CENTRE FOR
DRUG
RESEARCH

2

INTERNATIONAL
MONOGRAPH
SERIES

IMS

CLINICAL PHARMACOLOGY OF ANTI MALARIAL DRUGS

Pusat Penyelidikan Dadah dan Ubat-Ubatan
(Centre for Drug Research)
U.N. / W.H.O. / I.F.N.G.O. Research and Training Centre
Universiti Sains Malaysia
11800 Penang,
MALAYSIA.

WORLD
HEALTH
ORGANIZATION

CLINICAL PHARMACOLOGY OF ANTIMALARIAL DRUGS

Notes of a Workshop conducted at the
National Drug Research Centre
Universiti Sains Malaysia
Penang
23 January - 4 February 1989

under the auspices of the
Universiti Sains Malaysia
and the
Scientific Working Group on the Chemotherapy of Malaria
and the Research Strengthening Group of the
UNDP/WHO/World Bank Special Programme for
Research and Training in Tropical Diseases

Edited by

V. Navaratnam
E. B. Doberstyn, P. I. Trigg, W.H. Wernsdorfer

1993

Scientific Working Group on
The Chemotherapy of Malaria and
The Research Strengthening Group of the
UNDP/World Bank/WHO
Special Programme on Research and
Training in Tropical Diseases
in conjunction with

THE CENTRE FOR DRUG RESEARCH
UNIVERSITI SAINS MALAYSIA
MINDEN, PULAU PINANG
MALAYSIA

ISBN: 967-9979-33-4

ACKNOWLEDGEMENT

The editors wish to place on record the support and assistance of several persons, of whom only some have been mentioned here:

1. His Excellency Dr. Hiroshi Nakajima, Director-General, World Health Organization, Geneva.

2. Honorable Minister of Health, Malaysia.

3. The Director-General of Health, Ministry of Health, Malaysia.

4. Y. Bhg. Datuk Musa Mohamad, Vice-Chancellor, Universiti Sains Malaysia.

5. Dr. Tore Godal, Director, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

6. Dr. D. E. Davidson, Jr., Secretary, Steering Committee CHEMAL, TDR/WHO Geneva.

7. The Administrative and Secretarial Staff of the Centre for Drug Research for their assistance in the preparation of the manuscript.

INTRODUCTION

Under the joint sponsorship of the Universiti Sains Malaysia, Penang, and the UNDP/WHO/World Bank Special Programme for Research and Training in Tropical Diseases, a Workshop on the clinical pharmacology of antimalarial drugs was held in Penang on 23 January - 4 February 1989. This Workshop brought together scientists from a wide variety of disciplines and nationalities who had in common the interest and experience in the clinical pharmacology of antimalarial drugs.

The meeting reviewed the current state of the art in clinical pharmacology per se, pharmacokinetic methodology and its application to antimalarial agents, assay techniques and drug development from the early preclinical stages up to post-marketing surveillance. It also conveyed a perception of the potential and the limits of the current methodology and technology of clinical pharmacology, and helped identify areas requiring further study.

The use of various antimalarial drugs has certainly been improved based on the results of clinical pharmacological investigation, but there is much room for further improvement and for the exploration of new candidate drugs which will probably be required for as long as malaria remains a threat to man.

CONTENTS

CHAPTER 1 : CLINICAL PHARMACOLOGY*

CONTENTS

---

* This chapter is largely based on "Principles of clinical pharmacology and therapeutics" by A. Breckenridge and M.L'E. Orme which forms Section 7 of the Oxford textbook of medicine (D. Weatherall, ed.), Oxford University Press, 1989. The Publishers' and the Chief Editor's permission are gratefully acknowledged.

INTRODUCTION

The rational use of drugs dates from the latter part of the nineteenth century and may be attributed to three distinct developments. First was the birth of synthetic organic chemistry and the question of whether some new compounds might have medicinal value. Among the earliest chemicals recognized to be of therapeutic importance were the general anaesthetics and chemotherapeutic agents such as salvarsan. The second pillar of modern therapeutics was the elucidation of the mode of action of these chemicals by means of experiments in animals and in human. Third has been the development in the understanding of the basis of human disease expressed in terms of perturbation of underlying physiological control mechanisms and morbid anatomical changes.

From these disparate origins, clinical pharmacology has emerged as a discipline whose aim is, broadly speaking, the scientific study of drugs in humans. There are many aspects of clinical pharmacology. Pharmacokinetics, the mathematical description of the fate of drugs in the body, including the processes of drug absorption, distribution, metabolism and excretion, has probably attracted attention disproportionate to its importance because of the ability to make precise measurements of drug concentrations in biological fluids using refined analytical technology. Pharmacodynamics encompasses studies of the effects of drugs on the body and of the underlying modes of drug action; this still remains the main challenge in clinical pharmacology since available techniques are either invasive and thus generally inapplicable or are non-invasive and tend to be crude and imprecise. Toxicology is that aspect of pharmacology dealing with the adverse effects of drugs used in therapy and of chemical substances used in the household, in industry, or found in the environment. For political reasons this is the facet of clinical pharmacology most frequently shown to the public and is one reason for current trends away from allopathic medicine. A separate facet of clinical pharmacology is the testing of new drugs in humans by means of the clinical trial. Such studies are only as good as the methods used to assess drug effects, and the statistical methods to evaluate the results.

The rational use of drugs in clinical medicine obviously presents a challenge to the clinical pharmacologist. New fiscal measures to restrain the choice of drugs available to the practising physician both in the United Kingdom and elsewhere make the assessment of the value of individual therapeutic agents even more relevant than hitherto. Lists of drugs available for limited prescribing must be based on sound clinical pharmacological principles.

The principles underlying drug therapy are fundamentally similar for any condition in which drugs are used. Obvious variables include the nature and stage of the disease and the chemical nature and dose of the drug used. The aim of all

therapy is to administer the appropriate drug in the correct dose to produce the desired therapeutic effect with the minimum of adverse side-effects. This chapter outlines the principles on which the achievement of this aim rests.

1.1 BASIC CONCEPTS OF DRUG REACTION

1.1.1 Physicochemical characteristics of drugs

The three most important physicochemical properties of a drug are lipid solubility, degree of ionization, and molecular size.

Lipid solubility is the principal determinant of the ability of a drug to cross the membranes of cell walls, be they of the gastrointestinal tract, renal tubule, or blood-brain barrier. The relevance of lipid solubility can best be appreciated by considering the fate of drugs in the nephron. Filtered at the glomerulus, a lipid soluble drug is completely reabsorbed in the renal tubular system to remain in the body for an indefinite time. Drug metabolism can thus be viewed as a mechanism to change lipid soluble compounds into those with a higher degree of water solubility, i.e. greater polarity. The behaviour of water soluble metabolites in the renal tubules is quite different from that of the parent compound. By virtue of their new-found polarity, metabolites tend not to be reabsorbed in the renal tubules and will be eliminated in the urine. Lipid solubility can be measured by *in vitro* methods using partition of a drug between an organic and an aqueous solvent. Table 1.1 shows the partition coefficients of a series of beta adrenoceptor blocking agents between octanol and water.

TABLE 1.1 PRINCIPAL PHARMACOLOGICAL PROPERTIES OF SOME BETA ADRENOCEPTOR BLOCKING DRUGS

Drug	Cardio-selectivity	Intrinsic sympatho-mimetic activity	Membrane stabilizing activity	Log partition coefficient octanol/water
Acebutolol	±	+	+	1.87
Alprenolol	-	+	+	2.61
Atenolol	+	-	-	0.23
Metoprolol	+	-	±	2.15
Nadolol	-	-	-	0.71
Oxprenolol	-	+	+	2.18
Pindolol	-	++	+	1.75
Practolol	+	+	-	0.79
Propranolol	-	-	+	3.65
Sotalol	-	-	-	0.79
Timolol	-	-	+	2.10

This has clinical relevance since those beta blockers with a high degree of lipid solubility, e.g., propranolol and oxprenolol, tend to be well absorbed from the gut, to show a high first pass effect in the gut and the liver, and to have a relatively short half-life. They also gain easy access to the cerebral cortex and thus have the propensity to produce central side-effects. On the other hand, less lipid soluble beta adrenoceptor blocking drugs such as atenolol and sotalol are not so readily absorbed, are not extensively metabolized in the liver, and tend to be eliminated unchanged via the kidney. Further, they do not gain such easy access to the brain as more lipid soluble counterparts.

The extent to which a drug is ionized depends on the pKa of the drug and the pH of the medium in which the drug is dissolved. The pKa is defined as the pH at which 50% of the drug is ionized. If a weakly acidic drug is represented as HA, then:

$$HA = H^+ + A^- \qquad (1)$$

and therefore $$Ka = \frac{[H^+] + [A^-]}{[HA]} \qquad (2)$$

where Ka is the dissociation constant, pKa is the negative logarithm of Ka.

Logarithmic transformation of equation (2) gives:

$$pH = pKa + \log_{10}\frac{[A^-]}{[HA]} \qquad (3)\frac{[A^-]}{[HA]}$$

From this, it can be seen that changes in pH near the pKa of a drug such as phenobarbitone (pKa 7.4) will give rise to considerable changes in extent of its ionization. This is put to clinical use in patients overdosed with phenobarbitone in whom the urine can be made alkaline by sodium bicarbonate adminstration to facilitate its excretion. At urine pH of 8.0, over 95% of phenobarbitone will be ionized and thus will not be reabsorbed in the kidney.

For a basic drug, e.g. amphetamine or quinidine, (represented as BH), equation(3) is rearranged so that:

$$pH = pKa + \log_{10}\frac{[BH]}{[B^+]} \qquad (4)\frac{[BH]}{[B^+]}$$

By the same arguments as above, acidification of the urine will promote the elimination of basic drugs and this may also be of use of in treating an overdose.

The degree of drug ionization has implications for drug absorption from the gastrointestinal tract. Under the acid conditions in the stomach, it can be appreciated from equation (3) that acidic drugs such as salicylate or warfarin will exist preferentially in the non-ionized, lipid soluble form. Basic drugs such as chlorpromazine and tricyclic antidepressants will tend to be ionized and to be relatively lipid insoluble in the stomach on this pH partition hypothesis. However, the larger surface area of the small intestine dictates that both types of drug, pH and pKa notwithstanding, will tend to be absorbed maximally lower in the gastrointestinal tract than the stomach. The basis that only non-ionized drugs will preferentially cross the gastrointestinal tract is illustrated in Fig. 1.1.

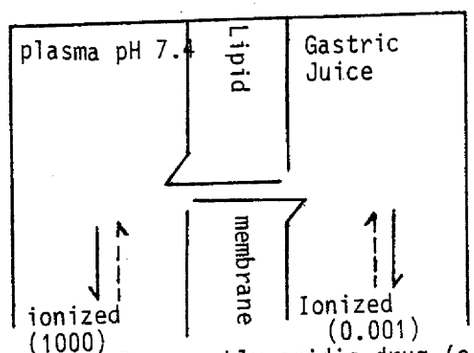


Fig. 1.1 Distribution of a weakly acidic drug (e.g. warfarin) between plasma and gastric juice. (The figures in brackets refer to the relative concentrations of warfarin in arbitrary units).

Molecular size is probably the least important of the three physical properties of a drug. Biliary excretion is largely determined by molecular size; in humans, compounds of relative molecular greater than 400 are excreted in the bile. Such excretion varies with species. This property can also be used therapeutically. Ampicillin is excreted in the bile and use is made of this in the treatment of biliary tract infections.

