there was delayed clearance of acetanilide, which was used as a 'test drug', suggesting that the cause of agranulocytosis in the

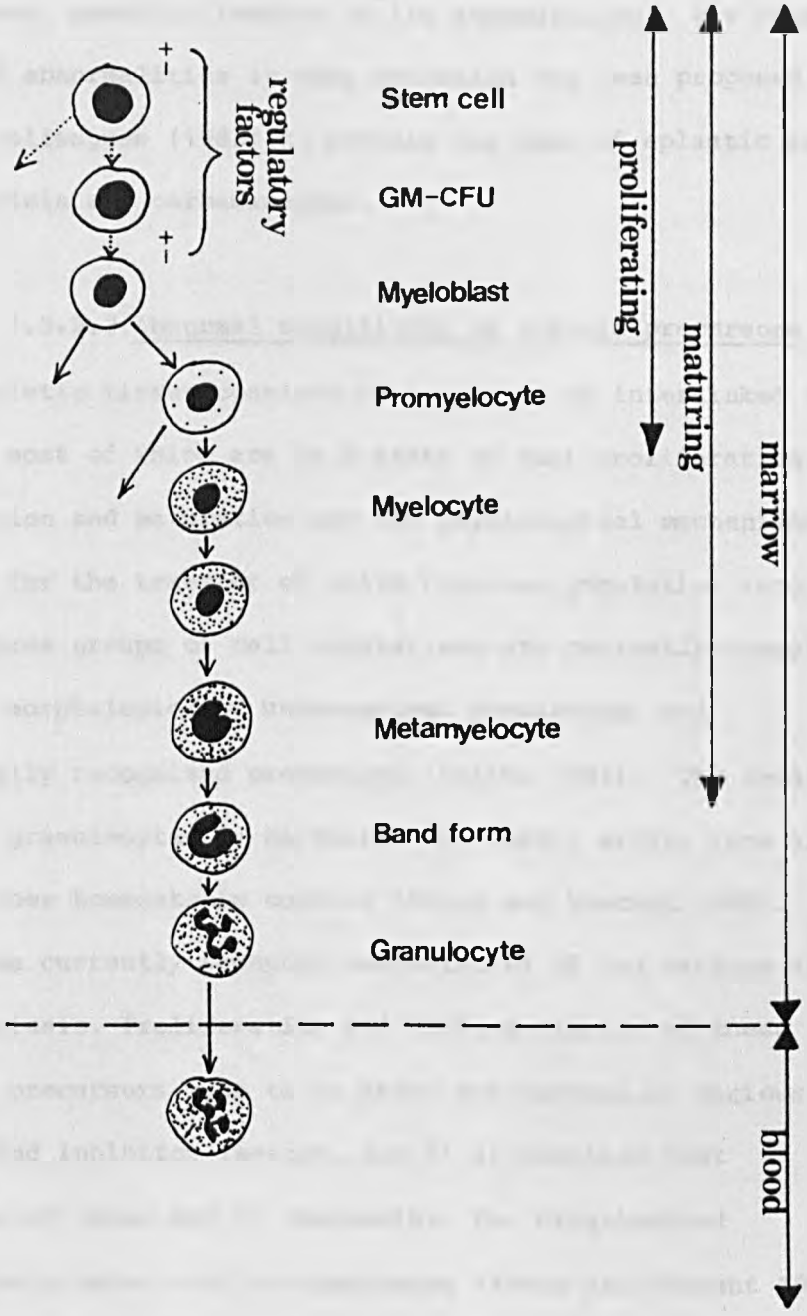


FIGURE 1.4 Differentiation of the pluripotential stem cell to the mature granulocyte (after Young and Vincent 1980)

test subjects might have been due to slow clearance of phenylbutazone, possibly leading to its accumulation. The suggestion of inherited abnormalities in drug oxidation has been proposed by Gerson and colleagues (1983) to explain one case of aplastic anaemia due to phenytoin and carbamazepine.

1.5.2.2. Abnormal sensitivity of myeloid precursors

Haemopoietic tissue consists of a complex of interlinked cell populations most of which are in a state of fast proliferation. Cell differentiation and maturation are the physiological mechanisms responsible for the transfer of cells from one population into another. Three groups of cell populations are currently accepted: stem cells, morphologically unrecognised precursors, and morphologically recognized precursors (Lajtha 1982). The level of circulating granulocytes is maintained in health within fine limits and under close homeostatic control (Young and Vincent 1980). Figure 1.4 shows the currently accepted nomenclature of the various stages of granulopoiesis. Proliferation and differentiation of these granulocyte precursors seem to be under the control of various stimulator and inhibitor factors, and it is possible that perturbation of these may be responsible for drug-induced agranulocytosis under some circumstances (Young and Vincent 1980). The best characterised of the stimulators are a family of glycoproteins referred to as colony stimulating factors (CSF) produced by monocytes, macrophages and certain tumour cell lines (Metcalf 1985 (a) and (b)). These CSF are of practical importance in the principal methods for the study of haemopoiesis, namely in-vitro growth of marrow colonies (see Chapter 6). Inhibitors of haemopoiesis have also been identified, and include prostaglandin E (Kurland and Moore 1977), but their role, if any, in drug-induced

agranulocytosis is as yet unclear.

Apart from theoretically possible perturbations of stimulator/inhibitor systems, idiosyncratic reponses to a drug might be the result of abnormalities in the precursor cells themselves. This might be the result of genetic factors, and a close analogy might be the mature erythrocyte deficient in glucose-6-phosphate dehydrogenase which, because of this deficiency, is rendered unduly sensitive to many drugs including primaquine (Tarlov et al 1962). However even in cases where drugs have been shown to inhibit bone marrow colony formation in vitro , details of the biochemical reactions leading to toxicity, such as have been determined for primaquine, are not usually understood. Drugs which have been shown to inhibit the formation of colonies from granulocyte/monocyte colony forming units (GM-CFU; the name given to the unidentified precursor cells) of susceptible individuals include quinine (Sutherland et al 1977), phenylbutazone (Smith et al 1977) and chloramphenicol (Howell et al 1975). Amodiaquine has been investigated using similar experimental models (Lind et al 1973; Rhodes et al 1986) and has been shown to inhibit the growth of GM-CFU of patients with amodiaquine-induced agranulocytosis, but not of normal controls (see Chapter 6).

1.5.2.3 Immune supression of granulopoiesis

This proposed mechanism of drug-induced agranulocytosis has largely been investigated as above using in vitro culture systems of human marrow. Relatively few drugs have been incriminated, these include quinidine (Kelton et al 1979) and phenytoin (Taetle et al

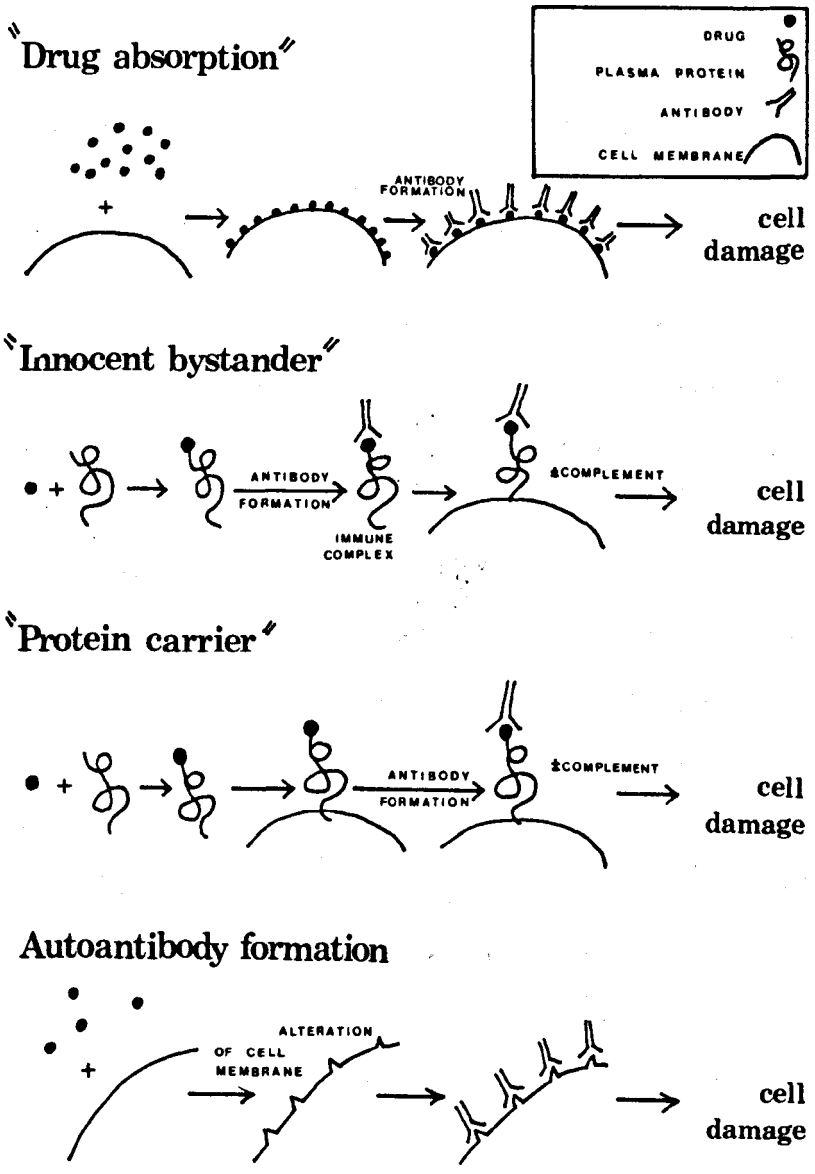


FIGURE 1.5 Possible immunological mechanisms involved in drug-induced agranulocytosis

1979). In the case of the latter drug the demonstrated antibody was non-complement-dependent, and in the case of one of the patients described, antibody-dependent cytotoxicity for mature neutrophils was demonstrated (Taetle et al 1979). The effects of such antibodies are dependent on the presence in the system of the specific drug (Young and Vincent 1980). The actual mechanisms whereby drug-dependent antibodies damage precursor cells is not known, but may be similar to those described for immune-mediated damage to mature circulating cells (Young and Vincent 1980; Vide Infra).

1.5.2.4. Immune destruction of mature granulocytes

This was the first mechanism identified as a cause of neutropaenia, the first drug so implicated being aminopyrine (Moeschlin and Wagner 1952). Since then drug-dependent leukocyte agglutinins have been reported with a number of drugs notably semisynthetic penicillins (Weitzmann et al 1978), propylthiouracil (Petz and Fudenberg 1975), and phenytoin (Taetle et al 1979). In one patient with quinidine-induced haemolytic anaemia and thrombocytopaenia, Ziegler and colleagues (1979) were able to demonstrate drug-dependent complement-fixing IgG antibodies to erythrocytes and platelets, which were strongly adherent to erythrocytes, suggesting that the drug was 'absorbed' on to the surface of the cell, and specific immunoglobulin bound to the absorbed drug which acted as a hapten. Drawing on data from many different studies Young and Vincent (1980) have proposed several mechanisms whereby drug-induced immunity could damage circulating cells (figure 1.5). The "drug absorption" model is illustrated by the example of quinidine. In the "innocent bystander" model the drug binds to plasma protein, and antibodies form against this complex.

The complex then passively binds to a cell membrane, activating complement and damaging the cell. In the final "protein carrier" model the drug binds to a plasma protein, and the complex binds to a cell surface. Specific antibodies are then formed against the 'antigen' and the cell is damaged with or without complement activation.

Schulthess and colleagues (1983) demonstrated a complement-dependent anti-granulocyte antibody in the plasma of one subject with amodiaquine-induced agranulocytosis (see Chapter 6). That this antibody was the sole cause of the toxicity in this case is doubtful, since marrow aspirates at the time showed almost complete absence of myelopoiesis. The amodiaquine-specific antibody was found to persist for ~ 7 days after withdrawal of the drug.

1.5.2.5. Autoantibody formation

In health, immune responses to autologous structures are strictly controlled by complicated mechanisms not yet fully understood. Burnet (1957) proposed that potentially self-reacting cells are eliminated from the immune system early in embryonic life, because contact between an 'immunocyte' and a specific antigen early in ontogeny leads to death of the cell. This is likely to be an oversimplification (Nossal, 1987), but may in part be true; contact between clones of T or B lymphocytes and antigen early in embryonic life can lead to "down regulation" of that clone, but this process (known as clonal anergy) may be reversible (Nossal 1987). Prevention of immune reactions to 'self' also seems to involve a type of T-lymphocyte referred to as the T-suppressor cell (or CD8 positive cell). The role of these cells is poorly understood but has been implicated in certain drug-induced blood disorders, most notably

those induced by α methyldopa and procainamide.

Alpha methyldopa was recognised as a cause of haemolytic anaemia by Carstairs and colleagues (1966), and thereafter it was noted that in the presence of patient serum neither the drug nor its metabolites needed to be present for the direct antiglobulin test to be positive either *in vivo* or *in vitro*. It was suggested that α methyldopa perturbed the immune system through a direct effect on lymphocyte function and not as an antigen, and this was investigated by Kirtland and colleagues (1980). These workers showed that α methyldopa perturbs the immune system by inhibition of suppressor T-cell function and postulated that this may be due to increased concentrations of lymphocyte cyclic AMP. The same workers suggested that these effects may lead to unregulated autoantibody production by B cells, leading to haemolysis.

Procainamide induces autoantibody formation in a high proportion of treated individuals (Blomgren *et al* 1972), particularly slow drug acetylators, and can cause a syndrome similar to systemic lupus erythematosus. This syndrome can involve agranulocytosis (Pisciotta 1978). Ochi and colleagues (1983) have shown that the major toxic action of procainamide is to inhibit suppressor T-cell activity therefore permitting autoantibody formation.

1.5.3 Amodiaquine-induced agranulocytosis

Thirty three cases of amodiaquine-associated agranulocytosis or neutropaenia are reported in the literature of which 6 (18%) ended fatally. Neutropaenia was reported as early as 1953 by Love and colleagues during the early assessment of the drug (2 cases), and between 1953 and 1984 a further 11 cases were reported (Kennedy 1955; Glick 1957; Perry *et al* 1962; Booth *et al* 1967; Lind *et al* 1973; Gillespie and Wagner 1977; Lepeu *et al* 1981; Schulthess *et al*

1983). Between 1984 and 1987, when amodiaquine use for malaria prophylaxis was frequent, a further 20 cases were published (Douer et al 1985; Hatton et al 1986; Neftel et al 1986; Carr 1986; Rhodes et al 1986; Ellis et al 1987). Stürchler and colleagues (1987) have studied, retrospectively, 451 travellers on prophylactic antimalarials. These workers found that subjects on amodiaquine, chloroquine or pyrimethamine had significantly lower total leukocyte counts than controls, but in all cases this was due to a diminution of lymphocyte rather than neutrophil numbers. Travellers taking amodiaquine (n = 92) had significantly higher aspartate transaminase levels than controls.

1.5.3.1 Demographic data on reported cases

Sixteen (48%) of the subjects reported in the literature were female and 17 (52%) male (see Table 1.1). Ages ranged from 2-76 years (mean 43 yrs), and all of those in whom race was stated were Caucasian. Indications for amodiaquine therapy were: malaria prophylaxis, 85%; systemic lupus, 7%; and sarcoidosis 7%; no cases of agranulocytosis are recorded where amodiaquine was in use for the treatment of malaria. Total doses of amodiaquine consumed ranged 1.2 - 11.2 g (3.6 $\pm$ 2.0g; $\bar{x} \pm$ SD). Duration of therapy prior to diagnosis of agranulocytosis ranged 2 - 12 weeks (6.6 $\pm$ 2.8 weeks; $\bar{x} \pm$ SD). Other drugs were taken with amodiaquine in 16 cases (48%): proguanil in 9 cases (27%), Fansidar in 4 cases (12%), penicillin in 2 cases (6%), cotrimoxazole in 2 cases (6%), pyrimethamine 1 case (3%) and oxytetracycline in 1 case (3%). Of the cases in which bone marrow aspirate was examined during the patient's illness (17 cases; 52%) all were found to have signs of absent or suppressed myelopoiesis.

TABLE 1.1 Demographic data on patients with amodiaquine-induced agranulocytosis

	Reference	Sex	Age (yr)	Indication for drug	Dose (g)	Length exposure (weeks)	Other drugs	Other pathology	Fatal ?
1	Love 1953	M		Sarcoid	4.2	4			
2		M		Sarcoid	4.2	4			
3	Kennedy 1955	F	2	Mal Pro	1.3	2			
4	Glick 1957	F	53	Lupus	11.2	8	Penicillin	Hepatitis	Yes
5	Perry 1962	M	33	Lupus	2.8	2		Hepatitis	
6	Booth 1967	M	20	Mal Pro	5.0	3			Yes
7		M	30	"	4.7	4			
8		F		"	1.2	4			
9		M		"	3.0	10			
10	Lind 1973	F	26	"	2.4	6			
11	Gillespie 1977	F	26	"	3.8	10	Tetracycline	Hepatitis	
12	Lepeu 1981	M	44	"	4.2	7			
13	Schulthess 1983	F	17	"	3.0	5			

(Mal Pro = Malaria Prophylaxis)

TABLE 1.1 (Continued)

	Reference	Sex	Age (yr)	Indication for drug	Dose (g)	Length exposure (weeks)	Other drugs	Other pathology	Fatal ?
14	Douer 1985	F	63	Mal Pro	2.4	6	Pyrimethamine	Rash	
15	Hatton 1986	M	51	"	2.4	6	Proguanil		
16		F	49	"	7.6	12			
17		M	62	"	3.6	9	Proguanil		
18		F	63	"	1.2	3	Proguanil		
19		M	35	"	2.8	7	Proguanil		
20		M	49	"	4.0	11	Proguanil		
21		F	62	"	2.8	7	Proguanil		Yes
22	Neftel 1986	M	71	"	3.6	6	Fansidar		Yes
23		M	76	"	3.2	6	Fansidar	Hepatitis	Yes
24		F	52	"	3.2	6	Fansidar	Hepatitis	
25		F	64	"	4.0	9	Fansidar	Hepatitis	
26		F	63	"	3.6	6		Hepatitis	
27		F	17	"	6.0	9			
28		M	39	"	1.8	5		Hepatitis	
29	Carr 1986	M	18	"					Yes
30		M	71	"	2.4	6	Proguanil		
31		M	54	"	3.2	8			
32	Rhodes 1986	F	21	"	2.4	12	Proguanil		
33	Ellis 1987	F	29	"	3.6	9	Proguanil		

(Mal Pro = Malaria Prophylaxis)

1.5.3.2 Proposed mechanisms

As can be seen from the brief review of the mechanisms of drug-induced agranulocytosis given above, one drug may produce agranulocytosis seemingly by several pathways. Bone marrow culture studies using patient marrow and control marrow have demonstrated amodiaquine concentration-dependent inhibition of colony formation in some hands (Lind *et al* 1973; Rhodes *et al* 1986). In other hands however similar methods have failed to demonstrate amodiaquine-concentration-dependent effects, but have demonstrated inhibition of colony formation by addition of 'acute phase' serum from a patient (Douer *et al* 1985) therefore suggesting an immune-mediated mechanism. The examination of serum for drug-dependent antibodies toxic to mature circulating granulocytes has been positive in one report (Schulthess *et al* 1983). Work will be presented in this thesis which investigates the effects on a bone marrow culture system of amodiaquine, its derivatives, and 'acute phase' plasma from amodiaquine-agranulocytosis sufferers (Chapter 6).

1.6 GENERAL PRINCIPLES OF PHARMACOKINETICS

1.6.1 Introduction

Part of the work to be described in this thesis is directed towards the improvement of understanding of the pharmacokinetics of amodiaquine. Pharmacokinetic analysis allows a quantitative description of the processes of drug absorption, distribution, biotransformation and excretion (Rowland and Tozer 1980). Determination of the various pharmacokinetic parameters of a drug allows concise representation of that drug's disposition and, to a degree, permits the clinician to predict the time course and extent of effect of a given dose of a drug. This knowledge is likely to help the clinician to maximise therapeutic benefit while avoiding

adverse effects. In the case of an unacceptably toxic drug, such as amodiaquine, knowledge of the drugs' pharmacokinetics may help the understanding of mechanisms of toxicity.

1.6.2 Absorption

To exert its effects a drug must gain access to its site of action which, in the case of an extravascularly administered agent, first requires that the drug is absorbed. This involves transfer of the drug in solution from the site of administration into the systemic circulation. Usually this transfer process occurs by passive diffusion of drug molecules down a concentration gradient although for some compounds absorption is an active process.

Oral administration is the most popular route of drug intake. Absorption of a drug from the gut may be influenced by a number of factors including the dissolution characteristics and lipophilicity of the drug, environmental pH, local blood flow and gastrointestinal motility. Pre-systemic metabolism in the gut wall or liver is a further major determinant of the fraction of drug transported, unchanged, into the systemic circulation (George and Shand 1982).

1.6.3 Distribution

Once present in the systemic circulation a drug is distributed throughout the body. The rate and extent of this reversible transfer process depends in part upon the physico-chemical properties of the drug and its binding to tissue components and plasma proteins, and in part upon blood flow to the various tissues. In due course, an equilibrium of drug concentrations throughout the body is achieved, and changes in drug concentration in the plasma are reflected in the tissues.

1.6.4 Elimination

The irreversible loss of drug from the body is achieved by the processes of biotransformation and excretion. Although hydrophilic compounds are readily excreted, primarily by the kidneys, most drugs are lipophilic compounds and therefore require conversion to more polar forms. These metabolites, while usually inactive, may have therapeutic or toxic properties.

1.6.4.1 Hepatic biotransformation

While many tissues are capable of metabolising drugs, it is the liver which is of primary importance. Such processes of hepatic biotransformation are generally divided into two phases: phase I reactions, which include drug oxidation, reduction and hydrolysis, result in the introduction or 'uncovering' of polar groups in the drug molecule; and phase II reactions in which either unchanged drug, or a metabolic product of a phase I reaction, undergoes coupling to an endogenous molecule such as glucuronic acid, or glutathione. Products of phase II reactions, referred to as drug conjugates, are inactive, highly polar compounds. The enzymes which catalyse phase I and II reactions are most often located in the endoplasmic reticulum of the hepatocyte, although other sources of enzyme have also been identified including mitochondria, lysosomes, nuclei and the cytoplasm. One particular group of enzymes largely located in the endoplasmic reticulum, has been extensively studied; this group, classified as cytochrome P-450 mixed function oxidase, has been shown to metabolise a wide range of structurally dissimilar compounds (Timbrell 1982).

1.6.4.2 Excretion

Although drugs may be excreted in other ways, the renal and

biliary routes are the most important. Free drug in the plasma water may be removed by glomerular filtration, but this is not possible for protein-bound drug since the large plasma protein molecules are not filtered. Some drugs may be removed by active secretion into renal tubules, this mechanism being most common in the cases of relatively strong acids and bases. Passive reabsorption, from the renal tubules into the systemic circulation, may occur to some drugs depending upon such factors as the drug's lipid-solubility, degree of ionization and molecular weight.

Some unchanged drugs and/or their metabolites may be actively excreted into the bile, and an important determinant of this is the molecular weight of the drug. Drugs with large molecular weights are more likely to be excreted into the bile (> 300 in the rat; > 350 in man). Once within the gut lumen the drug may be voided with the faeces, or may in some cases be reabsorbed, for example following hydrolysis of drug conjugates by gut bacteria, so called "enterohepatic circulation".

1.6.5 Pharmacokinetic parameters

1.6.5.1 Introduction

Pharmacokinetic parameters are derived from concentration changes of drug, or metabolite, over time (figure 1.6). Concentrations are usually measured in plasma, blood or urine and presented graphically. Time is plotted generally in a linear manner on the abcissa, and concentration is plotted on the ordinate using a logarithmic scale. Using such semilogarithmic plots the drug concentration/time profile seen after administration of a drug usually exhibits 3 phases: in the first, drug concentration rises as absorption takes place; in the second, drug concentrations decline due to distribution throughout the body; and in the third,

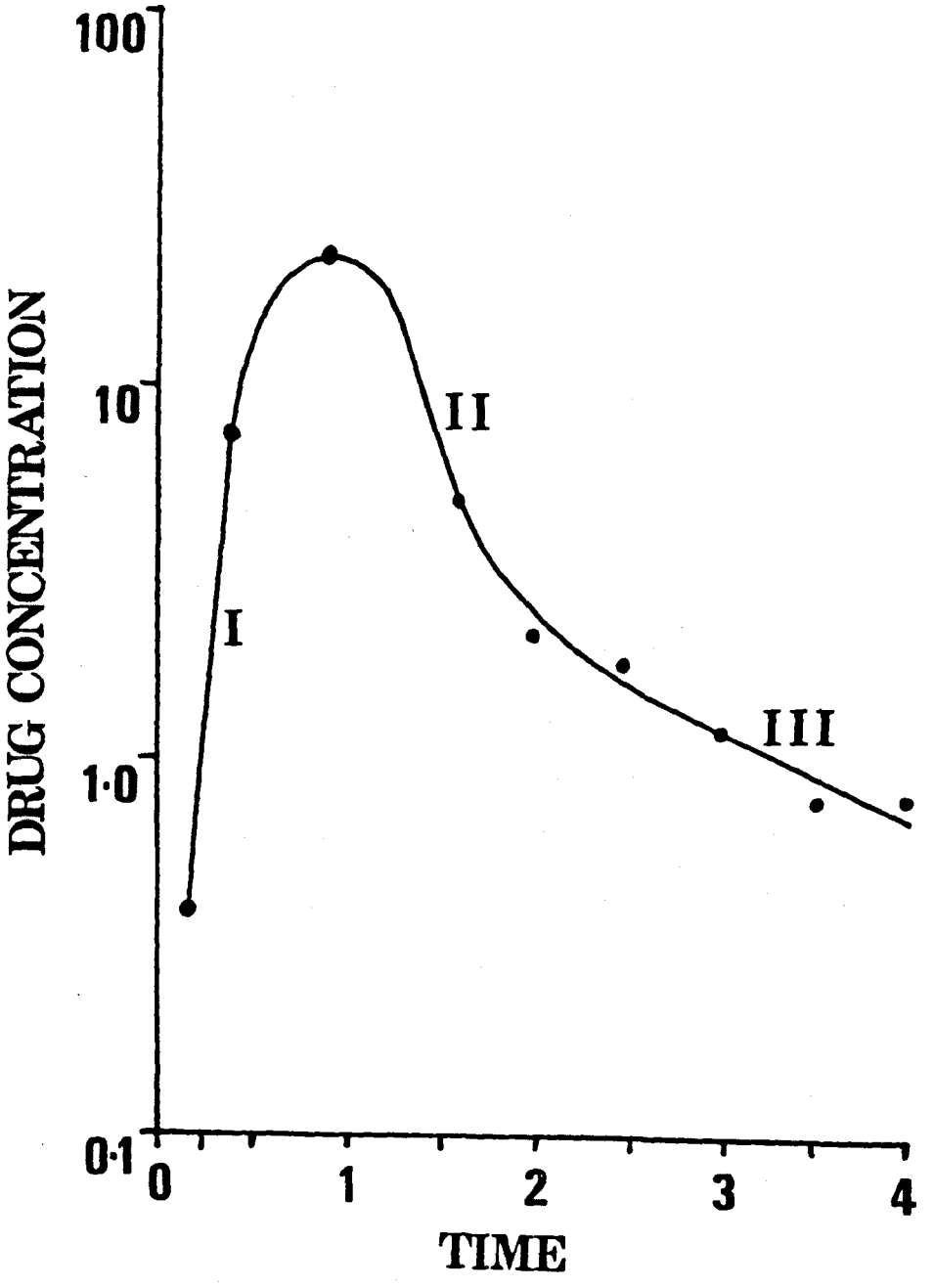


FIGURE 1.6 Concentration vs time curve following oral administration of drug; phase I = mainly absorption, phase II = mainly distribution, and phase III = mainly elimination.

phase there is a slower decline in drug concentration due to irreversible loss from the body (elimination phase). However, some of these processes may be so rapid that they cannot be determined experimentally, and in the case of most orally-administered drugs the 3 phases described above are rarely seen clearly.

For the majority of drugs at therapeutically-used concentrations, such processes occur at a rate proportional to the concentration of the drug (first order pharmacokinetics). However, the capacity of all enzymic processes is limited and the metabolism of some drugs can become saturated within therapeutically-used concentrations (for example phenytoin). Such compounds are said to exhibit zero-order, or non-linear, pharmacokinetics important clinical consequences of which include dose-dependent increases in half-life, and disproportionate increases in "steady-state" concentrations with small increases in the size of the maintenance dose.

1.6.5.2 Elimination half-life

The elimination half-life ($t_{\frac{1}{2}}$) of a drug is the time taken for its plasma concentration to fall by one half in the elimination phase. For drugs exhibiting first order pharmacokinetics this value is constant.

The value for $t_{\frac{1}{2}}$ can be determined from the slope of the drug-concentration *vs* time profile, during elimination phase. The elimination phase is characterised by the equation:-

i $\log Cp = \log Cpo - kt,$

where Cp = drug concentration at time t

Cpo = drug concentration at time 0 (obtained by extrapolation backwards to time 0 of the elimination-phase line)

k = the slope of the line

Conversion of equation i to natural logarithms gives:-

ii $\quad \text{LnCp} = \text{LnCpo} - \lambda z t$

where λz = the elimination rate constant, equal to 2.303 x k (2.303 being the value necessary to convert values of Log 10 to natural logarithm).

Since the $t_{\frac{1}{2}}$ is the time taken for concentration to fall by $\frac{1}{2}$, if Cp = Cpo ÷ 2 and t = $t_{\frac{1}{2}}$, substitution into equation ii gives:-

iii $\quad \lambda z . t_{\frac{1}{2}} = \text{Ln}2 = 0.693$

or

iv $\quad t_{\frac{1}{2}} = 0.693 \div \lambda z$

1.6.5.3 Clearance

The term clearance denotes the rate of removal of a compound from a given volume of biological fluid, per unit time, and is a useful parameter for the evaluation of elimination mechanisms. For a drug with linear pharmacokinetics clearance has a constant value irrespective of drug concentration. While clearance can be calculated directly for an organ (e.g. liver or kidney) given the values for organ blood flow (Q) and the extraction ratio (E) of the drug by the organ, such a 'physiological' approach is rarely used in human pharmacokinetic studies. Instead, clearance is derived indirectly.

At equilibrium:

v $\quad$ Rate of elimination = Cl x Cp

(where Cl = clearance and Cp = plasma concentration) and also

vi $\quad$ Rate of elimination = λz x A

(where A = is the amount of drug in the body)

and therefore

vii $Cl \times Cp = \lambda z \times A$

But since

viii $A = Cp \times VD$

(where VD = the volume of distribution)

ix $Cl \times Cp = \lambda z \times Cp \times VD$

or

$Cl = \lambda z \times VD$

and since

x $t_{\frac{1}{2}} = 0.693 \div \lambda z$, then $Cl = (0.693 \times VD) \div t_{\frac{1}{2}}$

From formula x it is apparent that changes in either clearance or volume of distribution can alter elimination half-life. However, calculation of clearance in this manner requires that the drug's volume of distribution is known, which is not necessarily so. Clearance values can be derived in another way, since:

xi $A = Cl \times AUC\ (0-\infty)$

Where AUC = the area under the drug concentration vs time curve; this value is measured by the trapezoidal rule (Gibaldi and Perrier 1982). The principle of this derivation is that the area under the curve from 0 to t is divided into trapezoids the areas of each of which is calculated by the formula

xii (Σ parallel sides $\div$ 2) x distance between them.

The areas of each trapezoid are summated giving the area under the curve from 0 to t. The additional area from time t to infinity is calculated from the ratio $Ct/\lambda z$, where Ct = the concentration at time t. Consequently

xiii $AUC\ (0 - \infty) = AUC\ (0 - t) + Ct/\lambda z$

As can be seen from formula xi the calculation of clearance from AUC data requires knowledge of the amount of the drug in the body; in the case of I.V. administration A = dose. However in the case of

oral administration, unless the drug is 100% bioavailable, the amount of drug in the body is unknown. Consequently systemic clearance cannot be calculated, following oral administration, using formula xi. The concept of oral clearance may however be applied to data obtained after oral administration of a drug whose bioavailability is unknown. Oral clearance is derived from dose $\div$ AUC(0 - ∞), and can be useful when assessing the effect of dose size on the pharmacokinetics of an orally-administered drug.

1.6.5.4 Organ clearance

Systemic clearance, as derived above, is the sum of both metabolic and excretory clearance processes, and is equal to the sum of all organ clearances. The clearance of a compound across an organ can be expressed in physiological terms:-

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

where Q = organ blood flow, CA = arterial drug concentration and CV = venous drug concentration. However such parameters are rarely known in the clinical setting, but organ clearance can often be calculated indirectly. In the case of renal clearance the value can be calculated knowing the urinary recovery of the compound, and relating this to the area under the concentration vs time curve, which is a measure of the amount of drug which has entered the systemic circulation, by the formula:-

xv $$Clr = Ae \div AUC$$

where CLr = renal clearance and Ae = urinary recovery of the drug.

1.6.5.5 Apparent volume of distribution

The value of this pharmacokinetic parameter rarely corresponds to a real physiological volume, but depends more upon the degree of

protein or tissue-binding of the drug, and its lipophilicity. The apparent volume of distribution (VD) relates the drug concentration to the amount of drug in the body after distribution equilibrium has been achieved, or

xvi $VD = A/Cp$

where A = amount of drug in the body (which, following i.v. administration, equals the dose), and Cp = plasma concentration. However the parameter is more usually calculated indirectly from plasma concentration vs time data as follows:-

xvii $VD = Cl \times \lambda z$

or

$$VD = \frac{DOSE}{AUC} \times \lambda z$$

where Cl = whole body clearance, λz = elimination rate constant.

It can be see that formula xvii is the same as formula x, and relates the parameters half-life, volume of distribution and clearance.

1.6.5.6 Bioavailability

Calculations of clearance and volume of distribution, as related above, can only be performed when the amount of drug in the body is known; in the case of I.V. administration, this value equals the dose. However, drugs administered extravascularly, may be subjected to degradation in the gut, incomplete absorption, pre-systemic metabolism by gut wall or liver and enterohepatic recirculation. The term bioavailability refers to the fraction of the administered dose of drug which reaches the site of measurement (blood or plasma) unchanged, and is calculated from:-

xviii $$F = \frac{DOSE\ (IV)}{DOSE(ORAL)} \times \frac{AUC\ (ORAL)}{AUC\ (IV)}$$

(where F = bioavailability).

The above method for determining bioavailability assumes that

the systemic clearance of the drug is the same for both routes of administration, and that the drug displays linear pharmacokinetics (since oral and i.v. doses may not be identical) (George and Shand 1982).

1.6.5.7 Summary

The calculation of pharmacokinetic parameters is essential for the quantitative description of changes in drug concentration as a function of time, dosage and route of administration. The pharmacokinetics of amodiaquine, following its oral administration (the most commonly-used route for this drug) will be determined for healthy subjects after a single dose, and after a variety of dose sizes, and will also be determined for malaria patients receiving the drug as part of their treatment for acute malaria.

1.7 THE SCOPE OF THIS THESIS

The antimalarial drug amodiaquine, having shown promise as a potentially useful agent against chloroquine-resistant *P. falciparum*, enjoyed a degree of popularity for both the treatment of such infections, and their prevention in travellers, during the first half of the 1980's. Because of a prior lack of interest in the drug, little was known of its clinical pharmacology, and the dose-recommendations in use were largely empirical. In order to optimise such dose-recommendations, detailed information was required about the drug's pharmacology, the first step towards which was the development of sensitive, selective and reproducible assay methods. A method for the measurement of amodiaquine in the plasma by high performance liquid chromatography (HPLC) had been described before the commencement of work for this thesis, but this method required

further development, and this is decribed in Chapter 2. Thereafter the clinical pharmacology of amodiaquine, in healthy subjects and in malaria patients, was investigated (Chapters 3 and 4).

Adverse effects from amodiaquine had been described early in its development, but once freely available, the drug was used mainly in the tropics, and largely away from scientific scrutiny. When used on a relatively large popuation from the developed world, severe adverse effects from the drug were soon noticed, and caused its withdrawal for malaria prevention. Investigations were needed, at this time, into the toxicity of the drug to discover, if possible, the mechanism(s) by which amodiaquine caused its most serious adverse effect, agranulocytosis; this seemed particularly important since the drug remained in use for the treatment of acute infections. Studies will be described in which the mass fate of amodiaquine was investigated in laboratory animals, and its tissue distribution assessed. Further studies will be described in which the effects of amodiaquine and its derivatives, were investigated using *in vitro* culture of normal human bone marrow.

CHAPTER 2 Methods

2.1 SIMULTANEOUS DETERMINATION OF AMODIAQUINE AND DESETHYLAMODIAQUINE IN BIOLOGICAL FLUIDS BY REVERSED-PHASE HPLC

2.1.1 Introduction

2.1.2 Chromatography

2.1.3 Sample treatment procedure

2.1.4 Extraction efficiency

2.1.5 Standard curves

2.1.6 Assay specifications

2.1.7 Infection-risk from samples of human biological fluids

2.2 MASS SPECTROMETRY OF URINARY DESETHYLAMODIAQUINE

2.2.1 Introduction

2.2.2 Extraction

2.2.3 Preparative HPLC

2.2.4 Mass spectrometry

2.3 RADIOCHEMICAL PURITY OF ^{14}C-AMODIAQUINE

2.3.1 Introduction

2.3.2 Assessment of radiochemical purity

2.3.3 Purification of ^{14}C-amodiaquine

2.4 DETERMINATION OF RADIOACTIVITY

2.4.1 Introduction

2.4.2 Determination of radioactivity in urine

2.4.3 Determination of radioactivity in blood, red cells, faeces and tissue homogenates

2.4.4 Assessment of quench correction of highly coloured specimens

2.5 IN-VITRO CULTURE OF HUMAN BONE MARROW CELLS

2.1 SIMULTANEOUS DETERMINATION OF AMODIAQUINE AND DESETHYLAMODIAQUINE IN BIOLOGICAL FLUIDS BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.1.1 Introduction

Until recently, understanding of the disposition of amodiaquine has been limited by the lack of suitably selective and sensitive methods for the determination of the drug and its metabolites in body fluids. Brodie (1947) used a fluorometric method for the measurement of chloroquine and related 4-aminoquinolines. In this procedure, the 4-aminoquinoline is separated from the biological fluid by organic solvent extraction, and is then exposed to ultraviolet irradiation for a constant time. This photochemical procedure converts the 4-aminoquinoline to unidentified highly fluorescent derivatives which may then be measured. This method was neither sensitive nor specific, and was unsuitable for the determination of amodiaquine after therapeutic doses.

Trenholme and colleagues (1974) also used a fluorometric method of detection to measure chloroquine and amodiaquine. The inherently poor native fluorescence of the compounds was improved by prolonged boiling, which therefore avoided the need for irradiation. The lowest detectable concentration of amodiaquine by this method was 50ng ml^{-1}, but as with previous fluorometric procedures, the method was not specific for the drug, and was therefore unable to distinguish between the parent compound and its metabolites.

Mihaly and colleagues (1985) described a sensitive, selective HPLC method for the measurement of amodiaquine in plasma, which involved the extraction of the compound from plasma into diethyl

ether, at high pH. Chromatographic resolution was by means of a reversed phase column, and detection was by ultraviolet absorption. The lower limit of detectability for amodiaquine in plasma was 5 ng ml^{-1}. However this method cannot be applied to the detection of amodiaquine in whole blood or packed red blood cells since the compound fails to extract. Although the drug can be extracted from urine using this method, an unacceptable amount of background interference results, presumably because of the extraction of endogenous compounds. Furthermore the method of Mihaly and colleagues (1985) is unsatisfactory for the measurement of desethylamodiaquine in plasma, having an unacceptably low extraction efficiency.

Churchill and colleagues (1985) reported an HPLC method for the determination of amodiaquine and desethylamodiaquine in whole blood and urine. However this method did not employ an internal standard, which is essential for the accurate determination of drug concentrations. Since the development of the methods described in this thesis, Pussard and colleagues (1986) have reported an HPLC method for the measurement of amodiaquine and desethylamodiaquine in whole blood, erythrocytes, plasma and urine, and Churchill and colleagues (1987) have reported an HPLC method using electrochemical detection. Lower limits of detectability using the method of Pussard and colleagues (1986) are comparable to those achieved by the methods described in this Chapter. However using electrochemical methods of detection Churchill and colleagues (1987) claim lower limits of detectability for amodiaquine and metabolites of 1ng/ml from biological fluids. While this is an improvement upon the methods described in this Chapter, such a low limit of detectability has not proved necessary for the

investigation of the clinical pharmacology of the drug.

The HPLC methods for the determination of amodiaquine and desethylamodiaquine in human and animal biological fluids, which are described in this Chapter were developed as an integral part of this research project. The methods are simple, sensitive, selective and reproducible and have been employed to study the disposition of amodiaquine in man and an animal model as will be described in subsequent chapters.

2.1.2 Chromatography

A Spectra-Physics HPLC system was used, which consisted of a solvent delivery system (model SP 8700) with an organiser module (model SP 8750) equipped with a Rheodyne valve injector, coupled to an ultraviolet absorbance detector (Millipore-Waters; model 441). Column effluent was monitored at a fixed wavelength of 340 nm throughout these studies. Use of a wavelength of 229 nm (which corresponds to λ_{max} for amodiaquine) resulted in a 70% increase in the peak height obtained after the injection of a fixed mass of amodiaquine onto the system; however this was accompanied by a disproportionate increase in the amplitude of background noise. Furthermore at 229 nm the injection of extracts of biological fluids produced many endogenous peaks on the chromatogram which co-eluted with either amodiaquine or desethylamodiaquine. This wavelength was therefore considered unsuitable.

Two combinations of stationary and mobile phases were assessed.

(a) A reversed phase pre-packed plastic column (μBondapak phenyl, 10μm particle size; 10 cm x 8 mm ID; Millipore-Waters UK) housed in a radial compression module (Z module; Millipore-

Waters UK) was used throughout these studies. Mobile phase consisted of a water-methanol mixture containing triethylamine (1%) and buffered with orthophosphoric acid. Increased proportions of water in the mobile phase produced lengthening of the retention times of amodiaquine and desethylamodiaquine and improved resolution between the two. The retention times of amodiaquine and desethylamodiaquine were not influenced by changes in pH of the mobile phase between 2.5 and 4.0 but that of the internal standard (6-methoxy-8-aminoquinoline) was very sensitive to changes in pH, its retention time lengthening with increasing pH.

For the determination of amodiaquine and desethyl-amodiaquine in human biological fluids mobile phase consisted of methanol:water (2.3:7.7 v/v) containing triethylamine (1%) buffered to pH 2.8 using orthophosphoric acid. Chromatography was carried out at a flow rate of 3.5 ml min^{-1}, which was associated with an operating pressure of 800-1000 PSI.

Because of the presence of endogenous peaks of short retention time in rodent urine and faeces, the proportion of water in the mobile phase was increased to methanol:water 1:4 (v/v) in the case of rodent urine, and 1.5:8.5 (v/v), in the case of rodent faeces. In order to reduce retention times and improve peak shape the flow rates were increased to 4.5 and 6.0 ml min^{-1} respectively. The latter flow rate was associated with an operating pressure of 1000-1500 PS1. Mobile phase pH was increased to 2.9 in order to adequately resolve amodiaquine and internal standard.

(b) Both because of the inherently poor U.V. absorbance of the drug, and the necessity of using a fixed wavelength other than

the compound's λ_{max}, the sensitivity of the above system is limited and this is exacerbated by the relatively low analytical recoveries achieved from several of the biological fluids assayed. Consequently a further mobile phase : stationary phase combination was assessed in an attempt to improve senstivity by improvements in peak shape.

A stainless steel column (Ultrasphere ODS, 5μM particle size; 15 cm x 4.6 nm 1D; Beckmann UK) was coupled to the previously described HPLC system in place of the usual stationary phase. The mobile phase consisted of acetonitrile: water (9:91 v/v) containing triethylamine (1%) buffered to pH 2.8 using orthophosphoric acid, and flowing at 2.0 ml min^{-1}. This resulted in 70% increases in peak height for both amodiaquine and desethylamodiaquine when compared to the previous system. However desethylamodiaquine co-eluted with chloroquine, and variations in the mobile phase failed to overcome this. Since chloroquine is very commonly detectable in samples from malaria suffers, often as a result of self-medication, this method was thought unsuitable for further evaluation.

Internal standard

6-Methoxy-8-aminoquinoline, employed by Mihaly et al (1985), was found to be a satisfactory internal standard and was used throughout the studies on healthy subjects, and rodents. Use of the 5μm Ultrasphere column required a different internal standard and amopyroquine proved the most satisfactory. Assay of red cell samples from malaria patients required changes in extraction procedure, described below, and a new internal standard was needed;

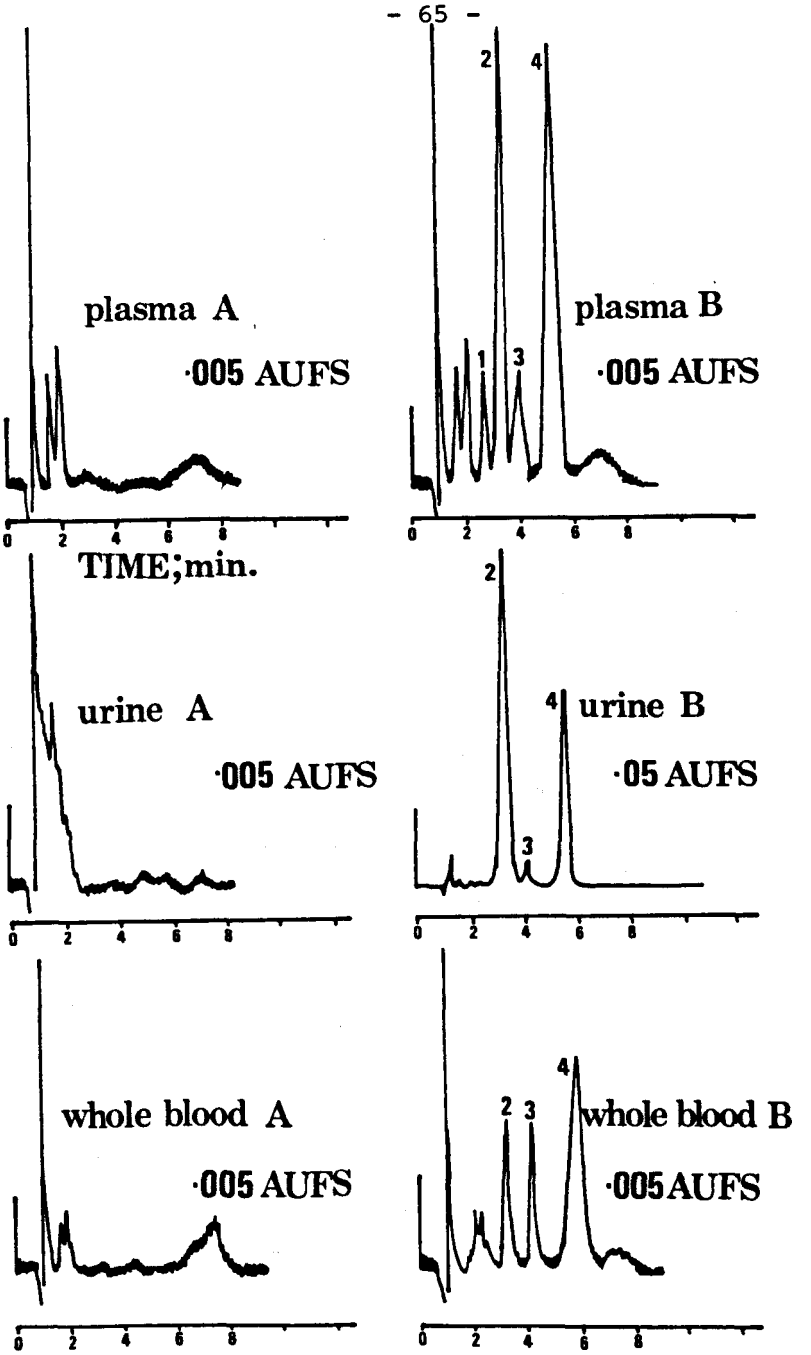


FIGURE 2.1 HPLC traces obtained from A drug-free human biological fluid, and B biological fluid obtained after oral amodiaquine

Key
1 = Unknown peak, possibly a metabolite
2 = Desethylamodiaquine
3 = Amodiaquine
4 = Internal standard, 6-methoxy-8-aminoquinoline.