Once drug conjugates have reached the gut via the bile, they may be broken down by the enzymes of gut bacteria, liberating free drug for reabsorption. This process of enterohepatic recirculation is described below. (See section 1.2.5).

1.1.2 Pharmacokinetic considerations

Pharmacokinetics is the mathematical description of the processes of drug absorption, distribution and elimination. The body can be considered for mathematical purposes as either a single compartment or a series of interconnected compartments each of a finite volume containing a drug at a definite concentration and whose ingress into and egress from the compartment is described by a series of rate constants.

1.1.2.1 One-compartment model

Figure 1.2 represents the body as a single compartment. This has a volume V which is also referred to as the apparent volume of drug distribution. In Fig. 1.2, K_{ab} is the rate constant of absorption and K_{el} the rate constant of elimination. Most kinetic processes can be described by first-order kinetics, i.e. the rate at which a drug enters or leaves a compartment is proportional to the concentration therein. This is illustrated in Fig. 1.3 where the plasma concentration of warfarin versus time is plotted over 100 hours after administration of a single dose of 35 mg. The vertical axis (warfarin concentration) is logarithmic, thus converting the exponential decay slope seen on an arithmetical scale into a straight line; the slope of this line gives the rate constant for elimination (K_{el}). The half-life of the drug in plasma is the time it takes for any concentration to fall by 50%.

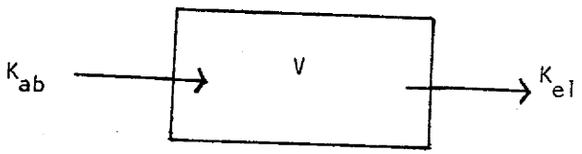


Fig. 1.2 Schematic diagram of the body as a one-compartment model.

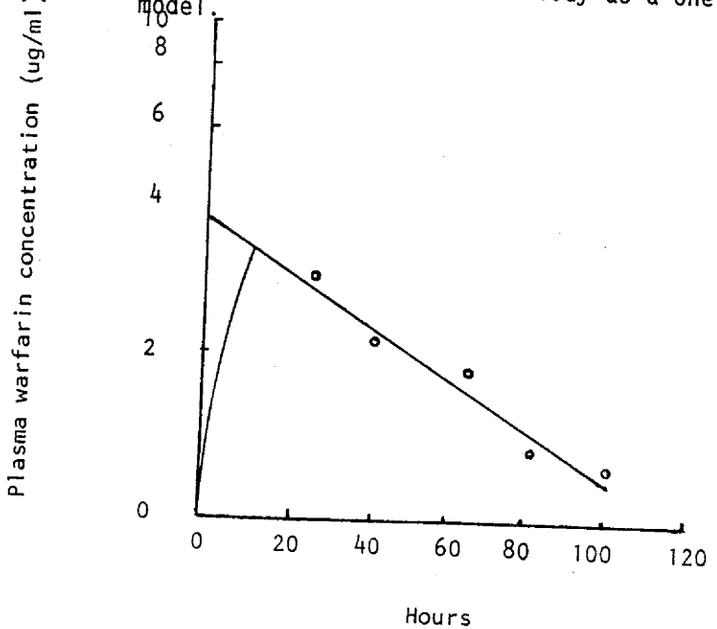


Fig. 1.3 Plasma concentrations of warfarin over a 100-hour period in a human being given a single dose of 35 mg. The vertical axis is on a logarithmic scale.

In a one-compartment model, the plasma concentration (C_p) at any time is proportional to the amount of drug in the compartment. Thus

$$\frac{dC_p}{dt} = KC_p \qquad (5)$$

On integration with respect to time,

$$C_p = C_{p_o} \exp(K_t) \qquad (6)$$

where C_{p_o} is the concentration at time zero. To calculate the plasma half-life of warfarin (i.e. the time taken for C_p to decline by 50% :

$$C_p = C_{p_o/2}$$

$$\text{or } C_{p_o} = C_{p_o} \exp(-K_{t1/2})$$

$$\text{therefore } t1/2 = 0.693/K \qquad (7)$$

If, in Fig 1.3, the decay slope of warfarin in plasma is extrapolated back to time zero, the plasma concentration at time zero is 4 ug/ml. The elimination plasma half-life of warfarin is approximately 50 hours.

The apparent volume of distribution (see 1.1.2.3) of warfarin using the one-compartment model system is calculated by dividing the dose (35 mg or 35 000 ug) by the value for C_o (4 ug/ml). The result is 8750 ml or 8.75 l. This is of the same order as the plasma volume.

1.1.2.2 Two-compartment model

When the plasma concentration of a drug versus time is plotted, the result may not be a linear decay as in Fig. 1.3, but may yield two linear portions as in Fig. 1.4, which shows the decline in plasma ethinyloestradiol after oral administration. The first phase can broadly be equated with the distribution of drug into the tissues of the body and the second phase represents elimination from the body. Rate constants for these two phases can be calculated as described above. The body can thus be considered as comprising two compartments, a central and a peripheral compartment which are connected as shown in Fig. 1.5. It is customary but not essential to consider absorption and elimination as occurring into and from the central compartment.

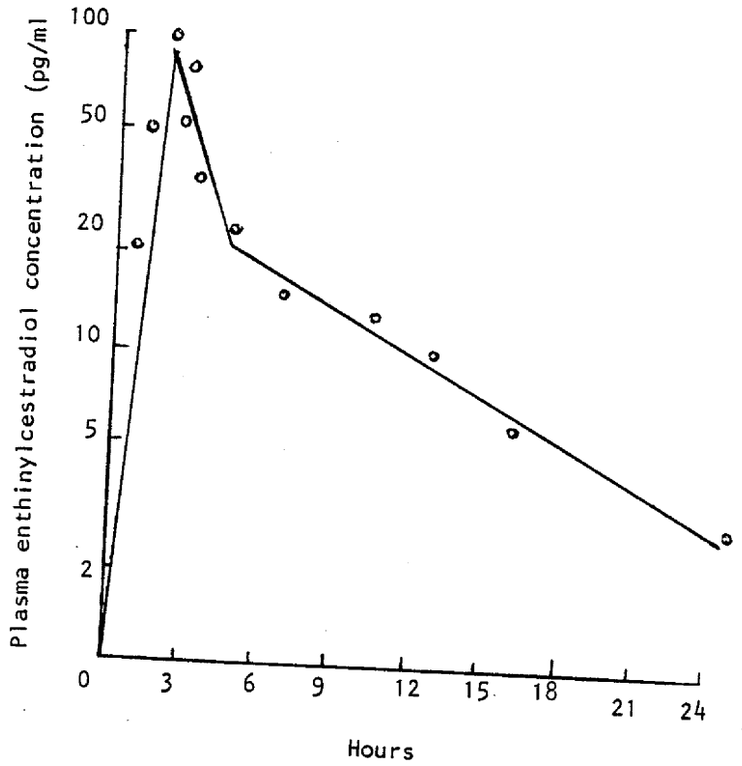


Fig. 1.4 Plasma concentrations of ethinyloestradiol over 24 hours in a woman given a single oral dose of 30 ug. The vertical axis is on a logarithmic scale.

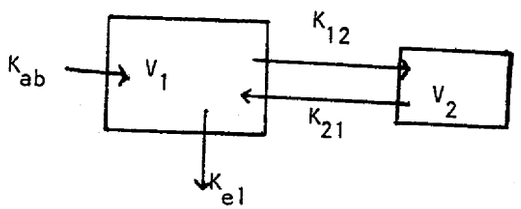


Fig. 1.5 Schematic diagram of the body as a one-compartment model. K_{12} and K_{21} are the transfer rate constants between the two compartments.

1.1.2.3 Apparent volume of distribution

The apparent volume of distribution is the sum of volumes of the compartments defined above. Irrespective of the model chosen, the apparent volume of distribution is merely a proportionality constant which describes the amount of drug in the body relative to that in the plasma at any one time. Thus the total amount of drug in the body is equal to its apparent volume of distribution multiplied by the plasma concentration. The apparent volume of distribution is a notional volume rather than one with anatomical significance. Drugs (such as warfarin) which have a low apparent volume of distribution tend to be located within the plasma, by virtue of their high affinity to plasma albumin. Drugs such as tricyclic antidepressants have a large apparent volume of distribution, i.e. their concentration in tissues relative to plasma is high. Table 1.2 gives the apparent volume of distribution for some commonly used drugs.

TABLE 1.2 APPARENT VOLUME OF DISTRIBUTION OF VARIOUS COMMONLY USED DRUGS

Drug	V_D(l/kg)
Frusemide	0.1
Warfarin	0.1
Phenylbutazone	0.1
Aspirin	0.15
Sulfafurozole	0.2
Nalidixic acid	0.3
Penicillin G	0.3
Diphenylhydantoin	0.6
Diazepam	0.7
Indomethacin	0.9
Lignocaine	1.3
Procainamide	2.0
Digoxin	6.0
Propranolol	15.0
Nortriptyline	20.0

One important therapeutic implication of the size of the apparent volume of distribution is the ease with which drugs may be removed from the body by haemodialysis after overdosage. A drug with a low volume of distribution located primarily in the plasma may be dialysed more readily than a drug with a high volume of distribution which is located primarily in the tissues. Thus aspirin (apparent volume of distribution 0.15 l/kg) may be dialysed, but nortriptyline (apparent volume of distribution 20 l/kg) may not, and any attempt to do so is doomed to fail.

The apparent volume of distribution may be calculated in several ways. As described already for warfarin, if one assumes rapid absorption after oral dosage, or more properly after intravenous dosage, the logarithmic decay of the drug in plasma versus time is linear. Then the dose of drug administered, divided by the plasma concentration at zero time gives the apparent volume of distribution.

A more general approach is to measure the total area under the plasma concentration time curve (AUC). Then :

$$V_D = \text{dose}/(\text{AUC} \times K_{el}) \qquad (8)$$

where V_D is the apparent volume of distribution and K_{el} is the rate of elimination. This formula is model independent.

1.1.2.4 Plasma clearance of drugs

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

At equilibrium:

$$\text{Rate of elimination} = Cl \times C_p \qquad (9)$$

where Cl = Clearance and C_p = plasma concentration

also rate of elimination = K x Ab
where K is the elimination rate constant and Ab is the amount of drug in the body i.e. the drug concentration x volume of distrubution or C_p x Vd, therefore clearance can be defined as:

$$CL = K_{el} \times V_D \qquad (10)$$

From equation (8) another expression for clearance is:

$$CL = \text{dose}/\text{AUC} \qquad (11)$$

1.1.2.5 Bioavailability

The amount of a drug reaching the systemic circulation, irrespective of route of administration, is termed its bioavailability. By definition, the bioavailability of a drug after intravenous administration is 100%. If the same drug is

given intravenously, and then orally, in the same dose to the same individual, the bioavailability is the ratio between :

AUC after oral dosing/AUC after intravenous dosing

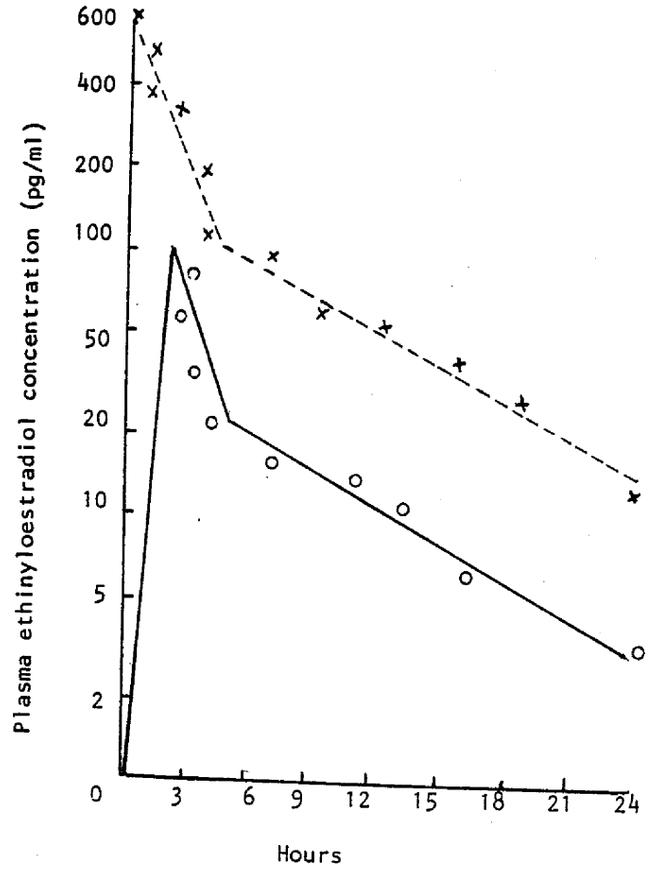


Fig. 1.6 Plasma concentrations of ethinyloestradiol over a 24 hour period in a woman given 30 ug by the intravenous (------) and oral (——————) routes. The vertical axis is on a logarithmic scale.

Figure 1.6 shows such data for ethinyloestradiol, which can be seen to have a low bioavailability - on average only 40% of the drug reaches the systemic circulation. Other evidence shows that ethinyl oestradiol is well absorbed from the gastrointestinal tract, but it is extensively metabolized in both gut wall and liver, thus accounting for its low bioavailability. This metabolic breakdown in gut wall and liver is termed the first pass effect.

1.1.2.6 Steady-state plasma concentration

Most therapeutic agents are not given as single doses but repeatedly on a regular basis. If a drug has a plasma half-life of 36 hours, irrespective of its route of elimination, and is administered twice daily, the drug will accumulate. However, if the elimination of the drug obeys first-order kinetics, as the concentration in the plasma increases, then the amount eliminated per unit of time will increase too. Thus a balance will eventually be reached when the amount of drug absorbed will equal the amount eliminated, i.e. a steady-state will be reached, around which the plasma concentration will fluctuate, depending on the amount of drug given. The rate at which the steady-state will be reached may be calculated from a knowledge of the plasma half-life of the drug. As seen in Table 1.3, 50% of the steady-state concentration will be reached in one half-life, 75% in two half-lives and so on. It takes approximately five half-lives of continuous administration (i.e. intravenous or repeated oral dosing) to reach some 97-98% of steady-state plasma levels.

TABLE 1.3 THE PLASMA CONCENTRATION AT DIFFERENT TIME POINTS AS A PERCENTAGE OF THE STEADY-STATE CONCENTRATION

Number of $t_{1/2}$	Plasma concentrations as %
1	50
2	75
3	87.5
4	94
5	97
6	98
7	99

As a consequence, the shorter the half-life of a drug, the sooner the steady-state will be achieved. For a drug such as phenobarbitone, with a plasma half-life of some 60 hours, it will take some 12-24 days for steady-state kinetics to be achieved. The shorter the half-life, the greater the fluctuation in plasma level between doses unless the drug is given very frequently. For example, heparin has a plasma half-life of 1-2 hours. If the drug were given six-hourly by bolus injections, there would be marked fluctuations in plasma levels and in therapeutic effect. Thus a continuous infusion of heparin is the preferred route of administration. If a drug given orally has a short half-life (e.g. propranolol or procainamide) then consideration of the development of a slow release formulation is appropriate. If, however, a drug has a plasma half-life of some 24-36 hours, as is the case for

tricyclic antidepressants, it makes no sense to consider slow release formulations. Thus for drugs which have long plasma half-lives (24 hours or longer) there is no need to give the drug very frequently. It is common in clinical practice to see drugs like phenytoin or imipramine being given three times daily. Compliance with therapy is poor for such a regime and therapy is likely to be more effective and better accepted if the drug is given once or twice daily. When once daily dosing is used, the high peak concentrations produced may cause side effects (e.g., ataxia with doses of carbamazepine greater than 400 mg). Further, if the drug is eliminated by the kidney and renal failure supervenes, either the dose of the drug or the frequency of administration, or both, should be decreased if the same steady-state plasma concentration is required as when the renal function was normal. This applies particularly to drugs such as aminoglycoside antibiotics where high plasma concentrations are associated with ototoxicity. In renal failure, prolongation of the plasma half-life of gentamicin means that with regular dosing it will take longer to achieve steady-state plasma levels. If an effect is required quickly, a loading dose may be administered.

1.2 INTERINDIVIDUAL DIFFERENCES IN DRUG RESPONSE

Drugs are, in general, organic molecules with varying degrees of lipid solubility. To aid elimination, as described above, they must be converted from lipid to water soluble compounds in the body, and this is performed by the process of metabolism. There are four main processes determining the fate of administered drugs: absorption, distribution, metabolism and excretion.

1.2.1 Drug absorption

Drugs are frequently given by mouth. After a drug has been swallowed as a tablet or capsule it must disintegrate and dissolve in the gastrointestinal fluids prior to absorption. Most drugs are absorbed in the small intestine but some acidic drugs, which will be un-ionized in the acid stomach contents, will be absorbed from the stomach itself. The balance between lipid and water solubility is an important determinant of a drug's absorption, since only un-ionized (and lipid soluble) drugs can cross the cell membranes from the gastrointestinal contents into the body itself. There are four possible mechanisms of drug absorption.

(a) Passive diffusion

This is the process by which most drugs are absorbed and involves the transfer of a drug down a concentration gradient from the gut to the blood stream with no expenditure of energy; the net transfer of the drug is directly proportional to the concentration gradient of the drug. The drug must initially achieve an aqueous solution at the surface of the cell and must then dissolve in the lipid of the cell membrane in order finally to pass into the aqueous phase on the other side of the membrane. There is no competition for absorption between drugs, even with those of similar structure.

(b) Active transport

Only drugs with a structure similar to a naturally occurring compound that undergoes active transport are absorbed by this method. The mechanism is highly specific for compounds such as sugars, amino acids and some vitamins. Alpha methyldopa and levodopa are two drugs that are probably absorbed by an active transport mechanism because of the similarity to the naturally occurring amino acid tyrosine.

(c) Filtration through pores

Pores are present between cells, but they are so small that only compounds with a relative molecular mass (Mz) of less than 100 may be absorbed in this way. Very few commonly used drugs are so small.

(d) Pinocytosis

The process whereby microscopic particles are engulfed by cells is not of major importance for drug absorption. An interesting development based on this principle is the preparation of drugs enclosed in a membrane such as a liposome, which may then be directly engulfed by the target cell. Liposomes, however, usually need to be administered systemically.

1.2.2 Factors affecting oral absorption

Factors affecting the absorption of drugs from the gastrointestinal tract are listed in Table 1.4.

TABLE 1.4 FACTORS AFFECTING THE ABSORPTION OF DRUGS FROM THE GASTROINTESTINAL TRACT

1. Formulation of drug
   - Disintegration time
   - Dissolution time
   - Presence of excipient

2. Patient characteristics
   - pH of lumen
   - Gastric emptying time
   - Intestinal transit time
   - Surface area of gastrointestinal tract
   - Presence of gastrointestinal disease

3. Presence of other substances in the gastro-intestinal tract
   - Interaction with other drugs or ions
   - Food

4. Pharmacokinetic characteristics of drug
   - Drug metabolism by gut bacteria
   - Drug metabolism by gut wall

The formulation of a drug may have dramatic effects on its absorption; for example, when the excipient contained in phenytoin capsules in Australia was changed, its bioavailability increased and this resulted in an epidemic of phenytoin intoxication. The presence of other drugs in the gut may also modify drug absorption. It is well known that the absorption of tetracycline is impaired by the presence of iron salts as well as other cations such as calcium or magnesium. Anion exchange resins such as cholestyramine may impair the absorption of drugs given at the same time, e.g. warfarin. It is often stated that food in the stomach has a deleterious effect on the absorption of drugs, but there is no consistent pattern in this effect. The absorption of some drugs (e.g. propranolol) is improved if taken with food. Perhaps the most important patient factor affecting drug absorption is the gastric emptying time. If gastric emptying is slow, then the absorption of acidic drugs from the stomach may be enhanced. In general, factors slowing gastric emptying will tend to slow the rate at which a drug is absorbed but will not normally reduce the amount of drug absorbed.

1.2.3 Alternative routes of drug administration

1.2.3.1 Intramuscular or intravenous administration

Drugs may be given by intramuscular injection because they are destroyed in the stomach (e.g. benzylpenicillin), because they are subject to extensive first pass effect (e.g. lignocaine), to aid compliance with therapy, or to speed up the rate of onset of the therapeutic effect. However, problems can arise if the drug is not soluble in water and may precipitate out of solution before absorption can occur (e.g. diazepam). Absorption after intramuscular administration may be delayed if the blood flow to skeletal muscle is reduced, e.g. in shocked patients given intramuscular morphine after myocardial infarction.

1.2.3.2 Buccal adminstration

This is used to ensure both a rapid onset of action (e.g. glyceryl trinitrate) by virtue of direct absorption into the systemic circulation, and absorption of a drug which would be destroyed by gastric acidity or by extensive first pass metabolism (e.g. morphine or buprenorphine). The pharmaceutical industry has, in the past few years, become increasingly aware of the advantages of this route of administration and a large number of formulations of therapeutic agents have been developed for utilization by this route (e.g. morphine, buprenorphine and nitrate derivates so as glyceryl or isosorbide).

1.2.3.3 Rectal administration

Drugs may be given as a suppository for the same reasons as for the buccal administration, but in general with less efficacy. A first pass effect is not completely avoided, due to the dual venous drainage of the rectum into the portal and

systemic systems. Since the surface area of the rectum is small, absorption may be slow. However, this can be put to advantage (when asthmatic patients are given aminophylline suppositories at night) such as to ensure a prolonged effect.

1.2.3.4 Percutaneous administration

Many drugs are well absorbed through the skin, especially if the skin is inflamed or diseased. A drug administered in this way is absorbed directly into the systemic circulation so avoiding first pass metabolism. Even normal skin will readily absorb lipid soluble drugs although absorption seems to be more rapid where the skin is less keratinized, e.g. on the upper arms, on the chest or behind the ears. Sustained release preparations for percutaneous administration are now available (e.g. glyceryl trinitrate) which deliver a fixed amount of drug over a 24-hour period. This route has a further advantage in that drug administration can be terminated quickly by removing the application from the skin. It is likely in the future that other drugs will be presented in formulations for percutaneous administration.

1.2.3.5 Pulmonary administration

Anaesthetic gases are typically absorbed in this way. The beta stimulants, salbutamol or terbutaline given by inhaler produce more rapid benefit and in a smaller dose than when given by the oral route. Sodium cromoglycate is not well absorbed from the gastrointestinal tract and it is only active in preventing asthma when the powder is inhaled. Patients need to be trained in the use of inhalers, and even then over 90% of the drug will be swallowed. Inhaled particles need to be 2-5 um in size to reach the smallest bronchioles.