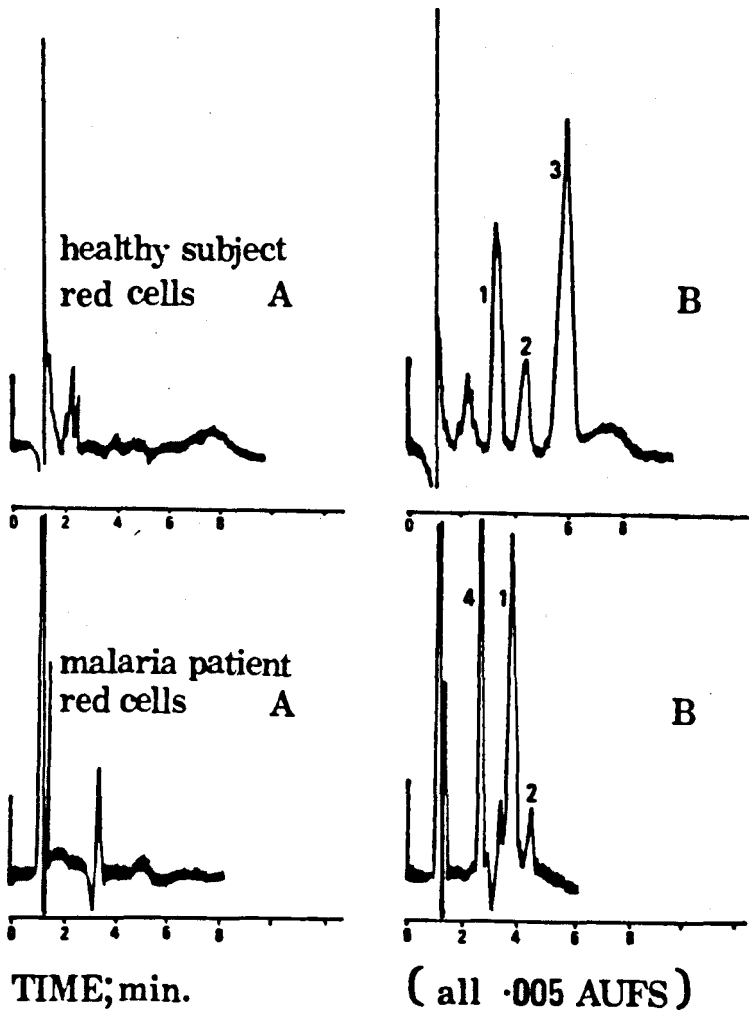


FIGURE 2.2 HPLC traces obtained from A drug-free human biological fluid, and B biological fluid obtained after oral amodiaquine

Key
1 = Desethylamodiaquine
2 = Amodiaquine
3 = Internal standard, 6 methoxy-8-aminoquinoline
4 = Internal standard, 7 chloro-4-[1-dimethylamino-4 pentylamino] quinoline.

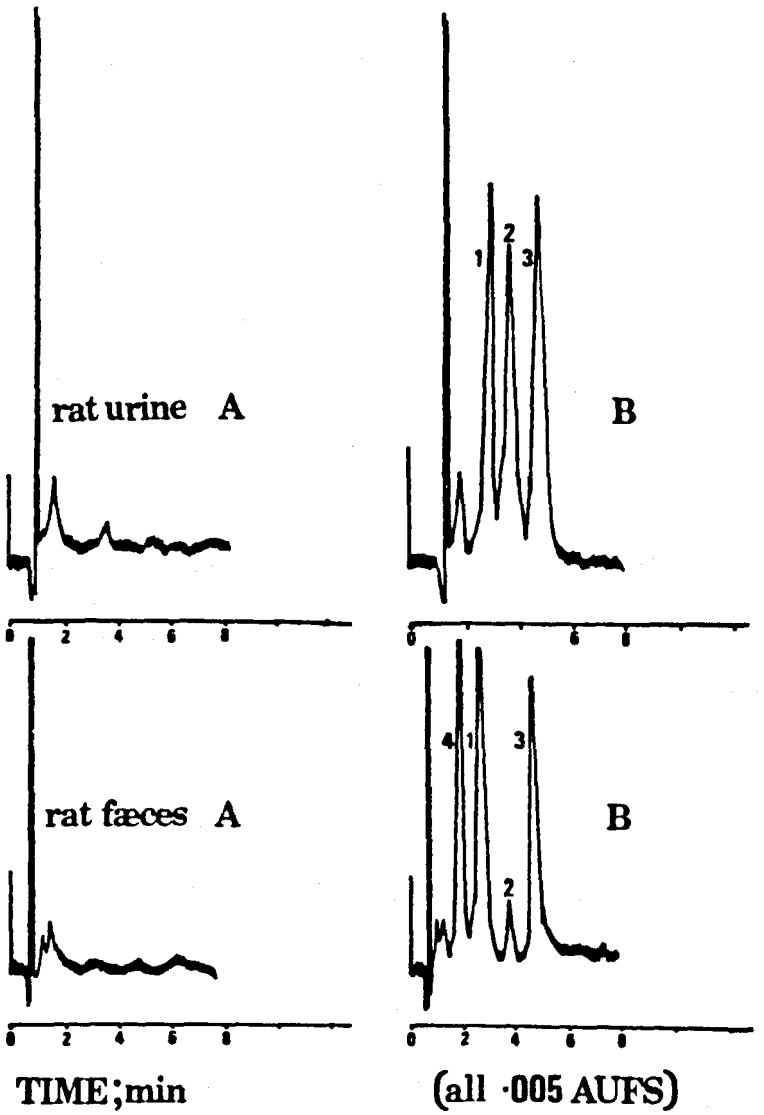


FIGURE 2.3 HPLC traces from A drug-free rodent biological fluids, and B biological fluids obtained following oral amodiaquine

Key
1 = Desethylamodiaquine
2 = Amodiaquine
3 = Internal standard, 6 Methoxy-8-aminoquinoline
4 = Unknown peak, possibly a metabolite.

7-chloro-4[1-diethylamino-4-pentylamino]quinoline was used.

2.1.3 Sample treatment procedures

Extraction of both amodiaquine and desethylamodiaquine from biological fluids proved difficult. Both compounds bind irreversibly to protein to a large degree (Maggs et al 1987a, b and 1988) and extraction efficiencies were often low for both compounds. The effect of different solvents, extraction pH and methods to induce haemolysis or protein precipitation were examined in order to optimize sample treatment procedures (tables 2.1 - 2.3). Typical traces, obtained by the methods described below, are given in figures 2.1 - 2.3.

a) Human biological fluids (plasma, whole blood, packed cells and urine)

It was found that pre-treatment of whole blood or red cells with acetonitrile was required before extraction into diethylether. The addition of acetonitrile (2 volumes) to blood or cells (1 volume) caused protein precipitation leaving a relatively clear liquid phase for subsequent extraction. Application of this method to plasma improved the extraction efficiency of amodiaquine into diethylether from 60% (Mihaly et al 1985) to 70%, and that of desethylamodiaquine from 20% to 50% (Table 2.4). Use of the acetonitrile pre-treatment step for urine samples generally produced little precipitation, but reduced the number and amplitude of endogenous peaks on subsequent chromatography.

TABLE 2.1 Choice of optimal pH for extraction of amodiaquine from aqueous solution into organic solvent

pH	Extraction Efficiency %
13.20	29
12.68	90
10.39	83
8.44	83
7.01	59

(n=1; concentration = 1μg ml^{-1})

TABLE 2.2 Choice of solvents for the direct extraction of amodiaquine (AQ) and desethylamodiaquine (AQm) from plasma (at pH 12.0)*

Solvent	Extraction Efficiency %	
	AQ	AQm
Diethyl ether	68	20
9:1 Hexane:ethylacetate	60	Not tested
95:5 Hexane:amyl alcohol	48	Not tested
Toluene	42	Not tested
Dichloromethane	34	Not tested

*No prior protein-precipitation step was employed

Method	Extraction Efficiency % Amodiaquine	Desethyl-amodiaquine	Comments
Repeated Freeze/Thaw	0	0	
Distilled H_2O (x 4v)	0	0	
1M NaOH (2.0ml)	0	0	
10% Triton-X-100 (0.5ml)	N/A	N/A	Too contaminated to allow injection
NCS Tissue solubiliser (0.5ml)	N/A	N/A	Too contaminated to allow injection
Trichloroacetic Acid	< 50	Not tested	Very variable extraction efficiency
30% WV H_2O_2 (50µl)	5	Not tested	
Taurocholic acid (1.0ml)	0	Not tested	
Acetone (2.0ml)	40	15	Injection causes rise in operating pressure
50% K_2HPO_4 (250µl)	50	30	Injection causes rise in operating pressure
Acetonitrile (2.0ml)	50	30	
50% K_2HPO_4 (250µl) Followed by acetonitrile (2.0ml)	40	10	

* In all cases extraction was from whole blood 1.0ml using diethyl ether at pH 12.0

TABLE 2.3 **Choice of protein-precipitation or haemolysis step, prior to extraction from whole blood (unparasitised)**

Extraction was performed in 15 ml capacity glass culture tubes, and pooled organic phases were evaporated to dryness in 10 ml capacity pointed centrifuge tubes. These had been soaked overnight in strong detergent solution (DeCon) and were then rinsed in tap water, distilled water and methanol before drying. When dry, the tubes were treated with dichlorodimethylsilane (5%) in toluene in order to minimise adsorption of amodiaquine or desethylamodiaquine. In order to avoid photodecomposition of the compounds all tubes were wrapped in aluminium foil.

To samples of biological fluid (1.0 ml) containing the internal standard (6-methoxy-8-aminoquinoline) (0.5 - 10µg; 50-100µl) was added acetonitrile (2.0 ml), followed by vortex mixing (15 s) and mechanical tumbling (15 min). After centrifugation (2000 g; 5 min) the liquid phase was transferred to a clean tube to which was added ammonia (SG 0.88; 2.0 ml) achieving a pH of 12.0. This mixture was then extracted twice with diethyl ether (5.0 ml x 2) by mechanical tumbling (15 min). After centrifugation (2000g; 10 min) and separation, the organic phases were combined and evaporated to dryness under a stream of nitrogen at 25°C in a water bath. (Temperatures > 25°C caused unpredictable variation in the subsequent amplitude of the internal standard peaks, presumably due to thermal decomposition). The residue was reconstituted in methanol (55 - 100 µl) and the total volume injected onto the system. Injection of reconstituted residues produced little or no rises in operating pressure when this method was used. Other methods of extraction which were studied were abandoned in part because of unacceptable rises in operating pressure after injection of extracts.

While the above extraction procedure proved satisfactory for

the determination of amodiaquine and desethylamodiaquine in blood and red cells from healthy subjects (Chapter 3) the concentrations of both compounds in red cells from malaria patients (Chapter 4) were often below the lower limits of detectability for the assay. The possibility that this observation was due to lower analytical recoveries of the compounds in the presence of malaria parasites was examined by the addition to malaria-infected red cells of known quantities of both compounds, followed by HPLC analysis as described above. Analytical recovery of both amodiaquine and desethylamodiaquine was not influenced by the presence of P. falciparum within the cells (see Table 2.4).

In order to increase the sensitivity of the assay from parasitised red cells it was necessary to improve analytical recovery. To this end a variation of the extraction method of Pussard and colleagues (1986) was examined, and found to recover more of both compounds than the previous method (Table 2.4). This method was followed therefore for the assay of both amodiaquine and desethylamodiaquine in parasitised red cells (Chapter 4). 6-Methoxy-8 aminoquinoline proved unsatisfactory as internal standard, probably because of breakdown during the acid back-extraction. A search was therefore undertaken for a new internal standard; 7-chloro-4[1-dimethylamino-4-pentylamino]-quinoline proved to be satisfactory.

As before, extractions were performed in silylated 15ml capacity glass culture tubes. To red cells (1.0ml) containing internal standard (500ng; 50µl) was added K_2HPO_4 (50% w/v; 500µl) and distilled water (1.0ml), followed by extraction into dichloromethane (8.0 ml) by mechanical tumbling (15 min). Following centrifugation (2000g; 10 min) the supernatant aqueous phase was

discarded, and the tube cleaned by the addition of NaOH (0.1M; 1.0ml) followed by mechanical tumbling (10 min). Following centrifugation (2000g; 5 min) the aqueous phase was discarded, and the remaining traces of alkali removed by the addition of distilled water (5.0ml) followed by mechanical tumbling (5 min). Following further centriguation (2000g; 5 min) the aqueous phase was discarded; to organic solvent was then added HCl (0.2M; 100µl) followed by mechanical tumbling (15 min). After centrifugation (2000g; 5 min) the supernatant acid was injected onto the HPLC system.

b) Rodent biological fluids (urine, homogenised faeces and homogenised tissue)

i **Faeces** 12 Hourly collections of rat faeces were homogenised in phosphate buffer (pH 7.5, 1:4 by weight). The pH of these samples was raised to 12.0 by the addition of ammonia (SG 0.88) as for human biological fluids. However it was found more convenient to extract faeces into dichloromethane, since the aqueous phase could be more easily removed from the surface of the organic solvent. Furthermore use of dichloromethane gave higher extraction efficiencies from faeces than use of diethylether.

Extractions were performed in silylated 15 ml capacity culture tubes. To sample (1.0 ml) containing internal standard (6-methoxy-8 aminoquinoline; 1-10µg; 100µl) was added ammonia solution (SG 0.88; 2.0 ml). This was then extracted once into dichloromethane (7.0 ml) by vortex mixing (15 s) followed by mechanical tumbling (15 min). After centrifugation (2000 g; 10 min) the supernatant aqueous phase was removed by suction. The remaining faecal pellet was removed from the surface of the organic solvent using a spatula.

Remaining particulate matter was removed by addition to the organic solvent of a mixture of ammonia and distilled water (1:10 v/v; pH 10-11), followed by vortex mixing and centrifugation. The supernatant aqueous phase, which contained remaining particulate matter was then removed by suction. The organic solvent was evaporated to dryness under a stream of nitrogen at 25°C. The yellow-coloured residue was reconsituted in methanol (100 µl) and injected onto the system. This was associated with a small rise in operating pressure. Extraction efficiencies for amodiaquine and desethylamodiaquine, were 60% and 50% respectively.

In an attempt to 'clean up' the samples prior to injection in order to avoid rises in operating pressure, a further step was added after extraction into dichloromethane. To the organic solvent (7.0 ml) was added hydrochloric acid (1.0 M; 1.0 ml); this was then mixed by mechanical tumbling (15 min). After centrifugation the supernatant aqueous phase was transferred to a clean tube. To this was added ammonia (SG 0.88; 2.0 ml) raising the pH to 11.5; this was then extracted twice with diethylether (5.0 ml x 2) by mechanical tumbling (15 min). After centrifugation and separation, the organic phase was pooled and evaporated to dryness under a stream of nitrogen. Resulting extracts were clean, and not associated with rises in operating pressure. However the extraction efficiencies of both amodiaquine and desethylamodiaquine fell to 27% and 16% respectively. It was therefore concluded that the 'clean-up' step was disadvantageous; the small increases in operating pressure which were produced by the 'dirty' samples did not lead to major problems.

ii **Urine** Pre-treatment of rodent urine with acetonitrile was not

found to be necessary. A large intrinsic peak was often seen upon subsequent chromatography, having a retention time of 7.8 min, but this eluted after, and was fully resolved from, the internal standard peak.

Extractions were performed in silylated 15 ml capacity culture tubes, and evaporation of organic phase was performed in silylated 10 ml capacity pointed centrifuge tubes. To the sample (0.5 ml) containing internal standard (6-methoxy-8 aminoquinoline; 1µg; 100µl) was added distilled H_2O (0.5 ml) and ammonia solution (SG 0.88; 2.0 ml) achieving a pH of 12.0. This was extracted once into diethylether (7.0 ml) by mechanical tumbling (15 min). After centrifugation (2000 g; 10 min) the organic phase was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 25°C. The residue was reconstituted with methanol (100 µl) and injected onto the system. Extraction efficiency for both amodiaquine and desethylamodiaquine was 50%

iii **Tissue** Quantification of the amount of extractable amodiaquine and desethylamodiaquine was required from rodent haemopoietic tissue. All tissues were homogenised in phosphate buffer (pH 7.5; 1:4 by weight) immediately after sacrifice. Several methods were studied in an attempt to extract the compounds from homogenised spleen. Pre-treatment of samples with acetone, followed by extraction into diethylether gave the best analytical recoveries.

To the sample (0.5 ml) containing internal standard (6 methoxy 8 aminoquinoline; 1µg; 100µl) was added acetone (1.5 ml). This was vortex mixed (15 s) and centrifuged (2000g; 10 min). The supernatant was transferred into a clean tube, to which was added

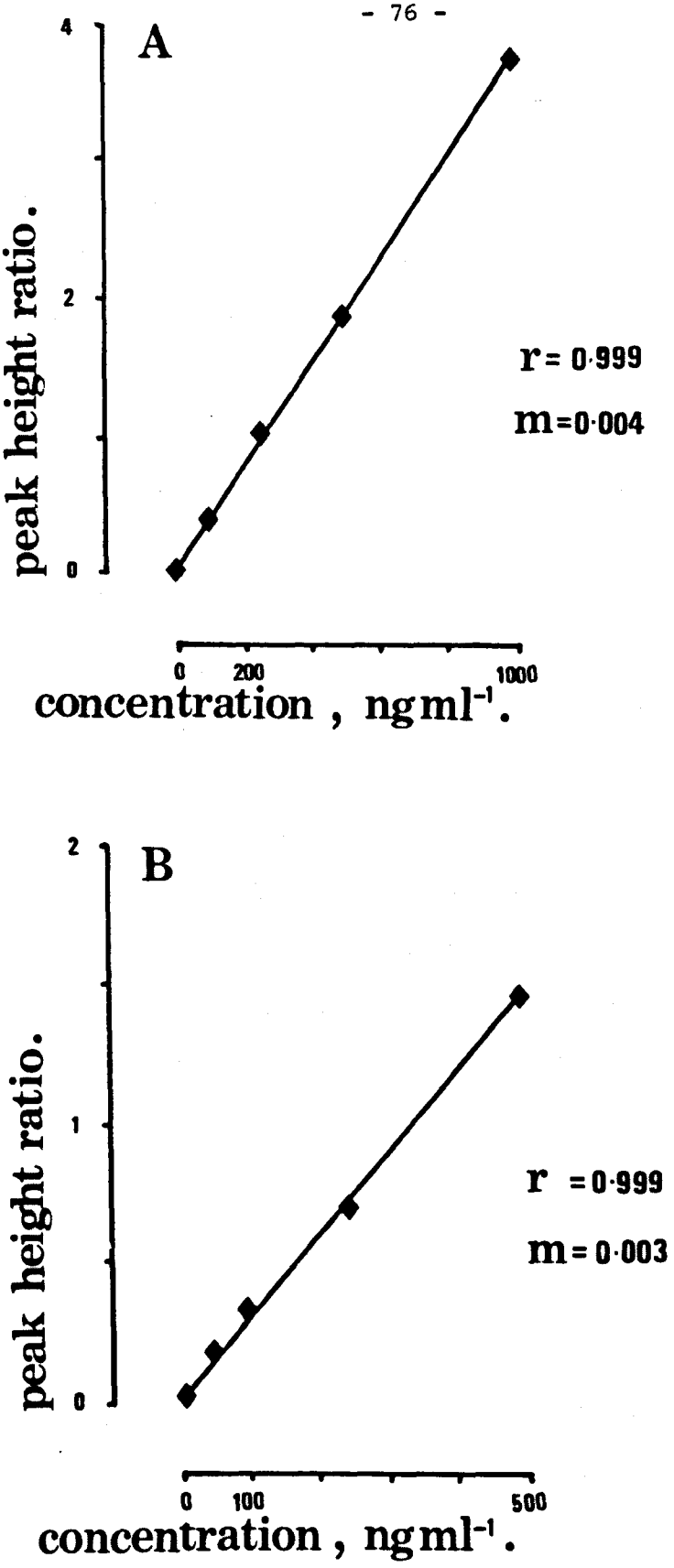


FIGURE 2.4 Typical standard curves for desethylamodiaquine (A) and amodiaquine (B) in human plasma.

ammonia (SG 0.88; 1.0ml). This was then extracted twice into diethylether (5.0 ml x 2) by mechanical tumbling (15 min). After centrifugation (2000g; 10 min) and separation, the organic phases were pooled and evaporated to dryness under nitrogen at 25°C. As before, the residues were reconstituted in methanol (100 µl) and injected onto the system. There were no rises in operating pressures following injection of these extracts. Extraction efficiencies for amodiaquine and desethylamodiaquine were 45% and 25% respectively.

2.1.4 Extraction efficiency

The extraction efficiencies of amodiaquine and desethylamodiaquine were calculated by comparison of peak heights from extracted samples of drug-free biological fluid spiked with a known mass of compound, with those of directly injected stock solutions. An additional check was available for amodiaquine by calculation of the recovery of radioactivity following the extraction of biological fluids spiked with ^{14}C-labelled drug. Analytical recoveries of both compounds are given in Table 2.4.

2.1.5 Standard curves

HUMAN SAMPLES

In the cases of plasma, whole blood and packed cells, standard curves were prepared by the addition of known quantities of both amodiaquine and desethylamodiaquine to a fixed concentration of internal standard in the appropriate drug-free biological fluid. It was possible to determine the concentrations of both compounds simultaneously. In urine, the concentrations of desethylamodiaquine were an order of magnitude larger than those of

TABLE 2.4 Analytical recoveries

Biological fluid	Extraction method	Recoveries (%) Amodiaquine	Desethyl-amodiaquine
HUMAN			
Plasma	)	70	50
Urine	)Acetonitrile	60	50
Parasite-free blood	)and	60	30
Parasite-free RBC	)ether	60	30
Parasitised RBC		60	30
Parasitised RBC	K_2HPO_4 and dichloromethane	60	50
RODENT			
Urine	Direct extraction into ether	50	50
Faecal homogenate	Direct extraction into dichloromethane	60	50
Tissue homogenate	Acetone and ether	45	25

the parent drug and therefore the standard curve concentrations differed by the same degree. Consequently, although both compounds were added to standard curve samples (in order that the standards should as closely as possible resemble the unknowns), in any one run it was only possible to measure either parent drug or metabolite. Each unknown was therefore assayed twice, once for each compound.

Samples were analysed as described above, the standard samples and one blank sample being included in each run with the unknowns. The peak height ratios of amodiaquine or desethylamodiaquine to internal standard, from the calibration curve samples, were plotted against the corresponding mass of compound. Calculation of amodiaquine and desethylamodiaquine concentrations in the unknown samples was made by comparison of their peak height ratios with those of the standard curve.

Example standard curves are shown in figure 2.4.

RODENT SAMPLES

Standard curves were prepared by the addition of known quantities of amodiaquine and desethylamodiaquine to fixed concentration of internal standard in the appropriate drug-free biological fluid. It was possible to determine the concentrations of both compounds simultaneously for all biological fluids. However in the case of faeces the range of concentrations of both amodiaquine and desethylamodiaquine encountered in the unknowns varied from 0.025 - 8.0 $\mu g\ ml^{-1}$ in the case of amodiaquine, and from 0.10 to 2.0 $\mu g\ ml^{-1}$ in the case of the metabolite. It was not possible therefore to determine accurately concentrations of either compound from both the highest and lowest of the unknowns using one

TABLE 2.5 Retention times

Compound	Mobile phase	Stationary phase	Flow ($mlmin^{-1}$)	Retention time (min)
Amodiaquine	Methanol: triethylamine pH 2.8-2.9	Microbondapak phenyl (10µM)	3.5	4.8
			6.0	4.0
	Acetonitrile: water: triethylamine pH 2.8	Ultrasphere ODS (5µM)	2.0	5.4
Desethylamodiaquine	Methanol: triethylamine pH 2.8-2.9	Microbondapak phenyl (10µM)	3.5	3.6
			6.0	3.0
	Acetonitrile: water: triethylamine pH 2.8	Ultrasphere ODS (5µM)	2.0	3.8
6-methoxy-8 Aminoquinoline	Methanol: triethylamine pH 2.8-2.9	Microbondapak phenyl (10µM)	3.5	5.6
			6.0	5.2
Amopyroquine	Acetonitrile: water:triethylamine pH 2.8	ultrasphere ODS (5µM)	2.0	4.4
7-chloro-4- [1-dimethylamino-4-pentyl amino] quinoline.	Methanol: triethylamine pH 2.8	Microbondapak phenyl (10µM)	3.5	2.4

standard curve. Consequently samples were assayed twice; the first time to determine approximate concentrations of both compounds, and a second time against either a low or high concentration standard curve to determine accurate values.

As described for the assay of human samples, a blank sample and standard curve samples were included in each run with the unknowns. Concentrations of amodiaquine and desethylamodiaquine in the unknowns was determined as for human samples by comparison of peak height ratios from unknown samples with those of the standard curves.

2.1.6 Assay specifications

The extraction procedures resulted in simple sample preparation. Chromatograms obtained from extracts of the various human and rodent biological fluids are shown in figures 2.1 - 2.3.

Amodiaquine, desethylamodiaquine and internal standard were detected as distinct peaks, which were chromatographically resolved to baseline. Retention times for the compounds, under differing chromatographic conditions, are given in Table 2.5. Chromatograms of the blank biological fluids were free of any interferring peaks; one or more endogenous components eluted with retention times of > 6 mins, but they were baseline separated from the internal standard and samples could be injected at ~ 7 minute intervals. There was no chromatographic interference from the commonly used antimalarial drugs chloroquine, primaquine, pyrimethamine, proguanil or cycloguanil.

Analytical recoveries for amodiaquine, and desethylamodiaquine from human plasma, urine, whole blood and red cells (acetonitrile method) are shown in table 2.4. The minimum detectable concentrations of amodiaquine using 1 ml of biological fluid was 5

ng ml^{-1} from human plasma and urine, and 12.5 ng ml^{-1} from whole blood and red cells; the corresponding values for desethylamodiaquine were 5 ng ml^{-1} from plasma and urine, and 25 ng ml^{-1} from whole blood and packed cells. Analytical recoveries for the two compounds from parasitised red cells, using the extraction method of Pussard and colleagues, are also shown in Table 2.4. The minimum detectable concentration of both compounds using 1ml of red cells was 5ng ml^{-1}.

The minimum detectable concentrations of amodiaquine from rodent biological fluids, using 0.5ml of fluid for urine and tissue, and 1.0 ml for faeces were 12.5, 25 and 12.5 ng ml^{-1} respectively; the corresponding values for desethylamodiaquine were 25, 50 and 25 ng ml^{-1} respectively. In each case, these concentrations produced a peak three times the baseline noise on the highest detector sensitivity used (x 0.005 A.U.F.S.).

Calibration curves were linear ($r > 0.99$) in the range 0 - 1000ng ml for both compounds in human plasma, whole blood and packed cells. In human urine calibration curves were linear for both compounds in the ranges 0 - 1µg ml^{-1} for amodiaquine and 0 - 20µg ml^{-1} for desethylamodiaquine. In the case of rodent biological fluids calibration curves were linear in the ranges 0 - 1000ng ml^{-1} and 0 - 10µg ml^{-1} of both compounds in faeces, 0 - 500ng ml^{-1} of amodiaquine and 0 - 1000ng ml^{-1} of desethylamodiaquine in urine, and 0 - 1000ng ml^{-1} of both compounds in tissue homogenate.

TABLE 2.6 Reproducibility of HPLC methods for human and rodent biological fluids

		HUMAN									RODENT			
		PLASMA		URINE		BLOOD		UNPARASITISED RED CELLS		PARASITISED RED CELLS	FAECES		URINE	
		LOW CONC	HIGH CONC	LOW CONC	HIGH CONC	LOW CONC	HIGH CONC	LOW CONC	HIGH CONC	ONE CONC ONLY	LOW CONC	HIGH CONC	LOW CONC	HIGH CONC
INTRA ASSAY	AQ#	5*	7	3	3	5	5	11	5	5.5	7	3	5	3
	AQm	4	4	3	3	6	6	8	9	7.0	9	5	5	6
INTER ASSAY	AQ	10	5	2	8	9	6	11	11	14	10	8	4	11
	AQm	10	6	5	4	11	11	10	11	10	8	8	6	9

*All figures represent coefficients of variation (%): n ranged from 5-8 replicates.

#AQ = Amodiaquine
AQm = Desethylamodiaquine

Reproducibility of methods was determined both within-day and day-to-day at 2 concentrations for both compounds, these being approximately the highest and the lowest concentrations expected from the unknowns. For within-day calculation of coefficients of variation, a sample of drug-free biological fluid was spiked with compound to a known concentration. This was then divided into aliquots and assayed according to the methods described above. For day-to-day reproducibility determinations, a sample of drug-free biological fluid was spiked with compound to known concentration and then divided into aliquots which were then stored in the same type of container used for the unknowns, and under the same conditions [i.e. - 20°C protected from light by aluminium foil wraps]. Aliquots were then assayed in pairs over at least 8 weeks.

Coefficients of variation were calculated from the mean and SD of the peak height ratios obtained from assayed aliquots according to the formula $CV = \frac{SD}{MEAN} \times 100$. The reproducibility data for all HPLC methods is shown in Table 2.6.

2.1.7 Infection risk from samples of human biological fluids

The serious virus infections hepatitis-B and Acquired Immune Deficiency Syndrome (AIDS) can be transmitted in a laboratory setting, mainly by the entry of virus particles through skin lesions. The risk of encountering infected subjects amongst healthy laboratory volunteers, such as participated in the studies described in chapter 3, was low. However, it was considered that those samples collected from Zambian patients (chapter 4) should be considered "high risk". Good laboratory practice, including the correct disposal of sharps and contaminated waste, was continued, and laboratory personnel had been vaccinated against hepatitis-B

prior to the commencement of the present work. No attempt was made to screen samples for the presence of antibodies against human immunodeficiency virus (HIV) (following advice from the local Public Health Laboratories), in view of the long delay in appearance of such antibodies, and the lack of effective tests for antibodies against HIV-2.

Heating of samples of biological fluid to 56°C for ≥ 0.5h is capable of inactivating HIV, and this was undertaken upon receipt of the plasma samples from Zambia. Heat treatment of the RBC samples from Zambia was not possible, since this caused coagulation of the material. When handling material from Zambian subjects, laboratory personnel wore disposable gloves. It was considered that virus particles would be inactivated during the extraction procedure with organic solvent.

In order to ensure that such heat treatment had no effect on amodiaquine or desethylamodiaquine, samples of plasma (1.0ml) containing both compounds (250ng ml^{-1}) were incubated in a water bath at 56°C for 0.25, 0.5 and 0.75h in duplicate. Samples were then subjected to HPLC analysis, as described previously and peak heights obtained from heated samples were compared with those obtained by extraction of similarly spiked plasma which had been stored on ice. The peak heights of amodiaquine and desethylamodiaquine were not effected by incubation at 56°C up to 0.75h, values ranging from 98 to 107% of the control values. The compounds were therefore judged to be stable under these conditions.

2.2 MASS SPECTROMETRY OF URINARY DESETHYLAMODIAQUINE

2.2.1 Introduction

Although concentrations of amodiaquine in the plasma (measured by Mihaly et al 1985) were high after I.V. administration of the drug, and were measurable for up to 24h, after oral administration concentrations of amodiaquine were low (Looareesuwan et al 1985). A large peak not present in pre-dose plasma or urine was seen in extracted samples following oral administration of amodiaquine, and its retention time (3.6 min) was identical to that of stock desethylamodiaquine. It seemed likely that this early peak was desethylamodiaquine, but confirmation of this required that the mass spectra of the authentic compound and the 3.6 min peak should be compared. Consequently ~ 100µg of the pure compound eluting at 3.6 min was needed.

2.2.2 Extraction

Urine (1.0 l) collected from one subject in the 24 h following oral administration of amodiaquine (600 mg) was extracted in aliquots (50 ml) using a glass separating funnel. To urine (1 vol) was added NH_3 (SG 0.88 1 vol) and diethylether (5 vol), and this mixture was shaken by hand (5.0 min), intermittently allowing equalisation of pressures. Aqueous and organic phases were separated by gravity and the aqueous phase discarded. To pooled organic phase was added $MgSO_4$ (100g), in order to remove contaminating water, and the mixture shaken by hand. The organic phase, now free from contamination with aqueous phase, was decanted into a clean container and evaporated to dryness using a rotary vacuum evaporator (Büchi Rotavapor-R) at 40°C. The residue was reconstituted in methanol (200 µl).

2.2.3 Preparative HPLC

Since triethylamine might be expected to obscure compounds of interest during mass spectrometry the standard mobile phase could not be employed. A triethylamine free mobile phase was therefore developed; this consisted of methanol:water (55:45 v/v) containing octane sulphonic acid (0.001M) adjusted to pH 2.8 with orthophosphoric acid, and flowing at 3.5 ml min^{-1}. Instrumentation was unchanged from the analytical HPLC system. Amodiaquine and desethylamodiaquine eluted as distinct peaks resolved to within 20% of baseline with retention times of 6.3 and 8.3 min respectively.

Aliquots of the urine extract were injected onto the system and the timed fractions corresponding to the unidentified peak were saved.

Extraction from mobile phase

The pooled phase (60ml) containing the eluted compound was extracted in 20 ml aliquots into diethylether. To mobile phase (1 vol) was added NH_3 (2 vols) and diethylether (5 vols) and was mixed by mechanical tumbling (15 min). After centrifugation (2000g; 10 min) and separation, the organic phase was pooled and evaporated to dryness under a stream of nitrogen at 35°C.

2.2.4 Mass Spectrometry

Purity of the extracted compound was confirmed by analytical HPLC, and the mass of compound was estimated at 200µg. This was then submitted to mass spectrometry by Mr. M. Prescott and Dr. R. Evershed (Department of Biochemistry, University of Liverpool). Electron impact mass spectra were run on a VG - Micromman 70 - 70F mass spectrometer interfaced with a Finnigan Incos Data System.

Electron energy was 70 meV. The resulting mass spectra of eluted compound and authentic desethylamodiaquine are given in chapter 3.

2.3 RADIOCHEMICAL PURITY OF ^{14}C-AMODIAQUINE

2.3.1 Introduction

[Quinoline-2-^{14}C] amodiaquine hydrochloride monohydrate (^{14}C-amodiaquine) was obtained from Amersham International (UK) having been prepared by the method of Burkhalter and colleagues (1948). The compound was said to be 92-96% pure on TLC criteria, and to have a specific activity of 7.15μCimg^{-1}. The material was stored at -20°C under nitrogen, and approximately one year elapsed between receipt and first use of the compound. At this time assessment of radiochemical purity, by the method set out below was ~ 87%; therefore the ^{14}C-amodiaquine was purified by TLC prior to use.

2.3.2 Assessment of radiochemical purity

Aliquots of ^{14}C-amodiaquine stock solution (100μl) of known total radioactivity were injected onto the HPLC system described earlier in this chapter. A single peak was seen with retention time equivalent to that of authenticated compound. Following injection of ^{14}C-amodiaquine onto the system, timed aliquots (0.5 min) of column eluent were collected in scintillation vials and were subjected to liquid scintillation counting. Radioactivity in the samples corresponding to the amodiaquine peak was summated and expressed as a percentage of the total.

2.3.3 Purification of ^{14}C-amodiaquine

^{14}C-amodiaquine was dissolved in ethanol (2.0ml) and applied as streaks to TLC plates (Kieselgel 60-ART 5748; Merck) which were

developed in triethylamine:methanol (50:50 v/v). The ^{14}C-amodiaquine was located by autoradiography, and the appropriate band of gel was scraped from the plate. Gel was suspended in ethanol in order to elute ^{14}C-amodiaquine, and was subjected to centrifugation (12000G; 10 min) to remove the gel. After rotary evaporation to remove ethanol, HCl was added (42.36µmole/10mg amodiaquine base) to convert the drug to its hydrochloride form. An ethanolic stock solution of this was stored at -20°C.

Recovery of ^{14}C-amodiaquine was determined by comparison of peak heights obtained from aliquots of ^{14}C-amodiaquine stock solution with those from known quantities of the drug injected onto the HPLC system. Of the ^{14}C-amodiaquine subjected to TLC 78% was recovered. Radiochemical purity was determined by the method given above, and was found to be 98%. The specific activity of the radiolabel was 8.57µCi mg^{-1} (base) after this purification procedure.

2.4 DETERMINATION OF RADIOACTIVITY

2.4.1 Introduction

^{14}C-Labelled amodiaquine was employed for tissue distribution and excretion studies using rodents (as described in chapter 5). Various scintillation cocktails were assessed regarding size of background counts from radiation-free scintillant, and the abilitity of different cocktails to mix with aqueous suspensions without the formation of two phases. Aqualuma plus (Table 2.7) was considered most satisfactory and was used throughout. Radioactivity was determined by liquid scintillation spectrometry using a microprocessor-controlled Beckman LS 1801 counter, equipped with automatic quench correction.

TABLE 2.7 **Assessment of background counts from scintillation cocktails**

Cocktail	Counts from 15ML (Radiation-free) (DPM)	Counts from 15ML plus 4ML mobile phase (radiation-free) (DPM)
1. Packard ES 299	39.6 ± 0.9	50 ± 3.6
2. In-house cocktail	48.4 ± 6.3	48 ± 1.9
3. Aqualuma plus*	37.9 ± 1.2	46 ± 0.9
4. Ria Luma	35.8 ± 0.8	Not tested
5. Optiphase safe	37.1 ± 1.5	Not tested.

(Figures are mean ± SD, n=4)

* Used throughout this thesis because of low background count, and ability to mix with aqueous solutions without the formation of two phases.

2.4.2 Determination of radioactivity in urine

To duplicate samples of urine (50µl) was added scintillant (10 ml) followed by vortex mixing (15s), and incubation at room temperature (12h) in order to reduce chemiluminescence. Four samples of radioactivity-free urine were similarly treated and carried through the run. After counting, the mean background reading was deducted from each of the unknowns.

2.4.3 Determination of radioactivity in blood, red cells, faeces and tissue homogenates

To triplicate samples (100 µl) was added NCS tissue solubiliser (500 µl) followed by incubation at 50°C overnight. While still hot, the digest was decolourised by the addition of hydrogen peroxide (500 µl) followed by vortex mixing for a few seconds and further incubation at 50°C for 20 min. To the decolourised digest was added glacial acetic acid (50 µl) (to reduce quenching) followed by scintillation cocktail (15ml). After vortexing (15 s) the specimen was incubated at room temperature in the dark (12 h) before counting, in order to reduce chemiluminescence, and similarly treated blank specimens were included in each run to assess background counts.

Limits Data were included for analysis where sample activity was > 2 x mean background activity. Counting times were varied in order to ensure that < 5% of values within any counting period were outside 2 SD of the mean (i.e. 2 SIG % < 5); this was generally achieved with a counting period of 10 min or less.

TABLE 2.8 To assess sample treatment procedure for highly coloured biological fluids, and accuracy of automatic quench-correction

* Volume of sample (µl)	Observed # activity (DPM X 10^4)	Expected activity (DPM x 10^4)	Percentage observed/ expected
25	1.5737	1.6733	94
50	1.7218	1.6733	103
75	1.7075	1.6957	101
100	1.6413	1.6845	97
125	1.6553	1.7182	96
150	1.6664	1.7070	98
		$\bar{x}$	98
		SD	3

* Rat faecal homogenate in all cases.

^{14}C-hexadecane

2.4.4 Assessment of quench correction of highly coloured specimens

In order to assess the adequacy of the automatic quench correction programme for highly coloured digests, observed counts obtained from faeces spiked with exactly known radioactivity were compared with those expected. To pre-weighed vials (n = 6) was added ^{14}C-labelled hexadecane (20 µl; specific activity 1.123×10^6 DPM g^{-1}; 0.773g ml^{-1}) and to further vials (n = 6) was added water (20 µl); the vials were then re-weighed to determine the exact weight of hexadecane added. Radioactivity-free faecal homogenate was then added to each vial in the volumes 25, 50, 75, 100, 125 and 150µl, so that each vial containing hexadecane had a matched control. The samples were then treated as above. Background counts were subtracted from the test samples and the resulting value was compared with the expected value (calculated from the known weights of hexadecane). The observed values were 98 ± 3% of those expected, confirming that the treatment procedure followed, and the automatic quench correction, were adequate (Table 2.8).

2.5 IN-VITRO CULTURE OF HUMAN BONE MARROW CELLS

2.5.1 Introduction

Techniques for culture of bone marrow cells *in vitro* have been developed over the last 20 years, at first for the growth of murine marrow cells (Metcalf *et al* 1967) and subsequently human marrow cells (Pike and Robinson 1974). These methods have been used primarily for the study of normal haemopoiesis and diseases which perturb this, but have also been employed to investigate adverse drug effects on marrow cell differentiation and proliferation.

It is possible to culture granulocyte/monocyte, erythrocyte

and megakaryocyte precursors *in vitro* using different methods. One of the principal requirements for cell proliferation *in vitro* is the addition of specific colony stimulating factors (CSF). The structures of several human CSFs, all glycoproteins, have now been described (Metcalf 1985 *a* and *b*). Sources of specific granulocyte/monocyte CSF (GM-CSF) include peripheral blood leukocytes and certain tumour cell lines.

Marrow-derived cells can be cultured in liquid medium, in which case cell proliferation is most usually measured by uptake of a radiolabelled nutrient. For this project, cells were cultured in semi-solid medium, and proliferation was assessed by manual counting of colonies under a light microscope. The term "colony" is used to describe a group of $\geq$ 50 cells, smaller groups of cells being referred to as "clusters" (Pike and Robinson 1974; see figures 2.5 - 2.8). The effects of a drug upon cell proliferation can be assessed by comparison of numbers of colonies resulting from culture in drug-free medium, with those resulting from culture in the presence of known concentrations of the drug. Care has to be taken to ensure that control plates resemble test plates in all ways other than presence of drug. Thus if a drug is dissolved in ethanol prior to addition to medium, then the same dilution of ethanol needs to be present in the controls.

Two methods of culture of granulocyte/monocyte colonies in semi-solid medium were assessed. The first (Pike and Robinson 1974) proved unreliable, because of a high degree of assay variation, and was abandoned. The second (Myers *et al* 1984) has been used throughout these studies. Both methods rely upon the manual counting of numbers of colonies, and this could be subject to observer bias. Consequently, although not standard practice in

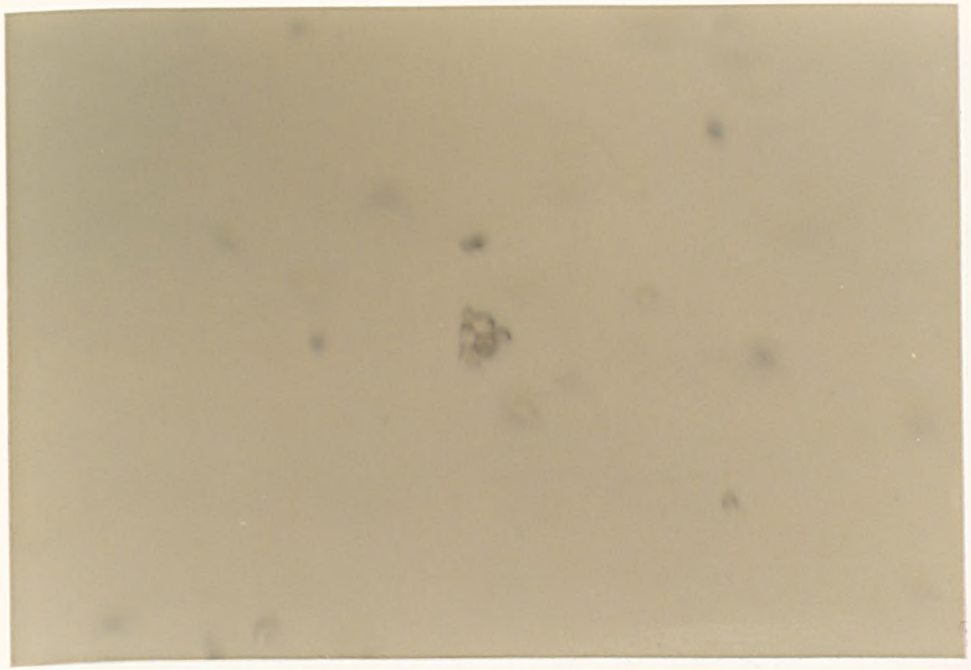


FIGURE 2.5 A small cluster of marrow cells 2 days after plating [x 200 magnification]

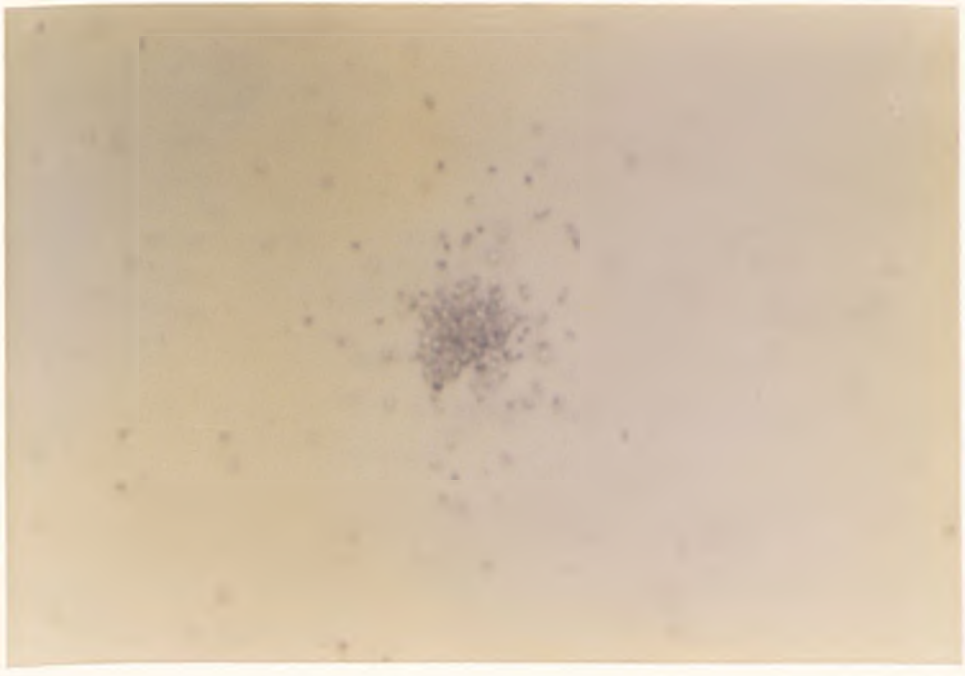


FIGURE 2.6 A colony 10 days after plating [x 100 magnification; the colony was in the same area of the plate as the cluster shown in Figure 2.5]

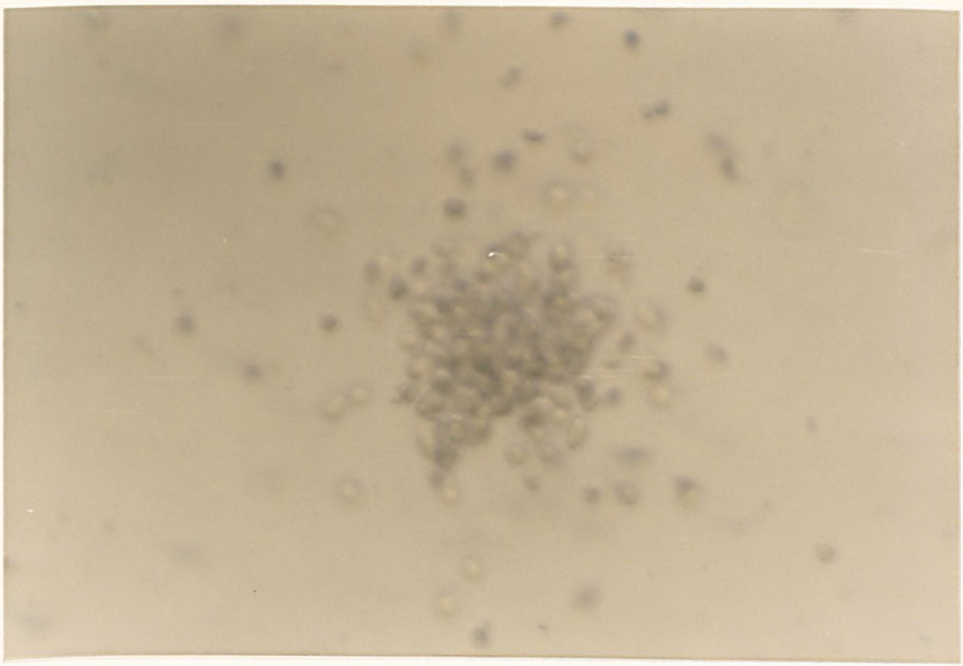


FIGURE 2.7 The same colony as shown in Figure 2.6, at higher magnification [x 200]

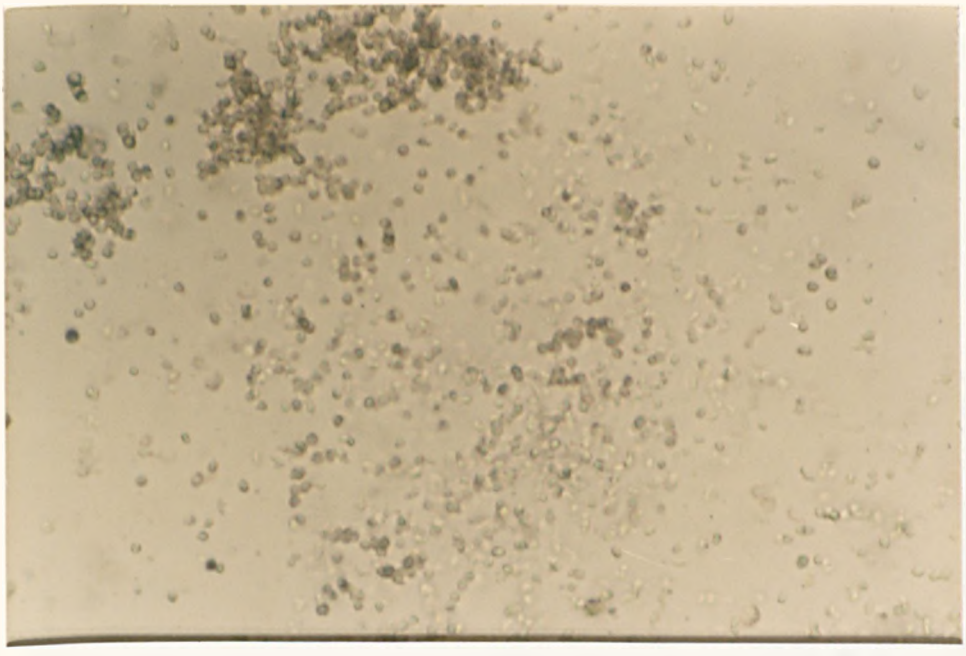


FIGURE 2.8 A large, relatively diffuse colony 14 days after plating [x 100 magnification]

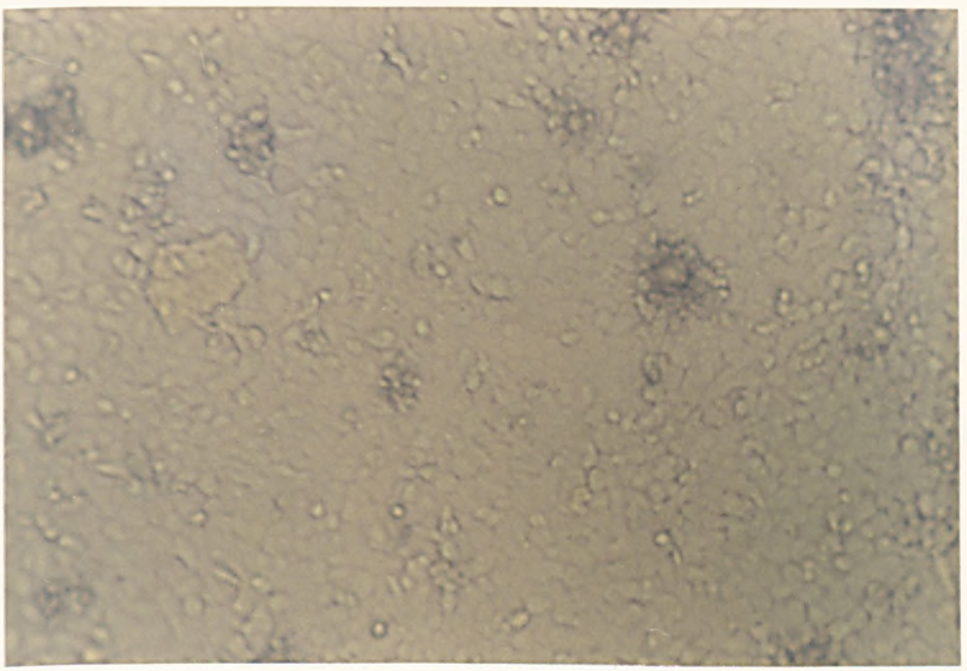


FIGURE 2.9 Cells of the C5637 bladder carcinoma cell line [x 100 magnification]

other laboratories, in all experiments performed as part of this work, plates were encoded and assessed "blind"; the investigator was in ignorance of the code until counting was complete.