Other routes of drug administration can be used, e.g., conjunctival, vaginal, and nasal, but they tend to be for specialist indications.

1.2.4 Binding and distribution of drugs

Following absorption, drugs are distributed via the blood stream to sites of action, e.g., receptors, to sites of storage in plasma or in tissues, and to sites of metabolism and excretion. The process of distribution largely depends on the physicochemical characteristics of the drug and on the blood flow to the various organs.

In the blood, drugs are often carried bound to plasma proteins, especially albumin. Basic drugs may also bind to acute phase proteins, such as a_1-glycoprotein. Forces involved in protein binding include ionic and hydrogen bonding. It is now recognized that there are at least two independent types of binding sites on human serum albumin and each site will bind a variety of drugs. As Table 1.5 shows, some drugs, e.g. tolbutamide, naproxen or indomethacin, will bind to both sites, while other drugs will only bind to one of the sites.

TABLE 1.5 PROTEIN BINDING SITES OF HIGHLY BOUND ACIDIC DRUGS ON HUMAN SERUM ALBUMIN

Site 1 ('warfarin site')		Site 2 ('diazepam site')	
Drug	% bound	Drug	% bound
Warfarin	99	Diazepam	98
Frusemide	91-99	Ethacrynic acid	85
Nalidixic acid	93-97	Cloxacillin	95
Phenytoin	87-93	Probenecid	85-95
Tolbutamide	95-97	Tolbutamide	95-97
Naproxen	98-99	Naproxen	98-99
Indomethacin	92-99	Indomethacin	92-99

The interaction between protein and drug is usually reversible and obeys the law of mass action:

drug + protein = drug-protein complex

The rate at which a drug-protein complex can dissociate is rapid with a half-life of only a few milliseconds. As stated earlier, only an unbound drug can diffuse into tissues according to current theory, and only an unbound drug can interact with a receptor to produce a pharmacological effect. The drug-protein complex thus acts as a store for the drug. For drugs which are rapidly cleared from the blood stream by the liver (e.g., propranolol), increased protein binding may increase the delivery of drug to the liver and hasten its elimination.

In theory, if two drugs that can bind to the same sites on human serum albumin are given together, they will compete for those sites. Thus, if a patient taking warfarin is given a non-steroidal anti-inflammatory drug such as indomethacin the non-steroid anti-inflammatory drug will tend to displace warfarin from its binding sites to form a new equilibrium.

1.2.5 Drug metabolism

Drugs are, in general, lipid soluble compounds that cannot be excreted as such by the kidney. The process of drug metabolism renders them more water soluble thus allowing excretion from the body. The main site of drug metabolism is the liver, but other tissues including skin, lung, blood and intestinal wall may also contribute. The gut wall is an important site of drug metabolism during the process of absorption, and drugs such as isoprenaline, ethinyloestradiol and morphine are, in part, converted there to inactive compounds.

The rate of metabolism of a drug in any individual is usually determined genetically, but can be changed by environmental factors (see 1.2.5.2). The rate of metabolism of any one drug varies widely from individual to individual, and a ten-fold variation in the rate of drug metabolism is not unusual. Metabolites formed are usually less pharmacologically active than the parent compound, but some drugs (e.g., cyclophosphamide) are only active through the production of a metabolite. A 'prodrug' (e.g., norethynodrel and ethynodiol diacetate - producing norethisterone, and talampicillin - producing ampicillin) is the name given to an agent which, through biotransformation, produces a therapeutically active substance. The manufacture of a prodrug is of value when, by reducing gastrointestinal toxicity, or minimizing first pass metabolism, it allows higher plasma concentrations of therapeutic substances to be achieved. Other drugs are active themselves, but, in addition, produce metabolites that are also pharmacologically active. In some of these cases the metabolite of the drug has a similar spectrum of activity to the parent drug (e.g., propranolol, procainamide or diazepam). However, the metabolite produced may differ in its pharmacological effects from the parent drug (e.g. pethidine whose metabolite norpethidine causes no analgesia but muscular twitching) or may have specific toxic effects (e.g. paracetamol, one of whose metabolites is responsible for causing liver necrosis).

1.2.5.1 Pathways of drug metabolism

A variety of biochemical reactions can take place during the metabolism of a drug to more water soluble compounds. They are of two types; phase I reactions are those whereby polar groups are introduced into the molecule by oxidation, reduction or hydrolysis; phase II reactions are synthetic and involve conjugation of the drug with glucuronic acid, glycine, sulfate or other groups. Some drugs may only undergo phase II reactions whilst others may first have to undergo a phase I reaction before a phase II reaction can take place. The enzymes that metabolize drugs in the liver are relatively non-specific compared with the enzymes in intermediary metabolism. Oxidation is the most frequent metabolic pathway and involves the transfer of molecular oxygen through the agency of cytochrome P_{450}. At one time it was thought that there was only one moiety of cytochrome P_{450}, but it now appears that there are many subtypes of the cytochrome in the liver, each responsible for the metabolism of different groups of drugs.

1.2.5.2 Factors affecting drug metabolism

Many factors can affect the rate of drug metabolism. Pharmacogenetic variations are dealt with in a later section. Important environmental factors that may affect drug metabolism are listed in Table 1.6.

TABLE 1.6 FACTORS AFFECTING DRUG METABOLISM

Factor	Response
Age	
Neonates	Reduced rate of drug metabolism
Elderly	
Environment	Enhanced rate of drug metabolism with occupational exposure to insecticides
Smoking	Enhanced rate of drug metabolism
Diet	Increased rate of drug metabolism by high protein/low carbohydrate diet Reduced rate of drug metabolism in malnutrition
Alcohol	
Acute ingestion	Inhibition of drug metabolism
Chronic ingestion	Increased rate of drug metabolism
Drugs	May increase or decrease rate of metabolism (enzyme induction or inhibition)

The maximal rate of drug metabolism is not fully developed at birth. Certain enzyme systems, particularly those involved in drug conjugation rather than those dealing with oxidation, develop slowly in the newborn. The ability to metabolize drugs may diminish with age, but this is a gradual process and the changes seen are small compared to the overall interindividual differences in drug metabolism that are known to occur.

Heavy cigarette smoking increases the rate of drug metabolism. It is known that smokers require higher doses of theophylline and pentazocine than non-smoking patients to produce a similar pharmacological effect. Prolonged occupational exposure to insecticides such as lindane or DDT will enhance drug metabolism. Diet may affect drug metabolism in a number of ways. High protein, low carbohydrate content diet will enhance the rate of drug metabolism and low protein, high carbohydrate diets may inhibit drug metabolism. In extreme malnutrition the rate of drug metabolism is reduced. Alcohol, in excess on a single occasion, will tend to inhibit drug metabolism but in chronic alcoholics, at least until liver damage ensues, the rate of drug metabolism is increased. Liver damage may result in diminished rates of drug metabolism.

Coadministration of other drugs influences the rate of drug metabolism and this is probably the most important environmental factor in clinical practice. A number of drugs are known to enhance the rate of drug metabolism in humans (enzyme inducers) and these, together with drugs that inhibit drug metabolism, are listed in Table 1.7.

TABLE 1.7 DRUGS THAT ARE KNOWN TO INHIBIT OR INDUCE DRUG METABOLISM IN HUMANS

Inhibitors	Inducers
Allopurinol	Barbiturates
Cimetidine	Carbamazepine
Chloramphenicol	Glutethimide
Phenylbutazone	Phenytoin
Sulthiame	Rifampicin

1.2.6 Excretion of drugs

1.2.6.1 Renal excretion of drugs

There are relatively few drugs (i.e. digoxin and gentamicin) in regular clinical use that are excreted unchanged by the kidney. Most tend to be, at least in part, metabolized and their metabolites are excreted in the urine. Active tubular secretion does occur for a few drugs. Bases, such as amphetamine, and acids, such as penicillin, probenecid, and salicylate, are carried across the renal tubular cell by an active transport mechanism against a concentration gradient. Probenecid will compete for the carrier mechanism with penicillin, thus inhibiting the renal clearance of penicillin and causing its plasma concentrations to increase.

The renal clearance of some drugs is affected by the pH of the urine. Weak acids such as phenobarbitone and salicylates are ionized by alkaline urine. Only un-ionized drugs can be reabsorbed into the body across the renal tubular epithelium. Similarly basic drugs such as amphetamine are excreted more rapidly in an acid urine. The urine can be made alkaline by the use of sodium bicarbonate and acid with ammonium chloride, and these principles can be helpful in the treatment of drug overdoses.

1.2.6.2 Biliary excretion of drugs

Drugs may be excreted by hepatic cells into the bile. They are occasionally excreted unchanged but more usually as conjugates (e.g., with glucuronic acid, sulfate or glycine). Polar metabolites are likely to be excreted in bile if their relative molecular mass (Mr) exceeds 400. Biliary excretion may serve as an alternative to renal excretion in patients whose kidney function is impaired, but it is unlikely that excretion in the bile will entirely compensate for deficiencies in renal excretion.

Some drugs are excreted in the bile and then undergo an enterohepatic circulation. For example, the contraceptive steroid ethinyloestradiol is absorbed from the small intestine forming sulfate and glucuronide conjugates in the gut wall and liver. A high proportion of these metabolites are excreted via the bile into the intestine. Bacterial flora hydrolyse these conjugates to liberate free ethinyloestradiol which is then available for reabsorption. Thus the enterohepatic recirculation is a mechanism which prolongs the action of the drug.

1.2.7 Pharmacogenetics

Pharmacogenetics (see also section 1.3) deals with the modification of drug responses by hereditary influences. Table 1.8 shows that some examples involve drug metabolism while others involve a variation in response to the drug.

The interplay between genetic and environmental influences on response is one of increasing interest. It has been calculated that for phenylbutazone at least two-thirds of the variation in rates of metabolism is due to genetic influences and the remainder to environmental effects. Another interesting example is that about 5% of people in the United Kingdom are poor hydroxylators of debrisoquine and this inability to metabolize the drug has a genetic basis. In other populations a smaller proportion of individuals are poor metabolizers of debrisoquine (cf. acetylation see below). It has recently been shown that if an individual is a poor metabolizer of debrisoquine, he or she will also be a slow metabolizer of metoprolol, encainide and phenformin. It seems likely that the hydroxylation of all these drugs is performed by the same form of cytochrome P_{450} in the liver. The clinical importance of this finding has yet to be fully explored, in part, at least, because none of the drugs so far implicated can be considered a first line drug in therapy.

The importance to therapy of genetic variations in conjugation can be seen in a number of examples shown in Table 1.8. A number of drugs such as isoniazid, procainamide and hydralazine are metabolized by acetylation. This process is performed by the enzyme N-acetyl transferase in the liver. The ability to acetylate isoniazid is inherited as an autosomal recessive trait and distribution histograms show a bimodal pattern. In a Caucasian population about 60% of the population are slow acetylators but in Japan the incidence falls to 10%. However, in the Canadian Eskimo races nearly 100% of the population are fast acetylators. The reason for this geographical variation is unknown. It is now clear that slow acetylators of isoniazid are more likely to develop toxic effects such as peripheral neuropathy than are fast acetylators. On the other hand, patients with tuberculosis, who are fast acetylators, may respond less well to isoniazid if the drug is given on a twice weekly basis. Fast acetylators seem to be more likely to develop liver damage following the use of isoniazid because this toxicity is caused by a metabolite acetylhydrazine which is produced in higher concentrations by

TABLE 1.8 GENETICALLY DETERMINED ABNORMAL DRUG RESPONSES

Condition	Response	Inheritance	Drugs involved
Slow and fast acetylators	Slow acetylators may show toxicity; fast acetylators show diminished response	Autosomal recessive	Isoniazid, hydralazine, procainamide, dapsone
Suxamethonium sensitivity	Prolonged apnoea	Autosomal recessive	Suxamethonium (succinylcholine
Porphyria	Abdominal pain paralysis	Autosomal dominant	Barbiturates
Favism	Haemolysis on exposure to certain drugs	Sex-linked incomplete dominant	E.g. primaquine, nitrofurantoin, sulfonamides
Malignant hyperthermia	Uncontrolled rise in body temperature	Autosomal dominant	Certain anaesthetic agents, e.g. halothane, suxamethonium
Glaucoma steroids	Glaucoma due to abnormal response to intraocular steroids	Autosomal recessive	Topical corticosteroids, systemic corticosteroids

rapid acetylators. Slow acetylators of procainamide and hydralazine are more prone to develop systemic lupus erythematosus than are fast acetylators.

Acute intermittent porphyria may be precipitated by drugs such as phenobarbitone. This disease is inherited as an autosomal dominant and is due to an abnormal inducibility of the enzyme -amino laevulinic acid synthetase.

About 100 million people in the world are at risk of developing drug induced haemolysis due to lack of glucose-6-phosphate dehydrogenase in the red cell. Haemolysis may be precipitated by namides (see Table 1.9.)

TABLE 1.9 SOME DRUGS CAUSING HAEMOLYSIS IN PATIENTS WITH GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

Primaquine	Sulfonamides
Quinine	Dapsone
Chloroquine	Nalidixic acid
Quinidine	Nitrofurantoin
Probenecid	Chloramphenicol
Aspirin	

Malignant hyperthermia is a recently recognized condition that occurs in approximately 1 in 20 000 anaesthetics and may be due to an abnormality of calcium binding by the muscular sarcolemma. Following the use of an anaesthetic drug such as halothane, suxamethonium or nitrous oxide, the body temperature may rise by $2^{o}C$ or more per hour.

1.3 MEASURING PLASMA CONCENTRATIONS OF DRUGS

A fundamental premise in pharmacology is that the intensity and duration of drug response is determined by the concentration of drug at the sites of action, i.e. the receptors. Since the concentration of a drug in plasma can usually be measured, it is important to know whether changes in this reflect changes in the concentration at the receptor sites. For drugs that act reversibly, the formation of drug-receptor complexes obeys the law of mass action. Thus:

drug + receptor = drug-receptor complex

At equilibrium, the rates of formation and dissociation of drug-receptor complexes are equal.

There are, however, many instances when pharmacological effects are not related to the plasma level of the drug. There are several possible reasons for this. First, a therapeutic substance may act, at least in part, through a metabolite, e.g. the activity of cyclophosphamide, the cytotoxic agent, resides in one or more metabolites formed from the parent substance by the hepatic microsomal enzyme system. -Methyldopa, the anti-hypertensive agent acts via -methylnoradrenaline which is two steps removed from the parent drug. Procainamide, the

antiarrhythmic agent, has an active metabolite N-acetyl procainamide. Second, drugs may act irreversibly, i.e. the quantity of active drug attached to receptors is not related to steady-state plasma concentrations. Such drugs often attach to their receptors and then bind covalently with them. A small amount of drug remains attached to these sites long after the rest of the drug has vanished from the body. Certain drugs act non-reversibly without bonding covalently but they are attached so tightly to receptors that they remain fixed to their sites after the concentration of unbound drug has declined to levels which are unmeasurable by present analytical techniques. Non-reversible drugs are inherently dangerous since their effects tend to accumulate even though the drug may not. Third, the assay method for plasma drug concentrations may be too insensitive to reflect an important pool of the drug, such as that for guanethidine stored in adrenergic neurones. In such instances it may be useful to forsake kinetic analyses based on plasma drug concentrations and examine urine excretion as it reflects better the kinetics of the drug.

There are several prerequisites for using the plasma concentration of a drug to monitor clinical effects:

(i) the drug should act by a reversible mechanism.

(ii) the drug should not have active metabolites.

(iii) the concentration of unbound drug in the plasma should reflect the concentration of unbound drug at the receptor sites. For drugs with a small apparent volume of distribution, it is reasonable to consider the plasma concentration as representing the amount of drug in the body. For drugs with a large apparent volume of distribution, the relationship between the plasma level and total amount of the drug may be more tenuous and difficult to ascertain. Tissue levels of the drug may be of more importance as a determinant of therapeutic effect.

(iv) the development of tolerance at the receptor sites should not be an important problem as with barbiturates and ethyl alcohol.

Fig. 1.7 Visual analogue scale for use in the assessment of pain.

(v) the pharmacological effects of the drug should be recorded in an accurate way. While the measurement of drug levels may pose certain technical problems, these are usually soluble. The question of measurement of drug effects is much more challenging. For psychotropic drugs, "rating" scales employing both subjective and objective assessment may have to be used. Visual analogue scales for drugs such as analgesics or anti inflammatory drugs may also be used (Fig. 1.7). Even the measurement of a physiological end point such as blood pressure may pose problems, e.g should lying or standing blood pressure be used, systolic or diastolic and if the latter, phase 4 or phase 5 Korotkoff sounds?

1.3.1 What to measure - total plasma concentration or free concentration?

In principle, it is free or unbound drug which is in equilibrium with receptor sites. There are variations in plasma protein binding of drugs although these are usually small in comparison with the differences which occur in rates of drug metabolism, suggesting that in most instances total drug concentration measurements are adequate. There are, however, several limitations to this approach.

When more than one drug is administered, displacement of one drug by another leads to an increase (albeit transitory) in the free drug concentration. A better correlation is obtained between the concentration of the free drug and effect than between total concentration and effect.

Plasma protein binding may be perturbed in patients with diseases. The unbound fraction of diphenylhydantoin is markedly increased in uraemia. It has been found that epileptics with uraemia respond both therapeutically and in terms of adverse effects at much lower total plasma concentrations of diphenylhydantoin than do epileptics with no renal disease.

Certain drugs tend to localize within the red cell. Propranolol and chlorthalidone have concentrations within the red blood cell considerably higher than in plasma, while chloroquine is extensively concentrated in white blood cells and malaria infected red cells. Whether the effect of drugs such as these is related to total blood level rather than to plasma levels remains to be explored in most instances.

1.3.2 Indications for monitoring drugs in plasma

1.3.2.1 Therapeutic monitoring

It is often more difficult to assess the clinical effect of the drug than to monitor the plasma concentration. This is not true for drugs such as antihypertensive agents, anticoagulants and hypoglycaemic agents where clinical observation (blood pressure) or simple laboratory tests (prothrombin time or blood sugar) must always form the basis of

dose adjustment. For drugs which have a narrow therapeutic ratio (e.g. lithium) or show dose-dependent kinetics (e.g. diphenylhydantoin), plasma concentrations are a better guide to both efficacy and potential toxicity than purely clinical observations although the latter must always play an extremely important role in dose adjustment.

1.3.2.2 Patient compliance

One of the most difficult problems in therapeutics is to decide whether a patient is taking his medication as prescribed. While certain dosage regimens (e.g. three or four times daily dosing) and certain patient characteristics (the old and those with poor doctor/patient relationship) predispose to poor compliance, many aspects of this important area have not been explored. If a patient responds poorly to a drug, it is useful to monitor its concentration in a biological fluid, e.g. plasma or urine. If a drug has a relatively long half-life and a low volume of distribution and no drug can be detected in plasma a few hours after alleged dosing, it is difficult to escape the conclusion that the patient has not taken the drug.

Measurement of drug (or metabolite) in the urine helps to distinguish between the patient who metabolizes a drug rapidly and a non-complier. In the former, the urine metabolite concentration over a specified time (e.g. 24 hours) should account for a defined and predictable amount of the ingested drug. In some situations attention is now being focused on the measurement of drug concentrations in saliva as a "non-invasive" procedure. In general, salivary concentrations of drugs reflect their unbound concentration in plasma, and salivary levels have been successfully used to monitor therapy with diphenylhydantoin, phenobarbitone, theophylline and isoniazid. One drawback to this method is that if the drug has an effect on salivary flow the interpretation of salivary concentrations may be difficult. Salivary concentrations may, of course, also be used in therapeutic monitoring although this technique may be most widely used to monitor drug compliance.

1.3.2.3 Renal or liver dysfunction

Drugs which are excreted predominantly by the kidney may pose toxicological problems in patients with increasing renal dysfunction but who require drug therapy, e.g., the aminoglycoside antibiotics which cause inner ear disease and also further renal dysfunction and digoxin which causes nausea, vomiting, and arrhythmias.

The handling of many drugs in patients with hepatitis or cirrhosis may be altered by the disease especially if these drugs undergo phase 1 metabolism. Thus the clearance of theophylline and phenytoin, both drugs which have a low therapeutic index, are diminished in cirrhosis. In such circumstances monitoring of drug concentrations is mandatory.

1.3.2.4 Drugs overdosage

If a definitive procedure such as haemodialysis or peritoneal dialysis is to be instituted for intoxication with drugs such as phenobarbitone or salicylate, it is wise to check the efficacy of the manoeuvre, e.g. monitoring plasma concentrations. In the case of intoxication with paracetamol, plasma concentrations above 200 ug/ml at 4 hours or 50 ug/ml at 12 hours after ingestion of the overdose are indications for administration of a specific antagonist such as n-acetyl cysteine (Fig. 1.8).

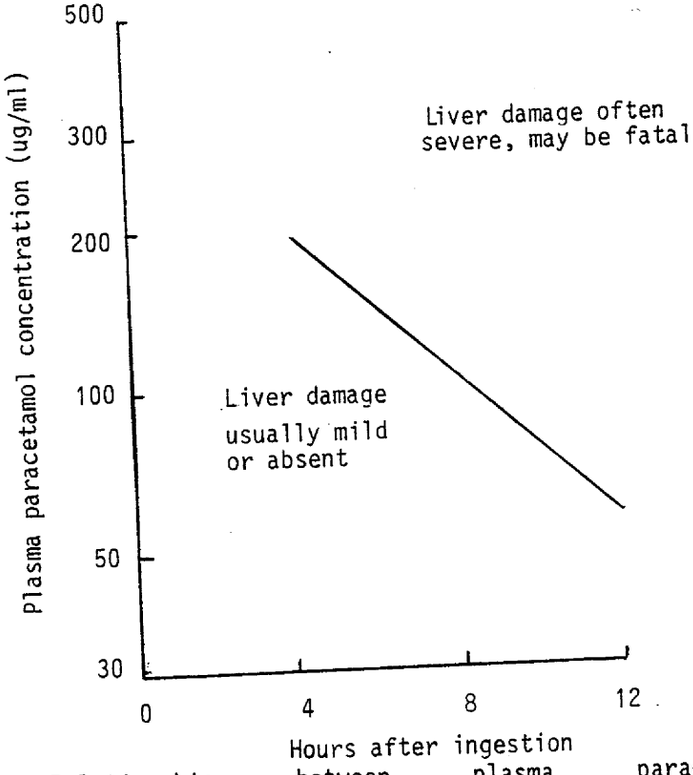


Fig. 1.8. Relationship between plasma paracetamol concentrations and time after ingestion to liver damage following an overdose of paracetamol. Treatment with sulphydryl compounds such as n-acetyl cysteine is indicated with values above the solid line. (After Prescott, L.F. (1981) Drug overdosage and poisoning. In: Avery, G.S., ed. Drug therapy, 2nd ed. Sydney, Adis Press p. 263.