2.5.2 Collection of bone marrow

Bone marrow was obtained from patients about to undergo major orthopaedic surgery (usually total hip replacement). Ethics Committee approval was obtained from both the Royal Liverpool and Broadgreen hospitals, and several surgeons at both establishments permitted their patients to be approached. Following explanation of the procedure and the reason for which it was to be undertaken (i.e. research purposes) patients gave written informed consent in the presence of a witness.

Following induction of general anaesthesia, the skin over the posterior iliac crest was cleaned with antiseptic solution. A Jamshidi marrow aspiration needle was then inserted into the medullary bone of the posterior iliac spine and marrow was aspirated by syringe (5.0 ml). Marrow was immediately transferred to a sterile tube containing transport medium (2.0 ml).

2.5.3 Determination of cell counts

Numbers of nucleated marrow cells (and peripheral blood leukocytes; relevant to the method of Pike and Robinson 1974) were determined prior to the addition of cells to culture medium. To an aliquot of cell suspension (50 µl) was added toluidine blue (1% in phosphate buffered saline; 50µl) and phosphate buffered saline (400 - 900µl) achieving a dilution of 1:10 or 1:20. The cells were incubated at 37°C (5 min) allowing adequate staining of nucleated cells. A small aliquot of the suspension was transferred to a

counting chamber (Neubauer chamber; Weber U.K.) and examined under an inverted light microscope.

2.5.4 Sterile procedure

Heat-stable solutions such as NaOH, $NaHCO_3$, deionised H_2O, and agar were sterilized by autoclave. Heat labile solutions such as L-glutamine were sterilized by filtration using a 0.22 µm pore-size filter (Millipore U.K.).

Glassware (including bottles for medium and agar) was sterilized by autoclave. Plastics including pipette tips and re-usable filter mounts were sterilized by dry heat (100°C; 4h).

To avoid contamination of plates with fungal spores and other micro-organisms, all culture work was undertaken in conditions of laminar flow employing a class II laminar flow cabinet (model 20229; MDH-intermed).

2.5.5 Batch-testing of foetal calf serum

Since individual batches of foetal calf serum are known to vary in their ability to support colony growth *in vitro*, 6 different batches were examined including an aliquot of that routinely used by the Department of Haematology (University of Liverpool) for granulocyte-monocyte cultures. The best of these was selected (Seralab batch 301121) and was used throughout these studies.

2.5.6 Method of Pike and Robinson (1976)

In this method marrow cells are cultured in semi-solid medium using peripheral blood leukocytes as the source of GM-CSF. Although widely used, the method proved unreliable, seeming to be prone to

inexplicable failure of colony growth. This method was used however for the batch testing of foetal calf serum referred to above.

2.5.6.1 Preparation of complete medium

To McCoys 5A medium (single strength) (81 ml) was added foetal calf serum (15 ml), 200 mM L-glutamine (0.4 ml), 21 mg ml^{-1} L- serine (0.04 ml), 10 mg ml^{-1} L-asparagine (0.16 ml), 7.5% $NaHCO_3$ (0.6 ml), 100 mM sodium pyruvate (1.0 ml), MEM vitamins (0.4 ml), MEM essential amino acids (0.8 ml), gentamicin solution (40,000 units ml^{-1}; 100µl; John Bull Labs., Warwick, U.K.), and MEM non-essential amino acids (0.4 ml). Complete medium was stored at 4°C for no longer than 5 days after preparation.

2.5.6.2 Preparation of transport medium

To complete medium (1.7 ml) was added preservative-free heparin (1000 units ml^{-1}; 0.3 ml). Transport medium was used within 24 h of preparation.

2.5.6.3 Preparation of feeder layers

Venous blood (10 ml) was collected from at least 2 volunteers and transferred to sterile plastic tubes containing two drops of preservative-free heparin (1000 units ml^{-1}). This was then incubated at room temperature (2.0 h), in order to permit sedimentation of red cells, followed by transfer of supernatant leukocyte-rich plasma to a clean sterile tube. The number of nucleated leukocytes per unit volume was then determined.

To complete medium (1.0 ml) was added cell-rich plasma containing 4×10^6 nucleated leukocytes. This cell suspension was then placed in a water bath at 37°C. To futher complete medium

(2.6 ml) at 37°C was added boiled 5% agar (0.4 ml; Difco-Bacto-Agar), and the mixture allowed to cool (~ 1 minute) at room temperature. To the agar/medium mixture (3.0 ml) was added the cell/medium mixture (~ 1.0 ml), to give a final agar concentration of 0.5%, and a final cell count of 1 x 10^6 cells ml^{-1}. Aliquots of this mixture (1.0 ml) were transferred to sterile plastic Petri dishes (35 mm diam; Gibco UK).

Note was kept of the cell donor for each of the sets of feeder layers. Cells from different donors were at no time mixed. Feeder layers were stored at 37°C in an atmosphere saturated with water vapour and containing 5% CO_2 in an incubator (Heraeus B506 EK/CO_2) for up to 3 days before use.

2.5.6.4 Preparation of bone marrow cell overlayers

Marrow suspended in transport medium was incubated at room temperature (2 h), in order to allow sedimentation of red cells, followed by transfer of nucleated-cell rich supernatant to a clean tube. The number of nucleated cells per unit volume was determined.

To complete medium (1.0 ml) was added cell-rich plasma (~ 40µl) containing 8 x 10^5 cells, and the cell suspension was placed in a water bath at 37°C. To further complete medium (2.6 ml) was added boiled 3% agar (0.4 ml) and the mixture allowed to cool (~ 1 min). To the agar/medium mixture (3.0 ml) was added the cell/medium mixture (~ 1.0 ml), bringing the final agar concentration to 0.3% and the final cell count to 2 x 10^5 cells ml^{-1}. Aliquots of this mixture (1.0 ml) were layered onto the prepared feeder layers, and incubated at 37°C in 5% CO_2 as above, for 10-14 days, followed by examination by inverted light microscope as described below.

2.5.7 Modification of the method of Myers and colleagues (1984)

The bladder tumour cell line C5637 has been shown by Myers and colleagues (1984) to produce GM-CSF, and conditioned-medium from these cells has been used by the same workers and others to promote bone marrow cell growth *in vitro*. The following method is essentially that described by Myers and colleagues (1984), except that RPMI-1640 medium was used in place of alpha medium.

2.5.7.1 Preparation of complete medium

To RPMI-1640 concentrate (x 10 strength; 50 ml) was added sterile deionised H_2O (430 ml), followed by sterile $NaHCO_3$ (7.5%; 16.7 ml). To this was added sterile NaOH (1M; 3-4 ml) to give a pH of 7.2. To the resulting single strength buffered RPMI-1640 (79 ml) was added L glutamine (200mM; 1.0 ml), foetal calf serum (20 ml), and gentamicin solution (40,000 units ml^{-1}; 0.1 ml; David Bull Labs., Warwick, U.K.). The resulting complete medium was stored at 4°C until use, and was retained for no longer than 5 days after preparation.

2.5.7.2 Preparation of transport medium

To complete medium (1.0 ml) was added further foetal calf serum (1.0 ml) and heparin (preservative-free; 1000 units ml^{-1}; 300µl). Transport medium was used within 24 h of preparation.

2.5.7.3 Preparation of medium containing GM-CSF (Conditioned medium)

Cells of the C5637 bladder tumour cell line (figure 2.9) were suspended in complete medium (3 x 10^6/20 ml). The suspension was

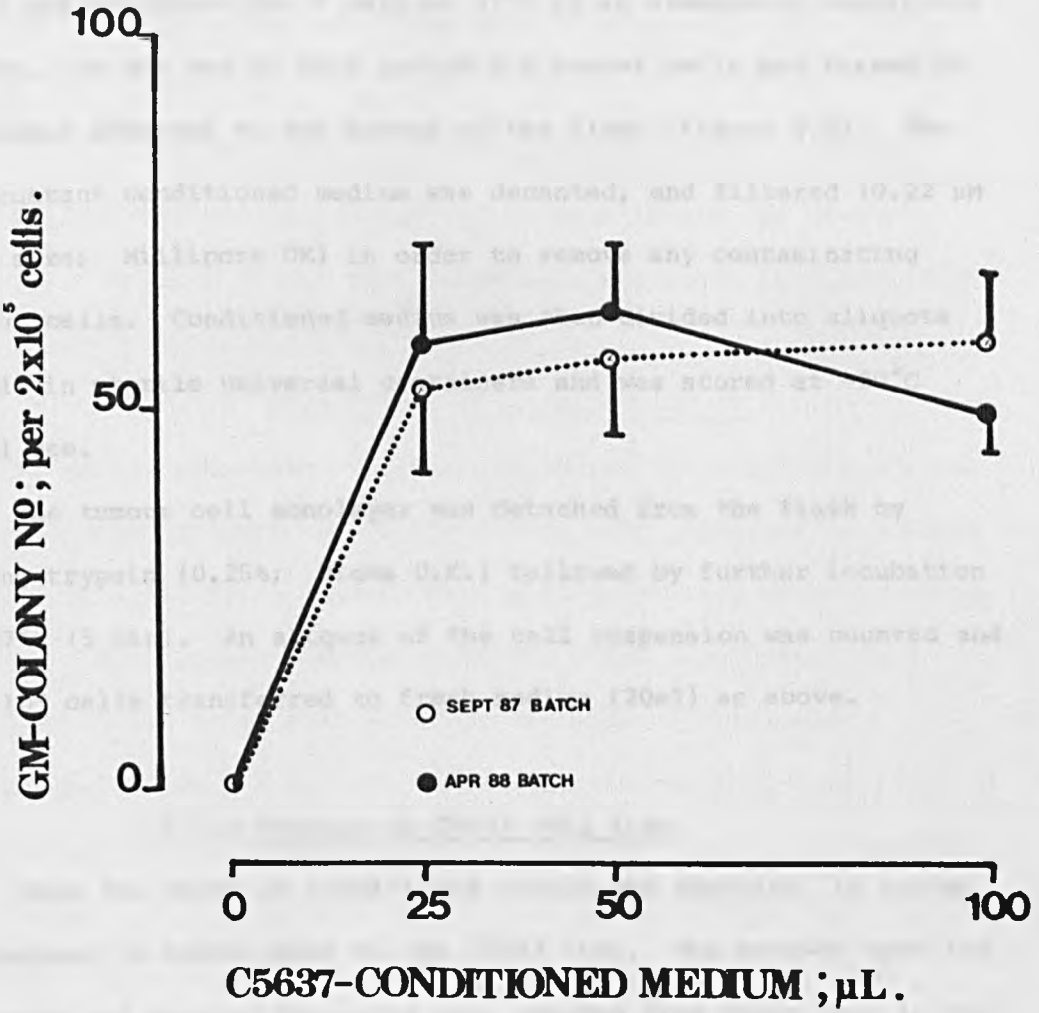


FIGURE 2.10 Typical results obtained when comparing batches of conditioned medium for GM-CSF activity (n=4 replicates, $\bar{x} \pm$ SEM)

transferred to a tissue culture flask (260 ml capacity; Gibco U.K.) and incubated for 7 days at 37°C in an atmosphere containing 5% CO_2. By the end of this period the tumour cells had formed an monolayer adherent to the bottom of the flask (figure 2.9). The supernatant conditioned medium was decanted, and filtered (0.22 μM pore size; Millipore UK) in order to remove any contaminating tumour cells. Conditioned medium was then divided into aliquots (10ml) in sterile universal containers and was stored at -20°C until use.

The tumour cell monolayer was detached from the flask by adding trypsin (0.25%; Sigma U.K.) followed by further incubation at 37°C (5 min). An aliquot of the cell suspension was counted and 3 x 10^6 cells transferred to fresh medium (20ml) as above.

2.5.7.4 Storage of C5637 cell line

When the stock of conditioned medium was adequate, it proved convenient to store cells of the C5637 line. The methods used for freezing and thawing the cells were adapted from those used in the Department of Haematology, University of Liverpool. To complete medium (10 ml) were added 3 x 10^6 cells and the suspension chilled to 0°C on ice. To the cell suspension was added dimethylsulphoxide (20% in foetal calf serum) dropwise to a total volume of 10 ml. The cell/DMSO mixture was aliquoted into sterile cryotubes, and frozen at -80°C.

In order to thaw the cell line, an aliquot (1.0 ml) was incubated at 37°C until it had just melted. To this was added single strength RPMI, containing 20% foetal calf serum, dropwise until a final volume of 10 ml was reached. This was centrifuged (1800g; 5 min) and the supernatant discarded. The cell pellet was

reconstituted in complete medium (10 ml) and incubated at 37°C in 5% CO_2 for 7 days to permit resumption of cell growth.

2.5.7.5 Assessment of batches of conditioned medium

All batches of conditioned medium were tested for GM-CSF activity. Results of one such batch test are shown in figure 2.10.

2.5.7.6 Preparation of marrow cultures

To marrow suspended in transport medium (~ 7.0 ml) was added sterile phosphate buffered saline (~ 7.0 ml). Aliquots (~ 3.5 ml) of this were carefully layered onto sterile density gradient medium (3.0 ml; Lymphoprep; Nyegaard, Norway), and were centrifuged (1800 G; 15 min). At the end of centrifugation cells were distributed between 4 layers: lowermost red cells, a hazy layer of density gradient medium containing low-density cells, a distinct "buffy coat" of low density cells, and supernatant buffer. Density gradient medium and "buffy coat" phases were pipetted into a sterile tube and were washed with phosphate buffered saline (10 ml). After centrifugation (1200 G; 5 min) and removal of buffered saline, the cell pellet was resuspended in phosphate buffered saline (10 ml). Following centrifugation (1200 G; 5 min) and removal of buffer, the cell pellet was resuspended in complete medium (1.0 ml). The nucleated cells were counted.

To complete medium (1.0 ml) was added 8×10^5 cells, and the cell suspension placed in a water bath at 37°C. To complete medium at 37°C (2.6 ml) was added boiled 3% agar (0.4 ml) and the mixture allowed to cool at room temperature (~ 1 min). To the agar/medium mixture (3.0 ml) was added the cell suspension (~ 1.0ml) bringing the final agar concentration to 0.3%, and the final cell count to 2

x 10^5 cells ml^{-1}. To a 35 mm Petri dish (Gibco UK) containing C5637-conditioned medium (100µl) was added an aliquot (1.0 ml) of the cell-agar suspension. Culture plates were then incubated in the usual manner for 10-14 days until counting (figures 2.5 - 2.8 show colonies obtained using this method).

2.5.8 Counting procedure

Plates were counted between days 10 - 14 using a binocular inverted microscope and a movable plate-holding frame (Wilovert II Ph; Will Wetzlar) at x 40 magnification (low power). The entire surface of each plate was examined systematically and colony numbers scored on a hand held clockwork counter. When each plate had been examined the code for the experiment was broken.

2.5.9 Compounds tested using the bone marrow cell culture technique

2.5.9.1 Synthesis of amodiaquine-p-quinoneimine

The quinoneimine of amodiaquine was synthesized by Dr. J. Maggs (Department of Pharmacology and Therapeutics, University of Liverpool). Briefly, to amodiaquine (0.4g) in chloroform (90ml) was added silver oxide (1.0g), and this mixture was stirred over anhydrous Na_2SO_4 (0.2g) at room temperature (0.5h). The mixture was filtered through paper, and the solvent evaporated *in vacuo* at 30°C. The resulting orange product (~ 0.2g) was found to be > 95% pure on HPLC criteria (using the method of Maggs; unpublished), and was identified as amodiaquine-p-quinoneimine by mass spectrometry (Maggs, unpublished observations).

2.5.9.2 Preparation and storage of stock solutions

Stock solutions of amodiaquine and desethylamodiaquine were prepared as the hydrochloride salts in aqueous solution (1.0mg ml^{-1}); similarly the stock solution of chloroquine was prepared as the sulphate salt in aqueous solution (1.0mg ml^{-1}). Amodiaquine, desethylamodiaquine and chloroquine stock solutions were stored at -20°C until use. The stock solution of amodiaquine-p-quinoneimine was prepared from the material yielded by the above synthetic method; after weighing, it was dissolved in ethanol (2.0mg ml^{-1}), and was stored under nitrogen at -20°C until use. No attempt was made to sterilize any of the 4 concentrated stock solutions, and this led to no problems with infection.

On the day of an experiment, concentrated aqueous stock solutions were quickly thawed. Under laminar-flow conditions, and using sterile pipette tips, 100μM solutions of each compound were prepared in complete medium. These solutions, which were stored in sterile plastic containers wrapped in foil, were made up fresh at the beginning of the day for each experiment. Further dilutions of the 100μM stock were prepared, under aseptic conditions, by addition of further complete medium.

2.5.9.3 Purity of stock solutions

The purity of 1mg ml^{-1} stock solutions of amodiaquine, desethylamodiaquine and chloroquine was assessed by the HPLC method described earlier in this chapter. Under the conditions of storage, these solutions were stable giving single peaks after injection of aliquots onto the HPLC system.

Amodiaquine-p-quinoneimine was assessed by either TLC or HPLC (using the method of Maggs, unpublished) before use on each occasion. For assessment by TLC a droplet of 2mg ml^{-1} solution

applied to the plate (Kieselgel 60-ART 5748; Merck) and was developed in dichloromethane:methanol (80:20 v/v). Plates were examined under Ultraviolet light. Amodiaquine-p-quinoneimine proved to be more stable than anticipated, but stock solutions required replacement at roughly six week intervals.

CHAPTER 3 THE DISPOSITION OF ORAL AMODIAQUINE IN HEALTHY SUBJECTS

3.1 Introduction

Several strains of chloroquine-resistant *P. falciparum* have been shown significantly more susceptible to amodiaquine than chloroquine, both *in vitro* (Rieckmann 1971; Spencer *et al* 1983) and *in vivo* (Watkins *et al* 1984; Looareesuwan *et al* 1985). Although this effect is not common to all chloroquine-resistant strains of the parasite (Watt *et al* 1987), such observations led to increased use of amodiaquine both in the treatment and prevention of *P. falciparum* infections in areas of widespread chloroquine-resistance.

Although available since 1949 amodiaquine had not previously been as frequently used as chloroquine, which is slightly cheaper, and information on the drug's pharmacology was scant. Dose-schedules, both for treatment and prophylaxis, were based upon data obtained using non-specific analytical methods (Trenholme *et al* 1974; Ritschel *et al* 1978) and an assumption that the disposition of amodiaquine was likely to be similar to that of structurally-similar chloroquine (Bruce-Chwatt 1980). With the increasing use of amodiaquine for malaria treatment and chemoprophylaxis in the years 1983-1986, came the clinical need for pharmacological data to plan dose-schedules which would rapidly achieve effective blood concentrations of the drug in acute malaria, and provide the traveller with maximal protection, while avoiding toxic levels of the drug in both cases. Development of an HPLC method for the measurement of amodiaquine in plasma allowed Mihaly and colleagues (1985) to study the disposition of the drug in two subjects following I.V. administration. However the oral route was much more commonly used, and the disposition of amodiaquine needed to be described

after a single oral dose.

Although in therapeutically-used doses most drugs exhibit linear relationships between dose-size and plasma concentration, this is not universal. For those drugs with zero-order pharmacokinetics, changes in dose size may alter parameters such as terminal half-life, and at steady-state may cause disproportionate changes in plasma concentration which may lead to toxicity (see Chapter 1). Some evidence suggests that chloroquine may exhibit zero-order pharmacokinetics (Frisk-Holmberg et al 1979), and this possibility had to be considered for amodiaquine, a close structural analogue. Were amodiaquine to exhibit non-linear relationships this could have had clinical significance, since dose size varies from 5-10 mg kg^{-1} per day, in the case of malaria treatment, and from 300-600mg per week in the case of prophylaxis. Therefore, the effect of dose size on the pharmacokinetics of amodiaquine in healthy subjects needed to be studied.

3.2 Experimental Design

3.2.1 Single oral dose study

Seven healthy males (non-smokers, on no other drugs) aged 22-46 years were studied after giving written informed consent. After an overnight fast pre-dose samples of blood and urine were obtained, and amodiaquine (600mg; Camoquin; Parke-Davis) was given by mouth with 200ml water. Blood was sampled thereafter at 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 48, 72 and 96h. A single urine collection was made between times 0-24h in 6 subjects. In one subject 12 hourly urine collections were made, up to 48h, followed by 24 hourly collections up to 96h. Further

24h urine collections were then made between the following times: 7-8, 16-17 and 28-29 days.

3.2.2 Dose variation study

Six healthy males (on no other drugs) aged 22-46 years each received, in random order and after an overnight fast, amodiaquine 200, 400 and 600mg by mouth with 200ml water. The interval between doses was > 6 weeks. Blood was sampled pre-dose and at times 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 48, 72 and 96h. Urine was obtained pre-dose and continuous 24h collections were made up to 96h. A further 24h collection of urine was made one week after dosing in all subjects.

3.2.3 Sample handling

Blood (10ml) was collected into plastic lithium heparin tubes and was centrifuged (1500g; 15 min) within 1h of collection. Plasma was transferred to plain plastic tubes, the buffy coat discarded and erythrocytes retained. All biological fluids were stored at -20°C, protected from light by aluminium foil wraps.

3.2.4 Ethical considerations

Both single dose and dose variation studies were planned and conducted prior to the demonstration that amodiaquine-use for chemoprophylaxis causes a high prevalence of agranulocytosis (Hatton et al 1986). Both studies received approval from the Mersey Regional Ethics Committee.

3.2.5 Partitioning of ^{14}C-amodiaquine in whole blood in vitro

In order to study further the affinity of amodiaquine for blood cells, whole blood from 3 healthy volunteers was incubated with ^{14}C-amodiaquine. At the end of the incubation period the blood was centrifuged over density gradient medium, and the

radioactivity of the various fractions determined by liquid scintillation counting.

To fresh whole blood from 3 subjects (5.0ml) in plastic lithium heparin bottles was added ^{14}C-labelled amodiaquine (98% radiochemical purity; 0.2μCi; 50μl); samples were then incubated in a water bath (37°C; 0.5h). Aliquots of whole blood (0.5ml) were saved and the remainder of each sample was carefully layered onto density gradient medium (lymphoprep; 3.0ml) and then centrifuged (1800g; 15 min). Samples were then seen to be in 4 phases: lowermost red cells, density gradient medium containing low-density cells, a "buffy coat" of low-density cells, and supernatant plasma. Following seperation of the phases, aliquots of each, and of whole blood, were subjected to liquid scintillation counting by the method given in chapter 2.

3.2.6 Pharmacokinetic calculations

Pharmacokinetic parameters (Cmax, tmax, $t_{\frac{1}{2}}$, λz, AUC) were calculated as described in chapter 1. Urinary recovery of excreted amodiaquine to 96h was calculated by summation of excreted drug in each of the continuous 24h urine collections. Urinary recovery of desethylamodiaquine to 168h was calculated from the area under excretion rate *vs* time curves up to 168h. Renal clearance (Cl_R) of desethylamodiaquine was calculated from Ac/plasma AUC(o-t), where Ac was the urinary recovery of compound to time t.

3.2.7 Statistical analysis

Comparisons between the AUC values obtained from plasma, blood and red cells, were made by Student's t-test for paired samples, accepting $p < 0.05$ as significant. The relationships between amodiaquine dose and AUC for both parent drug and

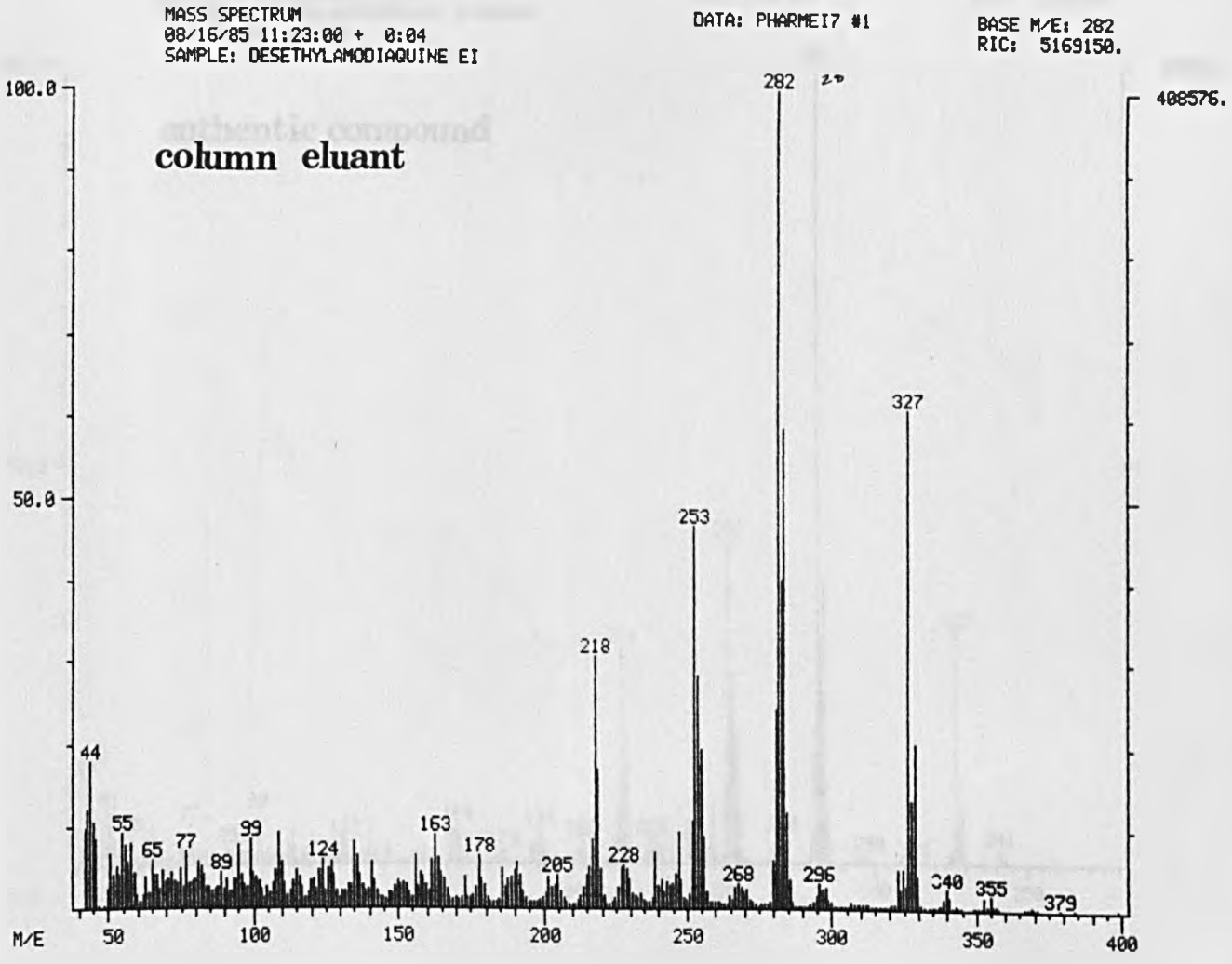


FIGURE 3.1 Mass spectrum obtained from the column eluant obtained by the methods referred to in Chapter 2 (2.2; page No. 86).

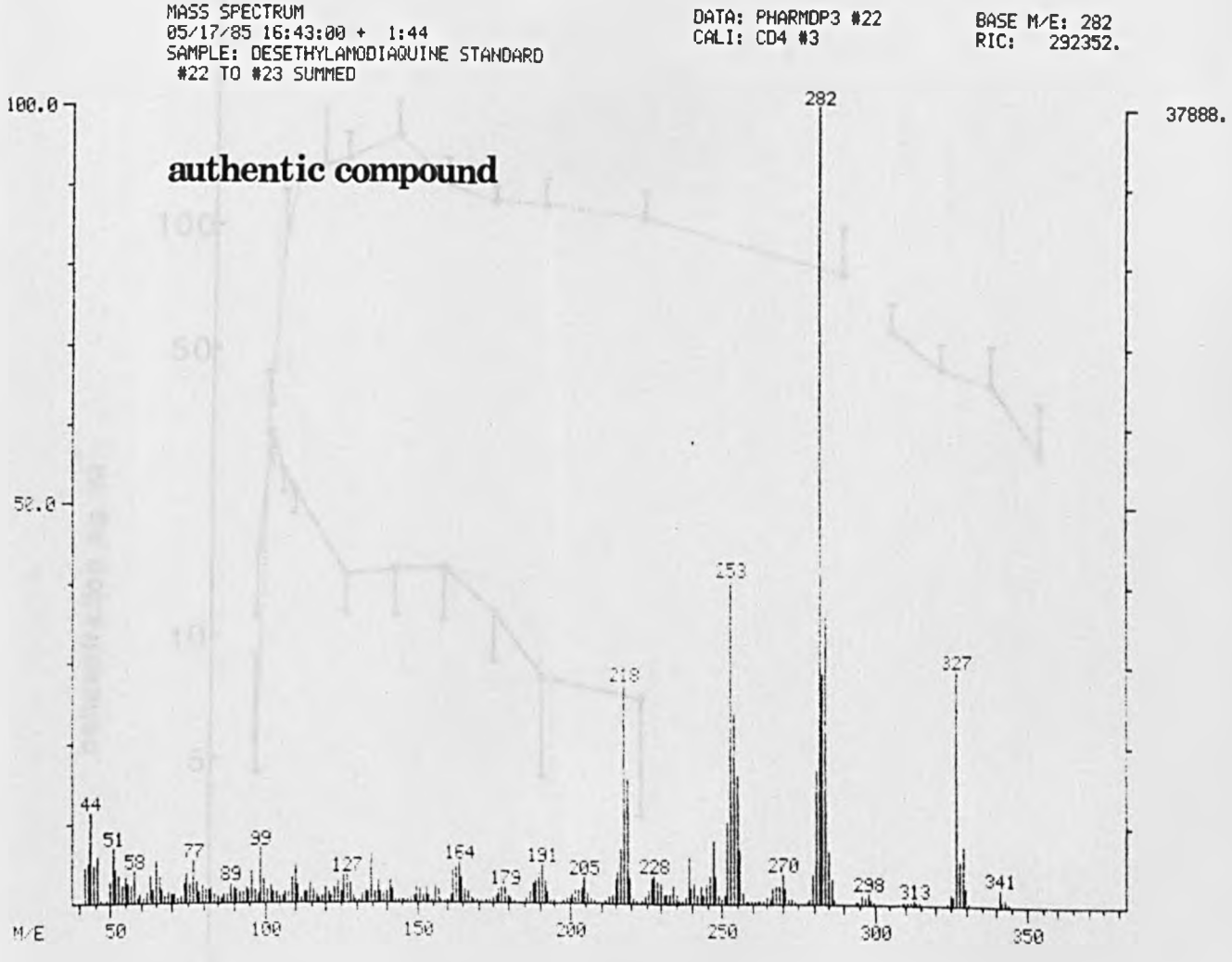


FIGURE 3.2 Mass spectrum of authentic desethylamodiaquine.

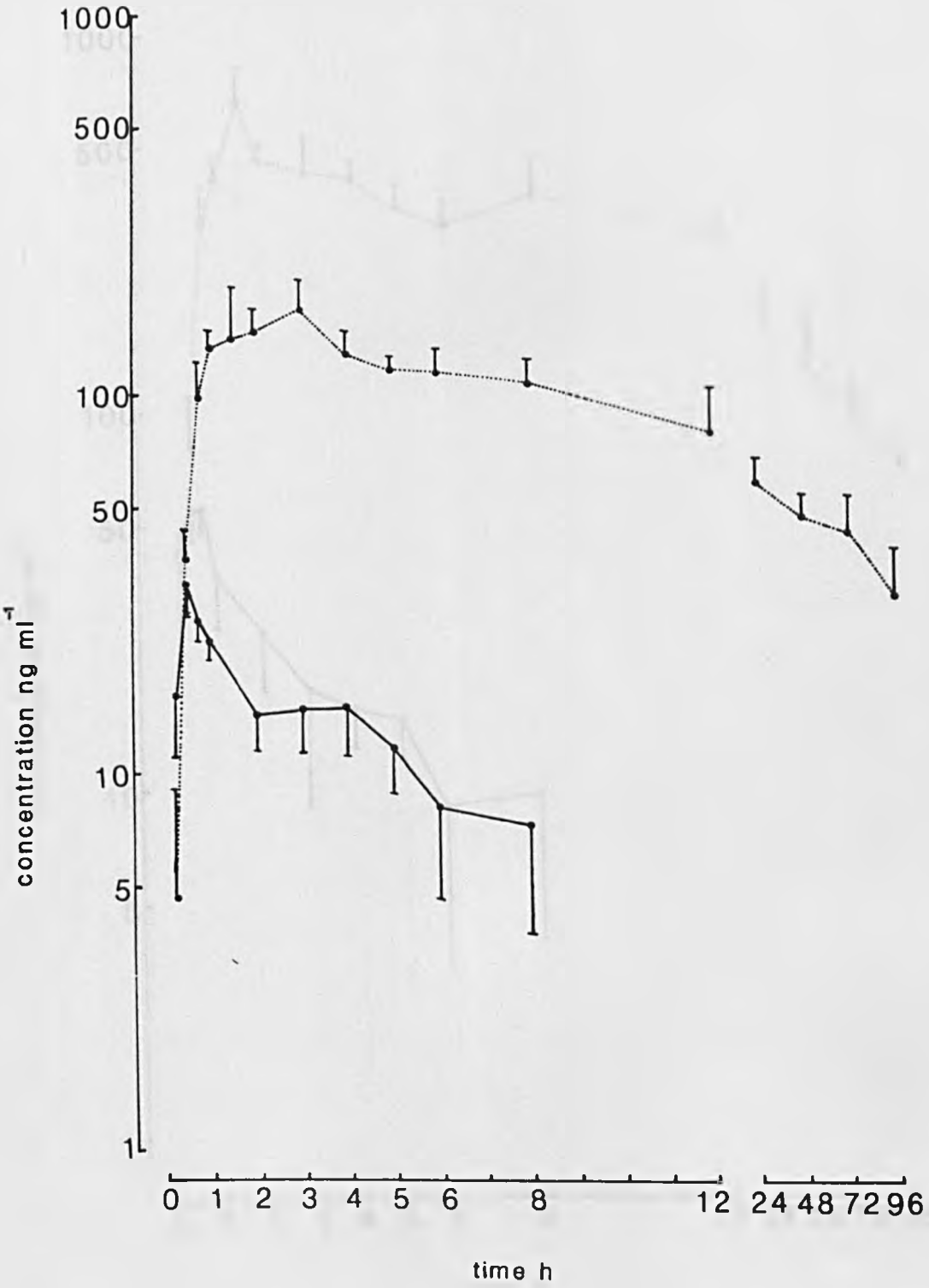


FIGURE 3.3 Plasma concentrations of amodiaquine (solid line) and desethylamodiaquine (dotted line) following oral amodiaquine (600 mg) (n = 7, $\bar{x} \pm$ SEM).

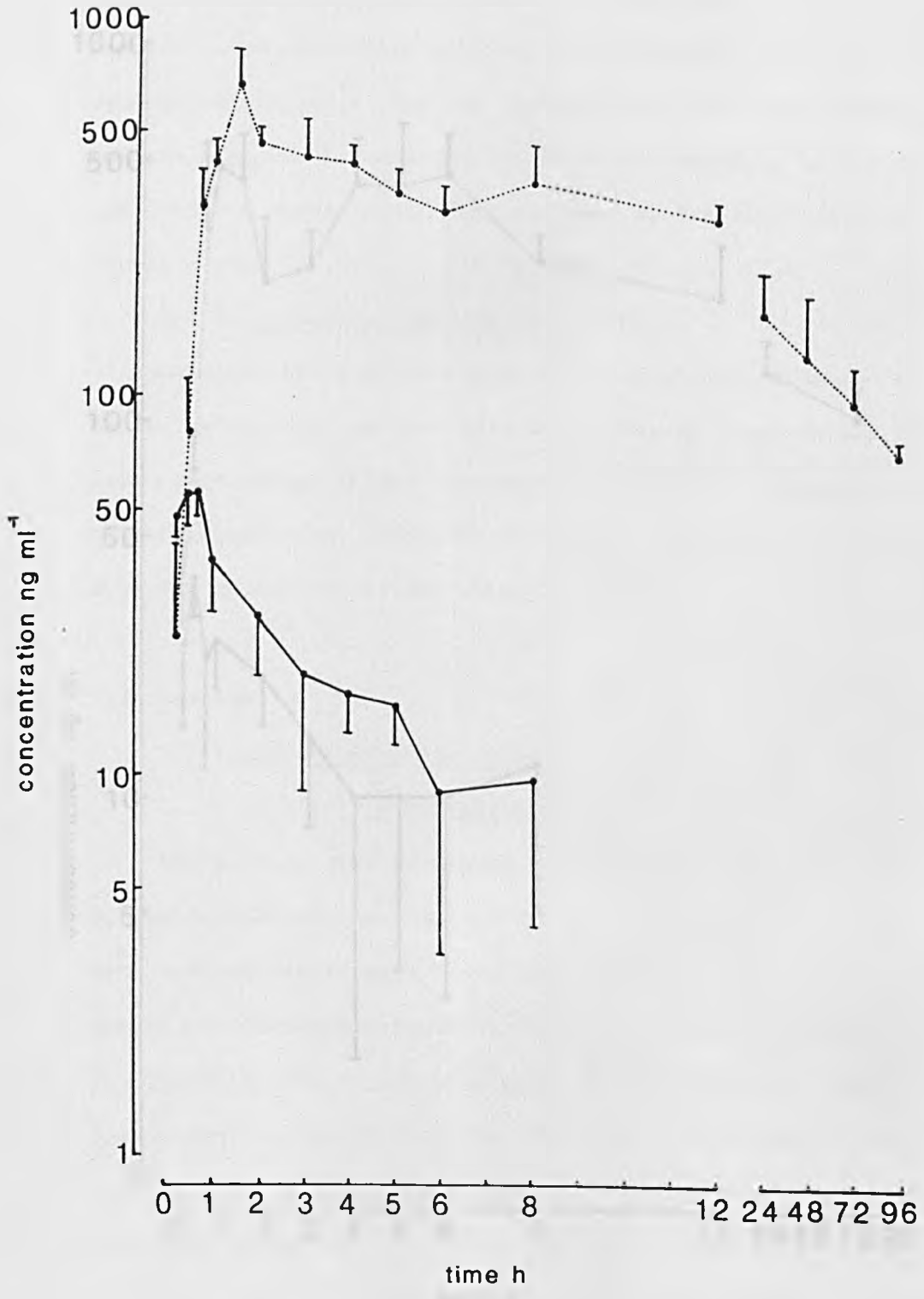


FIGURE 3.4 Whole blood concentrations of amodiaquine (solid line) and desethylamodiaquine (dotted line), following oral amodiaquine (600 mg) (n = 7; $\bar{x} \pm$ SEM).

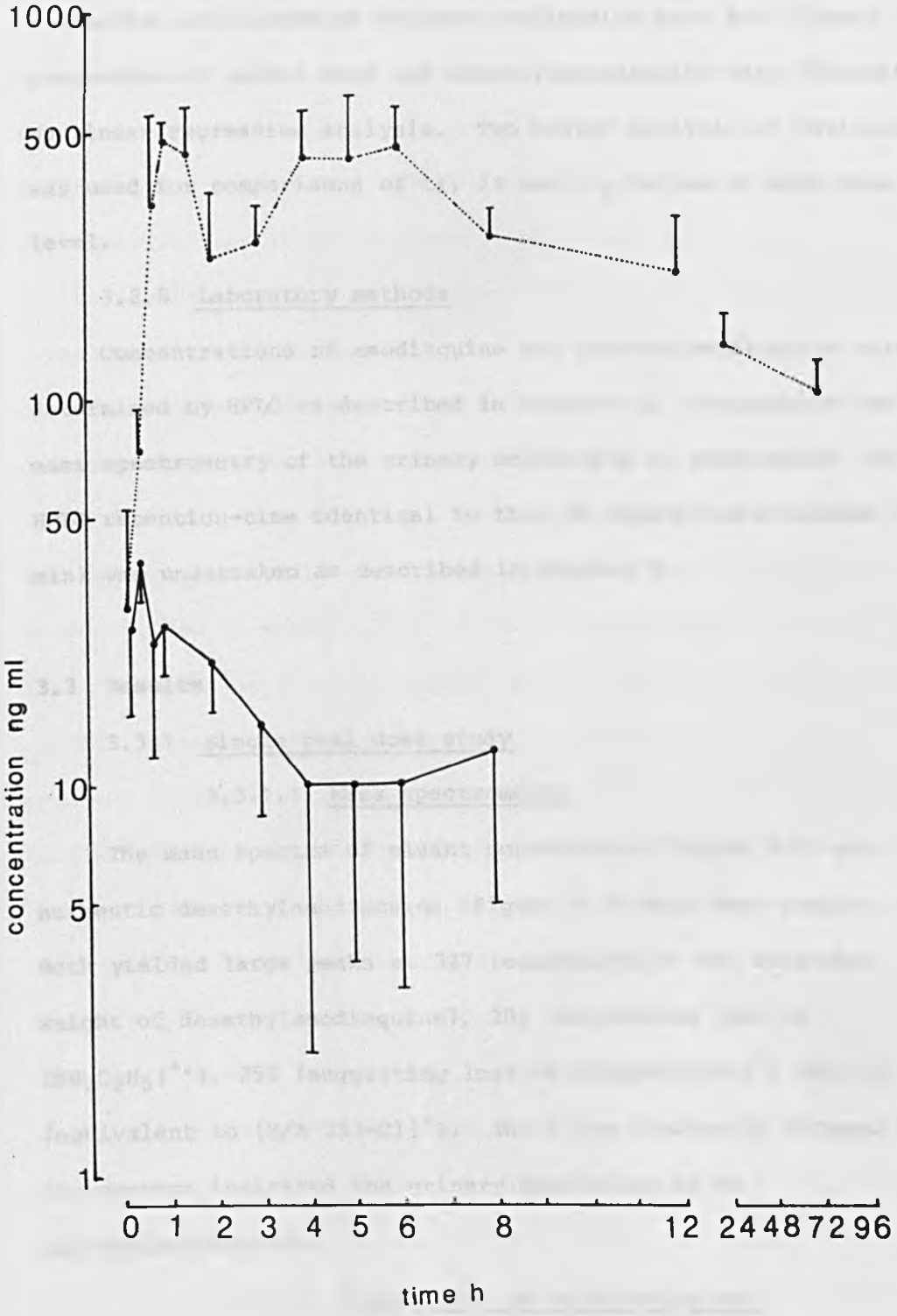


FIGURE 3.5 Red cell concentrations of amodiaquine (solid line) and desethylamodiaquine (dotted line), following oral amodiaquine (600 mg) (n = 7; $\bar{x}$ SEM).

metabolite were determined by linear regression analysis. Similarly, relationships between amodiaquine dose and urinary recoveries of parent drug and desethylamodiaquine were determined by linear regression analysis. Two factor analysis of variance was used for comparisons of $t_{\frac{1}{2}}$, λz and Cl_R values at each dose level.

3.2.8 Laboratory methods

Concentrations of amodiaquine and desethylamodiaquine were determined by HPLC as described in chapter 2. Preparation for mass spectrometry of the urinary metabolite of amodiaquine (with HPLC retention-time identical to that of desethylamodiaquine; 3.6 min) was undertaken as described in chapter 2.

3.3 Results

3.3.1 Single oral dose study

3.3.1.1 Mass spectrometry

The mass spectra of eluant concentrate (figure 3.1) and authentic desethylamodiaquine (figure 3.2) were very similar. Both yielded large peaks at 327 (equivalent to the molecular weight of desethylamodiaquine), 282 (suggesting loss of $[NH_2C_2H_5]^{+\bullet}$), 253 (suggesting loss of $[CH_2NHC_2H_5\text{-}O]^+$) and 218 (equivalent to $[M/Z\ 253\text{-}Cl]^+$). The close similarity between these two spectra indicated the urinary metabolite to be desethylamodiaquine.

3.3.1.2 Disposition of amodiaquine and desethylamodiaquine

Mean concentration *vs* time profiles for amodiaquine and desethylamodiaquine are shown in figures 3.3 to 3.5, and the pertinent pharmacokinetic estimates are listed in tables 3.1 and

3.2. Following oral administration amodiaquine underwent rapid absorption reaching peak concentrations in plasma, whole blood and red cells of 32 ± 3, 60 ± 10 and 42 ± 6 ng ml^{-1} (mean ± SEM) respectively, at 0.5 ± 0.03, 0.5 ± 0.1 and 0.5 ± 0.1h respectively. Thereafter the concentrations of amodiaquine in the circulatory phase declined rapidly, and amodiaquine was detectable for no longer than 8h. The apparent terminal $t_{\frac{1}{2}}$ of amodiaquine in plasma was 5.2 ± 1.7h.

Amodiaquine underwent rapid and extensive metabolism to desethylamodiaquine, peak concentrations of this metabolite being 181 ± 26, 561 ± 70 and 561 ± 143 ng ml^{-1} in plasma, whole blood and red cells respectively. Times to peak desethylamodiaquine concentration in plasma, whole blood and red cells were 3.4 ± 0.8, 2.2 ± 0.5 and 3.6 ± 1.1 h respectively. Thereafter concentrations of this metabolite in the circulatory phase fell slowly with apparent elimination rate constants (λz) of 0.015 ± 0.002, 0.016 ± 0.002, 0.015 ± 0.001 h^{-1} for plasma, whole blood and red cells respectively. The concentrations of desethylamodiaquine in plasma and whole blood at the end of the sampling period (96h) were 29 ± 8 and 74 ± 4 ng ml^{-1} respectively.

There were no significant differences between AUC (0,8) values for amodiaquine in plasma, whole blood or red cells (Figure 3.6). However AUC (0,24) for desethylamodiaquine in whole blood (6811 ± 752 ng ml^{-1} h) was significantly greater than that in plasma (2304 ± 37 ng ml^{-1} h; $p < 0.001$) (Figure 3.7). The whole blood to plasma concentration ratio for desethylamodiaquine was 3.1 ± 0.2.