1.3.3 Drugs whose plasma concentrations should be monitored

1.3.3.1 Anticonvulsants

Phenytoin is a difficult drug to use because of its capacity limited metabolism, i.e. if one doubles the dose, plasma concentrations may rise six-fold. It is now well established that monitoring the plasma concentration of phenytoin is helpful clinically and adjusting the dose to bring the plasma concentration into the range 10-20 ug/ml will reduce the frequency of seizures in most patients and will diminish drug toxicity. Some epileptic patients will have good epileptic control at plasma concentrations outside this range e.g. patients with renal failure (see 1.3.2.3). The main problem with phenytoin is probably underdosing. Workers in Sweden showed in a series of epileptic patients that more than 50% had plasma concentrations of phenytoin below the therapeutic range either because of poor compliance or rapid metabolism.

There is no persuasive evidence that monitoring plasma levels of phenobarbitone is of clinical value. A case can be made for monitoring carbamazepine levels in plasma (therapeutic range 2-6 um/ml, toxic effects seen above 8 ug/ml), although the presence of an active metabolite may render such data more difficult to interpret. Ethosuximide (therapeutic plasma concentration 40-80 ug/ml, toxic effects above 100 ug/ml) is also being monitored although wider experience is necessary to confirm its value (Table 1.10).

TABLE 1.10 DRUGS FOR WHICH THERAPEUTIC AND TOXIC PLASMA RANGES HAVE BEEN DEFINED

Drug	Therapeutic range		Toxic effect	
Digoxin	1-2	ng/ml	3	ng/ml
Digitoxin	10-25	ng/ml	40	ng/ml
Theophylline	10-20	ug/ml	25	ug/ml
Phenytoin	10-20	ug/ml	25	ug/ml
Lithium	0.5-1.5	mmol/l	1.5	mmol/l
Nortriptyline	50-140	ng/ml	200	ng/ml

1.3.3.2 Cardiovascular drugs

Monitoring of plasma concentrations of digoxin has been shown to be of value to check compliance and to adjust the dose in patients with renal failure. Considerable controversy exists as to how far plasma digoxin concentrations should be measured in routine clinical practice. Digitoxin, unlike digoxin, is metabolized by the liver rather than excreted by the kidney. It is used as an alternative to digoxin in patients with renal failure in some hospitals. The role of digitalis glycosides in the long-term management of congestive

heart failure is the source of continuing debate, although the consensus view now seems to be that long-term digoxin therapy in patients in sinus rhythm does improve cardiac output and exercise capacity. In patients with atrial fibrillation there is no question as to its value but when the patient is in sinus rhythm, diuretic therapy may be a more appropriate long-term monotherapy.

Procainamide is less used as an antiarrhythmic drug than previously. Although measurement of therapeutic plasma concentrations has been recommended, the presence of an active metabolite N-acetyl procainamide in plasma calls into question the value of measuring only unchanged drug. A similar reservation pertains to lignocaine. The importance of active metabolites remains to be clarified in routine therapeutic monitoring.

1.3.3.3 Bronchodilators

Many studies have confirmed the value of monitoring plasma theophylline concentrations in asthmatic patients, especially children. With attention to maintaining plasma concentrations within the prescribed range, theophylline is increasingly shown to be a most valuable first-line drug in asthma therapy. The therapeutic range is between 10 and 20 ug/ml.

1.3.3.4 Central nervous system drugs

Lithium, used in the management of mania and manic-depressive psychoses, has a low therapeutic index. Handled by the body in a similar manner to sodium, monitoring of plasma concentrations has been shown to be essential for its optimal use. (The therapeutic range is 0.5-1.5 mmol/l.) Coadministration of diuretics has been shown to cause perturbation of plasma concentrations. There is the further strong possibility that long term therapy with lithium may cause renal dysfunction making plasma level monitoring even more important.

Although several studies have demonstrated the value of measuring plasma concentrations of nortriptyline, this is not yet routinely carried out. The problems with antidepressant administration are the wide interindividual range of rates of metabolism, the difficulty in monitoring their clinical effect, the long duration (sometimes three or four weeks) before an optimum response is obtained after starting therapy, and the relatively narrow "therapeutic window" in which maximum benefit is seen.

1.3.3.5 Antibiotics

As mentioned above, aminoglycoside antibiotics, e.g. gentamicin, are excreted by the kidney. During renal failure accumulation of these drugs may result in ototoxicity and further renal damage unless appropriate dosage adjustment is made.

1.3.3.6 Drugs taken in overdosage

Paracetamol causes liver damage when taken in overdosage. A nomogram has been constructed (Fig. 1.8) using plasma concentrations relative to time after dosing as a basis for deciding whether administration of an antagonist, e.g. n-acetyl cysteine or methionine, should be administered to avert hepatotoxicity.

1.4 EFFECT OF DISEASE ON DRUG RESPONSE

Most initial clinical investigations of new drugs are performed in volunteers and the results are then applied to patients who may have a variety of diseases often quite distinct from the one for which the drug was designed. In some cases the presence of disease may alter the responsiveness of tissues to the drug, e.g., hypokalaemia enhances the toxicity of digitalis and morphine-like drugs have greater central nervous system depressant effects in patients with cirrhosis of the liver. Most information, however, is available on the effects of disease on the pharmacokinetics of drugs.

1.4.1 Drug absorption in disease

Drug absorptive processes are usually so efficient that it is rare for disease to have much effect. If gastric emptying is delayed, the rate of drug absorption will be slowed but the amount of drug absorbed will not change. This may mean a delay in the peak effect of the drug but little overall change in effect. Delayed gastric emptying may produce therapeutic failure with levodopa partly because the drug is metabolized in the stomach wall leaving less to be absorbed by active transport in the small intestine. In patients with malabsorption syndromes, drug absorption may be delayed but it appears that the disease has to be very severe before clinically significant changes in overall absorption will occur. In some cases malabsorption syndrome due to coeliac disease may actually lead to improved absorption of the drug and hence greater toxicity. For example, ethinyloestradiol is extensively conjugated in the gut wall with sulfate and the capacity for this conjugation is reduced in coeliac disease. Thus, first pass metabolism of this drug by the gut wall is reduced in coeliac disease leading to increased systemic bioavailability.

1.4.2 Drug distribution in disease

As described above, the distribution of drugs to their sites of action, their storage and elimination are mainly influenced by physicochemical characteristics of the drug and regional blood flow. Changes in plasma pH may, on occasion, result in a change in drug ionization sufficient to alter distribution of a drug whose pKa approximates that of plasma. This may contribute to the reduced effect and myocardial uptake of lignocaine in acidotic states. Reduction in blood flow in heart failure or following a myocardial infarction may also affect drug distribution.

Protein binding is also affected by disease. In severe hypoalbuminaemia, such as may occur in patients with the nephrotic syndrome or with cirrhosis, the binding of acidic drugs in plasma will be reduced. The protein binding of acidic drugs is also reduced in patients with impaired renal function. As this becomes reduced, a number of endogenous compounds are retained in the plasma and compete with drugs for the binding sites on plasma albumin. Drugs such as phenytoin, warfarin, phenylbutazone, sulfonamides and salicylates, show reduced binding to albumin in patients with renal impairment. This is important in the interpretation of plasma concentration data. Phenytoin is measured in plasma as the total concentration (i.e. free + bound) of which the free concentration is the pharmacologically active moiety. If under normal conditions a total plasma concentration of 15 ug/ml is required, the free concentration will be about 1 ug/ml. However, in a patient with impaired renal function a free concentration of 1 ug/ml may be achieved at a total plasma concentration of only 7.5 ug/ml or less. Under these circumstances it is obviously important to reduce the dose given.

Protein binding of basic drugs is not disturbed in renal failure. However, basic drugs (e.g. propranolol, chlorpromazine, quinidine or imipramine) will become more extensively bound because of the increased plasma concentrations of x_1-glycoprotein in inflammatory states.

1.4.3 Drug metabolism in disease

Since the liver is the main organ of where metabolism of drug occurs, it would not be too surprising to find that disease of the liver impairs this process. In general, liver disease needs to be fairly extensive before the metabolism of drugs is affected because of its large reserve capacity. It is now recognized that the metabolism of drugs in disease states will depend largely on the pharmacokinetic characteristics of the drugs. In terms of their hepatic clearance, drugs can have either high clearance or low clearance characteristics. The extraction ratio across the liver of high clearance drugs is large and the ability of the liver to eliminate them after intravenous administration is dependent more on liver blood flow than on the intrinsic ability of the liver to metabolize them. Thus, reduction in hepatic blood flow, as may occur in heart failure, will lead to reduced clearance of drugs such as lignocaine and propranolol given intravenously. In contrast, low clearance drugs are more dependent upon the intrinsic metabolizing ability of the liver and will be more affected by disease of the liver parenchyma than by changes in liver blood flow. Some examples of these changes are shown in Table 1.11.

TABLE 1.11 DRUGS WHOSE CLEARANCE MAY BE REDUCED IN LIVER DISEASE

High clearance drugs	Low clearance drugs
Lignocaine	Diazepam
Labetalol	Prednisolone
Chlormethiazole	Ampicillin
Propranolol	Theophylline
Pethidine	

1.4.4 Drug excretion in disease

Those drugs which are primarily cleared from the body by renal excretion show a prolonged half-life in patients with impaired function. Renal function may be reduced not only by disease but also by increasing age. With increasing degrees of renal failure such drugs may progressively accumulate in the body. It is generally assumed that drugs which are metabolized can safely be given in normal doses to patients with renal failure. This is true only if the metabolites have no pharmacological effect. In some cases polar metabolites will not be excreted readily by the patient who shows renal failure and any activity of the metabolite will be seen as increased therapeutic and toxic effects. The main active metabolite of procainamide, N-acetyl procainamide, accumulates in the plasma of patients with renal failure and has been the cause of arrhythmias. Norpethidine is a metabolite of pethidine that is not readily excreted in patients with renal function impairment. Norpethidine has little analgesic effect but may cause muscular irritability and twitching.

It is obviously of prime importance for safe therapy in patients with renal disease to know the fate and metabolism of administered drugs. In order to achieve a defined steady-state plasma concentration in these circumstances, three main points need to be understood.

(i) if a loading dose is given, this dose will not need to be changed provided that the distribution volume is unaltered in the disease state.

(ii) the maintenance dose of the drug should be smaller and/or the dose should be given less frequently.

(iii) the time taken to achieve steady-state plasma concentration, and therefore the optimum therapeutic effect, will be longer.

Several nomograms have been introduced into clinical practice to guide the physician in the choice of drug dosage in patients with renal failure but in general, these have not been shown to be of great clinical value. Table 1.12 shows the changes in plasma half-life of some drugs that may be seen in anuric patients.

TABLE 1.12 ELIMINATION HALF-LIVES (HOURS) OF SOME DRUGS IN NORMAL AND IMPAIRED RENAL FUNCTION

Drug	Normal (hours)	Anuria (hours)
Penicillin G	0.5	23
Cephaloridine	1.7	23
Gentamicin	2.5	35
Vancomycin	5.8	230
Tetracycline	8.5	90
Doxycycline	23	23
Digoxin	30	100
Digitoxin	170	170

1.5 DRUG INTERACTIONS

The likelihood of adverse reactions occurring increases with the number of drugs prescribed. It has been calculated that if five drugs are given simultaneously, there is a 75% chance of causing an adverse reaction due to the interactions perpetrated. In many respects, drug interactions have attracted an attention disproportionate to their clinical significance but, on the other hand, they may give clear insights into underlying pharmacological mechanisms. However, the important clinical examples are drawn from a relatively short list of drugs with narrow therapeutic ratios, i.e. whose doses must be adjusted within a small range and whose adverse effects are pronounced when this range is either exceeded or not attained. Such drugs include oral anticoagulants, anticonvulsants, antidepressants and antihypertensive agents. Some interactions may be beneficial and are deliberately perpetrated. Examples of drugs used in combination to achieve a therapeutic effect or minimize an adverse reaction in a way which could not be done with a single drug regimen included L-dopa and decarboxylase inhibitors used for Parkinson's disease, diuretics and beta-adrenoceptor blockers for hypertension, oestrogen-progestogen combinations for contraception, and trimethoprim with a sulfonamide as an anti-infective.

1.5.1. Mechanisms of interactions

In broad terms, the basis of underlying drug interactions may either be pharmacokinetic or pharmacodynamic. Pharmacokinetic interactions depend either on a change in free drug concentrations in plasma, or at the sites of drug action in the tissues. Pharmacodynamic interactions result from a modification of drug occupancy of a receptor site or a modification of an underlying physiological control mechanism. The additive effects of drugs with a similar action are often cited as a type of drug interaction but strictly speaking an interaction should result in either an enhancement or diminution of the combined separate effects of the drugs.

1.5.2 Sites of interactions

Interactions may be classified according to the sites at which they occur. Table 1.13 shows one such classification.

TABLE 1.13 SITES OF DRUG INTERACTION

(i)	Prior to administration
(ii)	Drug absorption Within the gut lumen By altering gut motility By altering gut flora Within the gut wall
(iii)	Protein binding
(iv)	Drug metabolism Stimulation Inhibition
(v)	Drug excretion Changes in urine pH Competition for active renal tubular excretion Fluid and electrolyte changes
(vi)	Interactions at receptors

1.5.3 Prior to administration

If drugs such as heparin or benzyl penicillin are mixed with infusion fluids of low pH (e.g. certain dextrose solutions), appreciable loss of drug activity may occur due to chemical inactivation. Further, since drugs may interact in the infusion bottle (e.g. carbenecillin is inactivated by gentamicin), administration of more than one drug in the same infusion should be avoided wherever possible. If it is essential that this should be done, the therapeutic consequences should always be considered.

A further example of a pharmaceutical interaction is when the excipients in the formulation of a tablet alter the release characteristics of an active drug. A recent example of this is the change in the formulation of digoxin which resulted in a change in bioavailability and consequently efficacy.

1.5.4 Drug absorption

(a) Within the gut lumen

Calcium, magnesium and aluminium interact with tetracycline to form a non-absorbable chelate and thus antacids containing these cations, or even milk should not be taken with tetracycline. Although they should be given as far apart in

time as possible. Cholestyramine, an ion exchange resin, will bind acidic drugs such as warfarin and digitoxin, preventing their absorption.

(b) By altering gut motility

If a drug which either enhances gastric emptying (e.g. metoclopramide) or diminishes it (e.g. propantheline, tricyclic antidepressants, or opiates) is given with a drug that is mainly absorbed in the small intestine, the rate of absorption of the second drug may be altered. This has been illustrated very clearly with paracetamol (Fig. 1.9).

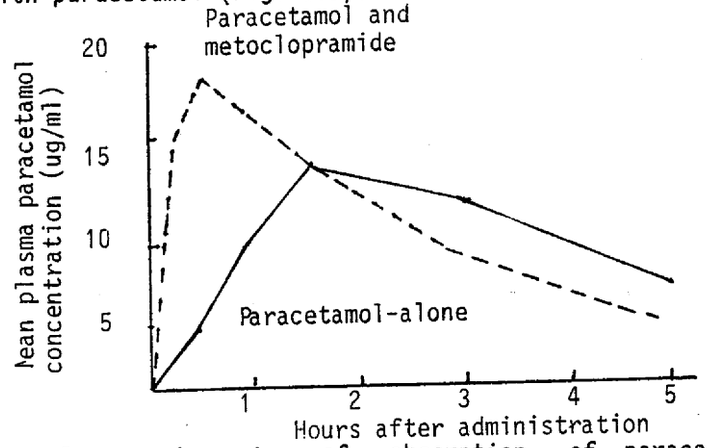


Fig. 1.9 Increased rate of absorption of paracetamol following the use of metoclopramide. (After Nimmo J. et al., 1973, British medical journal, 1: 587-589)

Metoclopramide increases gastric emptying and therefore the rate of paracetamol absorption. This combination is widely used for migraine. Conversely, propantheline decreases the rate of paracetamol absorption and thus, presumably, the onset of effective analgesia.

(c) By altering gut flora

In the past few years several reports have appeared of an increased incidence of intermenstrual bleeding and unwanted pregnancy in women given broad spectrum antibiotics and oral contraceptives. The putative mechanism of this effect is that conjugates of the contraceptive steroid ethinyloestradiol, are excreted in bile. Under control conditions, these conjugates are hydrolysed by gut bacteria and unchanged ethinyloestradiol is then reabsorbed by enterohepatic circulation. In the presence of antibiotics which either kill or alter gut flora, this hydrolysis will not occur, the enterohepatic circulation is interrupted and conjugated ethinyloestradiol excreted in the faeces. This mechanism is clearly established in animal models but its existence in humans is not proven. It seems likely that this is not a systematic interaction but will only occur in a

very small proportion of people who only excrete the conjugate of unchanged ethinyloestradiol in bile (as opposed to conjugates of its inactive metabolites) and whose gut bacteria are active in breaking down this conjugate. The number of subjects in whom this is likely to be important remains to be determined.

(d) Within the gut wall

The gut wall is an important organ for phase II metabolism, i.e. conjugation. For example, over 40% of ethinyloestradiol is metabolized to a sulfate conjugate within the gut wall in man and if vitamin C which competes for available sulfate, is administered with the steroid, plasma ethinyloestradiol concentrations are increased significantly. A similar mechanism is responsible for the interaction between isoprenaline (which forms a sulfate conjugate in the gut wall) and salicylamide (which competes for available sulfate). Under those circumstances, the amount of unchanged, and therefore active, isoprenaline that is absorbed increases.

1.5.5 Protein binding

As discussed previously, many drugs are transported in the blood bound to either albumin or specialized globulins. The significance of the displacement of drugs from binding sites as a cause of drug interaction has probably been exaggerated for several reasons. If a drug is 90% bound to plasma proteins, and if, in the presence of a displacing agent this percentage falls to 80%, there is a doubling of the concentration of the free drug. This free drug will then leave the plasma and be cleared by normal processes. Thus the total plasma concentration will fall until equilibrium is re-established, i.e. when 90% of the new total plasma concentration is bound (Fig. 1.10). As a result any effect of displacement will be transient and the final result may be an overall diminution in therapeutic effect.

A second consideration is the relative distribution of the drug between plasma and the tissues. A drug must be both highly bound, e.g. 90%, and have a low apparent volume of distribution, i.e. be predominantly localized in the plasma, for displacement to have any therapeutic significance. Displacement of warfarin (99% bound, V_D91) by 1% leads to a doubling of the free concentration.

Displacement of digoxin (20% bound V_D300 l) by 5% leads to a very small increase in free concentration both because of the high initial percentage free and because of its large apparent volume of distribution.

There are instances, however, when displacement of one drug by another could be dangerous. When the displacing drug is injected intravenously, the concentration of unbound drug could be increased instantaneously and thus highly perfused organs such as the brain, heart and liver may be exposed to

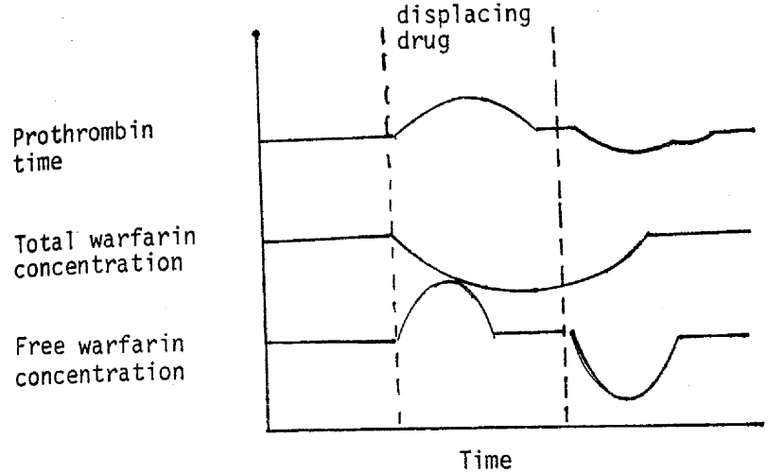


Fig. 1.10 Theoretical effect of protein binding displacement (after Koch-Weser, J. & Sellers, E.M. (1976) New England journal of medicine, 294: 311-316)

high free concentrations during redistribution. In other instances, if drug-protein complexes serve as transport carriers rather than drug depots (e.g. as with propranolol) displacement of the drug from binding sites in plasma could retard drug elimination and thus later the steady-state drug concentration of the unbound drugs. Not only may drugs be displaced from binding sites by other drugs with a higher affinity for the site but endogenous substances which may accumulate in disease may also compete for these sites. Bilirubin in liver failure, and various endogenous metabolites in renal failure, will displace drugs from albumin binding. Toxic effects from sulfonamides, phenytoin and warfarin have been documented in these instances.

Finally, it should be mentioned that several interactions, whose basis is alleged to be displacement, may have an entirely different mechanism. The enhancement of the pharmacological effect of warfarin by phenylbutazone (frequently cited as being due to displacement, since this can be demonstrated in vitro) is, in fact, more likely to be attributed to a stereo-selective alteration in the metabolite degradation of warfarin by phenylbutazone. Warfarin is a racemic mixture and phenylbutazone inhibits the metabolism of S-warfarin (the more potent isomer) while increasing the elimination of R-warfarin (the less potent isomer).

1.5.6 Drug metabolism

1.5.6.1 Stimulation

Many lipid soluble drugs cause non-specific stimulation of drug metabolism in both humans and experimental animals. The number of drugs shown to be inducing agents in humans is

relatively small(see Table 1.7). The administration of an inducing agent stimulates not only the metabolism of many drugs and physiological compounds which are substrates for micosomal enzymes but also the metabolism of the inducing agent itself. Microsomal enzyme induction occurs in many tissues, the gut wall, lung, kidney, skin as well as the liver. Depending on the dose and drug, induction usually develops over a period of several days or weeks and persists for a similar period following withdrawal of the inducing agent. The effect of some inducing agents such as chlorinated hydrocarbon insecticides is more persistent since these compounds are stored in body fat and remain for long periods within the body. Induction is often considered to equate with drug inactivation but if drug metabolites have a greater pharmacological activity or a different spectrum of action from the parent substances, the drug effects may be enhanced by induction. For example, hepatic necrosis following paracetamol overdosage is more severe in patients previously given inducing agents due to increased production of toxic metabolites of paracetamol. Similarly, prior use of inducing agents increases the risk of nephrotoxicity after methoxyflurane administration.