Recovery of desethylamodiaquine from the 0-24h urine collections was 6.8 ± 0.8mg; small peaks could be seen corresponding to amodiaquine on HPLC traces, but were poorly

TABLE 3.1 Pharmacokinetic variables for amodiaquine following 600mg by mouth (Mean ± S.E.M.)

	tmax (h)	Cmax (ng ml^{-1})	$t_{\frac{1}{2}}$ (h)	AUC (0,8) (ng ml^{-1} h)	AUC (0,∞) (ng ml^{-1} h)
Plasma	0.5 ± 0.03	32 ± 3	5.2 ± 1.7	99 ± 19	154 ± 38
Whole Blood	0.5 ± 0.1	60 ± 10	N/D	148 ± 25	N/D
Red Cells	0.5 ± 0.1	42 ± 6	N/D	167 ± 72	N/D

(N/D = Not determined)

TABLE 3.2 Pharmacokinetic variables for desethylamodiaquine following 600mg amodiaquine by mouth (Mean ± S.E.M.)

	tmax (h)	Cmax (ng ml^{-1})	λz (h^{-1})	AUC(0,24) (ng ml^{-1} h)	AUC(0,∞) (ng ml^{-1} h)
Plasma	3.4 ± 0.8	181 ± 26	0.015 ± 0.002	2304 ± 37*	8037 ± 1383
Whole blood	2.2 ± 0.5	561 ± 70	0.016 ± 0.002	6811 ± 752*	20074 ± 3270
Red cells	3.6 ± 1.1	561 ± 143	0.015 ± 0.001	5713 ± 1269	20260 ± 4000

* p 0.001 Plasma:whole blood

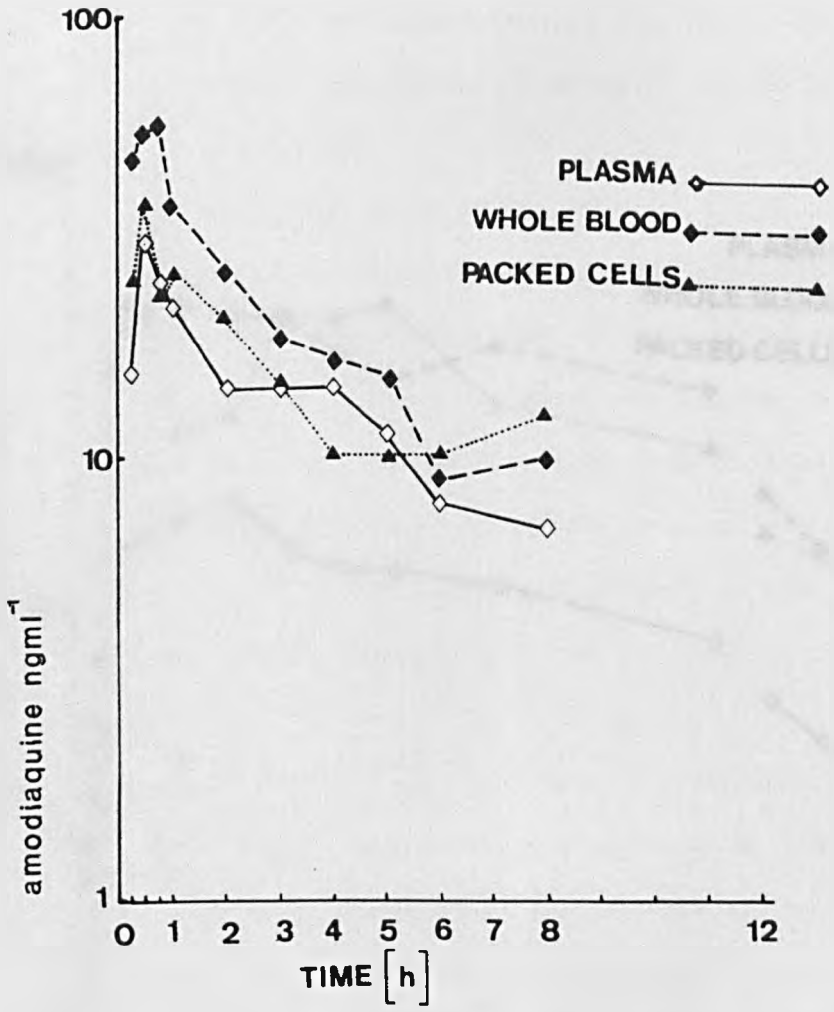


FIGURE 3.6 Mean plasma, whole blood and packed red cell concentrations of amodiaquine, following oral amodiaquine (600 mg).

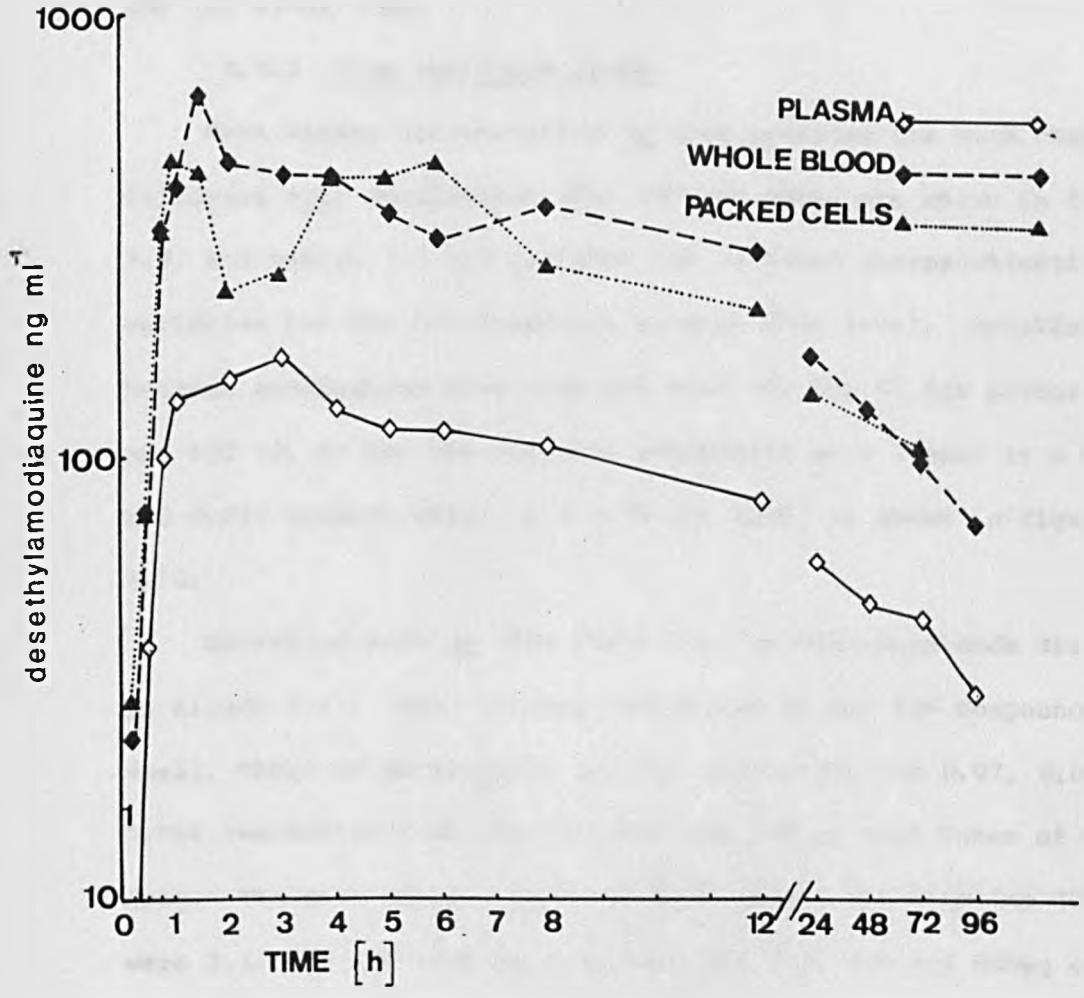


FIGURE 3.7 Mean plasma, whole blood and packed red cell concentrations of desethylamodiaquine, following oral amodiaquine (600mg).

resolved from the large desethylamodiaquine peaks, and could not be measured. In one subject timed urine collections had continued for 1 month. Figure 3.8 shows excretion rate *vs* time plots for both compounds from this subject; AUC (0,t) for amodiaquine was 3.0mg and for desethylamodiaquine was 45mg. It was possible to extrapolate the desethylamodiaquine curve, obtaining a value of AUC (0, ∞) of 57mg.

3.3.2 Dose variation study

Mean plasma concentration *vs* time profiles for both compounds following oral amodiaquine 200, 400 and 600mg are shown in figure 3.9, and tables 3.3 and 3.4 show the relevant pharmacokinetic variables for the two compounds at each dose level. Relationships between amodiaquine dose size and both AUC (0, 6) for parent drug and AUC (0, ∞) for the desethyl metabolite were linear ($r = 0.990$ and 0.972 respectively; $p < 0.05$ for both) as shown in figure 3.10.

Excretion rate *vs* time plots for for both compounds are shown in figure 3.11. Mean urinary recoveries of the two compounds were small, those of amodiaquine (0-96h) accounting for 0.07, 0.06 and 0.06% respectively of the 200, 400 and 600 mg oral doses of the drug. The mean urinary recoveries of desethylamodiaquine (0-168h) were 3.1, 9.6 and 15.6 mg following the 200, 400 and 600mg doses of amodiaquine. Relationships between amodiaquine dose size and urinary recoveries of parent drug (0-96h) and desethyl metabolite (0-168h) were linear ($r = 1.000$ and 0.999 respectively; $p < 0.01$ for both).

3.3.3 ^{14}C-amodiaquine concentrations in components of whole blood

Table 3.5 shows the recoveries of radioactivity from whole blood and its components expressed as percentage of the dose. Liquid scintillation counting of aliquots of whole blood from the

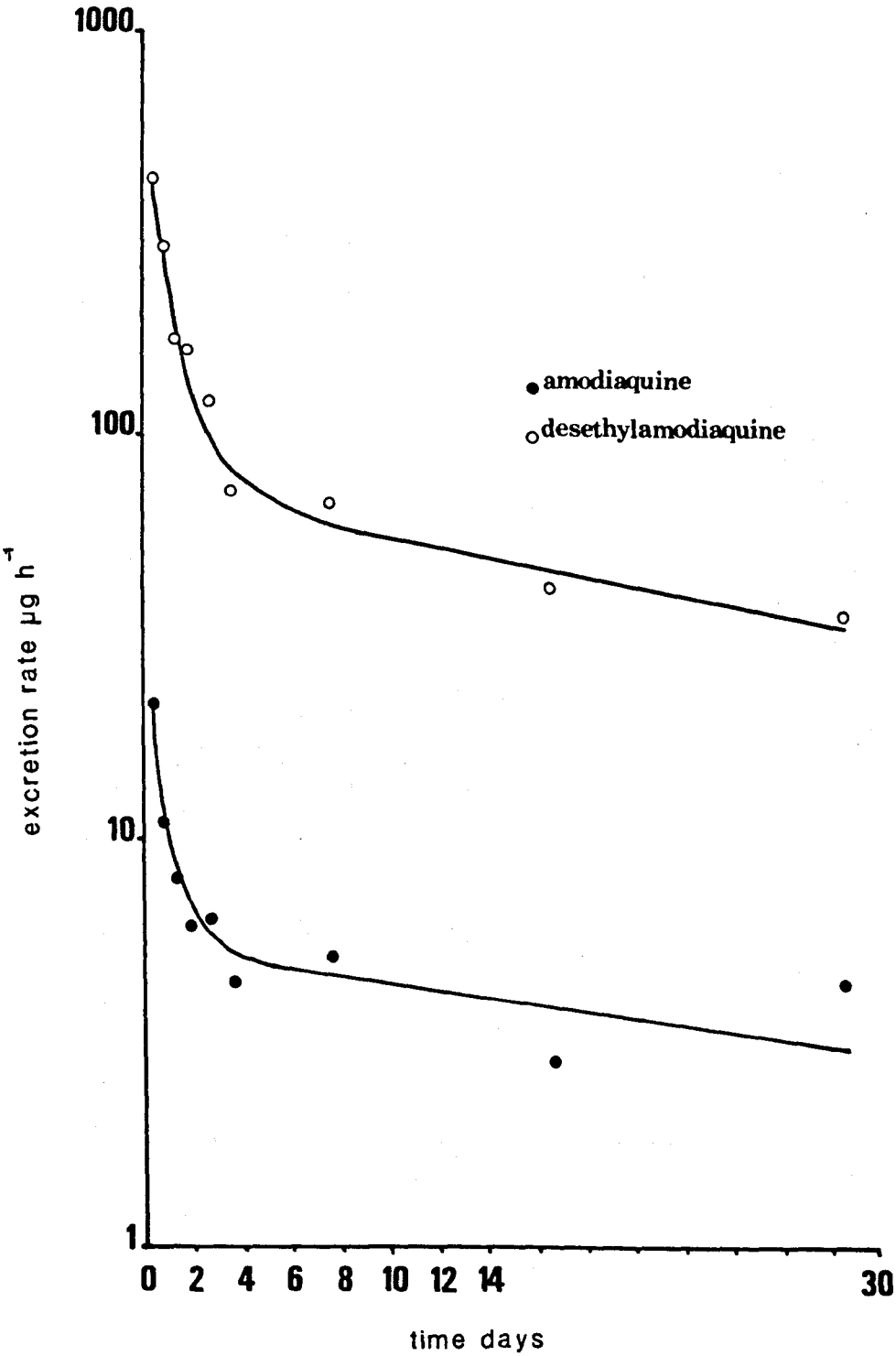


FIGURE 3.8 Urinary excretion rate of amodiaquine and desethylamodiaquine following oral amodiaquine (600 mg) (n = 1).

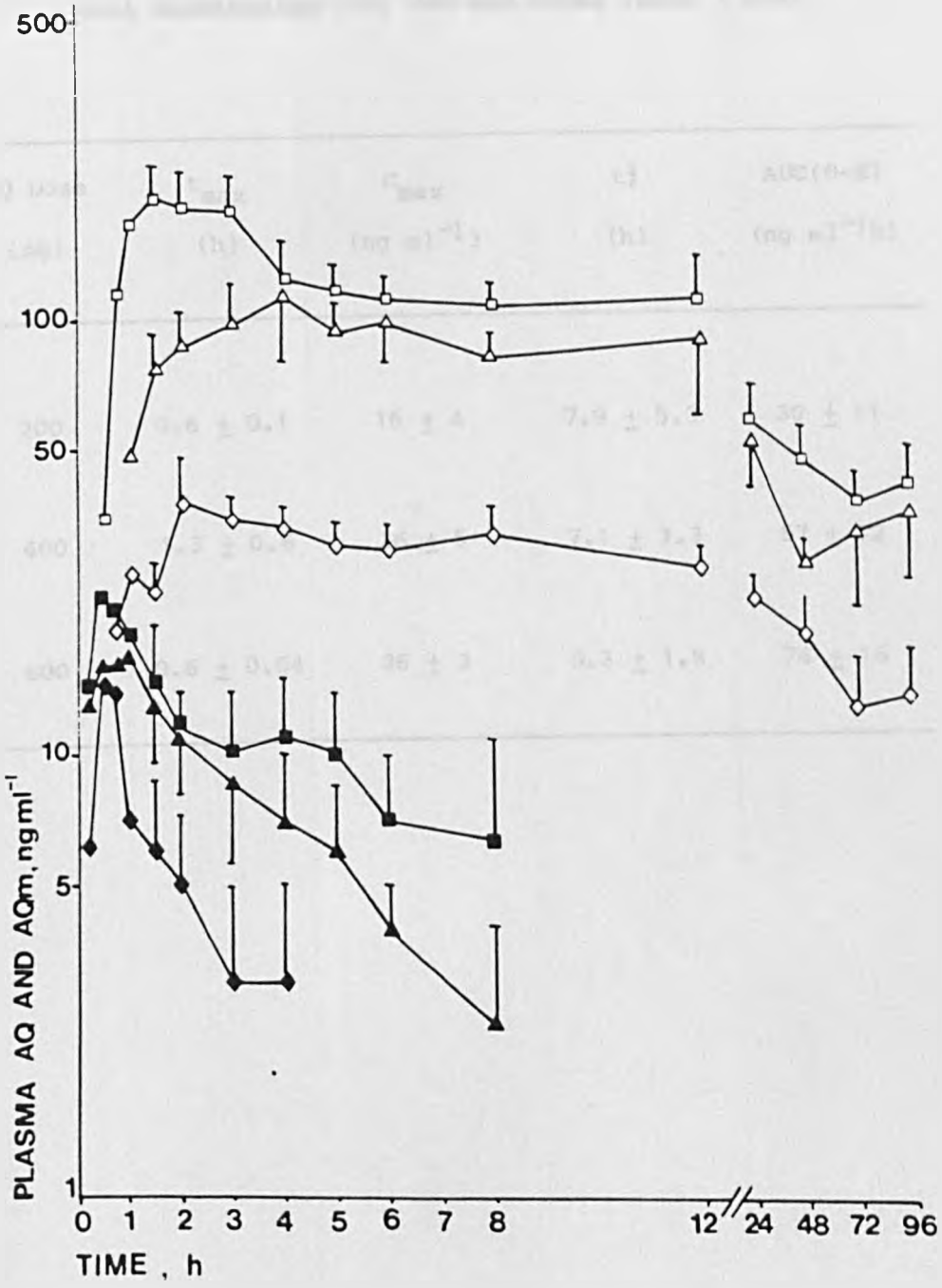


FIGURE 3.9 Plasma concentrations of amodiaquine (closed symbols) and desethylamodiaquine (open symbols) following amodiaquine 200 mg (diamonds), 400 mg (triangles) and 600 mg (squares);
(n = 6, $\bar{x}$ ± SEM).

TABLE 3.3 Pharmacokinetic variables for amodiaquine following oral amodiaquine 200, 400 and 600mg (mean ± SEM)

AQ Dose (mg)	t_{max} (h)	C_{max} (ng ml^{-1})	$t_{\frac{1}{2}}$ (h)	AUC(0-6) (ng $ml^{-1}h$)
200	0.6 ± 0.1	16 ± 4	7.9 ± 5.3	30 ± 11
400	1.3 ± 0.6	26 ± 5	7.1 ± 3.3	57 ± 12
600	0.6 ± 0.04	26 ± 3	5.3 ± 1.8	74 ± 16

TABLE 3.4 Pharmacokinetic variables for desethylamodiaquine following oral amodiaquine 200, 400 and 600 mg (mean ± SEM)

AQ Dose (mg)	t_{max} (h)	C_{max} (ng ml^{-1})	λz (h)	AUC(0, ∞) (ng $ml^{-1}h$)	Cl_R Lh^{-1}
200	5.5 ± 2	51 ± 7	0.013 ± 0.04	3300 ± 710	1.46 ± 0.1
400	5.0 ± 1	136 ± 27	0.011 ± 0.001	7520 ± 1090	1.07 ± 0.1
600	3.0 ± 1	204 ± 38	0.015 ± 0.002	9200 ± 2010	1.70 ± 0.2

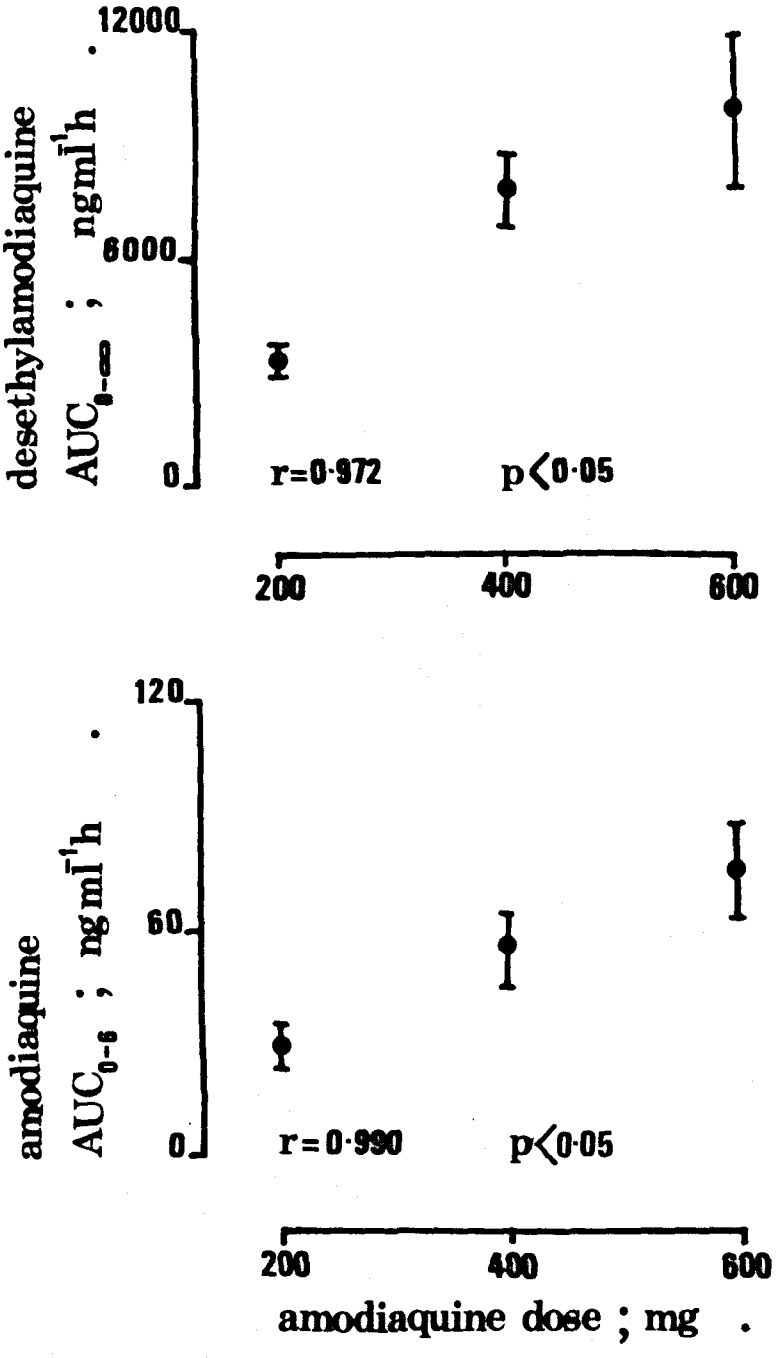


FIGURE 3.10 AUC vs dose relationships for amodiaquine and desethylamodiaquine, following oral amodiaquine 200, 400 and 600 mg ($\bar{x} \pm$ SEM).

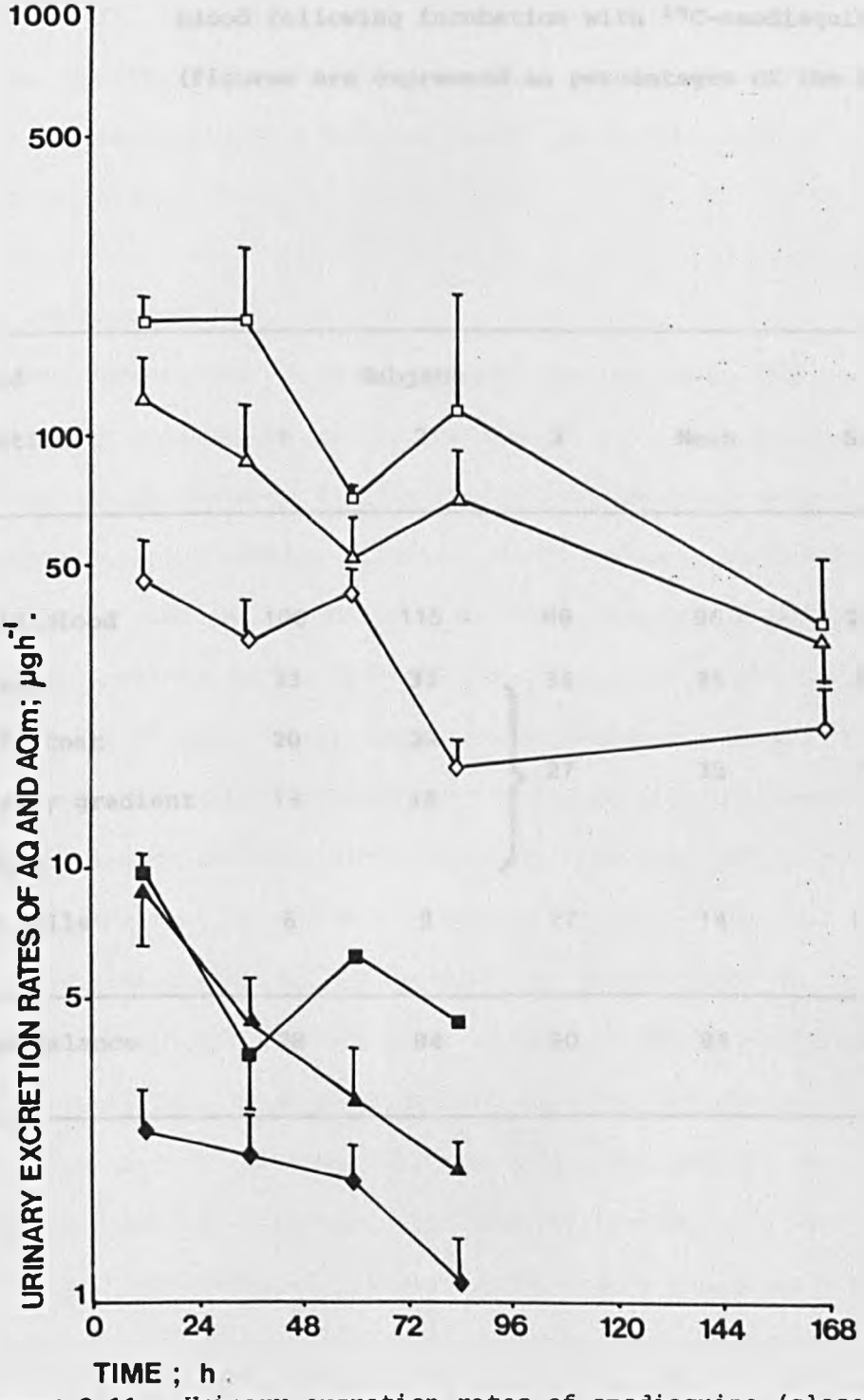


FIGURE 3.11 Urinary excretion rates of amodiaquine (closed symbols) and desethylamodiaquine (open symbols) (µg h^{-1}) following amodiaquine 200 mg (diamonds), 400 mg (triangles), and 600 mg (squares) (n = 6, $\bar{x} \pm$ SEM).

TABLE 3.5 **Recovery of radioactivity from fractions of whole blood following incubation with ^{14}C-amodiaquine (figures are expressed as percentages of the dose)**

Blood Fraction	Subject 1	2	3	Mean	SD
Whole Blood	106	115	68	96	25
Plasma	33	37	36	35	2
Buffy Coat	20	20	27	35	7
Density gradient medium	19	18			
Red cells	6	9	27	14	11
Mass Balance	78	84	90	84	6

3 subjects accounted for 96 ± 25% (mean ± SD) of the dose of radioactivity. Recovery of radioactivity from the blood fractions accounted for 35 ± 2%, 35 ± 7% and 14 ± 11% of the dose from plasma, low-density cells and red cells respectively; summation of these values gave a mass balance of 84 ± 6% of the dose of radioactivity.

3.4 Discussion

The current studies were designed and undertaken before the toxicity of amodiaquine was appreciated fully. Even so, none of the volunteers reached steady-state for the desethyl metabolite, and none reported adverse effects. Blood counts, checked during the current studies, showed no significant changes in peripheral neutrophil counts. Even in retrospect, the risk to the volunteers as a result of these current studies, was probably small.

Pharmacokinetic parameters for amodiaquine were summarised by Ritschel and colleagues (1978) based on data obtained from other workers (Berliner et al 1948; Trenholme et al 1974). The figures given suggested similarities between the dispositions of amodiaquine and chloroquine, such as long $t_{\frac{1}{2}}$ and high degree of bioavailability. In view of the effectiveness of the dose schedules used for amodiaquine, the widespread belief that the drug was relatively non-toxic and the low frequency of amodiaquine use up to 1983, detailed studies on the drug's disposition using specific assay methods, were not undertaken. An accurate description of the pharmacokinetics of amodiaquine became necessary initially because of the increasing popularity of the drug for malaria prophylaxis, and subsequently because of the demonstration of the drug's unacceptable toxicity when used for

this indication.

Although the present studies confirm that previously recommended chemoprophylactic dose-schedules for amodiaquine did not require major revision, substantial differences have been demonstrated between the pharmacokinetics of amodiaquine and those of chloroquine. After oral administration amodiaquine has been shown to be rapidly cleared from the plasma, with a short apparent $t_{\frac{1}{2}}$ (5.8h). However excretion of amodiaquine in the urine was slow, raising the possibility of a further elimination phase, with plasma concentrations below the lower limit of detectability for this assay (5 ng ml^{-1}). However, White and colleagues (1987) were able to detect plasma amodiaquine for up to 24h following IV infusion of the drug (10 mg kg^{-1}) in 10 subjects; the plasma concentration of the drug declined in a bi-exponential manner, and the $t_{\frac{1}{2}}$ of the apparent elimination phase was short (10h) with no measurable long terminal elimination phase. Furthermore using electrochemical detection to achieve a lower limit of detectable amodiaquine of 1 ng ml^{-1} Mount and colleagues (1987) describe blood concentrations of the drug similar to those in the present study; no estimate was given of $t_{\frac{1}{2}}$ for amodiaquine in this paper but may be calculated from the data given for one subject to be ~ 6.5h. If a long elimination phase is to be ascribed to amodiaquine in the plasma, an assay would be needed whose lower limit of detectability was considerably lower than those of current HPLC methods; furthermore since amodiaquine concentrations would be so low such a slow elimination phase of the unchanged drug is unlikely to be of practical importance.

After oral administration amodiaquine is rapidly metabolised, and 3 metabolites have been identified: desethylamodiaquine,

hydroxydesethylamodiaquine and bisdesethylamodiaquine (Churchill et al 1985 and 1986). In these present studies desethylamodiaquine has been identified by mass spectrometry as a major urinary metabolite. Desethylamodiaquine is known to possess antimalarial activity in vitro (Churchill et al 1985) but the other 2 identified metabolites probably have negligible activity (Mount et al 1987; Pussard et al 1987). After oral administration of amodiaquine, desethylamodiaquine rapidly appears in the plasma achieving much higher plasma concentrations than the parent drug; this observation has also been made by other workers (Churchill et al 1985; Mount et al 1987; Pussard et al 1986 and 1987). In some published work the parent drug has not been detected at all after oral administration, only desethylamodiaquine being seen (Salako et al 1985; Pussard et al 1987). This may reflect interindividual variation in drug disposition, or methodological problems.

It seems likely that desethylamodiaquine is responsible for much of the antimalarial effect achieved after oral administration of amodiaquine, since this metabolite is still detectable in the circulatory phase one week after dosing. However it is not strictly correct to refer to amodiaquine as a 'pro-drug' (Churchill et al 1985) since the parent drug has antimalarial activity (is indeed more active than desethylamodiaquine against certain strains of P. falciparum; Mount et al 1987), and probably does achieve an effective concentration in the circulatory phase for a short time after dosing.

Although peaks were seen to elute before desethylamodiaquine, in HPLC traces obtained from volunteer plasma samples, which may have represented the hydroxydesethyl or bisdesethyl metabolites of

the drug, these compounds were not measured in these studies. Neither has important antimalarial activity *in vitro* and both achieve blood concentrations much lower than those of desethylamodiaquine (Mount *et al* 1987). There have been no suggestions implicating either of these lesser metabolites of amodiaquine in the observed toxicity of the drug, and their routine measurement is likely to be unimportant. Amodiaquine-p-quinoneimine, an autoxidative breakdown-product of amodiaquine (Maggs *et al* 1987 *a* and *b*) cannot be detected using the HPLC methods described in this thesis, and plasma concentrations of this cannot be estimated. However it is likely that this reactive species would exist for only a very short time in aqueous solution, before forming adducts, or else undergoing reduction back to the parent drug. Attempts to measure this compound in the plasma would therefore be inappropriate.

Since desethylamodiaquine has not been given to volunteers, estimates of its $t_{\frac{1}{2}}$ are not possible, but the apparent elimination rate constant has been estimated. Interestingly this value (0.015 h^{-1}) is of the same order as the parameter given for amodiaquine by Ritschel and colleagues (1978) (0.014 h^{-1}), probably indicating that the non-specific assay method from which this figure was produced largely measured plasma desethylamodiaquine, and not its parent drug as had been assumed. From the data given by Mount and colleagues (1987) the elimination rate constant for desethylamodiaquine can be calculated to be 0.006 ± 0.003 h^{-1} (mean $\pm$ SD) from several profiles in one individual. However Pussard and colleagues (1987) measured desethylamodiaquine for 34 days after a single oral dose of amodiaquine (10mg kg^{-1}). These workers found that concentrations of the metabolite in the

circulatory phase declined in a polyexponential manner terminating in a slow elimination phase, the rate constant for which was ~ 0.002 h^{-1}; the authors felt able to estimate $t_{\frac{1}{2}}$ for desethylamodiaquine to be 9 - 18.2 days. This terminal phase of desethylamodiaquine elimination has not been assessed in this present study, but as pointed out by Pussard and colleagues (1987), only very small concentrations of the compound are present during this phase (< 35 ng ml^{-1}), and it has little effect on weekly dose schedules. Interestingly a similar slow elimination phase of desethylamodiaquine was seen in this present study in the urine of the one subject from whom collections were made up to 30 days (figure 3.8).

Chloroquine and desethylchloroquine concentrate in the cellular components of the blood (Bergquist and Domeij-Nyberg 1982). This present study has shown that desethylamodiaquine but not the parent drug appears to be similarly concentrated in blood cells. However one might expect that were desethylamodiaquine to accumulate in erythrocytes, then concentrations in this component of blood should be higher than those in whole blood. That this was not observed to be the case may reflect both the degree of plasma-trapping in the erythrocyte samples, and the possibility that desethylamodiaquine is maximally accumulated by leukocytes and platelets, as is the case for chloroquine (Bergqvist and Domeij-Nyberg 1982); leukocytes and platelets were present in whole blood samples but, following centrifugation, the buffy coat was removed from the surface of the erythrocyte fraction prior to storage and subsequent assay.

Radiolabelled desethylamodiaquine was not available, but the disposition of ^{14}C-labelled parent drug in whole blood *in vitro*

has been examined in the present studies. As seen in the volunteer studies amodiaquine failed to concentrate in the red cells, indeed the recovery of radioactivity from the red cells was under half that of plasma. However 35% of the administered radioactivity was observed to be present in the small number of low-density cells, composed largely of platelets and white cells. It seems likely that this affinity of amodiaquine for white cells and platelets is shared by the desethyl metabolite.

The 'target' of an antimalarial agent is the Plasmodium parasite. In the case of primary tissue schizontocides such as primaquine, effects are exerted within the hepatocytes, but in the case of 4-aminoquinolines such as amodiaquine and chloroquine, it is primarily the intra-erythrocytic blood schizont which is susceptible. The exact manner in which 4-aminoquinolines exert their toxicity on the parasite is controversial (see chapter 1) but their effect probably depends upon drug binding to part of the organism. The concentration of plasmodium-bound drug is likely to be in equilibrium with the free-drug concentration within the erythrocyte, which itself is likely to be in equilibrium with the total drug concentration within the erythrocyte. Although measurements of intracellular free-drug, or plasmodium-bound drug concentrations could be expected to most accurately predict the quantitative effect of the antimalarial, it is difficult to see how such measurements could easily be made. Consequently total erythrocyte-concentration of the drug is the closest possible approximation, and assuming that the drug is not subject to active transport, or irreversibly bound to its receptors (neither of which assumptions is necessarily true in the case of

4-aminoquinolines) this should provide a measure of the pharmacological effect.

The present study has demonstrated that amodiaquine exhibits linear pharmacokinetics within the clinically-used dose range. Relationships between dose of parent drug and AUC values for parent drug and metabolite were linear, and similarly the urinary recoveries of both amodiaquine and desethylamodiaquine displayed a linear relationship with the dose-size of the parent drug. Pharmacokinetic parameters such as $t_{\frac{1}{2}}$ for amodiaquine and λz for the metabolite showed no change with increasing dose size.

Urinary recoveries of both compounds were low. Peaks were to be seen in HPLC traces obtained from post-dose urine samples, eluting prior to desethylamodiaquine and these may have represented the bisdesethyl and hydroxydesethyl metabolites of amodiaquine, which were not measured. However it seems likely that the major route of elimination of amodiaquine and metabolites is biliary rather than renal, as is the case in the rat (see chapter 5, and appendix I).

3.5 Summary

1. Following oral administration of a single dose, amodiaquine achieves low concentrations in the circulatory phase, and is detectable for no longer than 8h.
2. Amodiaquine is rapidly converted to desethylamodiaquine which has lower clearance than the parent drug, and is detectable 96h post-dose.
3. The metabolite but not the parent drug, is concentrated in the cellular components of blood.

4. Amodiaquine exhibits first order pharmacokinetics within the dose range 200-600mg.

CHAPTER 4 The disposition of amodiaquine in malaria patients

4.1 INTRODUCTION

Like chloroquine and quinine, amodiaquine is a rapidly acting blood schizontocide, meaning that in acute malaria infections a rapid clinical cure can be achieved, and this may prove to be life-saving. Despite the high prevalence of toxicity observed when amodiaquine was used for malaria prevention (Hatton et al 1986), the drug has remained in use for the treatment of acute infections with chloroquine-resistant P. falciparum. Cross-resistance of such parasites between chloroquine and amodiaquine does occur, and while amodiaquine may prove useful in some parts of the world (Watkins et al 1984; Rieckman 1987) its clinical usefulness elsewhere is less certain (Khaliq et al 1987; Watt et al 1987). However in parts of sub-Saharan Africa where the transmission rate of P. falciparum is high, and especially in areas with much chloroquine resistance, such as East Africa, amodiaquine continues to be a relatively effective, cheap and apparently safe drug. Newer antimalarial compounds with activity against chloroquine-resistant P. falciparum, such as mefloquine and halofantrine, have the disadvantage of expense which may limit their usefulness. Therefore it may be that amodiaquine will continue to be used, in some parts of the tropics, for a while yet.

Malaria infection is known to perturb the disposition of several antimalarial drugs including quinine (White et al 1982), quinidine (Phillips et al 1985), chloroquine (Adelusi et al 1982), and possibly also mefloquine (Karbwang et al 1984); malaria infection might be expected to perturb the disposition of amodiaquine too. The assessment of such pharmacokinetic differences between healthy subjects and malaria patients can be difficult. The drugs preferentially employed in the treatment of this

infection are rapidly-acting, and therefore clearance of parasitaemia is often achieved within 48h of the commencement of therapy. Consequently not only is the patient's clinical condition improving over ~ 48h, but also the nature of his blood (in which drug concentrations are measured) changes over the same period of time, from heavy parasitaemia to complete clearance of parasites, and from high to lower concentrations of acute-phase proteins. Therefore, although malaria-induced perturbation of drug disposition may be relatively easily measured in the case of rapidly cleared drugs, such as the cinchona alkaloids, in the case of slowly-cleared drugs such as the 4-aminoquinolines such pharmacokinetic differences can be difficult to quantify. Nonetheless such studies are required in order to determine the effectiveness of the dose schedule employed, and in the case of amodiaquine, to determine whether or not there are significant differences in the disposition of the drug between healthy subjects and malaria patients which might help to understand why one group of subjects develops the observed toxicity, while the other seemingly does not.

4.2 EXPERIMENTAL DESIGN

4.2.1 Clinical study

This was undertaken by Dr. J.M. Kofi-Ekue (WHO team leader, Tropical Diseases Research Centre, Ndola, Zambia). Subjects presenting to Ndola Central hospital Zambia with febrile illness were screened by the examination of peripheral blood films for malaria parasites. Exclusion criteria included complicated malaria (WHO 1986), and refusal of written informed consent. Fourteen patients were recruited (12 male, 2 female; age range 15-55

TABLE 4.1 Patient details

	Age (Yr)	Sex	Weight (kg)	Height (cm)	Dose (g)
1	55	M	52	162	1.25
2	24	M	56	178	1.50
3	22	M	53	175	1.25
4	31	M	70	183	1.75
5	44	M	61	173	1.50
6	23	F	46	162	1.25
7	39	M	58	166	1.50
8	21	M	66	177	1.60
9	15	M	44	166	1.00
10	25	M	58	166	1.50
11	16	M	50	170	1.40
12	44	M	59	172	1.50
13	24	M	58	171	1.50
14	26	F	51	157	1.40

years; weight range 44-78kg). The organism was P. falciparum in 13 cases and P. malariae in 1 case. Patients received amodiaquine hydrochloride (Camoquin; Parke-Davis) by mouth in the following schedule: a loading dose of 10mg kg^{-1} (of the base) followed by 5mg kg^{-1} 6, 24 and 48h thereafter. Peripheral blood films were examined for P. falciparum daily up to day 7. Blood (5.0ml) was drawn, for estimation of drug concentrations, by venepuncture; the times of sampling were pre dose, 0.25, 0.5, 1, 2, 4, 6, 10, 12, 24, 28, 48, 52, 72, 120 and 168h.

4.2.2 Sample handling

Blood (5.0ml) was collected in plastic lithium heparin bottles and was centrifuged (2000g, 15min) within 10 minutes. Plasma was transferred to plain plastic tubes, and both plasma and packed cells were stored at -20°C, in the dark, until assay. The buffy coat was discarded. Samples were transferred from Zambia to Liverpool packed in dry ice.

4.2.3 Ethical considerations

Approval was obtained for the study from the Ethical Committee of the WHO Tropical Diseases Research Centre at Ndola, Zambia. All patients included gave written informed consent.

4.2.4 Calculations

Pharmacokinetic variables were calculated in the manner described in Chapter 2 of this thesis. Comparisons between the parameters obtained from this present study, and those obtained from the study on healthy subjects were made by Wilcoxon rank sum test for unpaired data.

4.2.5 Laboratory methods

Plasma concentrations of amodiaquine and desethylamodiaquine were determined by HPLC, using acetonitrile in a protein-precipitation step prior to extraction into diethyl ether, as described in Chapter 2 of this thesis. However, as related in Chapter 2, application of this method to the samples of packed red blood cells generated in this present study was unsatisfactory, since the red cell concentrations of both compounds in the samples from the early time points were often below the lower limit of detection. Analytical recovery of amodiaquine and desethyl-amodiaquine was improved, by adaptation of the HPLC method of Pussard and colleagues (1986) as related in Chapter 2 of this thesis, which permitted the detection of both compounds in parasitised red cells.

4.3 RESULTS

4.3.1 Clinical response

Asexual blood forms of the parasite were cleared in all 14 subjects (figure 4.1). Since blood films were examined only once daily parasite clearance rates can only be estimated; complete disappearance of asexual blood forms was achieved between 24 and 120h after the commencement of therapy, in the case of the 13 patients with P. falciparum infection, and by 48h in the case of the 1 patient with P. malariae infection. No cases of agranulocytosis or hepatitis were encountered, but mild adverse effects included pruritus, abdominal pain, headache and dizziness.

4.3.2 Disposition of amodiaquine and desethylamodiaquine

Mean concentration vs time profiles for amodiaquine and

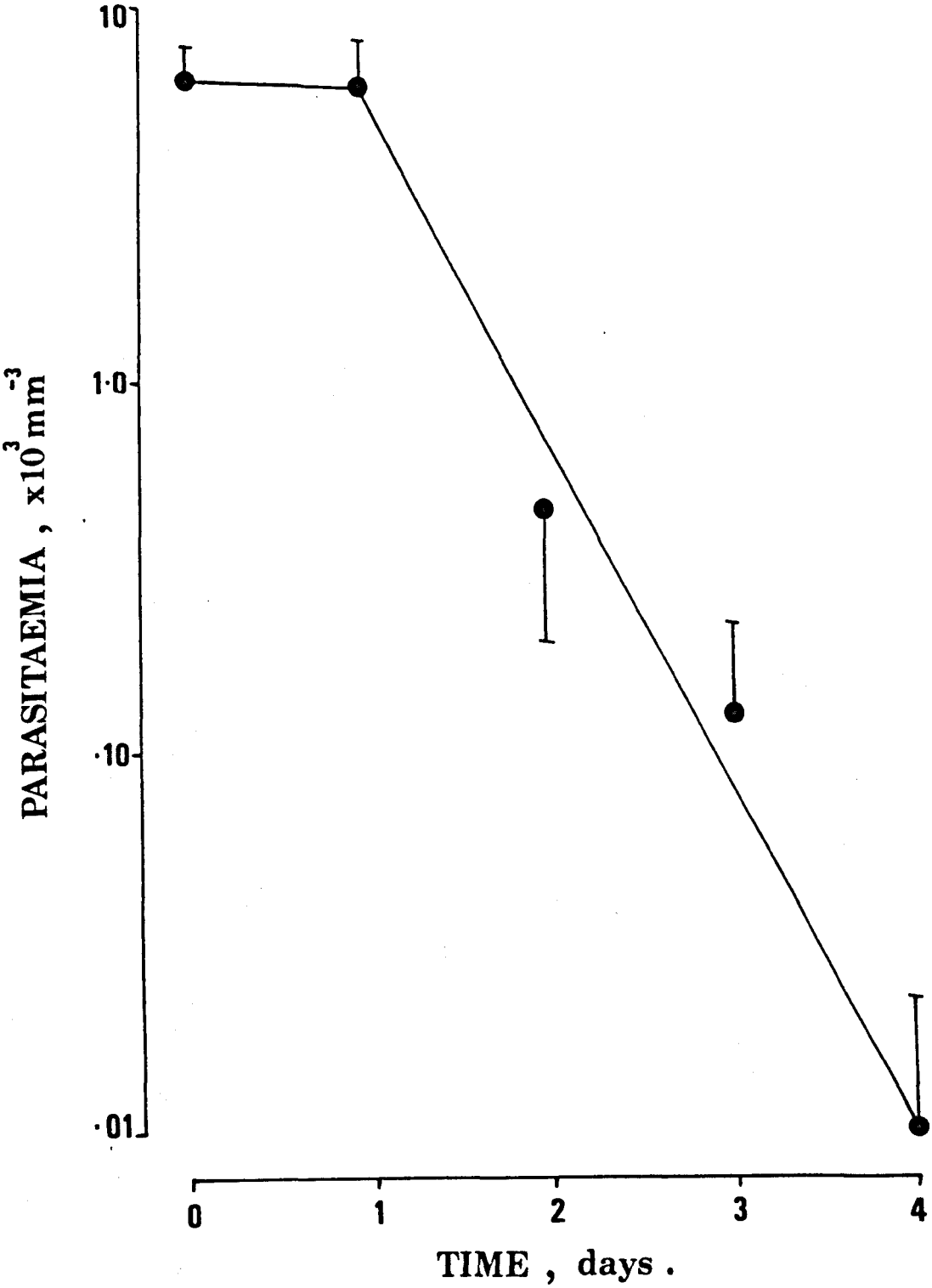


FIGURE 4.1 Clearance of peripheral blood asexual parasitaemia in 14 patients given oral amodiaquine ($\bar{x} \pm$ SEM)

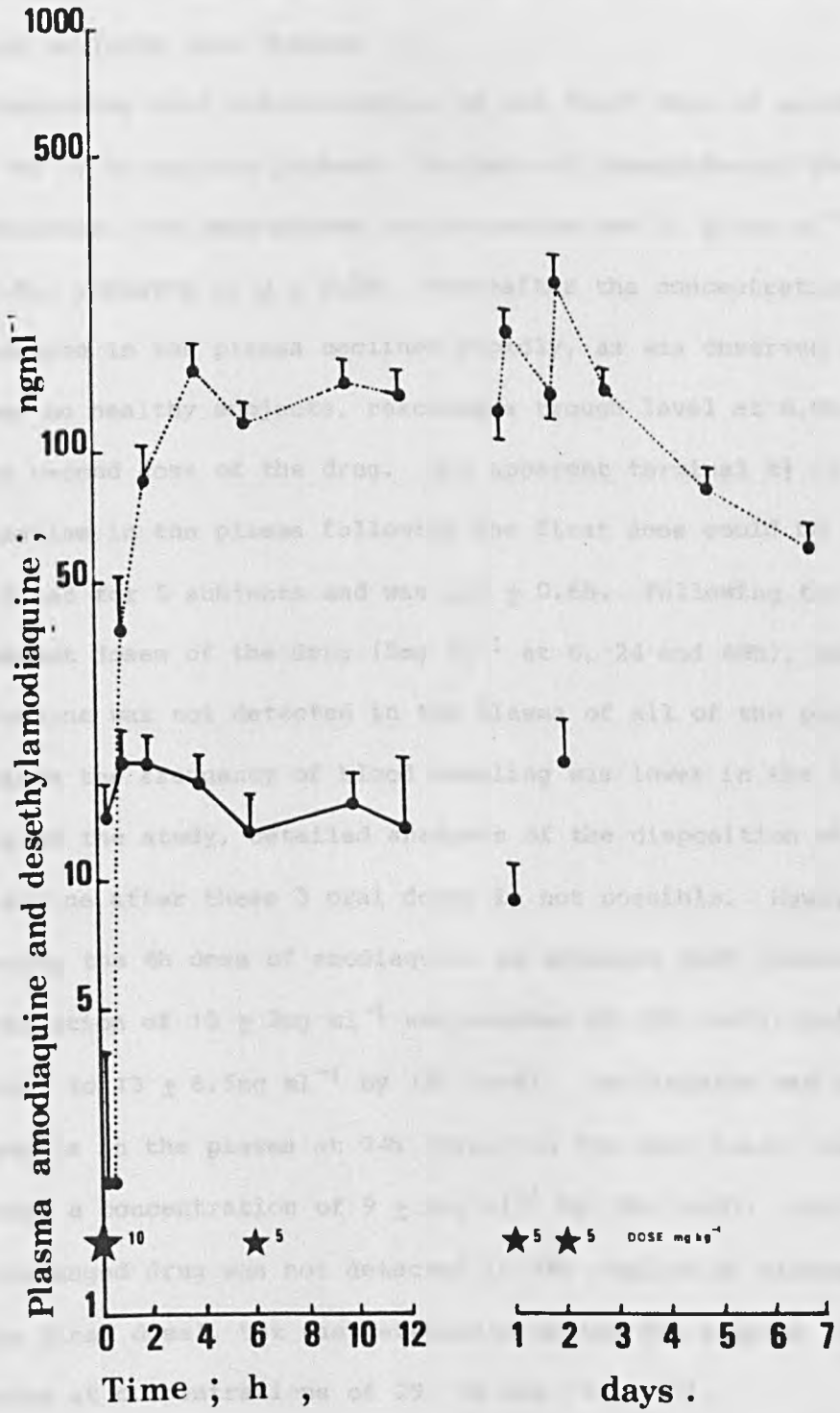


FIGURE 4.2 Plasma amodiaquine and desethylamodiaquine in malaria patients, following oral amodiaquine (10, 5, 5, 5 mg kg^{-1}) (n = 14, $\bar{x}$ ± SEM)

desethylamodiaquine in plasma (n=14) are shown in figure 4.2. Table 4.2 shows the pertinent pharmacokinetic variables derived from the plasma concentration data of this present study, and Table 4.3 contrasts these with the values obtained from the studies on healthy subjects (see Chapter 3).

Following oral administration of the first dose of amodiaquine (10mg kg^{-1}) to malaria patients the rate of absorption of the drug was variable; the peak plasma concentration was 21 ± 3ng ml^{-1} (mean ± S.E.M.) achieved at 2 ± 0.5h. Thereafter the concentration of amodiaquine in the plasma declined rapidly, as was observed in the studies on healthy subjects, reaching a trough level at 6.0h, prior to the second dose of the drug. The apparent terminal $t_{\frac{1}{2}}$ of amodiaquine in the plasma following the first dose could be calculated for 5 subjects and was 3.7 ± 0.6h. Following the subsequent doses of the drug (5mg kg^{-1} at 6, 24 and 48h), unchanged amodiaquine was not detected in the plasma of all of the patients, and since the frequency of blood sampling was lower in the later phases of the study, detailed analysis of the disposition of amodiaquine after these 3 oral doses is not possible. However following the 6h dose of amodiaquine an apparent peak plasma concentration of 15 ± 3ng ml^{-1} was reached at 10h (n=7), and had declined to 13 ± 6.5ng ml^{-1} by 12h (n=4). Amodiaquine was not detectable in the plasma at 24h (prior to the next dose), but achieved a concentration of 9 ± 2ng ml^{-1} by 28h (n=5); similarly the unchanged drug was not detected in 48h samples of plasma (prior to the final dose), but was detectable in the 52h samples of 3 subjects at concentrations of 29, 12 and 15ng ml^{-1}.

As had been observed in the studies on healthy subjects, amodiaquine underwent rapid, extensive conversion to desethyl-

TABLE 4.2 **Pharmacokinetic variables for amodiaquine and desethylamodiaquine, following oral administration of amodiaquine to malaria patients**

Amodiaquine	n	Dose $mgkg^{-1}$	tmax h	Cmax $ngml^{-1}$	AUC_{0-6} $ngml^{-1}$ h	$t_{\frac{1}{2}}$ h
Mean	14	10.1	2.0	21	77	3.7
SD		0.5	1.7	11	46	1.3

Desethylamodiaquine	n	Dose $mgkg^{-1}$	tmax h	Cmax $ngml^{-1}$	AUC_{0-6} $ngml^{-1}h$	λz h^{-1}
Mean	14	10.1	3.9	161	588	0.0115*
SD		0.5	1.2	72	267	0.0018

(*Data are from final elimination phase, between 52 and 168h)

amodiaquine, the peak plasma concentration of which was 161 ± 19ng ml^{-1} (following the first dose of amodiaquine), achieved at 3.9 ± 0.3h. Thereafter the plasma concentration of desethylamodiaquine fell slowly reaching an apparent trough level of 118 ± 13ng ml^{-1} by 6h (prior to the next dose of amodiaquine; 5mg kg^{-1}). By 10h an apparent peak plasma concentration of 146 ± 16ng ml^{-1} had been reached, and this fell to 123 ± 13ng ml^{-1} by 24h, prior to the next dose of amodiaquine (5mg kg^{-1}). At 28h an apparent peak plasma concentration of 194 ± 17ng ml^{-1} was achieved, and fell to 135 ± 14ng ml^{-1} by 48h, prior to the final dose (5mg kg^{-1}). By 52h a further apparent peak plasma concentration of desethylamodiaquine was reached at 251 ± 29ng ml^{-1}. Thereafter the plasma concentration of desethylamodiaquine fell slowly with an apparent elimination rate constant (λz) of 0.0115 ± 0.0005 h^{-1}; the concentration of this metabolite at the end of the sampling period (168h) was 60 ± 8ng ml^{-1}.

It is possible to compare certain of the pharmacokinetic variables obtained from the plasma concentration vs time data following the first dose of amodiaquine in this present study (i.e. up to 6.0h - immediately prior to the next dose) with those obtained following a single dose of amodiaquine to healthy volunteers (see Chapter 3 of this thesis). The doses of amodiaquine (in mg kg^{-1}) given to the two groups of subjects differed, being 8.1 ± 1.5mg kg^{-1} for the healthy subjects and 10.1 ± 0.5 mg kg^{-1} for the malaria patients (Table 4.3); however within this dose range amodiaquine has been shown to exhibit first-order pharmacokinetics (Chapter 3), and simple adjustment for dose enables a direct comparison to be made of pharmacokinetic variables between the two groups. As can be seen from table 4.3 the time

	Amodiaquine					Desethylamodiaquine				
n	Dose $mgkg^{-1}$	tmax h	Cmax $ngml^{-1}$	AUC_{0-6} $ngml^{-1}h$	$t_{\frac{1}{2}}$ h	n	Dose $mgkg^{-1}$	tmax h	Cmax $ngml^{-1}$	AUC_{0-6} $ngml^{-1}h$	λz h^{-1}
Malaria Patients											
14	10.1	2.0	21	77	3.7	14	10.1	3.9	161	588	#.0115
Healthy Subjects											
7	8.1	0.5	32	85	5.2	7	8.1	3.4	181	732	.0148
Significance											
		$p<0.01$	NS	NS	NS			NS	NS	NS	NS

(* NS = Not significant)

(# Data are from final elimination phase, i.e. 52-168h)

TABLE 4.3 **To compare pharmacokinetic variables from healthy subjects, and malaria patients (mean data)**

taken to reach the maximum plasma concentration (tmax) of unchanged drug was significantly longer in the malaria patients ($p < 0.01$), but there were no other significant differences between the two groups of subjects (Cmax and AUC_{0-6} values were adjusted for dose-size prior to statistical analysis). It was not possible to calculate the elimination rate constant (λz) of the desethyl metabolite, following the first dose of amodiaquine to malaria patients, since there were generally only two data points between the maximum plasma concentration achieved after the first dose of drug and the second dose (at 6.0h). However λz could be calculated from post-absorption and distribution phase data following the final dose of amodiaquine i.e. 52-168h. As can be seen from table 4.3, this value (0.0115) did not differ significantly from that obtained from healthy subjects (0.015).

Figure 4.3 shows the mean packed red cell (RBC) concentrations of desethylamodiaquine obtained from 7 malaria patients, plus mean plasma data from the same subjects, and relates these to the levels of parasitaemia. Figure 4.4 shows the RBC/plasma concentration ratios of desethylamodiaquine in these 7 subjects on day 1, when parasitaemia was $5.33 \pm 2.3 \times 10^3$ asexual forms per mm^3, and on the last time-point available from each subject (day 5 in 2 cases, day 7 in 5 cases) when there were no asexual forms of the parasite in the peripheral blood. Figure 4.5 shows changes of parasitaemia, and RBC/plasma ratios over time for these 7 subjects. The RBC/plasma ratios of desethylamodiaquine were significantly lower ($p < 0.05$) at the first time point (0.9 ± 0.5) than the final time point (2.8 ± 1.8). In contrast, the previous study on healthy subjects (Chapter 3) showed that RBC/plasma ratios did not alter significantly over the time course of the study being 2.5 ± 1.3 on

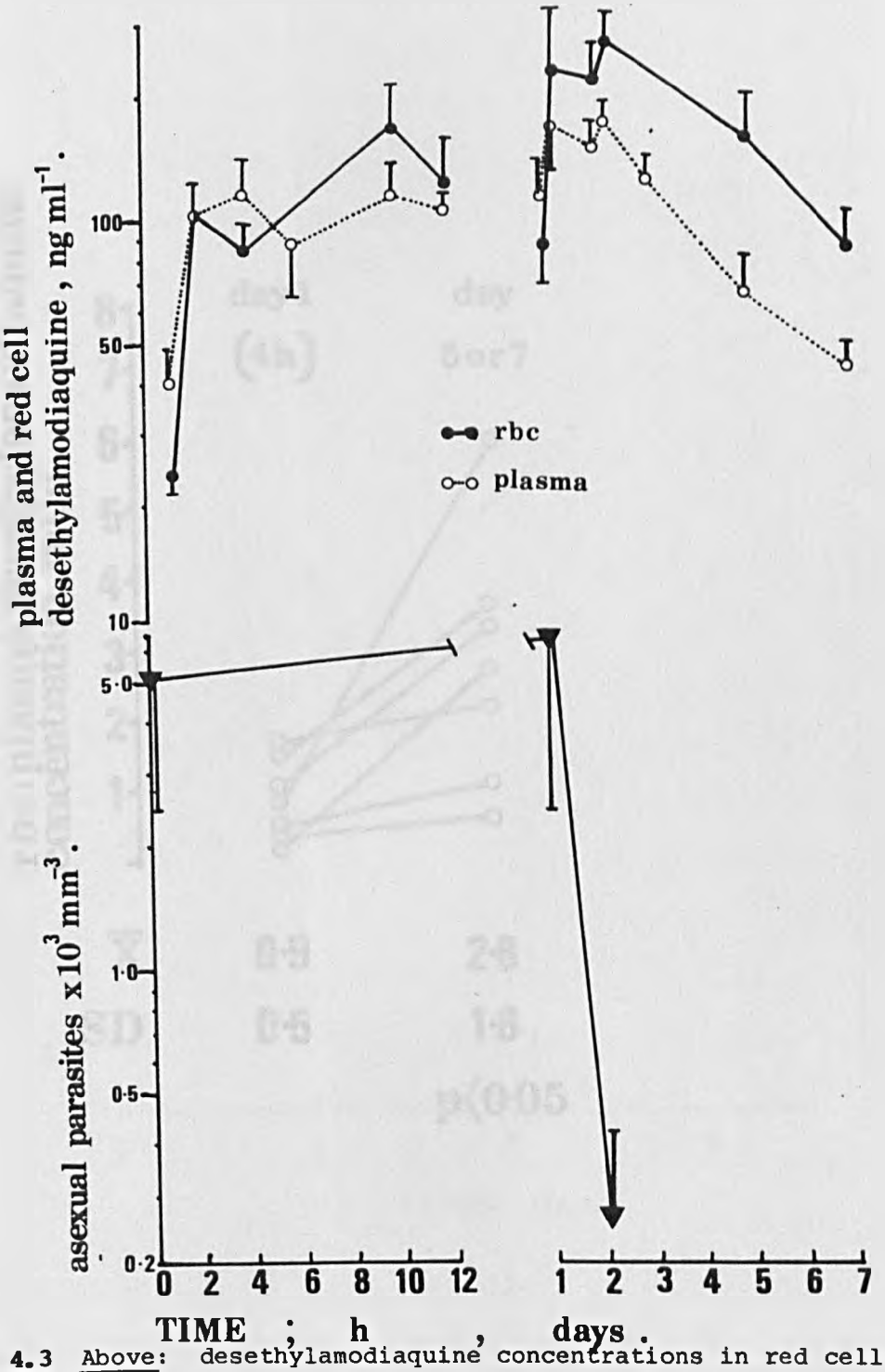


FIGURE 4.3 Above: desethylamodiaquine concentrations in red cells and plasma from malaria patients (n = 7, $\bar{x} \pm$ SEM).

Below: Parasitaemia in the same subjects (n = 7) $\bar{x} \pm$ SEM).

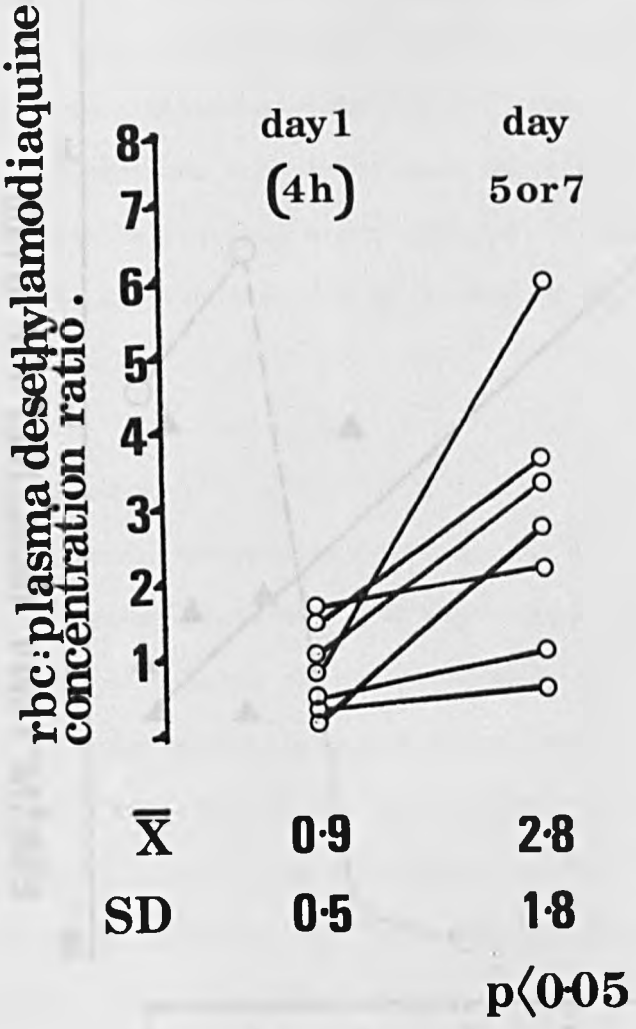


FIGURE 4.4 Desethylamodiaquine RBC: Plasma concentration ratios in malaria patients, on day 1 (n = 7) and either day 5 (n = 2) or 7 (n = 5) of amodiaquine therapy

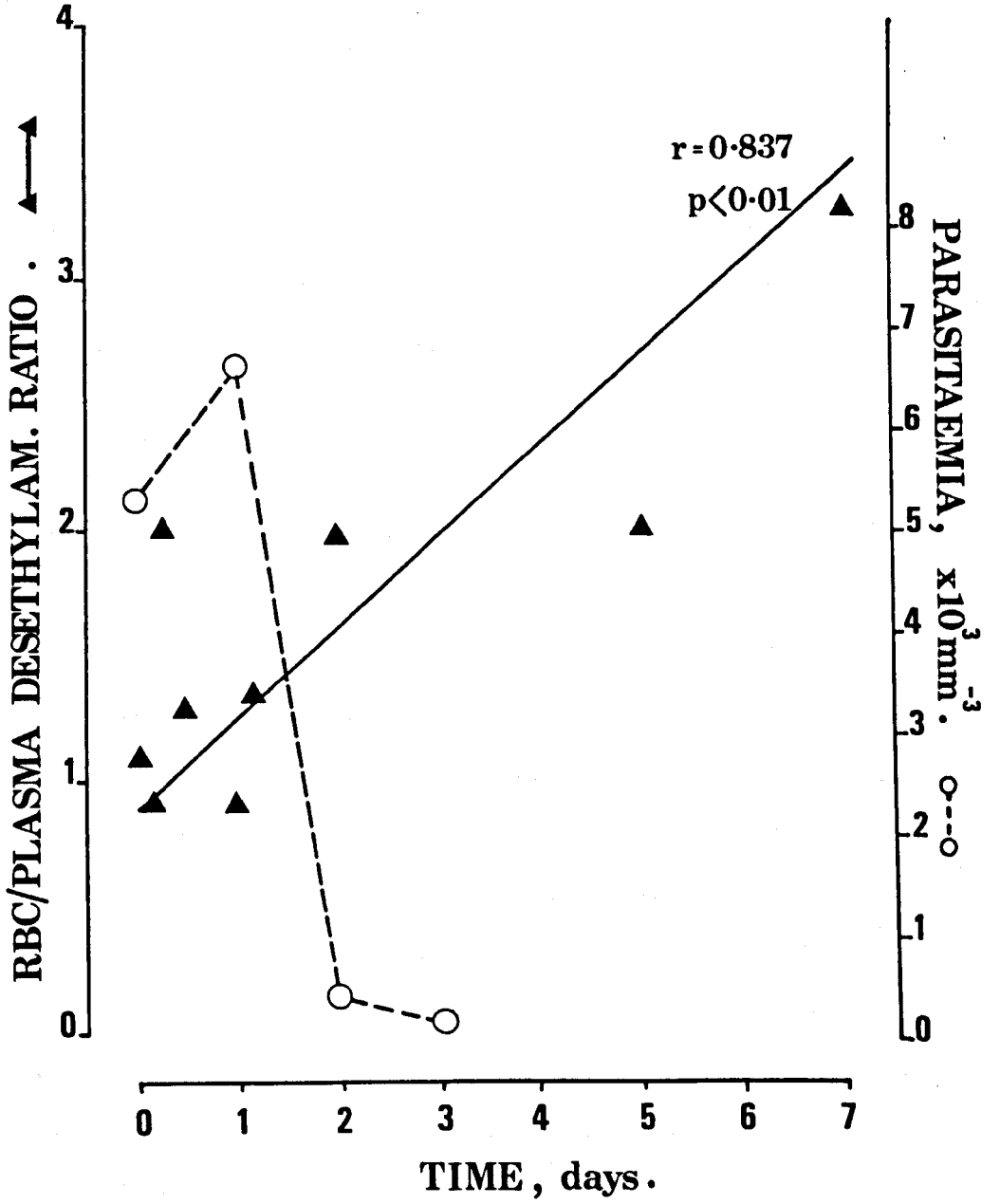


FIGURE 4.5 Relating changes in mean RBC: Plasma desethylamodiaquine concentration ratio to mean peripheral blood parasitaemia (n = 7)

1 and 3.8 ± 2.7 on day 3. RBC concentrations of the parent drug were detectable at few time points in any of the malaria patients, and in none of the samples from the later stages of the study, when parasitaemia had been cleared. Consequently comparisons between time points of high and zero parasitaemia, of RBC/plasma concentration ratios for amodiaquine, cannot be made. However since in general RBC amodiaquine concentrations were below the assay's limit of detectability in this present study, it seems likely that these concentrations were lower than those measured in the RBC samples from the previous study (Chapter 3), where the RBC concentrations of amodiaquine were roughly equal to those measured in the plasma.

4.4 DISCUSSION

Although amodiaquine was used widely as a chemoprophylactic agent for a brief time, the drug's principal use has been the treatment of falciparum malaria, a disease which can prove fatal, especially to children, pregnant women, and non-immune visitors. Like quinine and chloroquine, amodiaquine is a rapidly-acting blood schizontocide, but is more pleasant to take orally than either since it lacks a bitter taste - a characteristic which has led to amodiaquine being tolerated well by children. However amodiaquine is slightly more expensive than chloroquine (although cheaper than many antimalarials, probably including mefloquine), which has led to its less frequent use. The appreciation of the high risk of agranulocytosis and hepatitis associated with the prophylactic use of amodiaquine (Hatton et al 1986) does not seem to have affected the use of the drug for treatment of chloroquine-resistant falciparum malaria. However such advantages that amodiaquine has

over chloroquine in this setting, seem to be geographically variable; in East and West Africa amodiaquine is still used with good results (Watkins et al 1984; Walker, personal communication), while in the Philippines the drug is less effective than chloroquine (Watt et al 1987). The role of amodiaquine in the treatment of chloroquine-resistant falciparum malaria in the future is not certain, but it seems unlikely that it will be of great importance for very long.

Even so at the time of this present study amodiaquine was in fairly widespread use. The dose schedules were based empirically upon clinical response, and there were no pharmacokinetic data for the drug in malaria patients. Given that acute malaria can perturb drug disposition (White et al 1982; Adelusi et al 1982; Phillips et al 1985), such pharmacokinetic data as was available from studies on healthy subjects seemed unlikely to be of use. Consequently, the disposition of multiple doses of amodiaquine in patients with acute malaria (due to P. falciparum in nearly all cases) has been studied.

Plasma concentration vs time profiles of amodiaquine and desethylamodiaquine, in this present study, closely resembled those from the studies on healthy subjects (Chapter 3). There were certain limitations upon the design of this present study which have effected pharmacokinetic analysis: (a) the need for multiple doses of the drug in order to ensure cure, (b) the need for the second dose of drug to follow 6h after the first, (c) the need to reduce to a minimum the number of blood samples to be drawn from patients. It has however, been possible to compare pharmacokinetic variables obtained from plasma concentration vs time curves for amodiaquine and desethylamodiaquine following the first dose of

drug in this present study, with those obtained from studies on healthy subjects. Such parameters have often needed to be adjusted to account for the variation in dose (as a function of dose per unit body weight). No statistically significant differences were found between the two groups of subjects, other than the higher tmax value for amodiaquine found in this present study. This apparent delay in the absorption of amodiaquine by malaria patients may be because of delayed gastric emptying. However this finding is probably of little or no clinical significance, since the time taken for desethylamodiaquine to achieve its maximal plasma concentration was not significantly longer in this present study, and since the concentration of this active metabolite is an order of magnitude higher than that of its parent drug, the slower absorption of the latter would not have delayed onset of antimalarial efficacy.

Amodiaquine concentrations in the RBC samples from this current study could not be determined at most time points; RBC concentrations of desethylamodiaquine on the other hand could be measured, and there were major differences in the disposition of this active metabolite in the RBCs, between the present study, and that on healthy subjects. In the studies on healthy subjects (Chapter 3) RBC concentrations of desethylamodiaquine were consistently higher than plasma concentrations, the overall RBC/plasma ratio being 2.8 ± 1.8 ($\bar{x} \pm SD$); there was no significant trend of change in RBC/plasma ratios over the time course of the study. In the present study RBC concentrations of the metabolite were often lower than the plasma concentrations, at the early time points, but at the later time points RBC concentrations rose to exceed those in the plasma. The RBC/plasma

ratio of desethylamodiaquine concentration was significantly lower on day 1 than at the end of the study (day 5 or 7). The reason for this observation is not known, but seems to be related to the disappearance from the erythrocytes of asexual forms of Plasmodium.

In the case of chloroquine, Adelusi and colleagues (1982) also showed a change in RBC/plasma ratio over time, and related this to the clearance of parasitaemia. However these workers found that RBC/plasma concentration ratios fell over the time course of the study, from ~21 at the beginning of treatment to ~ 5 in the last sample (170h); the trend of RBC/plasma ratios for chloroquine in acute malaria would seem therefore to be the reverse of that observed for amodiaquine in the present study. Similarly RBC concentrations of quinine are higher during acute malaria infection than in healthy subjects, although the RBC/plasma ratio may still be below unity (Trenholme *et al* 1976; White *et al* 1983).

It is known that malaria parasites concentrate 4-aminoquinolines within parasitised red cells (Fitch *et al* 1975; Warhurst 1986) and so the observations of Adelusi and others (1982) on chloroquine-disposition in red cells would seem to follow on logically. On the other hand this present study seems to suggest that parasitaemia reduces red cell concentrations of desethylamodiaquine, which seems to be at variance with the observations of others (Fitch *et al* 1975). However *in vitro* investigation of amodiaquine accumulation by Plasmodium - infected RBCs has been undertaken with radiolabelled drug, and assessment of this accumulation has been made by scintillation counting (Fitch 1973; Fitch *et al* 1974). In other words the accumulated radiolabelled compound has not been identified, and need not necessarily have been amodiaquine or its desethyl metabolite. In

this present study the parasitised RBC samples from subjects treated with amodiaquine were subjected to extraction by organic solvent, and then reverse-extraction into HCl; amodiaquine and desethylamodiaquine were then identified by their retention times on the previously described HPLC system. The two compounds were then quantified by comparison with standard curves (as described in Chapter 2). Amodiaquine and desethylamodiaquine are unstable molecules which undergo non-enzymic autoxidation, in aqueous solution, to produce highly reactive quinoneimines (Maggs et al 1987 a, b and 1988); such quinoneimines would exist in aqueous solution for only short periods, but would form covalent bonds with available sulfhydryl groups eg on structural or enzymic proteins. Once covalently bound in this way, the quinoneimine(s) would not be expected to partition into organic solvent during the preparative extraction step prior to HPLC analysis. Conditions under which quinoneimine formation would be expected to be enhanced would include oxidation by enzymes such as oxidases and peroxidases, known to be present within Plasmodia (Fritsch et al 1987). Consequently although the total 4-aminoquinoline accumulation of parasitised RBCs exposed to desethylamodiaquine, is likely to be higher than that of unparasitised cells, the amount of unbound compound available for solvent extraction would be lower, as has been found to be the case in the present study. In order to investigate this further, in vitro experiments would need to be conducted in which parasitised and unparasitised red cells were incubated with radiolabelled desethylamodiaquine. By liquid scintillation counting of aliquots of cells, the degree of accumulation of total 4-aminoquinoline could be measured in the two cell types; subsequent solvent extraction and HPLC analysis of the

two types of RBCs would enable measurement of non-covalently bound desethylamodiaquine.

4.5 SUMMARY

1. Oral amodiaquine has been used to treat symptomatic falciparum malaria, in the dose schedule 10mg kg^{-1}, followed by 5mg kg^{-1} 6, 24 and 48h thereafter. A clinical cure was obtained in all 14 subjects, and no serious adverse effects were seen.

2. Plasma concentration vs time profiles for amodiaquine and desethylamodiaquine from this present study did not differ significantly from those obtained from healthy volunteers, save that the time taken for the peak plasma concentration of amodiaquine to be reached, following the first dose of the drug, was longer in this present study.

3. In contrast to previous studies on healthy subjects, ratios of RBC/plasma concentrations of desethylamodiaquine from this present study were significantly lower at the commencement of the study than at the end; the ratios obtained from the last time points of this present study were similar to those obtained from healthy subjects.

CHAPTER 5 Tissue distribution and excretion of amodiaquine in the rat

5.1. Introduction

The demonstration that adverse responses to amodiaquine were not isolated, rare events was delayed until the drug became commonly-used by physicians in developed countries. Prior to its increased popularity for malaria prophylaxis after 1984, amodiaquine had been used largely in the tropics, and was frequently taken without reference to a doctor. Cases of toxicity due to amodiaquine were soon noted in Europe however, and the scale of the problem was assessed (Hatton *et al* 1986; Neftel *et al* 1986). Although use of amodiaquine for malaria prophylaxis was abandoned, the drug remained in use for the treatment of chloroquine-resistant *P. falciparum* infections (Gustafsson *et al* 1987). Therefore, work was needed on the mechanism of toxicity, including the identification of those subjects most at risk from amodiaquine toxicity.

Although early work on the compound had included acute and chronic toxicity studies in animals and man (Wiselogle 1946; Grühzit 1946) tissue distribution data were scanty (Wiselogle 1946; Chambon *et al* 1968; Barrow 1974) and the mass fate of the compound was unknown. It was clearly important to investigate the drug's disposition in experimental animals, especially to study whether it accumulated, and if so, in which tissues.

5.2 Experimental Design

5.2.1 Oral administration of ^{14}C-amodiaquine

Initially, in order to assess the optimal dose of radiation, and duration of subsequent experiments, one male Wistar rat (250g) received ^{14}C-labelled amodiaquine (8.6 mg kg; 5µCi) in aqueous solution (2.0ml) by orogastric intubation. Thereafter it was

housed in a metabolism cage with access to food and water ad. libitum. Collections of faeces and urine were made 12 hourly up to 48h, and 24 hourly thereafter up to 192h post-dose. At 192h the animal was sacrificed, and its tissues retained for analysis.

In subsequent experiments male Wistar rats (220-250g; n = 6) were given ^{14}C-amodiaquine (8.6mg base kg^{-1}; 8μCi) in aqueous solution (2ml) by orogastric intubation. Animals were housed separately in plastic metabolism cages (Techniplast; Scanbur, Denmark) and were allowed access to food and water ad. libitum. Urine and faeces collections were made 12 hourly up to 72h. At 72h animals were sacrificed and the following organs retained for analysis: kidneys, liver, spleen, testes, heart, lungs, brain and eyes, and gut plus content. Red bone marrow (~ 100mg from each animal) was expressed from the tibiae and femora following their dissection from the animal. The residual carcass was retained for analysis.

5.2.2 I.V. administration of ^{14}C-amodiaquine

Male Wistar rats (230-250g; n = 6) under ether anaesthesia were given ^{14}C-amodiaquine (3.8mg base kg^{-1}; 2μCi) in 0.9% saline (0.4ml) I.V. via the tail vein. Satisfactory I.V. administration was determined by observation of venous filling, and lack of tissue induration at the end of injection. After recovery, animals were housed separately in metabolism cages, and were allowed access to food and water ad. libitum. Urine and faeces collections were made up to 72h.

Following both I.V. and oral administration of ^{14}C-amodiaquine at 72h, the inner surfaces of each cage were washed with methanol (~ 40ml), which was retained for scintillation counting.

5.2.3 Autoradiography

Autoradiography studies were undertaken in collaboration with the department of Biochemistry, University College Cardiff. Female Wistar rats (35g; n = 6) each received ^{14}C-amodiaquine (9.9mg base kg^{-1}; 2.48µCi) in aqueous solution (0.1ml) by orogastric intubation. Animals were sacrificed 0.5, 1, 3, 6, 24 and 48h thereafter. Autoradiographs were prepared in Cardiff by the method of Powell et al (1967); animal surfaces were exposed to X-ray film in contact with solid CO_2 for 3 weeks.

5.3 Laboratory Methods

5.3.1 Sample handling

After removal of contaminating food pellets and fur, faeces were weighed and homogenised in phosphate buffer (pH 7.4; weight of buffer added = weight of faeces x 4) using an Ultra-Turrax blender. All tissues other than red bone marrow were weighed and homogenised in phosphate buffer (pH 7.4; weight of buffer added = weight of tissue x 3) using glass mortars and teflon pestles. Red bone marrow was weighed and then freeze-dried; the residue was powdered using a spatula, and then reconstituted in phosphate buffer (pH 7.4; 500µl). After removal of organs the remaining carcass was dissected and homogenised in water using a Waring blender. Aliquots of urine, and homogenates were stored at -20°C in plastic containers, protected from light.

5.3.2 Analytical methods

Radioactivity was determined by liquid scintillation counting, and concentrations of amodiaquine and desethylamodiaquine were determined by HPLC analysis, both as described in chapter 2.

HPLC analysis of urine, faeces and tissue homogenates was performed on samples obtained after oral administration of radiolabel only.

5.3.3 Determination of radioactivity extractable into organic solvent from faecal homogenate

In order to establish the likelihood of acid compounds (such as carboxylic acid derivatives) or conjugated forms being major excretion products of amodiaquine in faeces, faecal homogenates were extracted into organic solvent under different conditions and extracts were then subjected to liquid scintillation counting.

5.3.3.1 Extraction of faecal homogenate at pH 0.45

To faecal homogenate (0.5ml) was added HCl (0.1M; 0.5ml), achieving a pH of 0.45, and dichloromethane (7.0ml) followed by mechanical tumbling (15min) and centrifugation (2000g; 10min), the supernatant aqueous phase was discarded, and the organic phase evaporated to dryness. The residue was reconstituted in methanol (500µl) and radioactivity was determined by liquid scintillation counting as described in chapter 2.

5.3.3.2 Extraction of faecal homogenate at pH 12.0 before and after treatment with deconjugating enzyme

To duplicate samples of faecal homogenate (0.5ml) was added either sulphatase/glucuronidase (50 units; Sigma) in sodium acetate buffer (pH 5; 0.5ml), or buffer without enzyme (0.5ml). Following mixing by inversion of the tubes, samples were incubated at 37°C in a water bath (24h). To all samples (1.0ml) was added NH_3 (2.0ml; 0.88SG), achieving a pH of 12.5, and dichloromethane

(7.0ml). Following mechanical tumbling (15 min) and centrifugation (2000g; 10 min) the supernatant aqueous phase and solid faecal residue were discarded, and the organic phase evaporated to dryness under N_2. To the residue was added methanol (500µl) which was subjected to scintillation counting (as described in chapter 2).

5.4 Calculations

5.4.1 Radioactive dose

The amount of radioactivity in each dose was calculated by liquid scintillation counting (of 3 x 50 - 100µl aliquots of the dosing solution); background counts were deducted from results. Syringes were weighed before and after administration.

5.4.2 Pharmacokinetics

Apparent elimination rate constants (λz) were calculated from the terminal phases of urinary and faecal excretion rate *vs* time curves by log-linear least squares regression analysis. The apparent terminal $t_{\frac{1}{2}}$ of amodiaquine was calculated from $0.693/\lambda z$. Recovery of total radioactivity was calculated from AUC(o,t) of radiation excretion rate *vs* time curves from urine and faeces. Likewise recoveries of amodiaquine and desethylamodiaquine were calculated from AUC(o,t) of excretion rate *vs* time profiles for the compounds in urine and faeces.

5.4.3 Statistics

Urinary and faecal recoveries of radioactivity following oral and I.V. administration of ^{14}C-amodiaquine were compared using unpaired t test accepting $P < 0.05$ as significant.

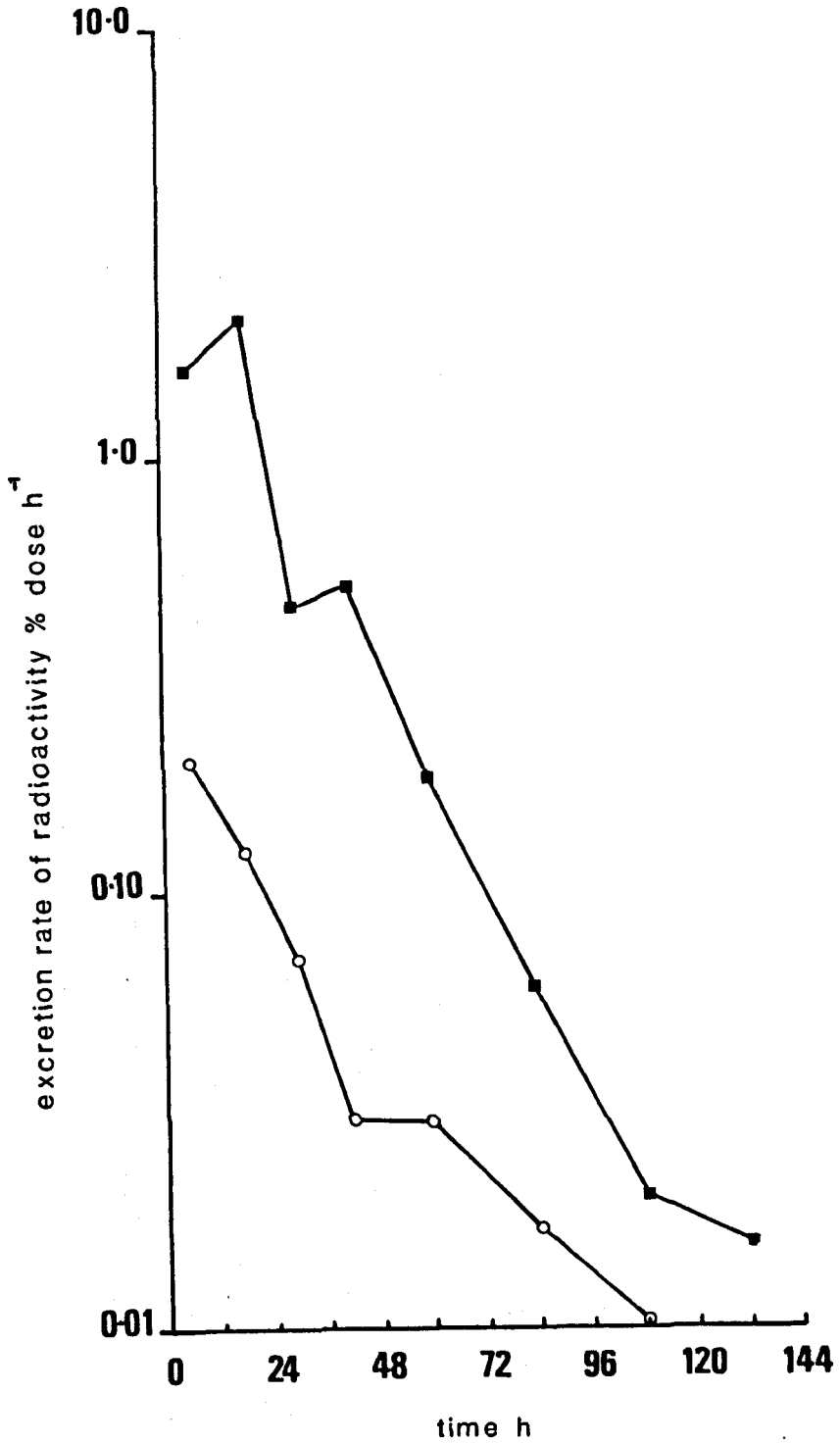


FIGURE 5.1 Excretion rates of radioactivity (% dose h^{-1}) in urine (open symbols) and faeces (closed symbols), following oral ^{14}C-amodiaquine (n = 1 animal)

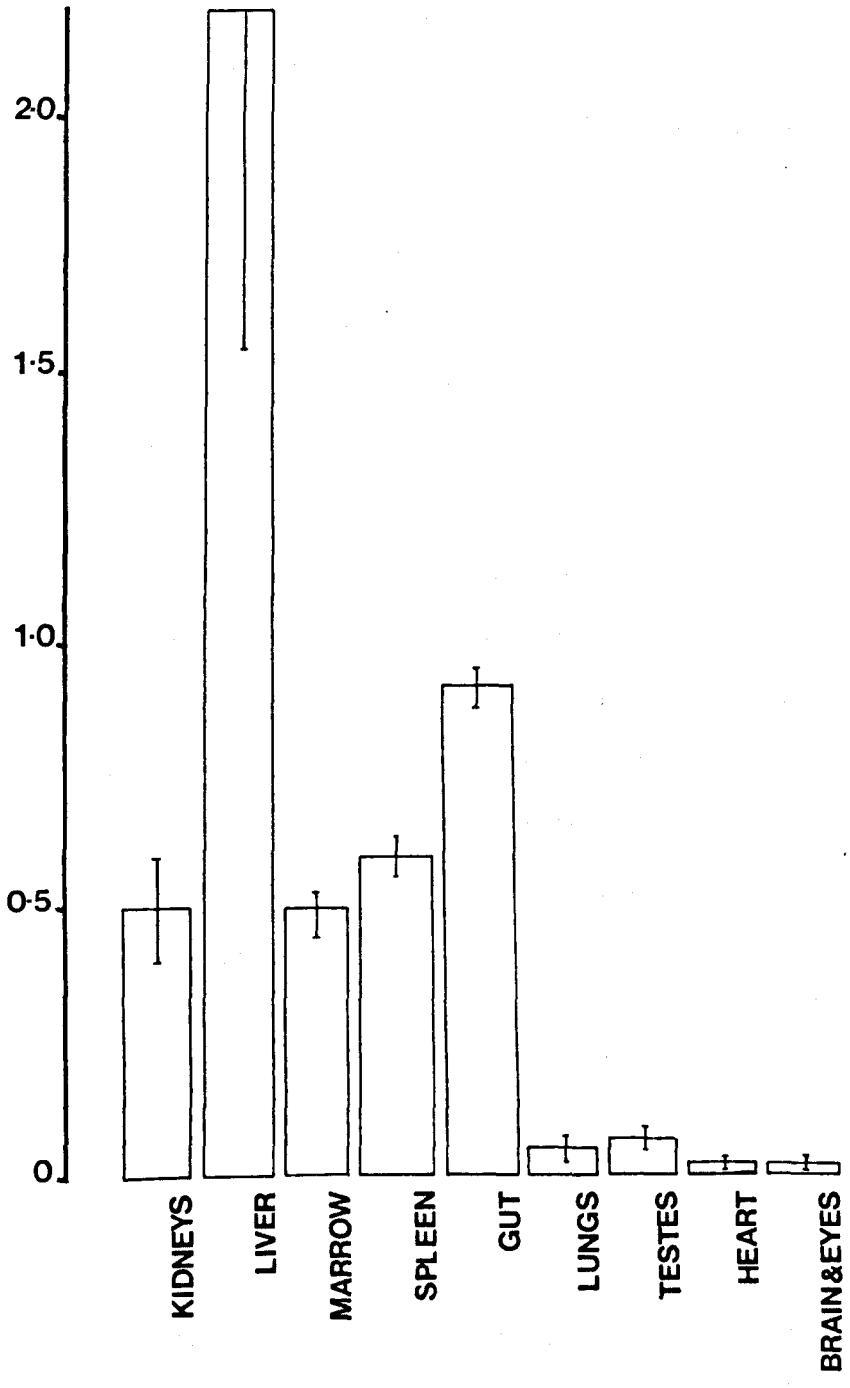


FIGURE 5.2 Tissue distribution of radioactivity 72h after oral [^{14}C]-amodiaquine (% dose) ($\bar{x} \pm$ SD)

5.5 Results

5.5.1 Oral administration of ^{14}C-amodiaquine

Following oral administration of ^{14}C-amodiaquine to the first animal 74% of the radioactivity was accounted for: 72.3% had been excreted by 192h (63.3% in faeces, 7.5% in urine and 1.5% in the cage wash), and 1.6% was recovered from the tissues (1% in liver, 0.3% in the gut, 0.1% in the kidneys, 0.1% in the lungs, 0.08% in heart and 0.03% in spleen). Figure 5.1 shows the percentage of the dose of radioactivity excreted per hour *vs* time, for both faeces and urine, up to 192h.

Based upon these data the dose of radioactivity administered to subsequent animals was increased, and animals were sacrificed at 72h. In these experiments (n = 6 rats) 90 ± 9% of the administered radioactivity was recovered (mean ± SD): 86 ± 8% had been excreted by 72h (77 ± 9% in faeces, 7 ± 1% in urine and 2 ± 2% in cage washes), and 4 ± 1% was recovered from the tissues. Figure 5.2 shows the tissue distribution of radioactivity at 72h. Although the ^{14}C content of the red bone marrow could be measured, the total mass of marrow in each animal was unknown; however Baker *et al* (1979) estimate the bone marrow to account for 3% adult body weight in the rat, which allows calculation of the ^{14}C content of marrow in each animal.

Following the removal of organs the residual carcass had been "homogenised", and its content of radioactivity was measured in the first 3 animals. This was found to be ~ 2% of the dose, but because of difficulties encountered in obtaining true homogeneity, this can be no more than an approximate value, and therefore is not included in figure 5.2.

The distribution of radioactivity in the tissues expressed as

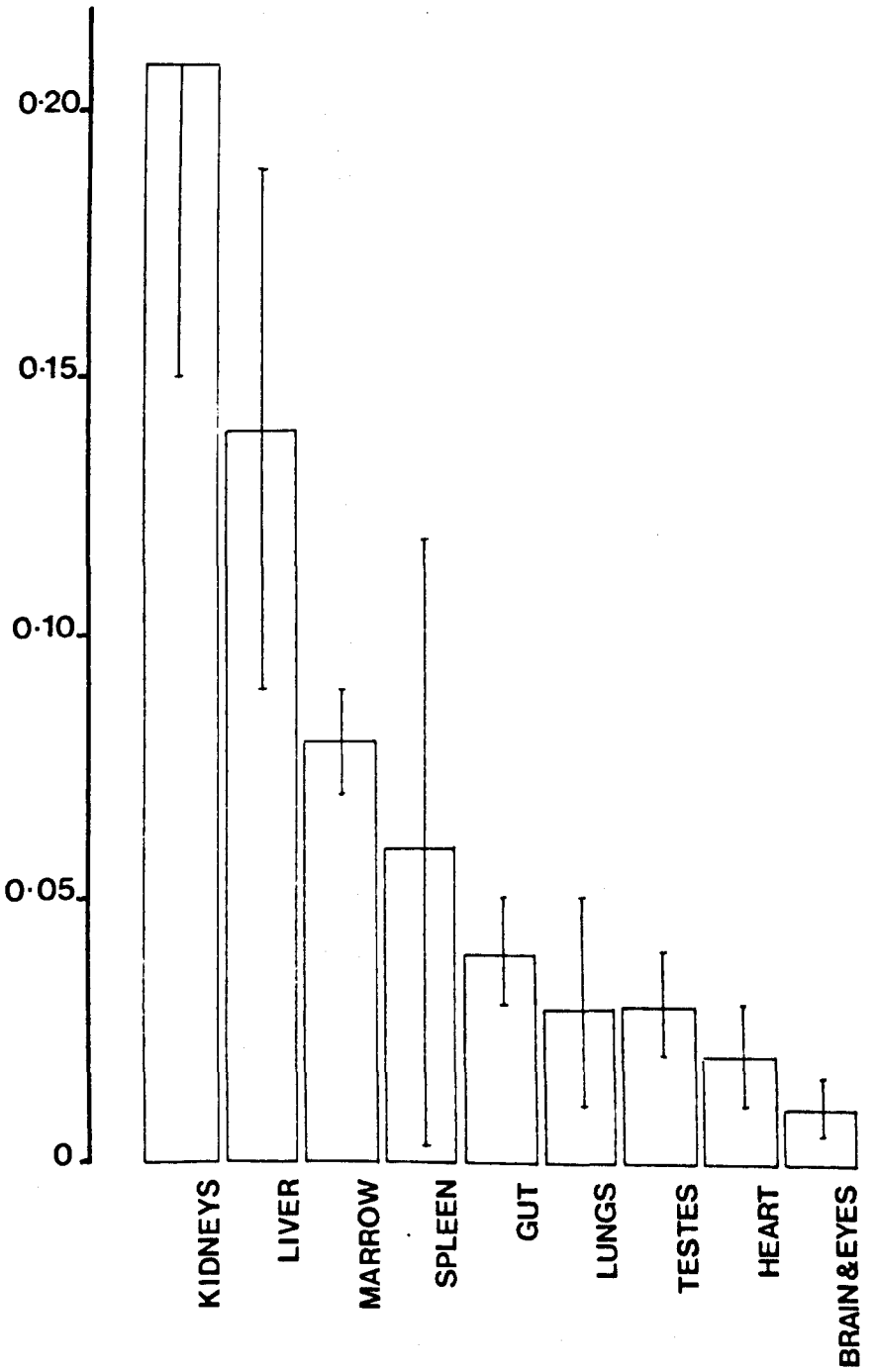


FIGURE 5.3 Tissue affinity for radioactivity, 72h after oral ^{14}C-amodiaquine (% dose μg^{-1} tissue) ($\bar{x} \pm SD$)

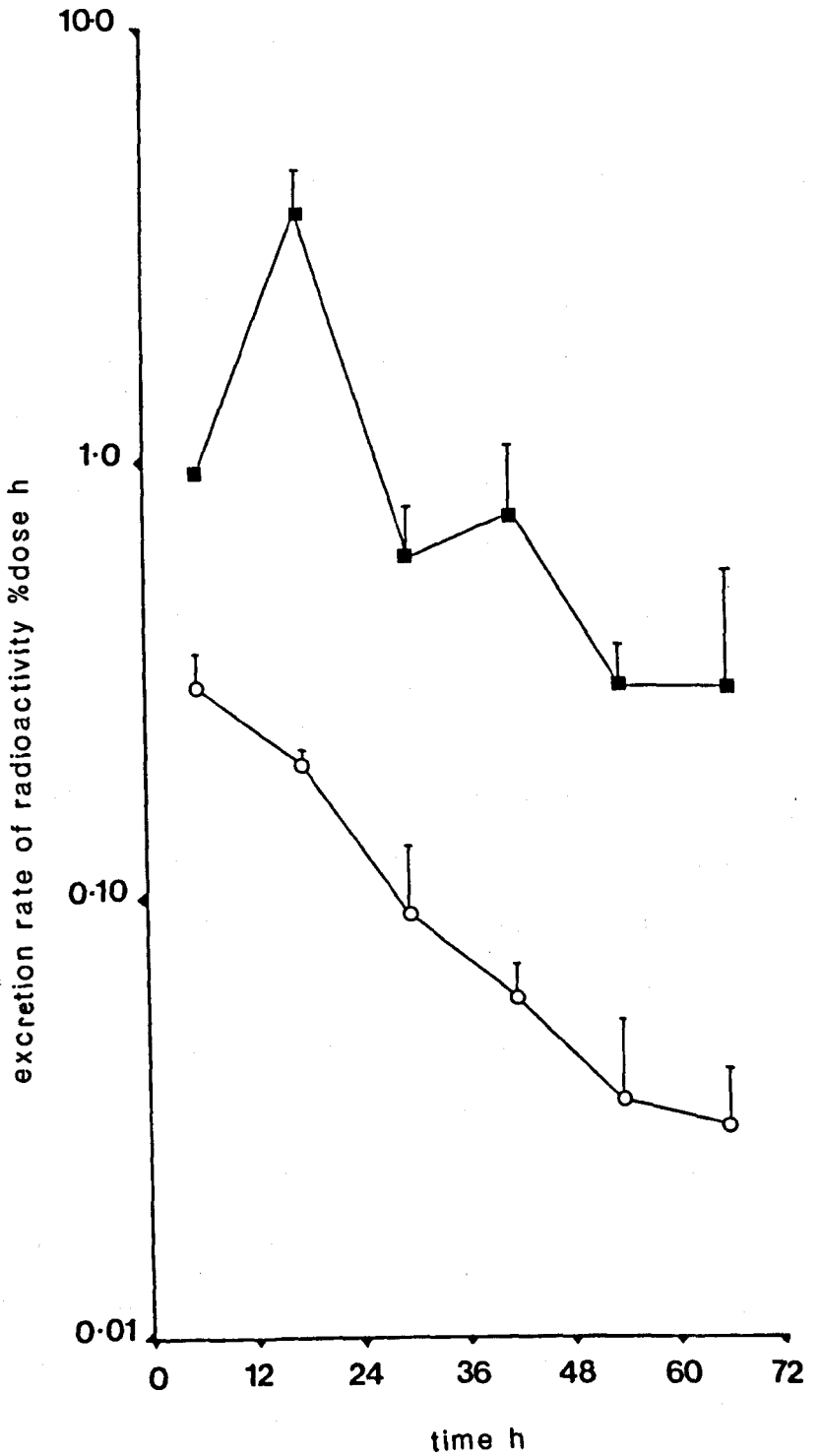


FIGURE 5.4 Excretion rates of radioactivity (% dose h^{-1}) in urine (open symbols) and faeces (closed symbols), following oral ^{14}C-amodiaquine (n = 6, $\bar{x} \pm SD$)

percentage dose per microgram of tissue (wet weight) is shown in figure 5.3. Tissues with highest affinities for radioactivity were kidneys, liver, marrow and spleen.

Figure 5.4 shows the percentage of administered radioactivity excreted per hour *vs* time for both urine and faeces. Faecal recovery of radioactivity was high (77 ± 9% dose); in the first 12h collection excretion rates of radioactivity varied widely, but peak radiation excretion rates were seen in the 12-24h collections of all 6 animals. The weight of faeces excreted by animals varied widely between collections. Urinary recovery of radioactivity was low (7 ± 1% dose); urinary radioactivity excretion rates were highest in the first 12h collection of each animal, and declined in a log-linear manner thereafter.

Figures 5.5, and 5.6 illustrate the excretion rates in urine and faeces of amodiaquine and desethylamodiaquine ($\mu g h^{-1}$) *vs* time. The urinary recovery of unchanged drug was 2.4 ± 0.5µg (~ 0.1% of the amodiaquine dose), and that of the metabolite was 18.5 ± 4.1µg. The peak urinary excretion rate of amodiaquine was seen in the first 12h collection of each animal, and amodiaquine was not detectable in the urine after 24h. In contrast, desethylamodiaquine was detectable in the urine to the end of collection. The peak urinary excretion rate of the metabolite was seen in the first 12h collections of all animals and thereafter declined in a log-linear fashion; λz for desethylamodiaquine in the urine was 0.041 ± 0.005 h^{-1} (n = 6).

The faecal recovery of the unchanged drug was 210 ± 79µg (~ 10% of the amodiaquine dose) and that of desethylamodiaquine was 123 ± 32µg. The peak excretion rate of amodiaquine in the faeces was seen in the 12 - 24h collections of all animals; after an

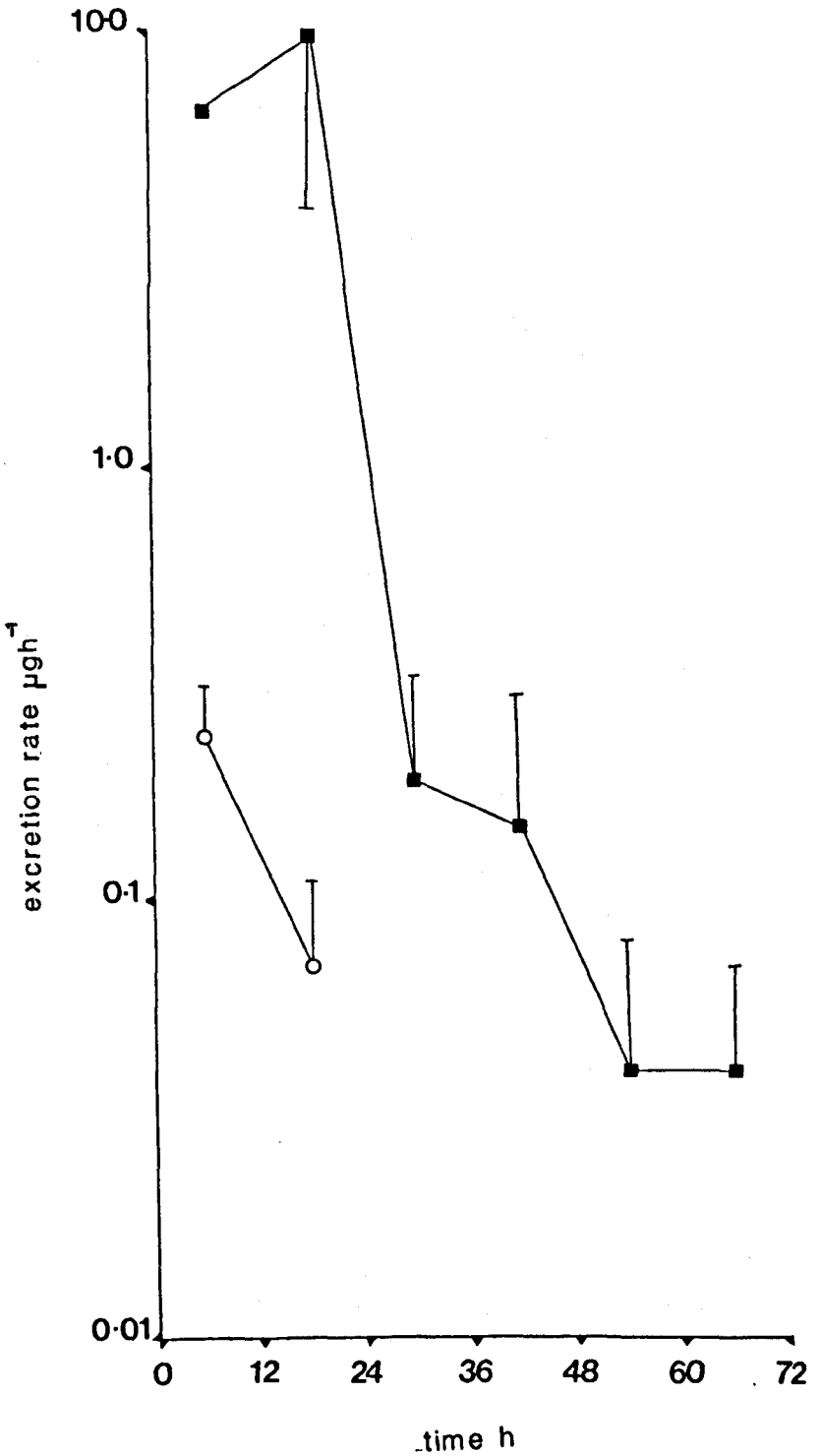


FIGURE 5.5. Excretion rates of amodiaquine (µg h^{-1}) in urine (open symbols) and faeces (closed symbols), following oral ^{14}C-amodiaquine ($\bar{x} \pm SD$)

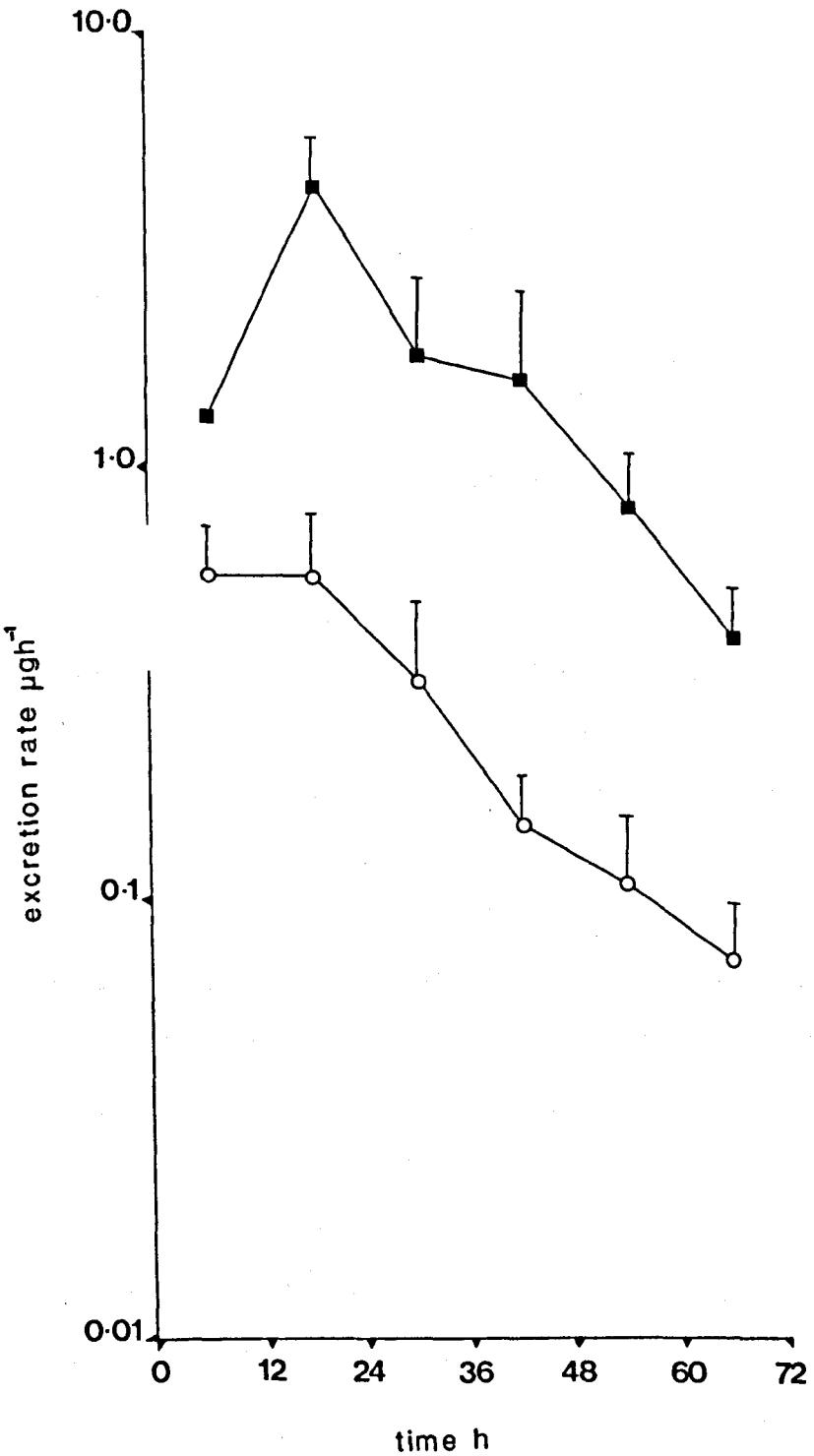


FIGURE 5.6 Excretion rates of desethylamodiaquine (µg h^{-1}) in urine (open symbols) and faeces (closed symbols) following oral ^{14}C-amodiaquine ($\bar{x} \pm SD$)

initial steep fall there was a slower elimination phase allowing calculation of $t_{\frac{1}{2}}$ which was 10 ± 4h (n = 4). The peak excretion rate of desethylamodiaquine in the faeces was also seen in the 12 - 24h collections, and declined in a log-linear fashion thereafter; λz for the metabolite in the faeces was 0.047 ± 0.005 h^{-1} (n = 3).

Desethylamodiaquine, but not amodiaquine, could be determined by HPLC of splenic homogenates of 5 of the 6 animals; total recovery was 1.54 ± 0.32µg. The "concentration" of desethylamodiaquine in splenic tissue was 1.28 ± 0.64 µg g^{-1}.

5.5.2 I.V. administration of [^{14}C]-amodiaquine

Following I.V. administration of ^{14}C-amodiaquine, radioactivity excretion rate *vs* time plots in urine and faeces (figure 5.7) were similar to those seen following oral administration (figure 5.4). Of the radioactivity given 103 ± 10% had been excreted by 72h: 91 ± 10% in the faeces, 11 ± 1% in the urine and 0.5 ± 0.2% in cage washes. Both faecal and urinary recovery of radioactivity were significantly higher ($p < 0.05$) following I.V. administration, than following oral administration.

5.5.3 Autoradiographs

Figure 5.8 shows autoradiographs obtained from animals sacrificed 0.5, 1, 3, 6, 24 and 48h following oral administration of ^{14}C-amodiaquine. At 0.5h most of the radioactivity was associated with the gastrointestinal tract and contents, but radioactivity was also seen in liver and kidney. From 1-48h autoradiographs showed progressively less radioactivity in the gastrointestinal tract, and greater amounts in the tissues. In the 24h autoradiograph, radioactivity can be seen in the vertebral

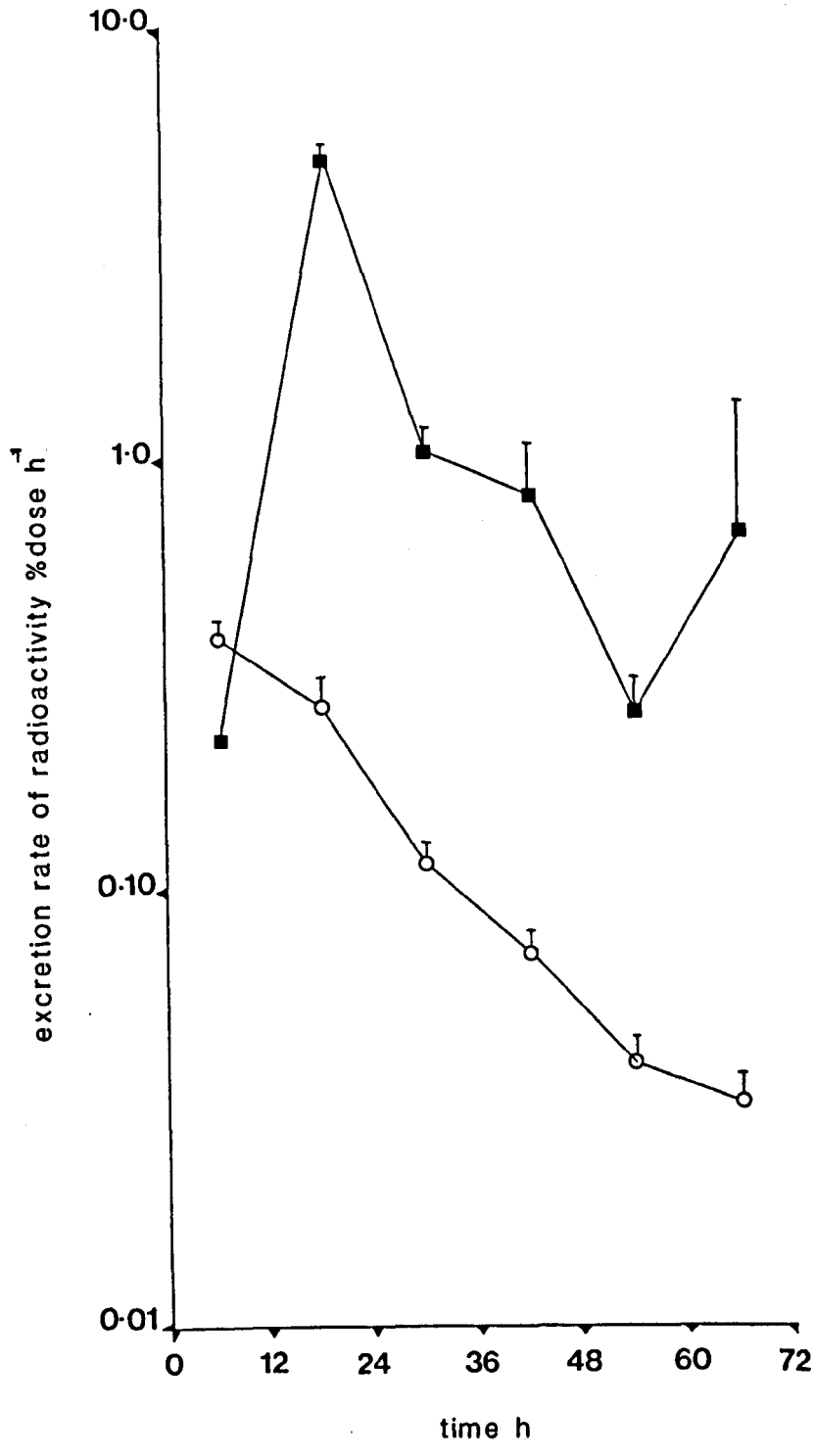
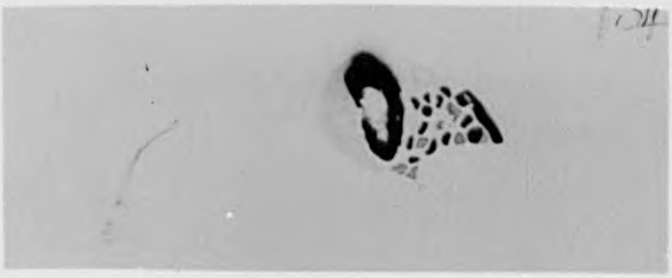


FIGURE 5.7 Excretion rates of radioactivity (% dose h^{-1}) in urine (open symbols) and faeces (closed symbols), following intravenous ^{14}C-amodiaquine (n = 6, $\bar{x} \pm SD$)

FIGURE 5.8 Autoradiographs obtained from animals sacrificed 0.5, 1, 3, 6, 24 and 48h (top to bottom) following oral administration of ^{14}C-amodiaquine



Animal	Collection	Total Faecal Radioactivity DPM x 10^6	Radioactivity extracted at pH 12.0 Before deconjugation DPM x 10^6	% Total	After deconjugation DPM x 10^6	% Total	Radiation Extracted at pH 0.45 DPM x 10^6	% Total
V	0-12	5.03	0.94	19	1.14	23	0.060	1.2
	12-24	5.77	0.76	13	1.13	20	0.100	1.7
	24-36	0.57	0.03	5	0.03	5	0.010	1.8
	36-48	2.10	0.20	10	0.26	12	0.050	2.4
	48-60	0.44	0.04	9	0.05	11	0.010	2.3
	60-72	0.33	0.03	9	0.04	12	0.005	1.5
VII	0-12	2.89	0.53	18	0.56	19	0.020	0.7
	12-24	9.67	0.88	9	1.61	17	0.120	1.2
	24-36	1.20	0.12	10	0.13	11	0.040	3.3
	36-48	1.38	0.07	5	0.10	7	0.010	0.7
	48-60	0.65	0.05	8	0.05	8	0.007	1.1
	60-72	0.59	0.04	7	0.05	9	0.010	1.7

Table 5.1

Radioactivity extractable from faecal homogenate into organic solvent under various conditions (Samples obtained from both animals after oral administration of ^{14}C-Amodiaquine.

bodies, and this is more evident by 48h. In the 48h autoradiograph significant amounts of radioactivity remained in the tissues, predominantly in liver, kidney, marrow, eye and gastrointestinal tract. The spleen was not included in the material subjected to autoradiography.

5.5.4 Radioactivity extracted from faecal homogenates into organic solvent

5.5.4.1 Radioactivity extracted at pH 0.45

Table 5.1 shows the percentage of total faecal radioactivity extracted into dichloromethane at pH 0.45 from 12 samples of faecal homogenate obtained after oral administration of ^{14}C-amodiaquine. Overall 1.63 ± 0.75% of total faecal radioactivity could be extracted at this pH (mean ± SD).

5.5.4.2 Radioactivity extracted at pH 12.0, before and after treatment with deconjugating enzyme

Table 5.1 also shows the percentage of total faecal radioactivity extracted into dichloromethane at pH 12.0, from the same 12 samples of faecal homogenate before and after treatment with glucuronidase/sulphatase. From untreated homogenate 10 ± 4.5% of total faecal radioactivity could be extracted into organic solvent (in contrast 50% and 60% of added desethylamodiaquine or amodiaquine respectively could be extracted from spiked samples of drug-free faecal homogenate). Following treatment with deconjugating enzyme this figure increased to 12.5 ± 5% of total faecal radioactivity.

5.6 Discussion

Prior to the present study the mass-fate of amodiaquine in the rat had not been clearly defined, and the extent and sites of organ accumulation of the drug had been little studied. The demonstration of a high prevalence of clinical adverse reactions to amodiaquine has prompted investigations of the drug's toxicity, and data on its mass fate and tissue distribution have become important. Wiselogle (1946) studied the tissue accumulation of amodiaquine in Rhesus monkeys, using a non-specific dye-complexing method. Wiselogle reported that highest concentrations were found in liver, spleen, lung, kidney and heart, in diminishing order of magnitude, after 30 doses of the drug (25 mg kg^{-1} base). Chambon and colleagues (1968) studied tissue accumulation of the drug in dogs also using non specific methods of analysis (Brodie et al 1947). Highest concentrations were again found in liver, kidney, spleen, heart and lung, one month after a single dose of amodiaquine (40 mg kg^{-1} base).

The most detailed investigation of the disposition of amodiaquine in animals was reported by Barrow (1974). In this study ^{14}C ring-labelled amodiaquine was synthesised, and administered to several species of rodent (Wistar, Sprague - Dawley and Hooded-Lister rats, mice and guinea pigs). The routes of administration used were oral, IV, and intraperitoneal (IP). Barrow then studied the excretion of radiation from intact animals, and bile duct-cannulated rats, and the tissue distribution of radioactivity in mice, rats and guinea pigs. However this author did not account for more than 51% of administered radioactivity in any one animal. Barrow (1974) reported that following both oral and IP administration radiation was excreted predominantly in the

faeces (20.5 - 41% of the dose by 216h), urinary excretion accounting for much less (3 - 10.5% of the dose by 216h). The tissue distribution of radioactivity 216h following oral administration of ^{14}C-amodiaquine was reported by Barrow to be 0.3 - 2.3% of the dose in kidney, 0.2 - 1.7% in liver, and 0.04 - 0.09% in spleen (Barrow 1974). In autoradiographs prepared from mice 48h after IV ^{14}C-amodiaquine, radioactivity was primarily associated with intestine, liver and kidneys. Barrow (1974) did not comment upon accumulation of radioactivity by the marrow of animals subjected to autoradiography.

The aims of the present study were to describe the mass-fate of amodiaquine following oral and IV administration to Wistar rats, and to assess the tissue distribution of retained radioactivity; this animal model might be expected to illustrate the drug's mass-fate in man, and might help the understanding of the its toxic effects. The size of the oral dose (8.6 mg base kg^{-1} body weight) was chosen to reflect clinically-used doses (equivalent to 600mg amodiaquine base to a 70kg man), and was much lower than the dose employed by Barrow (1974) (100 mg kg^{-1}). The size of the IV dose (3.8 mg base kg^{-1} body weight) was also much smaller than that employed by Barrow (1974) (30 mg kg^{-1}), and was chosen to avoid toxic reactions, since the LD50 for amodiaquine following IV administration to mice is 17 mg kg^{-1}, and the minimum toxic dose is 10 mg kg^{-1} (Grühzit 1964).

In this present study, following oral administration of ^{14}C-amodiaquine, 90% of the administered radioactivity was accounted for. Most of this was in the 0 - 72h faeces collection (77% of the dose) much less being present in urine or cage washes. Although Barrow (1974) also found that, following oral administration of

^{14}C-amodiaquine, radioactivity was excreted predominantly in the faeces over 216h, this author found less than 35% of the administered radioactivity in the faeces by 72h. The difference in the findings of this present study and that of Barrow (1974) may be due, in part, to the previously mentioned large differences in the sizes of the doses of amodiaquine studied. However the different results are probably better explained by differences in the methods used to determine faecal radioactivity. Barrow (1974) extracted faecal homogenates with ether at pH 9 - 10 for 24h; the ethereal and filtered aqueous phases were then subjected to liquid scintillation counting. The particulate faecal debris was evidently not examined. This present study determined faecal radioactivity in digests of whole faeces. The disparity of the results of this study and those of Barrow (1974) could be due therefore to the presence of much of the radiolabel, in unknown form, irreversibly bound to faecal debris.

Following oral administration of ^{14}C-amodiaquine, 4% of the radiation dose was still present in the tissues at 72h. Like Barrow (1974) the tissues with highest affinities for radiolabel were found by this study to be kidney and liver, the tissues with next highest affinities being marrow and spleen. This may relate to the observed toxic manifestations of amodiaquine in man where agranulocytosis and hepatitis have been the major problems, although renal toxicity has not been reported. From the recovery of radioactivity in each tissue it is possible to calculate the mass of accumulated total quinoline(s), these were ~ 5, 2.6, 1.5 and 0.9 µg quinoline g^{-1} tissue for kidney, liver, marrow and spleen respectively. It was possible to determine the concentration of desethylamodiaquine in splenic homogenate by HPLC,

and this value was ~ 1.3 μg g^{-1} tissue. However it is likely that other derivatives of amodiaquine, undetected by this HPLC method, are also present in the tissues and these may include quinoneimines which would be irreversibly-bound to protein (Maggs et al 1987 (a) and (b)). Maggs and colleagues (1987 (a) and (b)) have postulated that such oxidation products of the drug, and probably also of desethylamodiaquine, may be the derivatives of amodiaquine which are responsible for its toxicity.

The presence in the faeces of 86% of an orally administered dose of radiolabelled drug could be due to:- (a) incomplete absorption of the drug; or (b) absorption followed by excretion of the drug into the gut (most likely via the bile). The first explanation seemed unlikely, and indeed when ^{14}C-amodiaquine was administered IV 91% of the dose of radioactivity was found in the 72h collection of faeces, making it likely that the drug (or its derivative(s)) is subject to biliary excretion. Since the molecular weight of amodiaquine base is 356 this observation is not surprising. Biliary excretion of radioactivity by bile-duct cannulated rats was studied by Barrow (1974), following the oral administration of ^{14}C-amodiaquine to rats (n = 3), and found to account for 30% of the dose of radioactivity by 48h.

Using the HPLC method developed for this present study unchanged amodiaquine could be detected in the urine in only the first two 12h collections, but the faecal concentration of amodiaquine could be determined in all collections. Following an initial steep fall in the excretion rate of the drug there was a slower decline from which it was possible to calculate $t_{\frac{1}{2}}$ for 4 animals; this value was 10 ± 4h which is of the same order of magnitude previously reported in human subjects (chapter 3).

other metabolites of the drug (see chapter 2); these may be hydroxydesethyl-amodiaquine and bisdesethylamodiaquine which have been identified in man (Churchill et al 1985; Mount et al 1987). However identification and quantification of these metabolites in the rat would not solve the observed problem, since they too would be expected to be extracted into ether at alkaline pH.

In view of the low faecal recoveries of amodiaquine and desethylamodiaquine in this present study, faecal homogenates were extracted into ether at pH 0.45, at which pH possible acidic derivatives of the drug would be expected to partition into organic solvent; like Barrow (1974) this present study could extract little radioactivity under these circumstances. In an attempt to assess the presence of possible conjugated derivatives of amodiaquine in this present study, faecal homogenates were subjected to treatment with a deconjugating enzyme, but this treatment failed to increase the amount of radioactivity extractable into ether at pH 12 by more than 2.5%. These observations indicate that acidic derivatives of amodiaquine and glucuronide or sulphate conjugates of the drug (or metabolites) are not major excretion products in the faeces of this animal model.

The major form(s) of faecal excretion of radioactivity have not been identified in this study or elsewhere. However it has been demonstrated by Maggs and colleagues (1987 (a) and (b)) that amodiaquine is an unstable molecule which undergoes spontaneous autoxidation. It is possible that much of the radiolabel excreted in the faeces of rats is in the form of breakdown product(s) of amodiaquine irreversibly-bound to large molecules and thus unavailable for solvent extraction.

5.7 Summary

1. ^{14}C-amodiaquine has been administered to Wistar rats by the oral and IV routes, and the mass-fate of the drug determined. Following oral and IV administration 90 ± 9% and 103 ± 10%, respectively, of the administered radioactivity was accounted for.

2. Following both oral and IV administration the predominant route of excretion of radioactivity was faecal.

3. Following oral administration of ^{14}C-amodiaquine, 4 ± 1% of the dose of radioactivity was still present in the tissues at 72h. This was maximally accumulated by kidney, liver, marrow and spleen.

4. Total recovery of amodiaquine and desethylamodiaquine in urine and faeces were small, indicating that these were not major forms of excretion. Conjugated forms of the drug or its metabolites are also unlikely to be major excretion products in the faeces.

CHAPTER 6 The effects of amodiaquine and its derivatives on granulocyte-monocyte colony forming units (GM-GFU) from healthy subjects

6.1 INTRODUCTION

Reports of adverse reactions to amodiaquine between 1949 and 1981 were relatively few, but included 12 cases of agranulocytosis and 3 of coincident hepatitis (Love *et al* 1953; Kennedy 1955; Glick 1957; Perry *et al* 1962; Booth *et al* 1967; Lind *et al* 1973; Gillespie and Wagner 1977; Lepeu *et al* 1981); of these 12 subjects suffering adverse reactions 2 died. Between 1981 and 1987 amodiaquine was a popular choice for malaria prophylaxis in areas of extensive chloroquine-resistant *P. falciparum*; during this time adverse responses to amodiaquine were reported in a further 29 subjects (21 cases of agranulocytosis; 13 cases of hepatitis), 5 of whom died (Schultless *et al* 1983; Douer *et al* 1985; Hatton *et al* 1986; Neftel *et al* 1986; Carr 1986; Rhodes *et al* 1986; Larrey *et al* 1986; Amouretti *et al* 1986; Ellis 1987; Aymard *et al* 1987).

In all cases where marrow from agranulocytosis-sufferers was examined (n = 17), partial or total absence of myelopoiesis was seen. The mechanism by which certain subjects displayed idiosyncratic agranulocytosis, in response to the drug, was not understood, but was thought to be due to either an abnormal sensitivity of the myeloid precursor cells to direct amodiaquine toxicity, or to result from immune-mediated damage (see Chapter 1). The prevalence of amodiaquine-induced agranulocytosis was not known accurately, but was estimated by Hatton and colleagues (1986) to be about 1:2000 users of the drug for malaria prophylaxis.

Bone marrow cells from individuals suffering amodiaquine-induced agranulocytosis have been isolated and cultured in four studies, which attempted to investigate the mechanism of toxicity. In the first of these studies Lind and colleagues (1973) cultured granulocyte-monocyte colony forming units (GM-GFU) from a

26 year old woman suffering from amodiaquine-induced agranulocytosis, and also from a healthy control subject. In this study the growth of GM-CFU from the patient, but not from the normal control subject, was inhibited by amodiaquine at a concentration of 500ng ml^{-1} (~1.5μM). Work by Rhodes and colleagues (1986), in a similar study, reported significant inhibition of GM-CFU from a similar patient, but not from a control subject, by amodiaquine (500ng ml^{-1}). In neither of these reports were desethylamodiaquine, or the patient's acute-phase plasma studied.

Douer and colleagues (1985) subjected control GM-CFU and those obtained from a 63 year old woman (3 months after her recovery from amodiaquine-induced agranulocytosis) to culture in the presence of a fixed concentration of amodiaquine (500ng ml^{-1}). These workers reported that, in the absence of acute-phase plasma, amodiaquine (500ng ml^{-1}) had no effect upon either control GM-CFU or those from the patient; however the addition of acute-phase plasma (which had been drawn from the patient upon initial diagnosis) to the system in the presence of amodiaquine (500ng ml^{-1}) caused inhibition of proliferation of both the control and the patient's GM-CFU. Plasma drawn from the patient 3 months after her recovery did not produce this effect; likewise incubation of cells with "acute-phase" plasma in the absence of added drug failed to produce an effect. Douer and colleagues (1985) concluded that a drug-specific plasma component, presumed to be an antibody, was responsible for amodiaquine-induced agranulocytosis.

Ellis and colleagues (1987) cultured marrow cells from a 29 year old woman 10 weeks after her recovery from amodiaquine-induced agranulocytosis. Several compounds were tested including

amodiaquine (at a fixed concentration of 100ng ml^{-1}) and desethylamodiaquine (at a fixed concentration of 1500ng ml^{-1}) in the presence and absence of the patient's serum (which had been obtained 13 days after first presentation). These workers found that only the combination of desethylamodiaquine (1500ng ml^{-1}) plus patient's serum (volume not stated) caused significant inhibition of GM-CFU, and concluded that a serum component (again presumed to be an antibody) was responsible for the observed agranulocytosis, but in this case, the serum-dependent toxicity required the presence of desethylamodiaquine rather than the parent drug.

Circulating amodiaquine-dependent IgG antibodies to mature neutrophils have been demonstrated in one case of amodiaquine-induced agranulocytosis (Schulthess et al 1981). However it is clear that such cytotoxic antibodies directed against mature, circulating neutrophils are unlikely to be the main mechanism of toxicity, since in all cases examined there has been a reduction in, or complete absence of myeloid precursors in the marrow. The possible presence of cytotoxic antibodies in acute-phase plasma, directed against myeloid precursors has not been investigated.

These various studies have produced evidence for both abnormal sensitivity of myeloid precursors (obtained from patients known to have had agranulocytosis due to amodiaquine), and for immune-mediated toxicity to marrow cells. In only one of the studies cited was desethylamodiaquine examined, and in none was amodiaquine-p-quinoneimine examined. This latter compound, a highly reactive species, was demonstrated by Maggs and colleagues (1987(a) and (b)) to be an autoxidative product of amodiaquine in in vitro human liver microsome incubations. It was postulated by Maggs and colleagues (1987(a) and (b)) that amodiaquine-p-

quinoneimine might be generated *in vivo*, and might be responsible for toxicity to both marrow and liver (see chapter 1, section 1.4.3). Maggs and Park (1988) went on to examine the effects of both amodiaquine and its quinoneimine on normal human lymphocytes. It was found that while amodiaquine had no effects on cell viability up to a concentration of 100μM, its quinoneimine produced significant reduction of viability at a concentration of 10μM. It seemed possible that amodiaquine-p-quinoneimine might be as toxic to bone marrow cells as to lymphocytes, and supported the view that the quinoneimine played a part in the observed toxicity.

The aims of this present study were to investigate the effects on the proliferation of normal GM-CFU of amodiaquine, desethylamodiaquine and amodiaquine-p-quinoneimine, and to compare the effects of these compounds with those of chloroquine, a close structural analogue of amodiaquine which does not cause agranulocytosis frequently. A further aim was to study the effects on GM-CFU proliferation of acute phase plasma obtained from patients with amodiaquine-induced agranulocytosis.

6.2 METHODS

6.2.1 Assessment of the myelocytotoxicity of amodiaquine and its derivatives in comparison with chloroquine

Low-density marrow cells were obtained from 5 healthy subjects by the methods described in Chapter 2. These were incubated (37°C; 5% CO_2; 10-14 days) in semisolid medium containing fixed concentrations (0.1, 1.0, or 10.0μM) of amodiaquine, desethylamodiaquine, amodiaquine-p-quinoneimine or chloroquine. Each compound, at each concentration (4 replicates) was tested on all 5 marrow samples; experiments therefore took place on 5

separate days. In the cases of amodiaquine, desethylamodiaquine and chloroquine, which were in aqueous solution, control plates comprised marrow cells, cultured in drug-free complete medium. In the case of amodiaquine-p-quinoneimine, the stock solution of which was in ethanol, control plates comprised marrow cells, cultured in drug-free complete medium containing ethanol at a concentration equivalent to that present in the 10.0μM quinoneimine test plates.

In a further experiment low-density marrow cells (8×10^5) were incubated (37°C; 5% CO_2) in complete medium (1.0ml) in the presence of fixed concentrations (0.1, 1.0 and 10.0μM) of amodiaquine, desethylamodiaquine, amodiaquine-p-quinoneimine or chloroquine, for a period of one hour. Following incubation with these compounds, the cells were centrifuged (1200 RPM; 5 min) the supernatant fraction was removed, and the cells resuspended in drug-free medium (1.0ml); the washing process was then repeated, the cell pellet was resuspended in drug-free complete medium (4 x 1.0ml) and cells were cultured in semi-solid medium in the normal manner (4 replicates). "Aqueous" and "ethanolic" control plates were prepared by the incubation of marrow cells in drug-free medium and medium containing ethanol (in a concentration equivalent to that present in the 10.0μM quinoneimine incubation) followed by washing of the cells in a manner identical to that employed for the test groups.

6.2.2 Effects of glutathione-depleting agents on the myelocytotoxicity of amodiaquine and its quinoneimine

6.2.2.1 The glutatione depleting agents

Quinoneimines and semiquinoneimines are reactive compounds which often produce cell toxicity by arylation of cellular,

structural or enzymic, proteins. Glutathione possesses a sulphydryl group and is known to form thiol adducts with some quinoneimines, the best documented of which is that derived from paracetamol (Mitchell et al 1973 b); this reaction is thought to be protective, preventing some of the reactive compound from binding to cell proteins. To investigate further the cytotoxicity of amodiaquine and its quinoneimine, low-density cells, obtained from healthy marrow by the previously described methods, were pre-treated with agents known to produce depletion of cell glutathione content, and were then incubated with amodiaquine or its quinoneimine as before.

Three compounds were chosen for assessment, diethylmaleate, n-ethylmaleimide and buthionine-sulfoximine. The aim of this initial assessment was to determine the maximal concentration of the glutathione-depleting compound with which marrow cells could be incubated prior to exposure to amodiaquine, without abolition of colony formation.

Low density marrow cells were obtained using the previously described methods, and were incubated in a water bath (37°C) with fixed concentrations of diethylmaleate (0 to 1000μM), n-ethylmaleimide (0 to 20μM) or buthionine-sulfoximine (0 to 200μM) in complete medium. Following incubation (1.0h in the case of diethylmaleate, 0.5h for both n-ethylmaleimide and buthionine-sulfoximine) cells were washed twice by centrifugation (1200g; 5 min) and resuspension in complete medium. Cells were then cultured in the usual manner (3 replicates in the case of diethylmaleate, 4 replicates each for both of the other 2 compounds). Following its initial assessment, no further use was made of diethylmaleate, because of its additional unwanted effects on cell metabolism

(Costa and Murphy 1986).

6.2.2.2 Pretreatment of marrow cells with n-ethylmaleimide or buthionine sulfoximine, prior to incubation with amodiaquine or its quinoneimine

Low-density marrow cells from one subject (8×10^5) were incubated (37°C; 5% CO_2; 0.5h) with a fixed concentration of either n-ethylmaleimide (10μM) or buthionine sulfoximine (200μM). After centrifugation (1200 RPM; 5min) and removal of the supernatant, the cell pellet was washed in complete-medium (5.0ml), resuspended in semi-solid medium (4 x 1.0ml) containing fixed concentrations (0.1, 1.0 or 10.0μM) of either amodiaquine or its quinoneimine, and incubated for 10 days in the usual manner (4 replicates).

6.2.3 Effect of human plasma upon the myelocytotoxicity of amodiaquine

The effect of the incubation of low-density marrow cells with amodiaquine and human plasma was studied; samples of plasma were obtained from:- (a) subjects (n = 4) in the acute phase of amodiaquine-induced agranulocytosis (b) one subject with acute P. falciparum infection (c) healthy subjects on no drug therapy (controls) and (d) one healthy subject 96h after oral amodiaquine (600mg) (see table 6.1). Low-density marrow cells (8×10^5) were incubated in semi-solid medium (4 x 1.0ml) containing fixed concentrations of amodiaquine (0, 1, 2, 4 or 8μM) and human plasma (50μl in 1.0ml).

On any one day the effect on the GM-CFU of test plasma (from one of the agranulocytosis-sufferers, the malaria-sufferer, or the healthy subject on oral amodiaquine) was compared with that of control plasma at each of the amodiaquine concentrations. Where

TABLE 6.1 Sources of plasma samples

Subject No.	Sex	Initals	Clinical status at time of sampling	Plasma Amodiaquine (ng ml^{-1})	Plasma Desethylam. (ng ml^{-1})
1	Male	AB	Newly-diagnosed agranulocytosis thought due to amodiaquine	N/D	37
11	Female	CS		Unknown	Unknown
111	Male	HS		N/D	113
1V	Female	HR		N/D	14
V	Male	DT	Healthy subject took amodiaquine as part of a study	N/D	35
V1	Male	SP	African with falciparun malaria	N/D	74

N/D = none detected

TABLE 6.2 ABO Blood groups of plasma samples and marrow

Marrow donor	Test plasma	Control Plasma
O	(I) O	A
O	(I) O	A
O	(I) O	O
B	(II) O	O
AB	(III) A	A
O	(V) O	O
O	(VI) Not known	O
B	(VI) Not known	O

(Roman numerals in parentheses refer to those test subject described in table 6.1).

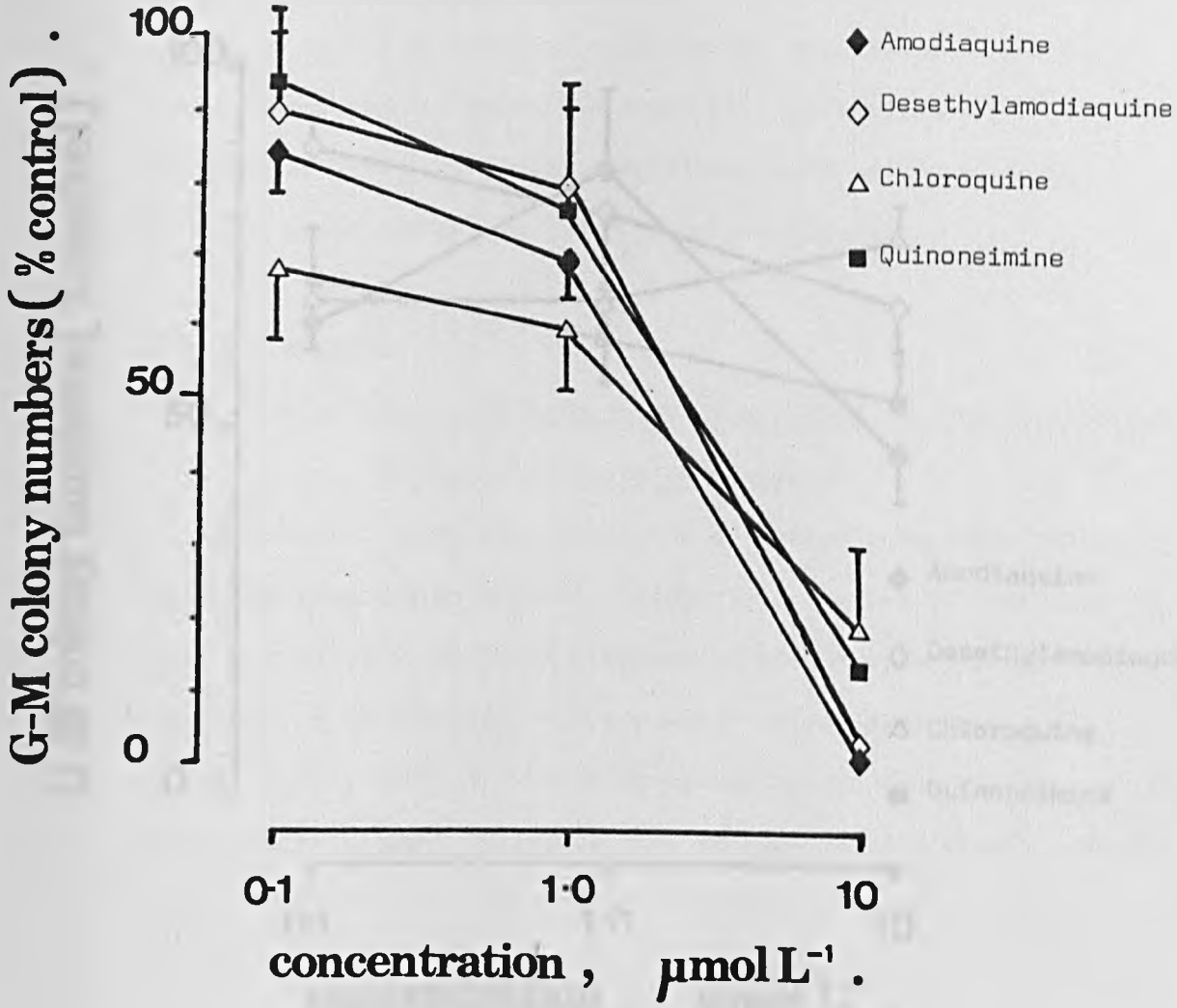


FIGURE 6.1 The effect on GM-CFU proliferation of ten day incubation with amodiaquine, its derivatives, or chloroquine

(n = 5 marrow donors; $\bar{x} \pm$ SEM)

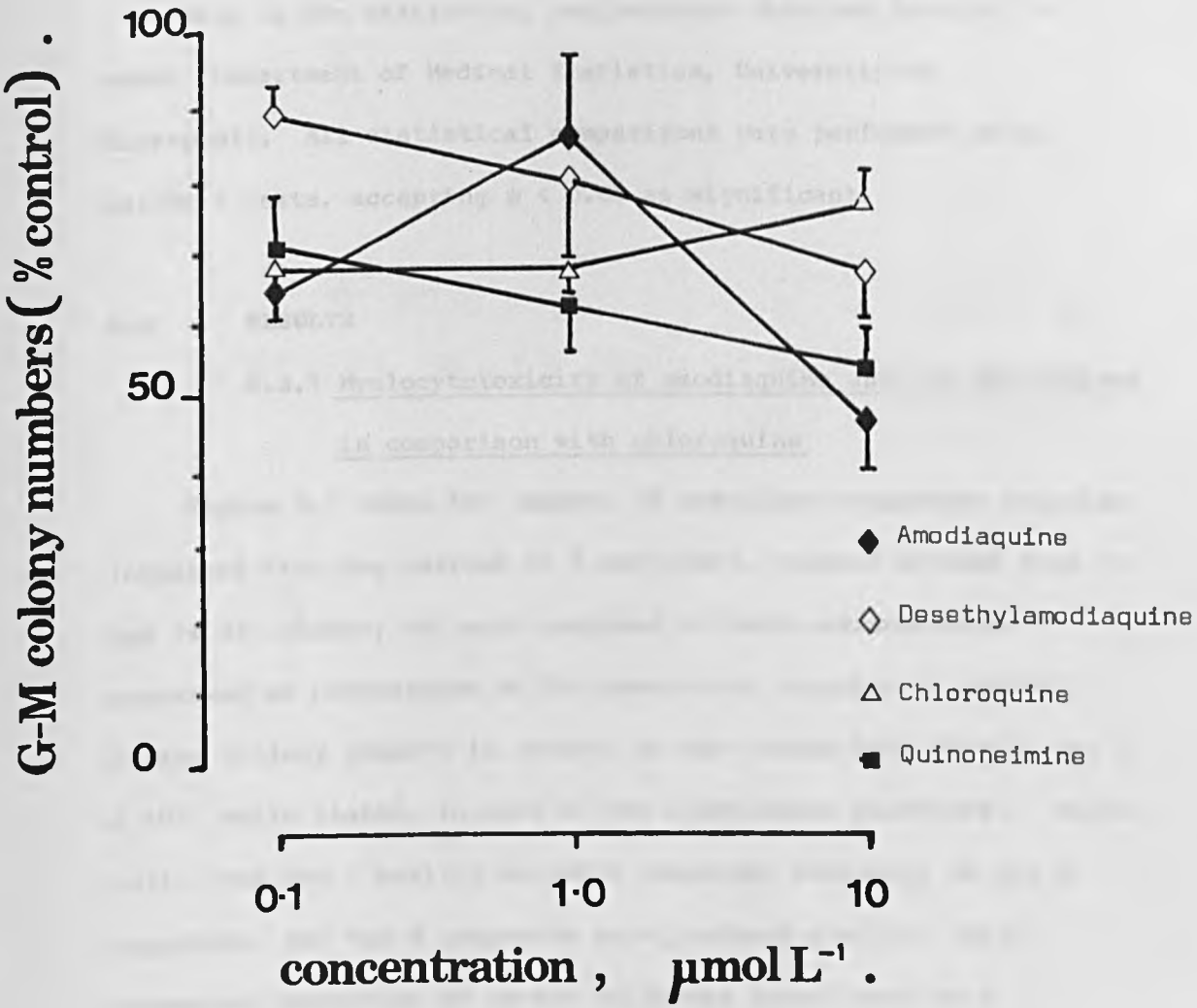


FIGURE 6.2 The effect on GM-CFU proliferation of one hour incubation with amodiaquine, its derivatives, or chloroquine

(n = 1 marrow donor, $\bar{x} \pm$ SEM 4 replicates)

possible the ABO blood groups of marrow donor, and test-plasma donor were matched. (table 6.2)

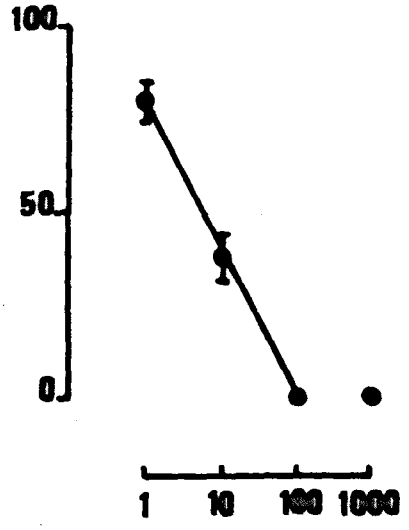
6.2.4 Statistical analysis

Help in the statistical analysis was obtained from Dr. D. Ashby (Department of Medical Statistics, University of Liverpool). All statistical comparisons were performed using paired t tests, accepting $p < 0.05$ as significant.

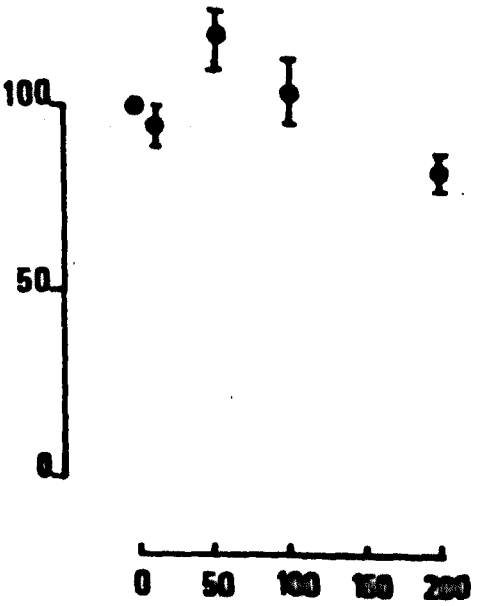
6.3 RESULTS

6.3.1 Myelocytotoxicity of amodiaquine and its derivatives in comparison with chloroquine

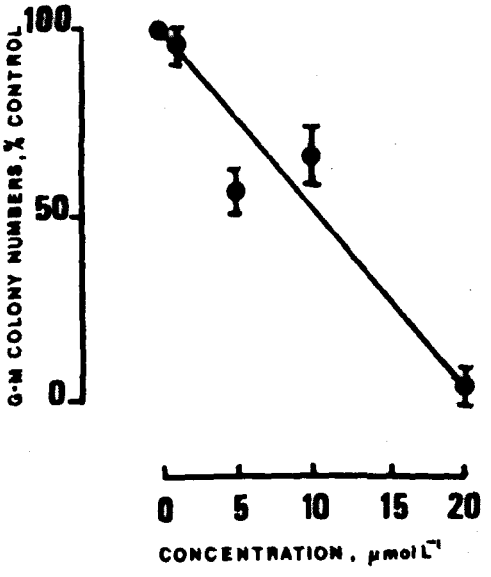
Figure 6.1 shows the numbers of granulocyte-monocyte colonies (obtained from the marrows of 5 subjects), counted between days 10 and 14 of culture, for each compound at each concentration, expressed as percentages of the numbers of colonies in control plates (colony numbers in control plates ranged from 30-110, per 2×10^5 cells plated, in each of the experiments described). Marrow cells from the 5 healthy subjects responded similarly to the 4 compounds, and the 4 compounds each produced similar, dose-dependent inhibition of GM-CFU which was significant at a concentration of 10μM ($p < 0.0001$ Figure 6.1). At 10μM amodiaquine no colonies were formed by GM-CFU from any of the 5 donors. In the cases of desethylamodiaquine, amodiaquine-p-quinoneimine and chloroquine, a concentration of 10μM permitted the formation of low numbers of colonies, but there was no statistically significant difference between the responses of GM-CFU to amodiaquine and chloroquine. At the lower concentrations, effects were less obvious, but amodiaquine and chloroquine produced significant inhibition of colony formation at 0.1 μM ($p < 0.05$ and 0.01 respectively) while all 4 compounds produced significant effects at 1.0 μM [amodiaquine $p < 0.001$; desethylamodiaquine $p < 0.01$;



DIETHYL MALEATE.



BUTHIONINE SULFOXIMINE.



n-ETHYL MALEIMIDE.

FIGURE 6.3 Effects of glutathione-depleting agents on GM-CFU from normal subjects

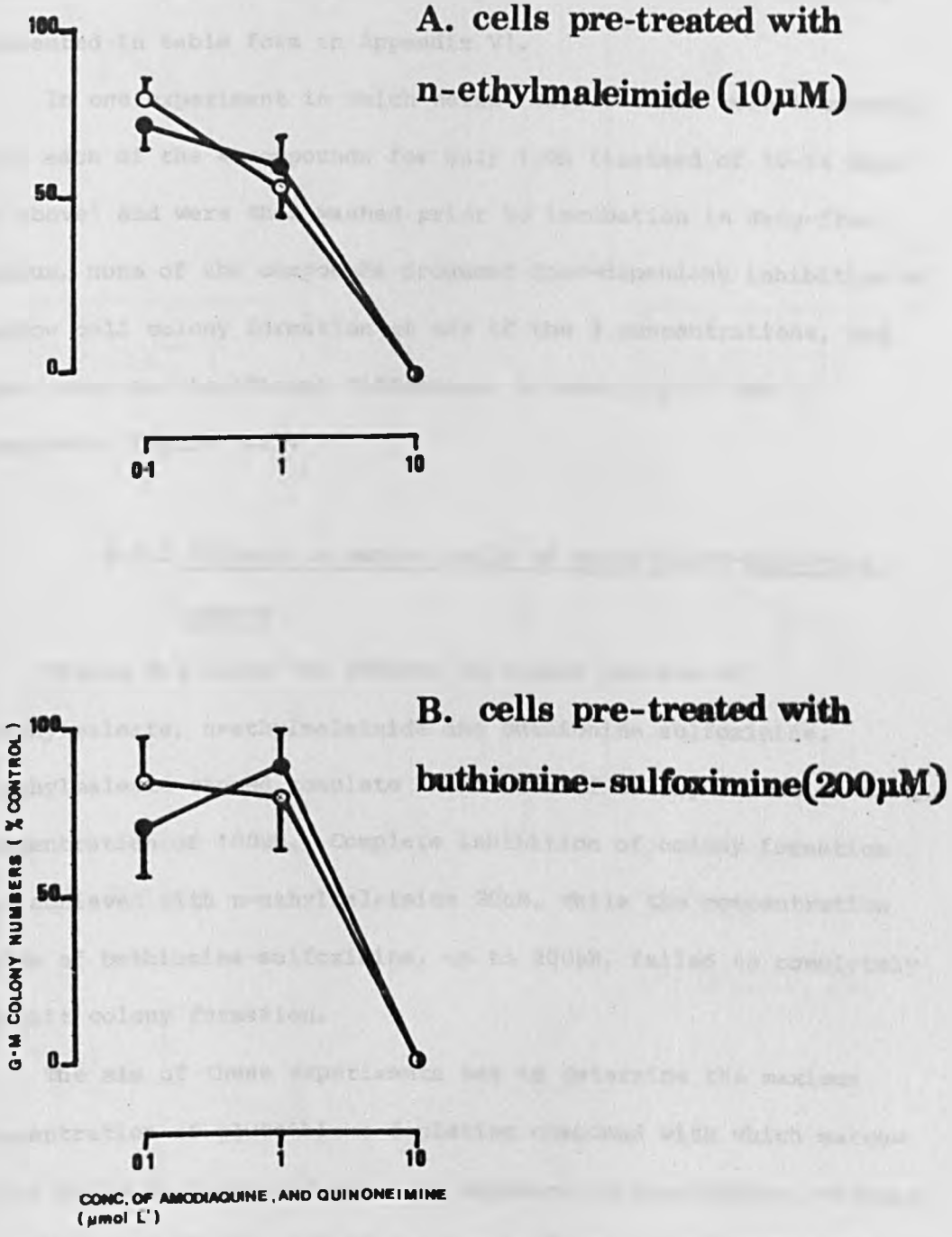


FIGURE 6.4 The effect on GM-CFU proliferation of 10 day incubation with amodiaquine ● and its quinoneimine ○ [1 marrow donor; 4 replicates $\bar{x} \pm$ SEM]

quinoneimine $p < 0.05$; chloroquine $p < 0.0001$]. [These data are presented in table form in Appendix V].

In one experiment in which normal marrow cells were incubated with each of the 4 compounds for only 1.0h (instead of 10-14 days as above) and were then washed prior to incubation in drug-free medium, none of the compounds produced dose-dependent inhibition of marrow cell colony formation at any of the 3 concentrations, and there were no significant differences between any of the 4 compounds (figure 6.2).

6.3.2 Effects on marrow cells of glutathione-depleting agents

Figure 6.3 shows the effects on colony numbers of diethylmaleate, n-ethylmaleimide and buthionine sulfoximine. Diethylmaleate caused complete inhibition of colony formation at a concentration of 100μM. Complete inhibition of colony formation was achieved with n-ethylmaleimide 20μM, while the concentration range of buthionine-sulfoximine, up to 200μM, failed to completely inhibit colony formation.

The aim of these experiments was to determine the maximum concentration of glutathione-depleting compound with which marrow cells could be incubated prior to exposure to amodiaquine, without inhibition of GM-CFU, and in the cases of n-ethylmaleimide and buthionine-sulfoximine, concentrations of 10μM and 200μM respectively were chosen for further study.

6.3.3 Effect of amodiaquine and its quinoneimine on bone marrow cells pre-incubated with n-ethylmaleimide or buthionine sulfoximine

Figure 6.4 (A) and (B) show the numbers of marrow cell colonies (obtained from the marrow of one subject), counted on day

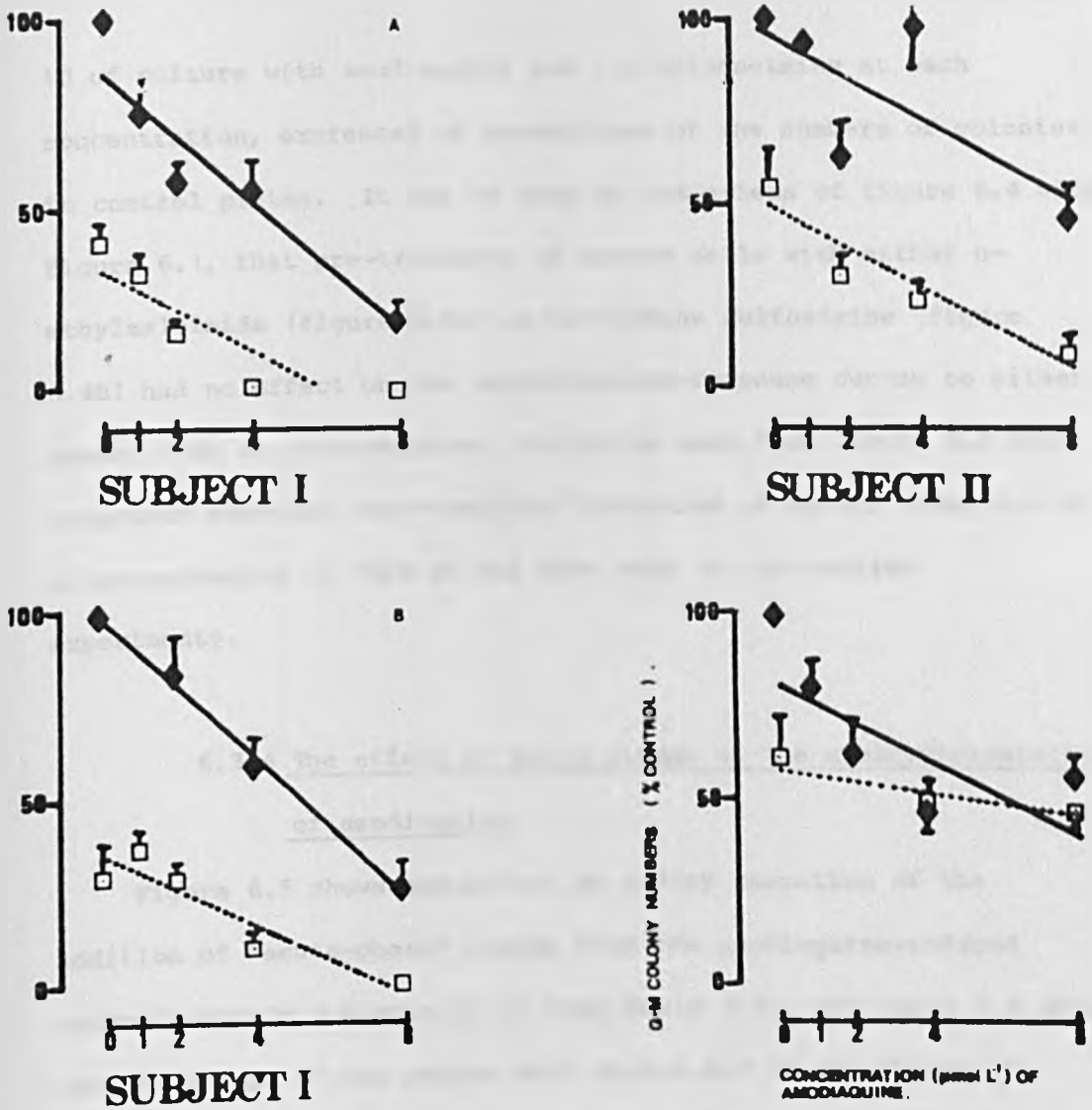


FIGURE 6.5 Effects of acute phase plasma, from subjects with amodiaquine-induced agranulocytosis, upon GM-CFU from normal subjects ($\bar{x} \pm$ SEM; 4 replicates) [Details of ABO blood groups, of GM-CFU donors, agranulocytosis patients and control subjects, are given in Table 6.2]

10 of culture with amodiaquine and its quinoneimine at each concentration, expressed as percentages of the numbers of colonies in control plates. It can be seen by comparison of figure 6.4 with figure 6.1, that pre-treatment of marrow cells with either n-ethylmaleimide (figure 6.4A) or buthionine sulfoximine (figure 6.4B) had no effect on the concentration-response curves to either parent drug or quinoneimine. As can be seen from figure 6.4 both compounds produced near-complete inhibition of colony formation at a concentration of 10μM as had been seen in the earlier experiments.

6.3.4 The effect of human plasma on the myelocytotoxicity of amodiaquine

Figure 6.5 shows the effect on colony formation of the addition of "acute-phase" plasma from the amodiaquine-induced agranulocytosis subjects I-III (see Table 6.1), and table 6.2 gives the ABO groups of the marrow cell donors and of the donors of control plasma. It proved possible to perform the experiment 3 times using plasma from subject I, but the plasma samples from subjects II and III were of too small a volume to permit repetition. Addition of plasma from subject IV to test plates caused macroscopic growth of bacterial colonies in all plates. As can be seen from figure 6.5 the presence of "acute-phase" plasma from these patients inhibited bone marrow colony formation by ~ 50% in the absence of added drug ($p < 0.01$). The presence of increasing concentrations of amodiaquine caused additive inhibition of colony formation, over that produced by "acute-phase" plasma alone.

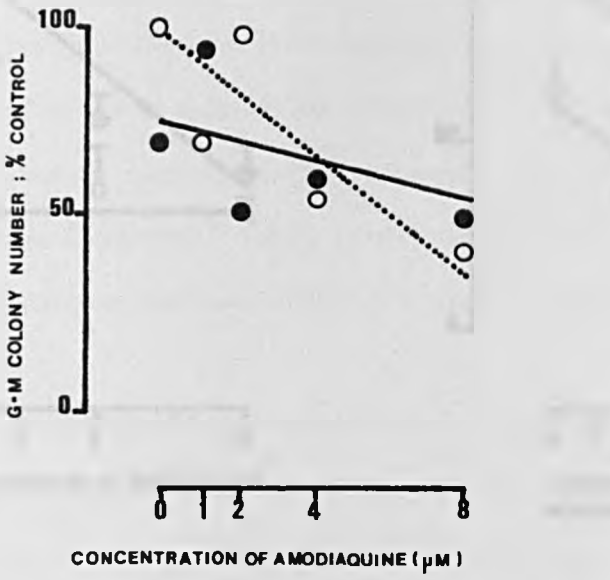


FIGURE 6.6. Effects on GM-CFU from a normal subject, of plasma from subject V, a healthy volunteer

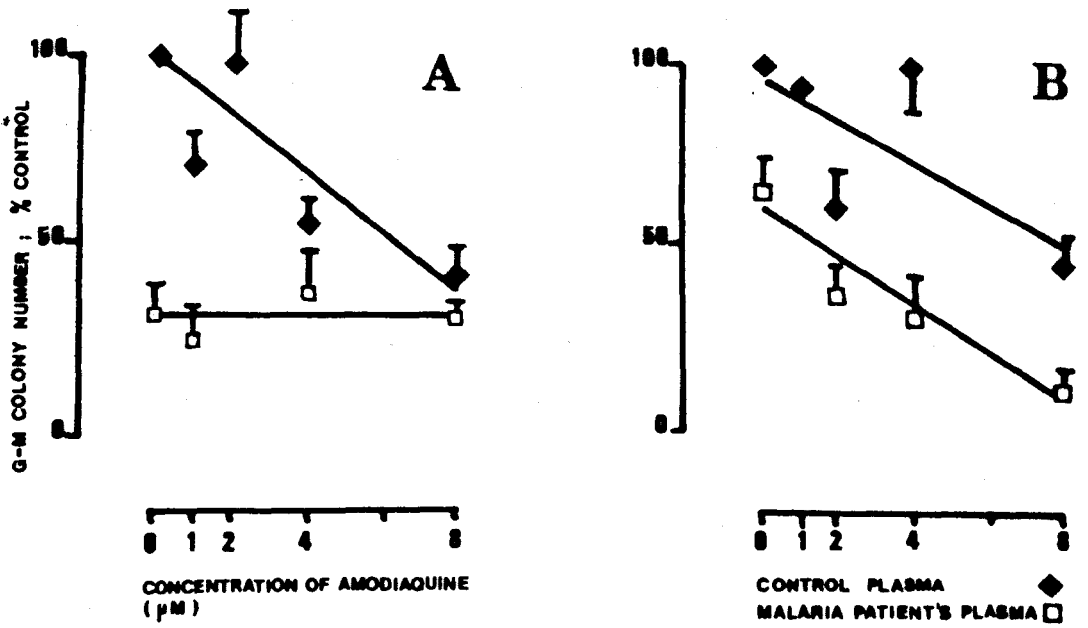


FIGURE 6.7 Effects on GM-CFU from healthy subjects of plasma from subject VI, a patient with falciparum malaria; A using pre-dose plasma, B using plasma drawn following treatment with amodiaquine

Figure 6.6 shows the effect on colony formation, of the addition of plasma from subject V. This healthy volunteer took amodiaquine 600mg by mouth as a part of the studies referred to in Chapter 3 of this thesis. Plasma was drawn from subject V pre-dose, and sequentially thereafter; for this experiment the pre-dose and 96h post-dose plasma samples were selected. On HPLC analysis of the 96h sample, amodiaquine was undetectable but the concentration of desethylamodiaquine was 35ng ml^{-1} (base). As can be seen from figure 6.6 there is no significant difference between the amodiaquine-concentration-response curves from the two plasma samples.

Figure 6.7 shows the effect, on colony formation, of the addition of samples of plasma from subject VI. This Zambian subject with acute falciparum malaria was treated with oral amodiaquine and blood samples were drawn as a part of the study described in Chapter 4 of this thesis. The pre-dose plasma sample and that drawn 168h following the first dose of drug were chosen for this experiment (amodiaquine was undetectable in the 168h sample; the concentration of desethylamodiaquine was 74ng ml^{-1} base). The 'pre-dose' and '168h' samples of plasma were studied on different days, using different sources of marrow cells. As can be seen from figure 6.7 both 'pre-dose' and '168h' plasma samples inhibited bone marrow cell colony formation in the absence of added drug. Addition of amodiaquine (1-8µg) to cultures using post-dose plasma from subject VI (figure 6.7B) gave additive inhibition of GM-colony formation, as had been seen using 'agranulocytosis-plasma'. In the case of pre-dose plasma from subject VI (figure 6.7A), addition of amodiaquine did not seem to inhibit colony formation any further. However this apparent effect probably

resulted from the relatively small numbers of colonies in control plates (mean, 33 colonies); inhibition of colony formation of 70%, in the absence of drug, reduced colony numbers to such a low level (mean, 10 colonies) that further effects were difficult to detect.

6.4 DISCUSSION

The overall incidence of idiosyncratic drug-induced agranulocytosis is not accurately known, largely because of the inadequate reporting of such adverse drug reactions. However a number of studies do give some perspective: in one paper from the United States 40% of reported cases of drug-induced blood dyscrasias were due to agranulocytosis (Huguley 1966). Böttinger and colleagues (1979) reported data on over 11,000 cases of adverse drug reactions seen in Sweden between 1966 and 1975; of these 199 were cases of agranulocytosis and 63 of these patients died. More recent data from the British Committee on Safety of Medicines shows that blood dyscrasias in general account for about 18% of all adverse drug reactions (CSM Update 1985). In the case of antimalarial compounds, and those drugs in general which are used for the treatment of tropical diseases, unless the drug is used by travellers from the developed world for prophylaxis, few organised attempts are made to assess the frequency of adverse drug reactions once the drug is freely available. In the light of recent experience with amodiaquine, where the high prevalance of agranulocytosis went unrecognised until the drug was used for prophylaxis, there is a need for better assessment of adverse drug reactions in tropical countries.

When considering the pathogenesis of idiosyncratic drug-induced agranulocytosis the central question is why a particular

patient developed the adverse effect when large numbers of other, seemingly similar, subjects did not. The various factors incriminated in drug-induced agranulocytosis have been reviewed in the introductory Chapter of this thesis. In the case of amodiaquine, agranulocytosis has been thought variously to be due to either an abnormal sensitivity to the drug of the myeloid precursor cells of affected subjects or to a drug-induced immune response in affected subjects leading to the adverse effect. Data has been published which would support either view (Lind et al 1973; Douer et al 1985; Rhodes et al 1986; Ellis et al 1987). However, generally, these papers have only studied the effects of the parent drug and have examined marrow cells from very small numbers of subjects.

The demonstration by Maggs and colleagues (1987) that amodiaquine is an unstable molecule which in aqueous solution spontaneously, and non-enzymatically, undergoes oxidation to form a potentially highly reactive quinoneimine, prompted this present study. The effect of amodiaquine-p-quinoneimine on human myeloid precursor cells clearly needed to be examined as the first step in the assessment of the role (if any) of this compound in the observed toxic response to amodiaquine. It was considered important too to study the effect of desethylamodiaquine on human marrow cells in vitro, since this metabolite is thought to be the principal compound involved in the therapeutic response to the drug (Churchill et al 1985). Similarly, it was clear that the parent drug needed to be examined using human bone marrow cell culture systems, but the other identified metabolites, bisdesethylamodiaquine and hydroxydesethylamodiaquine, have not been studied. This is in part because neither achieve as high a

plasma concentration as desethylamodiaquine, nor do either possess potent antimalarial activity, and in part because of the lack of availability of adequate quantities of either compound. Chloroquine was included for study because of its structural and functional similarities to amodiaquine, and because of its low liability to produce agranulocytosis clinically.

A major limitation of this present study is the lack of availability of bone marrow cells from patients suffering, or recovered, from amodiaquine-induced agranulocytosis. The prompt withdrawal of the drug for malarial prophylaxis meant that, thankfully, no further cases of amodiaquine-induced agranulocytosis were seen in Britain, and it was considered to be unethical to approach recovered subjects for bone marrow aspirates purely for research purposes. However, for the purposes of the present study, marrow cells from 'normal' subjects were adequate; in the first series of experiments the aim was to examine the relative toxicities of the 4 compounds studied, and in the second series of experiments the aim was to examine the possibility of the presence of drug-dependent components of the immune system in the samples of plasma available from patients with amodiaquine-induced agranulocytosis.

In the first series of experiments (figure 6.1) the effects on granulocyte-monocyte colony formation from normal human marrow cells of amodiaquine, desethylamodiaquine, amodiaquine-p-quinoneimine and chloroquine were studied. It had been expected that the quinoneimine would inhibit cell proliferation at a lower concentration than the parent drug, the desethyl metabolite or chloroquine, as had been observed by Maggs and Park (1988) in their experiments using peripheral blood lymphocytes, however this was

not seen. All 4 compounds produced significant inhibition of granulocyte-monocyte colony formation at a concentration of 1·0 μM and above (equivalent to 356 ng ml^{-1} amodiaquine, 328 ng ml^{-1} desethylamodiaquine, 354 ng gml^{-1} amodiaquine-p-quinoneimine, and 320 ng ml^{-1} chloroquine) which is the concentration that has been shown, using amodiaquine alone, to inhibit GM-CFU from patients recovered from amodiaquine-induced agranulocytosis; there were no significant differences between the 4 compounds. It is possible that the quinoneimine was subject to non-specific binding to elements of the nutrient medium, or that it was reduced back to the parent drug over the 10 day incubation period. (However it is relevant to note that in their experiments on the cytotoxicity of amodiaquine-p-quinoneimine, Maggs and Park (1988) examined low density human marrow cells as well as peripheral blood lymphocytes. Ten μM quinoneimine had no significant effect on the viability of the marrow cells, in contrast to lymphocytes; 50μM quinoneimine was needed before significant reductions were seen in cell viability.) Similarly it is possible that, in these present experiments, both amodiaquine and its desethyl metabolite exerted their toxic effect by autoxidation to quinoneimines once in solution. However of most importance is the overall similarity between the toxicity of chloroquine and that of the other compounds studied to the proliferation of granulocyte-monocyte precursor cells in this *in vitro* system. Chloroquine is only very rarely a cause of agranulocytosis and, although a 4 aminoquinoline like amodiaquine, possesses no phenolic ring in its side-chain, thus being incapable of giving rise to a quinoneimine. The lack of frequent cases of chloroquine-induced agranulocytosis does not seem to be due to differences in the distribution of chloroquine and

amodiaquine, since not only does chloroquine accumulate massively in certain blood cells (Bergqvist and Domeij-Nyberg 1983), but also ^{14}C-labelled chloroquine has been shown to accumulate in the bone marrow of rats (Lindqvist 1973). Even when the test compounds were incubated with the cells for only a short time before being washed off, no difference was to be seen between chloroquine and amodiaquine or its derivatives. That chloroquine is toxic to GM-CFU was observed by Rhodes and colleagues (1986); when the marrow cells of a patient recovered from amodiaquine-induced agranulocytosis were exposed to a variety of antimalarial drugs (in a culture system similar to that employed for these present studies) amodiaquine, chloroquine and sulfadoxine significantly inhibited colony formation at a concentration of approximately 1µ mol L^{-1} (none of the drugs in the concentrations used affected the normal control marrow cells). The observations of an apparent toxic effect of chloroquine *in vitro*, in these present experiments and elsewhere, when agranulocytosis due to chloroquine is only rarely a clinical problem, probably indicate the difficulty of extrapolation of such *in vitro* data to the clinical setting.

Were amodiaquine to exert its toxicity to marrow cells via the direct toxicity of its quinoneimine it could be expected that such damage would largely result from covalent binding of the quinoneimine to cell macromolecules, such as enzymes and structural proteins. One of the principal cell defence mechanisms against such damage involves conjugation of reactive compounds with glutathione, forming inactive sulphydryl conjugates. A well known example of drug toxicity which involves this mechanism of cell defence is overdosage with paracetamol (Mitchell *et al* 1973 *a* and *b*). In this situation a relatively small fraction of the paracetamol dose is

metabolised by a cytochrome P-450 to a reactive intermediate (a quinoneimine) which is detoxified by conjugation with glutathione. It is only when the cellular 'pool' of glutathione is exhausted that toxicity from the reactive compound is produced. It is known that dietary deficiency may perturb cell glutathione content (McLean and Day 1975) and that inborn errors of glutathione synthesis exist (Spielberg et al 1977); such individuals with low cell glutathione-content might be expected to be more susceptible to damage by amodiaquine-p-quinoneimine.

In an attempt to increase the sensitivity of normal granulocyte-monocyte precursor cells to amodiaquine and its quinonimine, cells were pre-treated with compounds known to deplete cell glutathione stores. Spielberg and colleagues (1980) used n-ethylmaleimide to deplete human lymphocyte glutathione content in vitro. These workers found that 5μM and 10μM depleted glutathione content by 60% and 91.3% respectively. Buthionine-sulfoximine is a potent inhibitor of the enzyme γ glutamylcysteine synthetase, which in involved in glutathione synthesis. At a concentration of 200μM buthionine-sulfoximine was observed by Griffith and Meister (1979) to produce virtually complete inhibition of γ glutamylcysteine synthetase in vitro. Buthionine-sulfoximine has also been used in experiments on intact cells in vitro as a depletor of glutathione-content (Lavelle and Patterson 1987). Two problems were encountered in this present study when using such glutathione-depleting agents: (a) depletion of cell glutathione content alone has an effect on cell viability and could be expected to have effects on the proliferation of GM-CFU. Since the method employed by the present study used inhibition of cell proliferation as an assessment of drug toxicity, complete inhibition of such

proliferation by glutathione-depleting agents was not desirable. Consequently the maximal concentration of glutathione-depleting agent which permitted GM-CFU proliferation had to be identified. (b) Although the concentration of n-ethylmaleimide and buthionine-sulfoximine which were employed were likely to have produced depletion of cell glutathione content, the length of time for which this effect persisted after the compounds were washed from the cells was not known, but was likely to be short (of the order of 0.5 - 1.0h). It would have been possible to have incubated cells with both glutathione-depleting agent and amodiaquine (or quinoneimine) for the full 10-14 day period, but unpredictable chemical reactions between the two compounds might well have occurred, making interpretation of results difficult. In the present study the glutathione-depleting agent was exposed to the marrow cells for a short time and was then washed off prior to a 10-14 day incubation of the cells with either amodiaquine or the quinoneimine. As can be seen by comparison of figures 6.1 and 6.6 pre-incubation with glutathione-depleting agents did not alter concentration response curves to either amodiaquine or amodiaquine-p-quinoneimine.

Humoral components of the immune system (antibodies) may be responsible for drug-induced agranulocytosis (Young and Vincent 1980), and circulating amodiaquine-dependent IgG antibodies directed against mature peripheral blood neutrophils were demonstrated by Schulthess and colleagues (1981) in serum from one subject suffering from amodiaquine-induced agranulocytosis. Douer and colleagues (1985), using in vitro culture of GM-CFU, demonstrated that colony formation from both one patient with amodiaquine-induced agranulocytosis, and an allogeneic control were

inhibited by the combination of acute-phase serum from the patient, plus amodiaquine. More recently Ellis and colleagues (1987), employing a similar technique of marrow cell culture, and using marrow from a further patient with amodiaquine-induced agranulocytosis, demonstrated inhibition of colony-formation by the combination of desethylamodiaquine and acute phase serum. For this present study, no patients were available with amodiaquine-induced agranulocytosis, but plasma samples were available from 4 such patients, and these samples had been drawn shortly after the initial diagnosis of agranulocytosis had been made. The effect of the 'acute phase' plasma samples was studied on normal GM-CFU. The plasma sample from subject I was large enough to permit the experiment to be conducted 3 times, while the samples from subject II and III permitted only one experiment each; the plasma sample from subject IV was clearly contaminated with bacteria. Because donor GM-CFU were to be incubated with plasma, the ABO blood groups of cell donors and 'control plasma' donors were ascertained. ABO blood groups of cell donors and 'agranulocytosis plasma' donors were matched if possible; if this was not possible, then a donor of 'control plasma' was chosen whose ABO group was the same as that of the 'agranulocytosis plasma' donor. In the event, even when ABO groups of cell donor and 'control plasma' donor were widely different, GM-CFU proliferation did not seen to be affected adversely.

As can be seen from figure 6.5 granulocyte-monocyte colony formation was inhibited by ~ 50% in the presence of 'acute phase' plasma in the absence of added drug. Plasma from a healthy volunteer who took oral amodiaquine as part of an earlier study (figure 6.8) had no such effect on colony formation, indicating

that the effect observed using the 'acute phase' plasma samples was unlikely to have been due to the presence in the plasma of a highly toxic metabolite or derivative of the drug. However each of the agranulocytosis patients had been acutely ill at the time the plasma samples had been obtained. In other words there was another difference between the donors of the acute-phase plasma samples and control plasma samples other than the consumption of amodiaquine, namely the state of general health of the subject. In acute infection many known changes occur to the constituents of the plasma, and these include the presence of such glycoproteins as tumour necrosis factor (Tracey et al 1987; Scuderi et al 1986) which could be expected to perturb the in vitro proliferation of granulocyte-monocyte precursors. Consequently in order to examine the relevance of the response of marrow cell proliferation to acute-phase plasma from the agranulocytosis patients, similar experiments were performed using plasma obtained from a Zambian subject ill with acute falciparum malaria. As can be seen from figure 6.7 plasma from this patient, whether drawn pre-dose or drawn 168h after commencement of amodiaquine therapy, produced the same response in the marrow cell culture system as had been observed using the agranulocytosis patients' plasma. This experiment indicated the sensitivity of granulocyte-monocyte precursors grown in vitro to plasma from an ill subject, and demonstrated the lack of drug-specificity of the previous results. Clearly a part of the observed response to the plasma samples from subjects I, II and III may have been due to the presence of drug-specific antibody, but this cannot be deduced conclusively from these present experiments. Since this present study was completed Christie and Park (in press) using the same

plasma samples from subjects I-IV, have demonstrated the presence of high titres of anti-amodiaquine antibodies.

6.5 SUMMARY

1. The effects have been studied of amodiaquine, desethylamodiaquine, amodiaquine-p-quinoneimine and chloroquine on the proliferation in vitro of granulocyte-monocyte colony forming units (GM-CFU) obtained from the bone marrow of healthy subjects.

2. All 4 compounds were equally toxic, producing near-total inhibition of colony formation between 1-10μmol L^{-1}.

3. Pre-incubation of marrow cells with 2 compounds, known to deplete cellular glutathione stores, failed to increase the toxicity of either amodiaquine or amodiaquine-p-quinoneimine.

4. Incubation of marrow cells with plasma (5%; v/v) obtained from patients during the acute phase of amodiaquine-induced agranulocytosis caused ~ 50% inhibition of colony formation in the absence of added amodiaquine. However this effect could be reproduced using drug-free plasma from a subject with acute malaria infection, indicating the effect to be neither drug-dependent nor drug-specific.

CHAPTER 7 Discussion, and recommendations for further research

This final chapter will consider the main findings of the thesis, and will discuss the problems which have been encountered during the project, and the ways in which these have been dealt with. Finally, recommendations will be made of those areas of work which merit further investigation.

Prior to the present studies, the pharmacokinetics of amodiaquine were thought to be similar to those of chloroquine, but this misapprehension was based on data which had been produced by drug analytical methods which were neither specific nor sensitive. As a part of the work toward this thesis, HPLC methods were developed which allowed selective, sensitive and reproducible measurement of amodiaquine and desethylamodiaquine in biological fluids. These were then used to investigate the clinical pharmacology of the drug. The present studies, and those of others reported during the course of the work (and referred to in previous chapters), have shown that the disposition of amodiaquine differs from that of chloroquine in many important respects. In particular, amodiaquine only achieves low concentrations in the plasma when administered by mouth, and is cleared rapidly. Desethylamodiaquine, which has been shown by others to be an active antimalarial agent *in vitro* (Churchill *et al* 1985, Mount *et al* 1987, Pussard *et al* 1987, Childs *et al* 1987), is the principal plasma metabolite, and is cleared more slowly than its parent drug. These observations suggest that metabolism to desethylamodiaquine, most likely in the liver, is a major route of amodiaquine clearance. It seems likely that amodiaquine, unlike chloroquine which has a bioavailability of 0.85 (Gustaffson *et al* 1983), is subjected to extensive first-pass metabolism, resulting in the observed low plasma concentrations of unchanged drug.

Even though amodiaquine is probably subject to extensive first-pass metabolism it would not be correct to refer to it as a "pro-drug" (Churchill et al 1985). This expression is generally applied to agents which possess little or no efficacy until they undergo biotransformation, usually in the liver, to an active form (or forms); a good example of this is proguanil, which is largely inactive until metabolised to its triazine derivative, cycloguanil. In vitro, the antimalarial efficacy of amodiaquine has been shown to be at least the equivalent of its desethyl metabolite, indeed usually slightly greater (Mount et al 1986; Pussard et al 1987; Childs et al 1987). Furthermore, although amodiaquine is most usually administered by mouth, following which most, but not all, of the antimalarial effect is likely to be due to the desethyl metabolite, the drug has also been used IV. White and colleagues (1987) have successfully used IV infusions of amodiaquine (10mg base kg^{-1}) to treat falciparum malaria in 10 adult Thais; no desethylamodiaquine was detected in the plasma of these subjects, in whom the therapeutic benefit was largely, or totally, due to the unchanged drug.

In the case of most drugs, in therapeutic doses, relationships between maintenance dose size and steady state drug concentrations are linear, and elimination half-life does not vary when the dose is altered. However in the case of some compounds clearance mechanisms, usually enzymic hepatic biotransformation, can become saturated within the therapeutically used dose range. This may lead to disproportionate rises in steady-state plasma level should the maintenance dose be increased. If the drug also has a narrow therapeutic index, this rise in plasma level may lead to toxicity. Amodiaquine itself, achieving as it does only low

plasma levels following oral administration, and undergoing rapid clearance from the plasma, would be unlikely to accumulate in the body on a weekly dosing schedule (as employed for malaria prophylaxis). However, the desethyl metabolite, which is cleared much more slowly, could accumulate. Although acute toxicity data for desethylamodiaquine are not available, it seems likely that they would resemble those of the parent drug. Like other 4 aminoquinolines, amodiaquine and desethylamodiaquine are probably compounds with relatively narrow therapeutic indices; when amodiaquine was given IV by White and colleagues (1987) no serious toxicity was seen, but there were significant falls in the systemic blood pressure of four subjects, and the electrocardiographic QRS complexes were widened in six. While a degree of accumulation of desethylamodiaquine could be expected to occur during the treatment of malaria (studied in chapter 4 of this thesis), long-term consumption of the drug, in the setting of malaria prevention (where a greater total mass of drug is usually taken), might be expected to lead to greater accumulation. Furthermore it was the population taking amodiaquine for malaria prevention which was shown to have high risk of agranulocytosis (Hatton et al 1986; see also chapter 1, section 1.6, of this thesis). While amodiaquine was not suspected of exhibiting non-linear pharmacokinetics, the suggestion had been made by Frisk-Holmberg and colleagues (1979) that chloroquine, a close structural-analogue, did behave in this way. The possibility that amodiaquine pharmacokinetics might be non-linear, and that this might have contributed to the drug's toxicity, when taken for malaria prevention, needed to be investigated. No evidence was found, however, which would support this view, and the possibility

has been discounted.

Amodiaquine remains in current use for the treatment of chloroquine-resistant falciparum malaria in many part of the world, particularly east Africa. Its use for this indication is likely to be limited by the cross-resistance of the organism, and by the availability of more efficacious agents in the near future. However toxicity from amodiaquine, in the setting of malaria treatment, does not seem to be a major problem. Given that the drug is likely to remain in use for this indication, at least for a short while, and given its known propensity to produce adverse reactions when taken for prophylaxis, investigation of the drug's disposition in malaria patients needed to be made. It was expected that the changes to chloroquine disposition observed during acute malaria (Adelusi et al 1985), would also be seen with amodiaquine. Most importantly it was expected that, during malaria parasitaemia, amodiaquine, and perhaps also its desethyl metabolite, would accumulate in the parasitised red cells, as was demonstrated to be the case for chloroquine (Adelusi et al 1985). In the event, as described in chapter 4 of this thesis, red cell levels of amodiaquine were generally undetectable in samples from the malaria patients. Red cell: plasma ratios of the desethyl metabolite were generally below unity at times of parasitaemia, seeming to rise as parasites were cleared, and eventually achieving levels of ~3:1, equivalent to the observed values from healthy subjects. This marked difference in the red cell disposition of the compound between healthy subjects and malaria patients was not mirrored by the plasma data. Plasma concentration vs time profiles for both parent drug and desethylamodiaquine from malaria patients were very similar to

those obtained from healthy subjects, and the derived pharmacokinetic parameters too were similar (in as far as they were comparable, given the differences between the dose schedules for the two groups of subjects). Of course, it should be remembered that the measured plasma concentrations of both drug and metabolite were total values, and not values of free, or unbound, compound. It is possible that, due to changes in plasma protein concentrations during acute malaria, free concentrations of both amodiaquine and desethylamodiaquine are quite different from those to be measured in healthy subjects. Since plasma protein binding of such weakly basic compounds as amodiaquine and desethylamodiaquine is likely to involve acid α_1-glycoprotein, and since the concentration of this rises in malaria, the trend would probably be to reductions in free drug levels during malaria.

It is difficult to know what significance, if any, can be placed upon the differences observed in these present studies, between the disposition of amodiaquine in healthy subjects and malaria patients, in terms of propensity to develop adverse effects to the drug. No data have been presented in these present studies which could directly explain the tendency for healthy subjects to develop serious adverse effects, while malaria patients seem to be spared. It is possible that the observed difference in drug-handling between the two groups plays a part in the toxic mechanism, but it seems more likely that it is duration of treatment and total mass of drug consumed, both greater on the whole in malaria prophylaxis, which determine the adverse effect profile.

The discovery of the high risk of agranulocytosis in subjects taking amodiaquine for malaria prevention prompted the studies

described in chapter 5 of this thesis, where the mass fate and tissue distribution of the drug were investigated using laboratory animals. This seemed particularly important because, were the adverse-effects of the drug due to tissue accumulation, leading to direct toxicity, recommendations on dosage might have been possible to reduce such accumulation. Chloroquine, which is structurally similar to amodiaquine, has been shown by McChesney and colleagues (1965) to accumulate in the tissues of Rhesus monkeys. In this study, 35% of the radioactivity, given to the monkeys as ^{14}C-chloroquine, was present in the tissues at 96h post-dose. The liver was one of the main organs of accumulation, but the marrow was not examined. Lindquist (1973) reported the tissue accumulation of radioactivity, after IV injection of ^{14}C-chloroquine into pigmented mice, finding that the marrow accumulation of radioactivity was of the same order of magnitude as that in the liver. Barrow (1974) reported the results of excretion studies following the administration of ^{14}C-amodiaquine to rodents, but much of the administered radioactivity was unaccounted for, and few data on tissue distribution were given. The present studies have shown that although radioactivity did accumulate in the tissues of rats following oral [^{14}C]-amodiaquine, this accounted for less than 5% of the dose. The tissues with major affinity for radioactivity included liver and marrow however, and these organs are the sites of the observed toxicity in humans.

The toxicity of many stable compounds is attributable to their oxidative metabolism to chemically reactive intermediates. The demonstration by Maggs and colleagues (1987 a, and b and 1988 a) that reactive compounds could be generated from amodiaquine,

and that this process did not require the catalysis of an enzyme, is likely to prove of major importance in the understanding of toxic responses to amodiaquine. Maggs and Park (1988 b) went on to show that amodiaquine-p-quinoneimine (10μM) was more toxic than the equimolar parent drug against human peripheral blood lymphocytes. However lymphocytes are not the "target" cells in drug induced agranulocytosis, and it was clear that the response of human marrow cells to amodiaquine and its derivaties needed to be studied. Ideally, marrow from two populations, healthy controls and patients recovered from amodiaquine induced agranulocytosis, would have been studied. The aim would have been the quantification of differences, if any, between the marrow cells from controls and agranulocytosis patients, in their responses to the amodiaquine derived compounds. However it was appreciated from the outset that marrow from the latter group would not be available since the drug had, by that time, been withdrawn for prophylaxis, and even had the drug continued in use the incidence of cases of agranulocytosis due to amodiaquine would probably have been too small to allow recruitment of such patients. Consequently in the present studies only 'normal' marrow cells were available. The technique which was used to investigate cell proliferation in the presence of added compound, is well established. Indeed *in vitro* culture of marrow cells has been used by other workers to study the effects of amodiaquine (reviewed in chapter 6) in individual cases of drug-induced agranulocytosis. The present studies compared the effects on marrow cell proliferation of amodiaquine, desethylamodiaquine, amodiaquine-p-quinoneimine and chloroquine. All four compounds were found to be equally toxic, and there were no significant

differences between them. This is in keeping with the work of Maggs and Park (1988 b), for although these workers showed significantly greater toxicity on lymphocytes of the quinoneimine when compared to the parent drug (both at 10μM), bone marrow cells were found, by these workers, to be less sensitive. Using bone marrow cells 10μM quinoneimine had no effect, and even at 50μM the reduction in viability, reported by Maggs and Park (1988 b), was only from 98 ± 2 to 91 ± 2% (significant at the 0.5% level). It is difficult however, to be fully confident of observations on the effect upon cell viability or proliferation, of such a reactive compound as a quinoneimine. It is likely that amodiaquine-p-quinineimine undergoes partial reduction back to the parent drug soon (seconds) after introduction to an aqueous solution (Maggs, personal communication). It is possible therefore that the essentially negative observations, concerning the effects of amodiaquine-p-quinoneimine on human marrow cells, of Maggs and Park (1988 b) and these present studies are the result of methodological problems. And yet if this were so, one would have expected that no difference would have been found between amodiaquine and its quinoneimine upon lymphocyte viability, when in fact Maggs and Park (1988 b) showed the quinoneimine to be more toxic.

The effect of acute phase plasma, from subjects with amodiaquine-induced agranulocytosis, on bone marrow cell proliferation has been also studied as part of the work in this thesis. As described in chapter 6 each of the plasma samples produced ~ 50% inhibition of GM-CFU proliferation, in the absence of added drug. However, this seemingly major effect could be reproduced using plasma from a subject with acute malaria

infection, raising the possibility that the effects observed using the agranulocytosis patients' plasma were not drug-induced. The effects observed may all have been due to the presence in the plasma samples of compounds which inhibit cell proliferation, such as tumour necrosis factor. All of the agranulocytosis patients, and the malaria patient, were systemically ill at the time that the plasma samples were obtained, and various inhibitory factors may have been present in the plasma. After completion of these present studies, remnants of the same plasma samples were subjected, by Christie, to ELISA for amodiaquine dependent antibodies, which were found in all samples (Christie *et al* in press). It is possible therefore that the observations made in the present studies were indeed drug-related, but the method used (i.e. *in vitro* culture of marrow cell colonies) was not adequately specific for this purpose.

During the course of the experimental work toward this thesis the status of amodiaquine has changed significantly. At the commencement of the work amodiaquine, hitherto little used when compared with chloroquine, showed great promise for both the prevention and treatment of chloroquine-resistant falciparum malaria in many parts of the tropics. Unfortunately enthusiasm for the compound was abruptly diminished by the discovery that its preventative use was associated with a high risk of agranulocytosis. Consequently, while the initial aim of this present research was the provision of clinically useful data on the drug's disposition, the aims of the research came to include investigation of the most serious of amodiaquine's adverse effects, agranulocytosis. Although it is obviously regrettable that amodiaquine caused serious toxicity, and despite the fact

that this curtailed many of the clinical studies which were planned, the resulting studies into the drug's toxicology have proved fruitful. The planned studies of the drug's disposition after repeated oral doses were abandoned on ethical grounds [data from the one subject studied are presented in appendix II]. However, together with the work of others, the studies into the toxicology of amodiaquine, presented in chapters 5 and 6 of the thesis, have proved instructive to the author, and have provided information on the drug's mechanism of toxicity.

Information on the absolute bioavailability of amodiaquine would be helpful to clinicians while the drug remains in use. This would be particularly the case if others are to use amodiaquine by IV infusion, as was undertaken by White and colleagues (1985). A study was planned to measure the bioavailability of amodiaquine by means of the simultaneous administration of the drug (600mg) by mouth and of a small dose of ^{14}C-amodiaquine by IV injection. This method avoids intraindividual day-to-day variations in drug disposition, and the need to administer large, and possibly toxic, doses of drug IV. The method depends in particular upon two criteria:
(i) that the drug exhibits linear pharmacokinetics, and
(ii) that the plasma concentration of the radiolabelled compound is detectable for long enough to permit its AUC value to be calculated. Such a "bioavailability study" was conducted on one healthy male subject, and the results are given in appendix I of this thesis. As can be seen from the results in appendix I, total plasma radioactivity fell below twice background-activity within 0.5h of the completion of injection, making meaningful calculations of bioavailability impossible. Consequently, the

study was not pursued further. Successful measurement of the absolute bioavailability of amodiaquine using this technique would require the use of a higher dose of IV radioactivity, and therefore would probably require a radiolabel with higher specific activity than the one used in the present studies. However, in the light of the data presented in Chapter 5 of this thesis, which showed tissue accumulation of the drug using an animal model, such a bioavailability study would not be ethical.

The choice of drug for the treatment of chloroquine-resistant falciparum malaria can be difficult. Amodiaquine continues to fill a role, in sub-Saharan Africa, as an oral agent effective against many chloroquine-resistant strains. However, amodiaquine is now known to be toxic and, if it is to be used to best effect, more information is required of its pharmacology and toxicology. No cases have been reported of serious toxicity with the drug when used for the treatment of acute malaria. However this may merely reflect poor reporting of adverse drug reactions in third world countries. It is clear that, if amodiaquine is to continue in clinical use, prospective studies are required to examine the risk to patients.

It is relatively unusual for the mechanism of toxicity of a drug to be understood in detail. Such mechanisms, once outlined, may prove to be useful models when considering the toxicity of other drugs. Furthermore, at the beginning of an era of specifically designed drug molecules, such as the newer angiotensin converting enzyme inhibitors, it would be clearly advantageous to maximise knowledge of adverse drug effect mechanisms, in order to allow a degree of prediction. Amodiaquine may well provide some of this useful information. Much work has

already been reported on its mechanism of toxicity, but much remains to be done. On the level of basic pharmacology, further work is required into the metabolic fate of the drug. Although the animal model, reported in chapter 5 of this thesis, provided information on the mass-fate of amodiaquine, its major forms of excretion remain unidentified. The parent drug and its desethyl metabolite accounted for less than 15% of the dose, and little evidence was found of conjugates as major forms of excretion.

The recent demonstration by Christie and colleagues (in press) that both amodiaquine and its quinoneimine are immunogenic in rats has provided further areas in which work is needed. In addition to more detailed studies in animal models, a prospective clinical study is now needed to examine the prevalence of the development of an antibody response to amodiaquine in patients being treated for malaria. This study would be a logical extension of the prospective search for clinical adverse reactions to the drug.

The demonstration, in chapter 6 of this thesis, that desethylamodiaquine accumulation in red cells _in vivo_ is effected by the presence of Plasmodium requires further investigation. It is possible that the presence of the parasite in the red cell increases the degree to which amodiaquine and its metabolites form reactive species, and thus become covalently bound. In order to study this further, a radiolabelled form of desethylamodiaquine would be required, and would be incubated with infected and uninfected red cells _in vitro_. Scintillation counting would allow an assessment of the degree to which the compound had been taken up into the cells, while HPLC analysis would allow quantitation of non irreversibly bound compound. Such studies, involving the

incubation of human biological fluids with radiolabelled compound *in vitro*, could be extended to consider the plasma protein binding of amodiaquine and its derivatives. Such information as there is concerned only the parent drug, and this area clearly requires further work.

The studies presented in this thesis have expanded our knowledge of the pharmacology of amodiaquine, and have posed many new questions. While these studies are currently of clinical relevance, the main relevance of much of the work is in the elucidation of the drug's toxicology. If the toxic mechanism of amodiaquine is to be better understood, and therefore become a useful model for the understanding of other drugs, much further work, both laboratory and clinical, is required.

REFERENCES

Adelusi SA, Dawodu AH, Salako LA (1982); Kinetics of the uptake and elimination of chloroquine in children with malaria. Br J Clin Pharmac; 14: 483-7.

Amouretti M, Raymond JM, Baldit C, Duman F, Couzigou P, Beraud C (1986); L'amodiaquine (Flavoquine) est hepatotoxique [LETTER). Gastroenterol Clin Biol; 10: 691.

Barrow A (1974); The disposition and metabolism of amodiaquine in small mammals. Xenobiotica; 4: 699-80.

Bepler CR, Baier HN, McCracken S, Rentschler CL, Rogers FB, Lansbury JA (1959); A 15 month controlled study of the effects of amodiaquine (Camoquin) in rheumatoid arthritis. Arthritis Rheum; 2: 403-13.

Bergquist Y, Domeij-Neiberg B (1982); Distribution of chloroquine and its metabolite desethylchloroquine in human blood cells, and its implication for the quantitative determination of these compounds in serum and plasma. J Chromatogr; 272: 137-48.

Berliner RW, Earle DP, Taggart JV, Zubrod CG, Welch WJ, Conan NJ, Baumann E, Scudder ST, Shannon JA (1948); Studies on the chemotherapy of the human malarias. The physiological disposition, antimalarial activity and toxicity of several derivatives of 4-aminoquinoline. J Clin Invest; 27: 98-107.

Blomgren SE, Condemi JJ, Vaughan JH (1972); Procainamide-induced lupus erythematosus. Clinical and laboratory observations. Am J Med; 52: 338-48.

Booth K, Larkin K, Maddocks I (1967); Agranulocytosis coincident with amodiaquine therapy. Br Med J; 3: 32-33.

Bottiger LR, Furhoff AK, Holmberg L (1979); Drug-induced blood dyscrasias. A ten year material from the Swedish Adverse Drug Reactions Committee. Acta Med Scand; 205: 457-61.

Brodie BB, Udenfriend S, Dill W, Chenkin T (1947); The estimation of basic organic compounds in biological material. J Biol Chem; 168: 319-25.

Bruch-Chwatt, LJ (1980); Essential Malariology; William Heinemann; London.

Burckhalter JH, Tendick FH, Jones EM, Jones PA, Holcombe WF, Rawlins AL (1948); Aminoalkylphenols as antimalarials. II. (Heterocyclic-amino)-2-amino-0-cresols. J Am Chem Soc; 70: 1363-73.

Burnet FM (1957); A modification of Jerne's theory of antibody production using the concept of clonal selection. Aust J Sci; 20: 67-9.

Carr, R (1986); Neutropaenia and prophylactic amodiaquine. (LETTER). Lancet; I: 556.

Carrington HC, Crowther AF, Davey DG, Levi AA, Rose FL (1951); A metabolite of Paludrine with high antimalarial activity. Nature; 168: 1080.

Carstairs KC, Worlledge SM, Dollery CT, Breckenridge AM (1966); Methyldopa and haemolytic anaemia. Lancet; I: 201.

Chambon MM, Vo P, Remenant JM (1968); Enquete experimentale sur le metabolisme de quelque medicaments antimalariques; chloroquine et amodiaquine. Bordeaux Med; 8: 1471-7.

Childs GE, Boudreau EF, Milhous WK, Wimonwattrawatee T, Pooyindee N, Limchitte S, Pang L, Webster HK; In vitro activity of amodiaquine and desethylamodiaquine against isolates of Plasmodium falciparum. Trans Roy Soc Trop Med Hyg; IN PRESS.

Christie G, Park BK; The detection of anti-amodiaquine antibodies by ELISA. Br J Clin Pharmac; IN PRESS.

Churchill FC, Patchen L, Campbell C, Schwartz I, Nguyen-dinh P, Dickinson C (1985); Amodiaquine as a pro-drug: importance of metabolite(s) in the antimalarial effect in humans. Life Sci; 36: 53-62.

Churchill FC, Mount D, Patchen L, (1986); Isolation, characterisation and standardisation of a major metabolite of amodiaquine by chromatographic and spectroscopic methods. J Chromatogr; 377: 307-18.

Ciak J, Hahn FE, (1969); Chloroquine: mode of action. Science; 151: 347-9.

Coatney GR, Cooper WC, Eddy NB, Greenberg J (1953); Survey of antimalarial agents. US Gov Printing Off, Washington DC.

Coatney GR (1963); Pitfalls in a discovery; the chronicle of chloroquine. Am J Trop Med Hyg; 12: 121-28.

Costa LG, Murphy SD (1986); Effect of diethylmaleate and other glutathione-depletors on protein synthesis. Biochem Pharmac; 35: 3383-8.

CSM update (1985); Blood dyscrasias. Br Med J; 291: 1269.

Cunningham JL, Leyland MJ, Delamore IW, Evans DAP (1974); Acetanilide oxidation in phenylbutazone-related hypoplastic anaemia. Br Med J; III: 313-7.

Douer DD, Schwartz E, Shaked N, Ramot B (1985); Amodiaquine-induced agranulocytosis; drug inhibition of myeloid colonies in the presence of patients serum. Israel J Med Sci; 21: 331-3.

Ejercito A, Dugue M (1948); Preliminary report on CAM-AQI dihydrochloride (Miaquine, Camoquin) in the treatment of human malaria. J Philippine Med Ass; 24: 633-42.

Ellis ME, Steed AJ, Addison GM (1987); Neutropaenia associated with dual antimalarial chemoprophylaxis - use of bone marrow culture as an aid in further drug management. J Infection; 15: 147-52.

Evans DAP, White TA (1964); Human acetylation polymorphism. J Lab Clin Med; 63: 394-403.

Falco EA, Hitchings GH, Russell PB, Vanderwerff H (1949); Antimalarials as antagonists of purines and pteroylglutamic acid. Nature; 164: 107-8.

Fitch CD (1973); Chloroquine-resistant Plasmodium-falciparum: differences in the handling of ^{14}C amodiaquine and ^{14}C chloroquine. Antimicrob Ag Chemother; 3: 545-8.

Fitch CD, Yunis N, Chevli R, Gonzalez Y (1974); High affinity accumulation of chloroquine by mouse erythrocytes infected with Plasmodium berghei. J Clin Invest; 54: 24-33.

Fitch CD, Gonzalez Y, Chevli R (1975); Amodiaquine accumulation by mouse erythrocytes infected with Plasmodium berghei. J Pharmacol Exp Ther; 195: 397-403.

Fitch CD, Chevli R, Banyal HS, Phillips G, Pfaller MA, Krogstad DJ (1982); Lysis of Plasmodium falciparum by ferriprotoporphyrin IX, and a chloroquine-ferriprotoporphyrin IX complex. Antimicrob Ag Chemother; 21: 819-22.

Fitch CD (1986); Antimalarial schizontocides: ferriprotoporphyrin IX, interaction hypothesis. Parasitol Today; 2: 330-1.

Friedman MJ, Roth EF, Nagel RF, Trager W (1979); P. falciparum physiological interactions with human sickle cells. Exp Parasitol; 47: 73-80.

Friman G, Nystrom-Rosander C, Jonsell G, Bjorkman A, Lekas G, Svendsrup B (1983); Agranulocytosis associated with malaria prophylaxis with Maloprim. Br Med J; 286: 1244-5.

Frisk-Holmberg M, Bergquist Y, Domeij-Neiberg B, Helstrom L, Jansson F (1979); Chloroquine serum concentration and side-effects; evidence for dose-dependent kinetics. Clin Pharmacol Ther; 25: 345-50.

Fritsch B, Dieckmann A, Menz B, Hempelmann E, Fritsch KG, Fritsch G, Jung A (1987); Glutathione and peroxide metabolism in malaria parasitised erythrocytes. Parasitol Res; 73: 515-7.

George CF, Shand DG, (1982); Pre-systemic drug metabolism in the liver. In "Pre-systemic drug elimination, clinical pharmacology and therapeutics". George and Shand; Butterworths, London UK.

Gerson WT, Fine DG, Spielberg SP, Sensenbrenner LL (1983); Anticonvulsant-induced aplastic anaemia; increased susceptibility to toxic drug metabolites in-vitro. Blood; 61: 889-93.

Gillespie P, Wagner F (1977); Amodiaquine agranulocytosis. Med J Aust; 1: 298-9.

Glazko AJ, Wolf LM (1957); Laboratory studies on Camoquin. Data on file, Parke-Davis Research Laboratories.

Glick L (1957); Fatal agranulocytosis during treatment with amodiaquine. Br Med J; 1: 932.

Griffith OW, Meister A (1979); Potent and specific inhibition of glutathione synthesis by buthionine-sulfoximine (S-n-butyl homocysteine sulfoximine). J Biol Chem; 254: 7558-7560.

Gruhzit OM (1961); Data on file, Parke-Davis Research Laboratories.

Gustaffsson LL, Walker O, Alvan G, Beerman B, Estevez F, Gleisner L, Lindstrom B, Sjoquist F (1983); Disposition of choroquine in man after single intravenous and oral doses. Br J Clin Pharmac; 15: 471-9.

Gustaffsson LL, Beerman B, Aden-Abi Y (1987); Handbook of drugs for tropical parasitic infections. Taylor and Francis, London, New York and Philadelphia.

Guttman P, Erhlich P (1981); Uber die Wirkung des Methianblau bei Malaria. Berl Clin Wochenschr; 28: 953-956.

Halawani A, Baz I, Morkos S (1947); A preliminary report on the antimalaria CAM-AQI. J Roy Egyptian Med Ass; 30: 99.

Hatton C, Peto T, Bunch C, Pasvol G, Russell S, Singer C, Edwards G, Winstanley P (1986). Frequency of severe neutropaenia associated with amodiaquine prophylaxis against Malaria. Lancet; 1: 411-3.

Hobbs HE, Sorby A, Freedman A (1959); Retinopathy following chloroquine therapy. Lancet; II: 478-9.

Holme JA, Dahlin SD, Nelson SD, Dybing E (1984); Cytotoxic effects of N-acetyn benzoquinoneimine, a common arylating intermediate of paracetamol and N-hydroxyparacetamol. Biochem Pharmac; 33: 401-406.

Howell A, Andrews TM, Watta TWE (1975); Bone marrow cells resistant to chloramphenicol in chloramphenicol-induced aplastic anaemia. Lancet; I: 65-69.

Huguley CM (1966); Haematological reactions. JAMA; 196: 408-10.

Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR, Brodie BB (1973); Acetominophen-induced hepatic necrosis, II role of covalent binding; J. Pharmac Exp Ther; 187: 195-201.

Kale O (1982); Clinical trials of amodiaquine in onchocirciasis. Bull WHO; 60: 929-32.

Karbwang J, Looareesuwan S, Phillips RE, Wattanagoon Y, Molyneux M, Nagachinta B, Back DJ, Warrell DA (1987); Plasma and whole blood mefloquine concentrations during treatment of chloroquine-resistant falciparum malaria with the combination mefloquine-sulphadoxine-pyrimethamine. Br J Clin Pharmac; 23: 488-81.

Kelton JG, Hugan AT, Mold N, Logue GL, Rosse WF (1979); The use of in vitro techniques to study drug induced pancytopaenia. New Eng J Med; 301: 621-4.

Kennedy AF (1955); Absolute neutrophil leukopaenia after uncontrolled use of amodiaquine. Br Med J; 2: 475-6.

Khaliq AA, Fox E, Sarwar M, Strickland T (1987); Amodiaquine fails to cure chloroquine-resistant Plasmodium falciparum in the Punjab. Trans Roy Soc Trop Med Hyg; 81: 157-9.

Kirtland HH, Mohler DN, Horwitz DA (1980); Methyldopa inhibition of supressor-lymphocyte function. New Eng J Med; 302: 825-32.

Kurland J, Moore MAS (1977); Modulation of haemopoiesis by prostaglandins. Exp Haematol; 5: 357-73.b

Kuroda K (1962); Detection and distribution of chloroquine metabolites in human tissues. J Pharmacol Exp Ther; 137: 156-61.

Lajtha LG (1982); Cellular kinetics of haemopoiesis. In "Blood and its disorders"; Hardisty and Weatherall, Blackwell, London.

Larrey D, Castot A, Pessayre D, Merigot P, Machayeky JP, Feldmann G, Lenoire A, Rueff B, Benhamou JP (1986); Amodiaquine-induced hepatitis. An Int Med; 104: 801-3.

Lavelle TJ, Patterson LH (1987); Effect of doxorubicin on glutathione-depleted hepatocytes. Biochem Soc Trans; 15: 454-5.

Lepeu G, Codine P, Janbon F, Bertrand A (1981); Agranulocytose a l'amodiaquine [LETTER]. Nouv Presse Med; 10: 2827-8.

Lind DE, Levi JA, Vincent PC (1973); Amodiaquine-induced agranulocytosis: toxic effect of amodiaquine in bone marrow cultures in-vitro. Br Med J; 458-60.

Lindquist NG, (1973); Accumulation of drugs on melanin. Acta Radiolog; suppl 325.

Looareesuwan S, Phillips R, White NJ, Karbwang J, Benjasurat Y, Attanath P, Warrell DA (1985); Intravenous amodiaquine and oral amodiaquine-erythromycin in the treatment of chloroquine-resistant falciparum malaria. Lancet; 2: 805-808.

Love J, Foulk R, Williams RG, Mitchell RB (1953); Evaluation of amodiaquine (Camoquin) in the treatment of relapsing vivax malaria. Am J Med Sci; 225: 26-33.

Maggs JL, Colbert J, Winstanley PA, Orme ML'E, Park BK (1987 a); Irreversible binding of amodiaquine to human liver microsomes: chemical and metabolic factors. Br J Clin Pharmacol; 23: 649P.

Maggs JL, Kitteringham NR, Breckenridge AM, Park BK (1987 b); Autoxidative formation of a chemically reactive intermediate from amodiaquine, a myelotoxin and hepatotoxin in man. Biochem Pharmacol; 36: 2061-2.

Maggs JL, Tingle MD, Kitteringham NR, Park BK (1988 a); Mechanisms of formation of protein-arylating intermediates from amodiaquine, a myelotoxin and hepatotoxin in man. Biochem Pharmacol; 37: 303-11.

Maggs JL, Park BK (1988 b); Cytotoxicity of the quinoneimmine of amodiaquine. Br J Clin Pharm; 25: 143P.

Martindale (1982); Reynolds JEF. Pharmaceutical Press, London.

Manson-Bahr, Sir P; The story of Malaria (1963) Int Rev Trop Med 2: 329-390.

McChesney EW, Wyzan HS, McAuliff JP (1956); The determination of 4-aminoquinoline antimalarials: re-evaluation of the induced fluorescence method with specific application to hydroxychloroquine analysis. J Pharm Sci; 45: 640-5.

McChesney EW, Conway WD, Banks WF, Rogers JE, Shekosky LM (1966); Studies on the metabolism of some compounds of the 4-aminoquinoline series. J Pharmacol Exp Ther; 151; 482-93.

McChesney EW, Fasco MJ, Banks WF (1967); The metabolism of chloroquine in man, during and after repeated oral dosage. J Pharmacol Exp Ther; 158: 323-31.

McLean AEM, Day PA (1975); The effect of diet on the toxicity of paracetamol, and the safety of paracetamol-methionine mixtures. Biochem Pharmacol; 24: 37-42.

Metcalf D, Bradley TR, Robinson WA (1967); Analysis of colonies developing in-vitro from mouse bone marrow cells stimulated by kidney "feeder layers" and leukaemic serum. J Cell Physiol; 69: 93-107.

Metcalf D (1985 a); The granulocyte-macrophage colony stimulating factors. Cell; 43: 5-6.

Metcalf D (1985 b); Molecular control of granulocyte and macrophage production. In "Experimental approaches for the study of hemoglobin switching; Alan R. Liss, Inc.

Mitchie M, Manogue K, Spriges D, Revhaug A, O'Dwyer S, Dinarello C, Cerami A, Wolf S, Wilmore D (1988); Detection of circulating TNF after endotoxin administration. NEJM; 318: 1481-6.

Mikkelson RB, Tanabe K, Wallach DFH (1982); Membrane potential of Plasmodium-infected erythrocytes. J Cell Biol; 93: 685-9.

Mihaly G, Nicholl D, Edwards G, Ward S, Orme M, Breckenridge A (1985); High performance liquid chromatographic analysis of amodiaquine in human plasma. J Chromatogr; 37: 166-71.

Mitchell JR, Jollow JD, Potter WZ, Davis DC, Gillette JR, Brodie BB (1973 a); Acetominophen-induced hepatic necrosis. I: role of drug metabolism. J Pharmacol Exp Ther; 187-94.

Mitchell JR, Jollow JD, Potter WZ, Davis DC, Gillette JR, Brodie BB (1973 b); Acetominophen-induced hepatic necrosis. IV: protective role of glutathione. J Pharmacol Exp Ther; 187: 211-7.

Moeschlin S, Wagner K (1952); Agranulocytosis due to the occurrence of leukocyte-agglutinins. Acta Haematolog; 8: 29-41.

Morbidity and Mortality Weekly Report. US Department of Health and Human Services (1985); Revised recommendations for preventing Malaria in travellers to areas with Chloroquine-resistant Plasmodium-falciparum. 34[14].

Mount DL, Patchen LC, Nguyen-Dinh P, Barber AM, Schwartz IK, Churchill FC (1987); Sensitive analysis of blood for amodiaquine and its major metabolites by high performance liquid chromatography with electrochemical detection. J Chromatogr; 383: 375-86.

Myers CD, Katz FE, Joshi G, Millar JL (1984); A cell line secreting stimulating factors for CFU-GEMM culture. Blood; 64: 152-55.

Neftel K, Woodtly W, Schmid Frich P, Fehr J (1986); Amodiaquine-induced agranulocytosis and liver damage. Br Med J; 292: 721-3.

Nossal G (1987); Current concepts in immunology: the basic components of the immune system. New Eng Med J; 316: 1320-5.

Ochi T, Goldings EA, Lipsky PE, Ziff M (1983); Immunomodulatory effect of procainamide in Man. J Clin Invest; 71: 36-45.

Payne E, Sharp E, Nickel K (1949); Parenteral use of Camoquine hydrochloride as an antimalarial. Am J Trop Med; 29: 353-68.

Penido H, DeSouza FE, Bezerra FPG (1947); Report given before the sixth Brazilian Congress of hygiene.

Perry HO, Bartholomew LG, Hanlon DG (1962); Nearly fatal reaction to amodiaquine. J.A.M.A.; 179: 598-601.

Peto T, Gilks C (1986); Strategies for the prevention of malaria in travellers; comparison of drug regimens by means of risk: benefit analysis. Lancet 1: 1256-1261.

Petz LD, Fudenberg HH (1975); Immunological mechanisms in drug-induced cytopaenias. Prog Haematol; 9: 185-206.

Phillips RE, Warrell DA, White NJ, Looareesuwan S, Karbwang J (1985); Intravenous quinidine for the treatment of severe falciparum malaria: clinical and pharmacokinetic studies. New Eng J Med; 312: 1273-8.

Pike B, Robinson WA (1974); Human bone marrow colony growth in agar gel. J Cell Physiol; 76: 77-84.

Pinichpongse S, Doberstyn E, Cullen J, Yisunri L, Thongsombun Y, Thomasarn K (1982); An evaluation of 5 regimens for the outpatient treatment of faciparum malaria in Thailand 1980-81 Bull WHO 60(6) 907-912.

Pisciotta V (1978); Drug-induced agranulocytosis. Drugs; 15: 132-43.

Pomeroy H, Warren C, Mills D, Clark G (1959); The effect of amodiaquine (Camoquin) on the course of Rheumatoid arthritis. Arthritis Rheum; 2: 396-402.

Powell G, Curtis G, Dodgson K (1967); Distribution of 35-S labelled sulphuric acid esters administered to mice and rats. Biochem Pharmac; 16: 1997-2001.

Pussard E, Verdier F, Blayo MC, Pocidalo JJ (1985); Biotransformation de l'amodiaquine et prophylaxie due paladisme a P. falciparum. C R Acad Sci Paris; III: 383-5.

Pussard E, Verdier F, Blayo MC (1986); Simultaneous determination of chloroquine, amodiaquine and their metabolites in human plasma, red blood cells, whole blood and urine by column liquid chromatography. J Chromatogr; 374: 111-8.

Pussard E, Verdier F, Faurisson F, Scherrmann JM, Le Bras J, Blayo MC (1987); Disposition of monodesethylamodiaquine after a single oral dose of amodiaquine and three regimens for prophylaxis against plasmodium falciparum Malaria. Eu J Clin Pharm; 33: 409-14.

Rieckmann K (1971); Determination of the drug sensitivity of plasmodium falciparum. J.A.M.A.; 217: 573-8.

Rieckmann K, Sax JA, Campbell G, Mrema JS (1978); Drug sensitivity of Plasmodium falciparum. An in vitro micro technique. Lancet; 1: 22-23.

Rieckmann K (1987); Amodiaquine in malaria treatment. Trans Roy Soc Trop Med Hyg; 81: 1040 [LETTER].

Ritschel WA, Hammer GV, Thompson GA (1978); Pharmacokinetics of antimalarials and proposals for dosage regimens. Int J Clin Pharmacol; 16: 395-401.

Rhodes EG, Ball J, Franklin IM (1986); Amodiaquine-induced agranulocytosis: inhibition of colony growth in bone marrow by antimalarial agents. Br Med J; 292: 717-8.

Rowland M, Tozer TN (1980); Clinical pharmacokinetics. Lea and Febiger, Philadelphia.

Salako L, Idowu D (1985); Failure to detect amodiaquine in the blood after oral administration. Br J Clin Pharmac; 20: 307-11.

Schmidt LH, Vaughan D, Mueller D, Crosby R, Hamilton R (1977); Activities of various 4-aminoquinolines against infection with chloroquine-resistant strains of Plasmodium falciparum. Antimicrob Ag Chemother; 11: 825-43.

Schonhofer F (1942); Uber bedeutung der Chinoidenbinding in Chinolinverbundungen fur die Malariawirkung Hoppeseyleiz. Z F Physiol Chem; 21: 995-8.

Schulemann W, Mietzsch F, Wingler A (1930) U.S. patent No. 1,766,403.

Schulthess HK, Von Felten A, Gmur J, Neftel K (1983); Amodiaquin-induzierte Agranulozytose bei Malariaprophylaxe: nachweis eines amodiaquin-abhangigen zytotoxischen Antikorpers gegen Granulozyten. Schweiz Med Wschr; 113: 1912-13.

Scuderi P, Sterling KE, Lam KS (1986); Raised serum levels of tumour necrosis factor in parasitic infections. Lancet; 2: 1364-5.

Simeon A, Chkatre K (1947); Preliminary report on a new synthetic antimalarial. Ind Med Gaz; 82: 255.

Smith CS, Chinn S, Watts RWE (1977); The sensitivity of human bone marrow granulocyte-macrophage precursor cells to phenylbutazone, oxyphenbutazone and gammahydroxyphenylbutazone in-vitro with observations on the bone marrow colony-formation, in phenylbutazone-induced granulocytopaenia. Biochem Pharmacol; 26: 847-52.

Spielberg SP, Kramer LI, Goodman SI, Butler J, Tietze F, Quinn P, Schulman JD (1977); 5-oxoprolinuria, biochemical observations and case report. J Pediatr; 91: 237-41.

Spielberg S (1980); Acetominophen toxicity in human lymphocytes in-vitro. J Pharmacol Exp Ther; 213: 395-8.

Spencer HC, Kariuki DM, Koech DK (1983); Chloroquine-resistance in Plasmodium falciparum from Kenyan infants. Am J Trop Med Hyg; 32: 922-5.

Spencer HC, Oloo AJ, Watkins WM, Sixsmith DG, Churchill FC, Koech DK (1984) Amodiaquine more effective than chloroquine against P. falciparum malaria on the Kenyan coast. Lancet 1 956-957,

Stephens JWW (1937); Blackwater Fever: a clinical survey and summary of observations made over a century. University Press of Liverpool; Hodder and Stoughton London.

Sturchler D (1984); Malaria prophylaxis in travellers: the current position. Experentia 40: 1357-62.

Sturchler D, Schar M, Gyr N (1987); Leucopenia and abnormal liver function in travellers on malaria chemoprophylaxis. J Trop Med Hyg; 90: 239-43.

Surrey AR, Hammer HF (1950); Preparation of 7-chloro-4[4-(N-ethyl, n-2-hydroxyethylamino)-1-methylbutylamino)-quinoline and related compounds. J Am Chem Soc; 72: 1814-5.

Sutherland R, Vincent PC, Raik E, Burgess K (1977); Quinine-induced agranulocytosis: toxic effect of quinine bisulphate on bone-marrow cultures in-vitro. Br Med J; I: 605-7.

Taetle R, Lane TA, Mendelsohn J (1979); Drug-induced agranulocytosis: in-vitro evidence for immune supression of granulopoiesis and a cross-reacting lymphocyte antibody. Blood; 54: 501-12.

Tarlov AR, Brewer GC, Carson PE, Alving AS (1962); Primaquine sensitivity. Arch Int Med; 109-34.

Thompson PE, Weston K, Glazko AJ, Fisken RA, Reutner TF, Bayles A, Weston JK (1958); Laboratory studies on amopyroquine (propoquin). Antibiot Chemother; 8: 450-60.

Timbrell JA (1982); Principles of biochemical toxicology. Taylor and Francis, London.

Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A (1987); Anti-catechin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature; 330: 662-4.

Trenholme GM, Williams RL, Patterson EC, Frischer H, Carson PE, Riechmann KH (1974); A method for the determination of amodiaquine. Bull WHO; 51: 431-4.

Trenholme GM, Williams RL, Riechmann KH, Frischer H, Carson PE (1976); Quinine disposition during malaria and during induced fever. Clin Pharmacol Ther; 19: 459-67.

Warhurst DC (1986); Antimalarial schizontocides: why a permiase is necessary. Parasitol Today; 2: 459-67.

Watkins WM, Spencer H, Kariuki D, Sixsmith D, Boriga D, Kipingor T (1984); Effectiveness of amodiaquine as treatment for chloroquine-resistant Plasmodium falciparum infections in Kenya. Lancet; 1: 357-9.

Watkins WM, Brandling-Bennett AD, Nevill CG, Carter JY, Boriga DA, Howells RE, Koech DK; Chlorproguanil-dapsone for the treatment of nonsevere falciparum malaria in Kenya: a pilot study. Trans Roy Soc Trop Med Hyg; IN PRESS.

Watt G, Long G, Padre L, Alban P, Sangalang R, Ranoa C, Laughlin L (1987); Amodiaquine less effective than chloroquine in the treatment of falciparum malaria in the Philippines. Am J Trop Med Hyg; 36: 3-8.

Weitzman SA, Stossel TP, Desmond M (1978); Drug-induced immunological neutropaenia. Lancet; I: 1068-71.

White NJ (1982); Clinical pharmacokinetics of antimalarial drugs. Clin Pharmacokin; 10: 187-215.

White NJ, Looareesuwan S, Edwards G, Phillips RE, Karbwang J, Nicholl D, Bunch C, Warrell DA (1987); Pharmacokinetics of intravenous amodiaquine. Br J Clin Pharmac; 23: 127-35.

White NJ (1988); The treatment of Falciparum Malaria. Parasitol Today; 4: 10-14.

Wiselogle FY (1946); A survey of antimalarial drugs 1941-1945, vol 1 JW Edwards, Ann Arbor, Michigan.

Withington E, (1909); Greek therapeutics and the Malaria Theory. In "Malaria and Greek history" Jones, WHS; Man Univ Publ History Series VIII.

World Health Organisation (1973); Chemotherapy of Malaria, and resistance to antimalarials. WHO Tech Rep Ser; 529: 30-54.

World Health Organisation, Weekly Epidemiological Record: Geneva (1984); 59: 234.

World Health Organisation, malaria action programme (1986); Severe and complicated malaria. Trans Roy Soc Trop Med Hyg; 80: SUPPL 1-50.

Young GAR, Vincent PC (1980); Drug-induced agranulocytosis. Clin Haematol; 9: 483-504.

Ziegler Z, Shadduck RK, Winkelstein A, Stroupe TK (1979); Immune hemolytic anemia and thrombocytopenia secondary to quinidine: in-vitro studies of the quinidine-dependent red-cell and platelet antibodies. Blood; 53: 396-402.

APPENDIX I

BIOAVAILABILITY STUDY

INTRODUCTION

The absolute bioavailability of amodiaquine is unknown. This has not been important since, with early exceptions, the drug has been given almost exclusively by mouth. However, White and colleagues (1987) gave amodiaquine by IV infusion as treatment for chloroquine-resistant falciparum malaria, and at that time data on the drug's bioavailability would have been useful. Therefore, as part of the dose-proportionality study described in chapter 3 of this thesis, it was planned to assess the bioavailability of amodiaquine. Simultaneous with the 600mg oral dose of the drug, a single IV bolus of ^{14}C-amodiaquine was to have been given to each subject. HPLC analysis, and liquid scintillation counting of eluted fractions would have permitted measurement of labelled and unlabelled drug concentrations, and would therefore have allowed an assessment of bioavailability. This method (which has been applied to other drugs) allows avoidance of intra-subject day-to-day variation in drug disposition, and permits the use of very small IV doses of drug, thus minimising the risk of adverse effects. Permission was obtained from the Mersey Regional Ethics Committee, and the DHSS Radioisotopes Panel for the performance of this study.

METHODS

I. One healthy male Caucasian (aged 46, wt 72kg) gave written informed consent. Following an overnight fast, heparinised IV cannulae were inserted into veins on both forearms. Amodiaquine (600mg base; Camoquin Parke-Davis) was given by

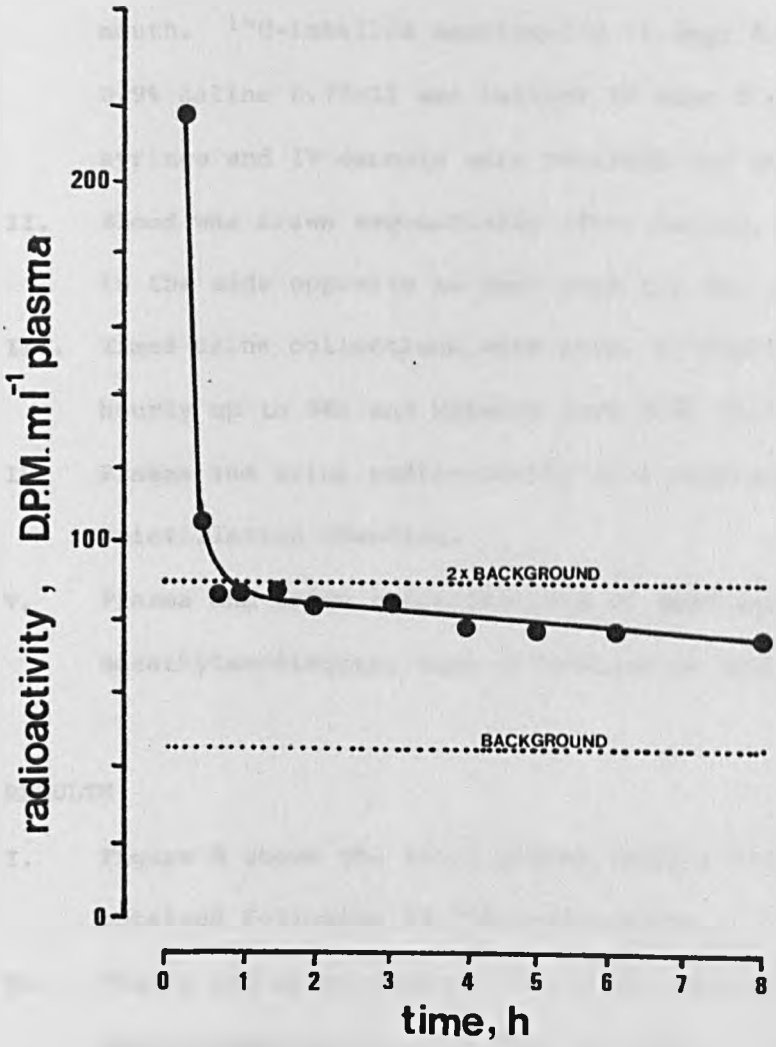


FIGURE A Plasma radioactivity vs time curve following IV injection of [^{14}C]-amodiaquine in one healthy subject

mouth. ^{14}C-labelled amodiaquine (1.5mg; 6.6 micro Ci, in 0.9% saline 8.77ml) was infused IV over 2 minutes; the syringe and IV cannula were retained for weighing.

II. Blood was drawn sequentially after dosing, from the cannula in the side opposite to that used for the IV infusion.

III. Timed urine collections were made, 12 hourly up to 48h, 24 hourly up to 96h and between days 7-8, 16-17 and 28-29.

IV. Plasma and urine radioactivity were determined by liquid scintillation counting.

V. Plasma and urine concentrations of amodiaquine and desethylamodiaquine were determined by HPLC.

RESULTS

I. Figure A shows the total plasma radioactivity vs time curve obtained following IV ^{14}C-amodiaquine.

II. Plasma and urine concentrations of amodiaquine and desethylamodiaquine have been included in chapter 3 of the thesis.

III. Urinary recovery of radioactivity (estimated from AUC of the excretion rate vs time curve) was 28%, up to 30 days post-dose.

CONCLUSIONS

I. Since plasma radioactivity declined, within 0.75h, to less than twice background counts, further analysis of samples, using preparative HPLC, to determine the concentration of ^{14}C amodiaquine was thought to be pointless.

II. Bioavailability cannot be calculated from these data.

III. Only a minority of the radiolabel was excreted in the urine

of this one subject. This is in-keeping with the animal data presented in chapter 5 of this thesis, although the urinary recovery of radiolabel in this one human subject was higher than was seen in the animal model.

IV. No further attempts were made to determine bioavailability by this technique, since it was considered that success would have required the use of a much larger dose of radioactivity than had been given to the first subject. This was not thought to be ethically acceptable given the low urinary recovery of radiation, and the consequent possibility that much of the dose had been retained within the tissues.

APPENDIX II

PLASMA CONCENTRATION DATA DURING MALARIA-PROPHYLAXIS DOSE SCHEDULE

INTRODUCTION

The provision of clinically-useful data on the pharmacology of amodiaquine would ideally include its disposition during a weekly dosing schedule, as used for malaria chemoprophylaxis. Such a study was planned, but was not undertaken because of the, by then apparent, high risk of serious toxicity. However, it did prove possible to study one healthy subject following his final dose of amodiaquine as part of a weekly chemoprophylactic regimen.

METHODS

I. DT, a healthy male Caucasian aged 42, took amodiaquine (400mg; Camoquin Parke-Davis) once weekly, and proguanil (200mg) once daily for malaria prophylaxis, as recommended by his physician, during an eleven week period. He remained free from clinical malaria.

II. Upon his return to Britain, the antimalarial regimen was continued. Sampling, for the determination of plasma amodiaquine, was undertaken before and following the final dose of the drug.

III. Blood was drawn prior to the final dose of amodiaquine, and at the following times thereafter: 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 48, and 120 hours.

IV. Plasma amodiaquine and desethylamodiaquine were determined by HPLC. Plasma concentrations of proguanil and cycloguanil were not determined.

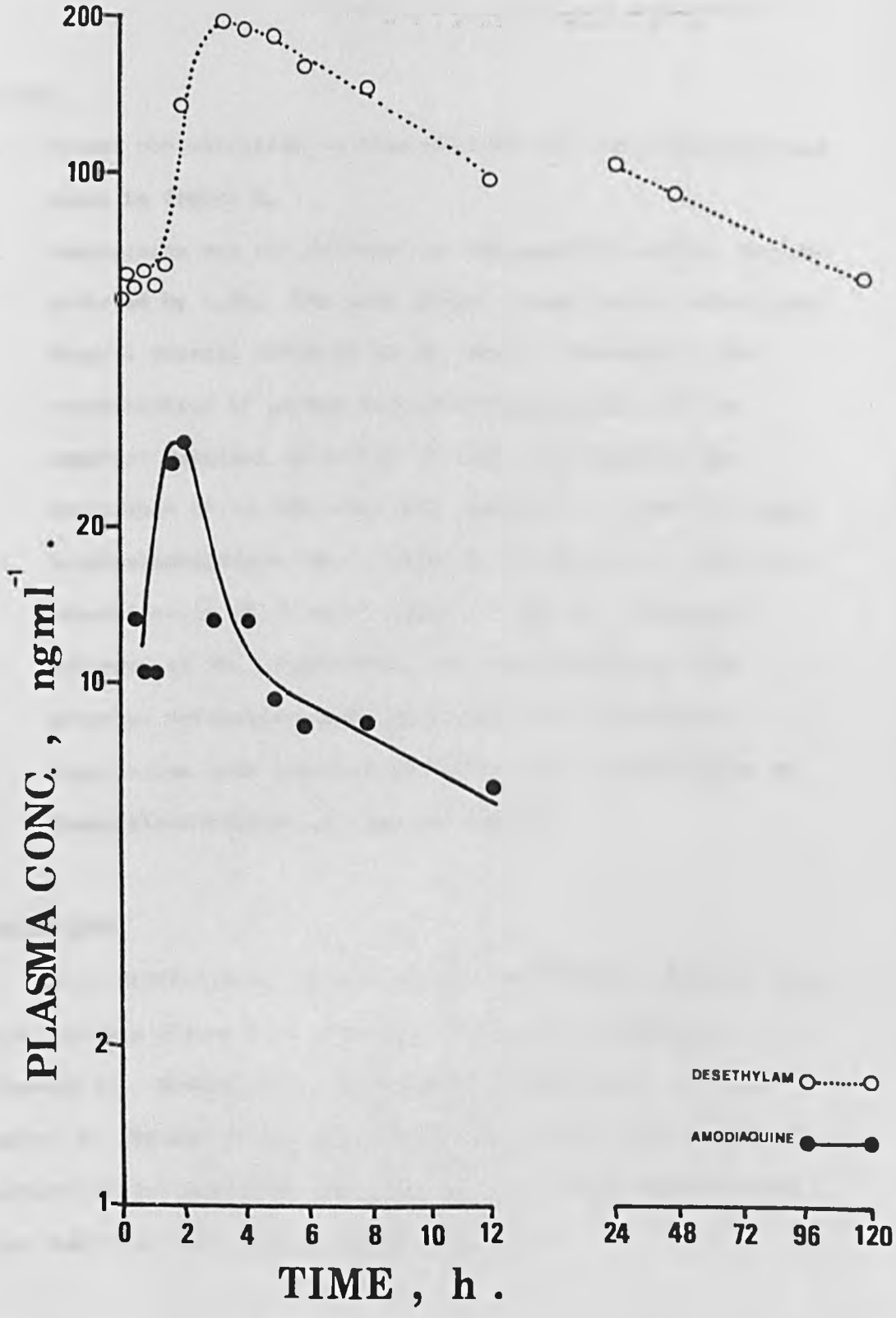


FIGURE B Plasma concentration vs time curves for amodiaquine and desethylamodiaquine following oral amodiaquine 400mg; final dose of prophylactic regimen (n = 1).

RESULTS

I. Plasma concentration vs time profiles for both compounds are shown in figure B.

II. Amodiaquine was not detected in the pre-dose sample, but was detected by 0.5h. The peak plasma concentration (Cmax) was 30ng/ml (base), achieved at 2h (Tmax). Thereafter the concentration of parent drug declined rapidly with an apparent terminal half-life of 7.5h. Amodiaquine was detectable up to 24h, when its concentration was 6.5 ng/ml.

III. Desethylamodiaquine was present in the pre-dose plasma at a concentration of 56 ng/ml (base). Cmax was 199 ng/ml, achieved at 3h. Thereafter, the concentration of the desethyl metabolite declined slowly, with an apparent elimination rate constant of 0.009. The concentration of desethylamodiaquine at 120h was 63ng/ml.

CONCLUSIONS

It is difficult to compare these current single-subject data with the mean plasma data obtained from healthy volunteers (chapter 3). However, the disposition of amodiaquine in this subject at the end of a chemoprophylactic regimen (which had included daily proguanil) does not seem to differ significantly from that seen following a single oral dose.

APPENDIX III

REAGENT SOURCES

1. Amodiaquine dihydrochloride monohydrate:- Parke-Davis, Pontypool UK.
2. Amodiaquine hydrochloride (tablets):- Parke-Davis, Pontypool UK.
3. ^{14}C-Amodiaquine (quinoline-2-^{14}C-amodiaquine hydrochloride):- Amersham International, Amersham UK.
4. Bacto agar:- DIFCO laboratories, Detroit, USA.
5. Bisdesethylamodiaquine hydrochloride:- Parke-Davis, Pontypool UK.
6. Bladder carcinoma cell line C5637:- Gift from Dr. E. Rhodes (University of Liverpool, UK).
7. Chemicals other than solvents (analytical grade):- BDH Chemicals Ltd., Dorset UK.
8. Chloroquine sulphate:- May and Baker, Dagenham UK.
9. 7-Chloro-4[1-dimethyalino-4-pentylamino]-quinoline:- Gift from Dr. O. Walker (University of Ibadan, Nigeria).
10. Desethylamodiaquine hydrochloride:- Parke-Davis, Pontypool UK, and World Health Organisation, Geneva, Switzerland.
11. Foetal calf serum:- Seralab (batch 301121), Crawley Down UK.
12. Gemtamicin:- David Bull Laboratories, Warwick UK.
13. Glucuronidase-sulphatase (4260 units/ml):- Sigma, Poole UK.
14. McCoys 5A culture medium:- Flow Laboratories, Rickmansworth, UK.
15. 6-Methoxy-8-aminoquinoline:- Aldrich, Gillingham UK, and World Health Organisation, Geneva Switzerland.
16. NCS Tissue solubilizer:- Amersham International, Amersham UK.

17. RPMI-1640 culture medium:- GIBCO Laboratories, Amersham UK.

18. Solvents (all HPLC grade):- Fisons, Loughborough UK.

19. Vitamins, amino acids and other nutrients:- Flow Laboratories, Rickmansworth, UK.

APPENDIX IV

EQUIPMENT SOURCES

1. HPLC hardware:

   Solvent delivery system, SP8700:- Spectra Physics, St. Albans UK.

   Organizer module, SP8750:- Spectra Physics, St. Albans UK.

   U.V. Absorbance detector, 441:- Millipore-Waters, Harrow UK.

   Z Module:- Millipore-Waters, Harrow UK.

2. Columns:

   Rad-Pak phenyl:- Millipore-Waters, Harrow UK.

   Ultrasphere ODS (5 micron):- Beckman, Warrington UK.

3. LS/1801 Scintillation counter:- Beckman, Warrington UK.

4. Tissue culture hardware:

   20229 Laminar Flow cabinet:- MDH-Intermed, UK.

   Petri Dishes (35mm):- GIBCO Laboratories, Uxbridge UK.

   Cryotubes:- Flow Laboratories, Rickmansworth UK.

   Tissue culture flasks:- GIBCO Laboratories, Uxbridge UK.

APPENDIX V

Tabulated results of human marrow cell culture experiments (Chapter 6 of this thesis).

The figures given are mean ± SD of GM-colony numbers

Expt - AMODIAQUINE

	Dose											
	Nil Count			0.1 µm Count			1.0 µm Count			10.0 µm Count		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Date												
230787	4	48.00	12.68	4	44.50	16.34	4	28.25	14.06	4	0.00	0.00
130887	3	51.00	3.46	3	37.33	7.57	4	31.25	5.25	4	0.25	0.50
170887	3	48.00	14.00	4	45.50	16.92	4	35.25	18.21	4	0.00	0.00
240987	4	58.50	18.23	4	49.25	19.50	4	44.75	9.54	4	0.00	0.00
081087	4	54.00	8.72	4	39.00	10.10	4	39.50	13.99	4	0.00	0.00
All	18	52.17	11.94	19	43.42	14.08	20	35.80	13.01	20	0.05	0.22

Expt - CHLOROQUINE

	Dose											
	Nil Count			0.1 µm Count			1.0 µm Count			10.0 µm Count		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Date												
230787	4	48.00	12.68	4	33.00	6.22	4	33.25	8.92	4	28.50	6.45
130887	3	51.00	3.46	3	24.00	9.00	4	21.25	4.03	4	9.75	2.50
170887	3	48.00	14.00	3	58.00	22.65	4	26.25	15.90	4	0.00	0.00
240987	4	58.50	18.23	4	31.25	10.63	4	30.25	8.26	4	7.25	6.08
081087	4	54.00	8.72	3	38.33	9.87	3	41.00	12.29	4	0.00	0.00
All	18	52.17	11.94	17	36.35	15.54	19	29.84	11.35	20	9.10	11.33

Expt - DESETHYLAMODIAQUINE

	Dose											
	Nil Count			0.1 µm Count			1.0 µm Count			10.0 µm Count		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Date												
230787	4	48.00	12.68	4	34.00	10.86	4	26.25	12.89	4	1.75	1.26
130887	3	51.00	3.46	4	27.75	8.38	2	44.50	3.54	4	0.00	0.00
170887	3	48.00	14.00	3	64.00	25.06	3	47.00	7.00	4	0.00	0.00
240987	4	58.50	18.23	4	59.75	18.25	4	50.00	10.17	4	0.00	0.00
081087	4	54.00	8.72	4	45.00	18.07	3	39.33	6.03	4	0.00	0.00
All	18	52.17	11.94	19	45.16	20.31	16	40.81	12.45	20	0.35	0.88

Expt - QUINONEIMINE

	Dose											
	Nil Count			0.1 µm Count			1.0 µm Count			10.0 µm Count		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Date												
230787	4	59.00	15.81	4	53.75	9.91	3	21.67	2.89	4	14.25	5.19
130887	4	48.25	6.13	4	33.00	3.16	3	36.33	11.72	4	0.00	0.00
170887	4	38.25	4.50	4	47.25	10.37	4	51.50	9.81	4	0.00	0.00
240987	4	46.00	7.39	4	45.25	8.54	4	39.00	12.83	4	12.00	5.60
081087	3	112.00	25.94	4	102.75	9.54	3	62.33	7.09	3	0.00	0.00
All	19	58.00	27.59	20	56.40	25.94	17	42.53	16.09	19	5.53	7.39