Many of the important parameters of enzyme induction such as the time course of induction, dose dependence and structure activity relationship were determined using barbiturates.

If barbiturates are given to a patient taking warfarin or an oral contraceptive steroid, the clinical effect of these will be reduced over a period of two to three weeks because their plasma concentrations will fall due to the increased rate of metabolism.

When the barbiturate is stopped, the rate of metabolism slowly returns to its previous level but this may take several weeks. Figure 1.11 shows an example of the interaction between amylobarbitone and warfarin.

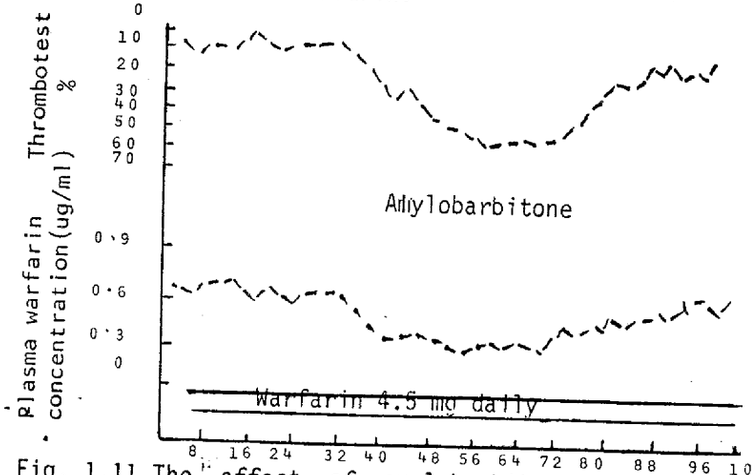


Fig. 1.11 The effect of amylobarbitone on the plasma warfarin concentration and anticoagulant control (measured as thrombotest) in a patient taking 4.5 mg warfarin daily.

With the decrease in use of barbiturates, except for their use in epilepsy, rifampicin is probably a more important inducing agent nowadays. Rifampicin, in particular, has been shown to diminish the efficacy of oral contraceptives and of oral anticoagulant therapy and to alter the rate of metabolism of various natural substrates such as vitamin D and cortisol.

An interesting facet of enzyme induction is the resultant increase in liver blood flow due to an increase in portal venous flow rather than hepatic arterial flow. For drugs which are highly cleared by the liver, e.g. propranolol, the increase in liver blood flow is a more important determinant of their increased elimination than an increase in liver microsomal enzyme activity when an inducing agent is given.

Not all increases in rates of metabolism are due to induction. There is some evidence of activation of drug metabolizing enzymes in certain experimental situations, e.g. with certain steroids. Activation implies a more rapid effect and is probably due to structural alteration in the drug metabolizing enzyme without necessarily increased de novo protein synthesis which occurs in enzyme induction.

1.5.6.2 Inhibition

Inhibition of drug metabolism may result in prolonged and exaggerated drug responses and an increased risk of toxicity. The time course of such inhibition is quite different from that seen with induction as it depends on the rate of elimination of the drug whose metabolism has been inhibited. This potentially rapid effect is of greater clinical significance than induction. Inhibition of drug metabolism may be competitive or non-competitive; an example of the latter type is the destruction of cytochrome P_{450} by quinalbarbitone. Azathioprine is converted in the body to 6-mercaptopurine which is metabolized by xanthine oxidase. Allopurinol inhibits this enzyme and, if azathioprine and allopurinol are prescribed together, the dose of the former must be reduced two to three fold to minimize bone marrow toxicity. Monoamine oxidase inhibitors (MAOI) are weak inhibitors of drug oxidation but are the source of an important drug interaction with noradrenaline. In the presence of MAOI, noradrenaline accumulates in all adrenergic nerve endings including those in the gut wall. When an indirectly acting catecholamine (i.e. one which releases catecholamines such as phenylpropanolamine which is a common constituent of many cold cures, or tyramine which is present in many foods) is taken, noradrenaline is released into the circulation and the blood pressure may rise abruptly. A recent example of interest is the effect of the H_2 receptor antagonist, cimetidine, shown to be a potent inhibitor of metabolism of warfarin (Fig. 1.12), diazepam and other drugs. This effect is probably due to its imidazole structure since other H_2 antagonists with different chemical structures do not show this. Inhibition of metabolism may have a stereospecific basis, e.g. as with phenylbutazone and S-warfarin.

Recently primaquine has been shown to be a potent inhibitor of drug metabolism. This inhibition would seem to be a selective process since 45 mg of primaquine given to humans will markedly inhibit the metabolism of antipyrine when given as a 600 mg dose but not the metabolism of ethinyloestradiol given in the smaller dose of 50 ug.

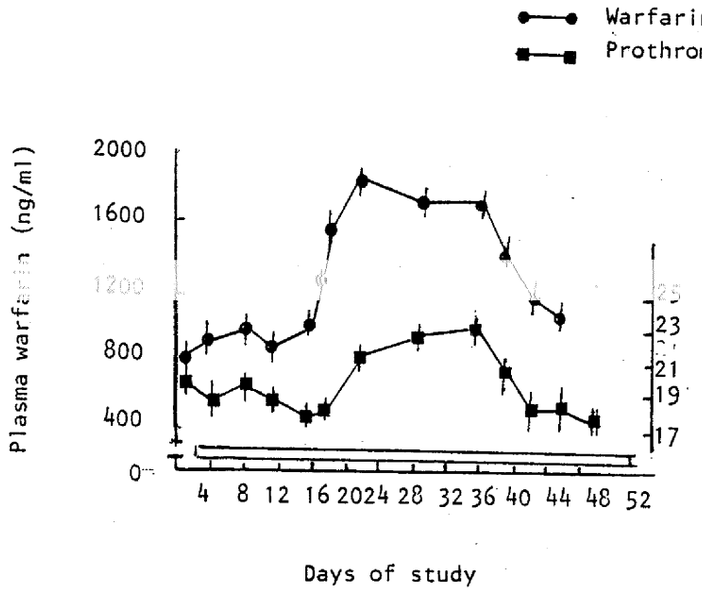


Fig. 1.12 The effect of cimetidine on the plasma warfarin concentrations and prothrombin times in seven volunteers. (After Serlin M.J. et al. (1979) Lancet, ii: 317-319.)

1.5.7 Interactions at the level of the kidney

(a) Changes in urine pH

The renal clearance of weak acids (i.e. those with pKa 4.0-7.5) is increased in alkaline urine, and that of weak bases (those with pKa 7.5-10.0) increased in acid urine due to the impermeability of renal tubules to ionized molecules and their permeability to non-ionized molecules. These facts are used in the treatment of overdosage with phenobarbitone (pKa 7.4) and aspirin (pKa 3.5), i.e. alkalization of urine increases their elimination. In contrast, amphetamine overdosage (pKa 9.9) is treated by urine acidification. The half-life of the antifilarial drug diethylcarbamazine (pKa 7.8) can be increased to up to three-fold in alkaline urine after therapeutic dosing and this has produced therapeutically useful results.

Of course, if drugs are eliminated by hepatic metabolism rather than renal excretion, a change in urine pH in the normal clinical situationn is irrelevant, irrespective of the pKa.

(b) Competition for active renal tubular excretion

The proximal part of the renal tubule has an active transport mechanism which is used by many drugs. There is probably one system for acidic drugs and another for basic compounds. Examples of acidic drugs using this transport mechanism include penicillin, probenecid, thiazide diuretics, methotrexate, and some sulfonamides. Probenecid is sometimes given to block the renal tubular secretion of penicillin to augment its activity. Another interaction currently causing great interest is the augmentation of digoxin toxicity by quinidine. Quinidine (a weak base) blocks the renal tubular secretion of digoxin but this interaction, which is not clearly defined, may have another component at the level of digoxin distribution.

(c) Fluid and electrolyte changes

Changes in electrolyte balance may have profound effects on the therapeutic effect of drugs acting on the myocardium, the central nervous system and the kidney. Hypokalaemia (caused by steroids or carbenoxolone) for example, markedly enhances the toxicity of digoxin, prolongs the paralysis produced by non-depolarizing muscle relaxants, and may antagonize the action of acetylcholine by producing hyperpolarization of the motor end-plate. Fluid retention caused by phenylbutazone antagonizes antihypertensive drug effects. Interestingly, other non-steroidal anti-inflammatory drugs, which act by inhibiting prostaglandin synthesis, may antagonize the antihypertensive effect of the beta blocker, propranolol. Certain prostaglandins have vasodilator properties and this may be the basis of this interaction.

1.5.8 Interactions at receptors

There are many examples of pharmacodynamic interactions where one drug alters the effect of another by virtue of its greater affinity for a receptor site. The antagonism of warfarin by vitamin K_1, of morphine by naloxone, of acetycholine by tubocurarine or atropine and of dopamine by chlorpromazine are all such examples. In some instances the underlying mechanism is slightly more complicated. The muscle relaxation produced by tubocurarine can be reversed by neostigmine which inhibits cholinesterase and thus increases the concentration of acetylcholine at nerve endings.

Interference with intracellular transport mechanisms constitutes a further type of receptor interaction. The adrenergic neurone blocking drugs, guanethidine, bethanidine, and debrisoquine, lower blood pressure by being actively taken up into sympathetic nerve endings where they replace the neurotransmitter noradrenaline.

Several drugs will inhibit their uptake and thus antagonize their antihypertensive effect. The best known antagonists of these adrenergic neurone blockers are the tricyclic antidepressants, although chlorpromazine and amphetamine have a similar attenuating action.

Some interactions are said to occur "by altering receptor sensitivity", e.g. those between clofibrate and warfarin, or anabolic steroids and warfarin. This is not an especially valuable concept. The mechanism is frequently postulated when no obvious kinetic or dynamic explanation is apparent. As pharmacological knowledge advances, the underlying basis of many of these interactions may become obvious and will fit into the schemes suggested above.

1.6 ADVERSE DRUG REACTIONS

1.6.1 Incidence

The reported incidence of adverse reactions varies depending on the method of data collection used. If trained personnel ask each patient specific questions, higher prevalence figures are reported than if reliance is placed solely on patients volunteering the information. In various reports, therefore, the prevalence of adverse reactions ranges between 7% and 30%. It has been estimated that between 1% and 3% of all hospital admissions are due to adverse drug reactions. The number of reactions leading to death is also difficult to quantify since reports come from hospitals with patients who were seriously ill from other causes so that the contribution of the adverse drug reaction to the fatal outcome is frequently impossible to determine.

TABLE 1.14 DRUGS MOST COMMONLY IMPLICATED AS THE CAUSE OF ADVERSE DRUG REACTIONS

Antibiotics	Heparin
Aspirin	Insulin
Digoxin	Prednisone
Diuretics	Warfarin

In spite of the very large number of prescribed drugs, most adverse reactions are attributed to a relatively small group of drugs. In most series some 6-10 drugs are most commonly implicated (Table 1.14).

1.6.2 Epidemiology

Some important determinants of drug reactions are given below.

(a) Age and sex

Adverse effects are more likely to occur in the very young and the elderly, probably because of their relative inability to eliminate drugs. More adverse reactions are recorded in women than men in the ratio 2:1 and a similar ratio applies to the incidence of fatal drug reactions. This may, in part, be attributable to the use of contraceptive steroids but in certain societies there is a greater tendency for women to seek medical attention and thus receive drugs.

(b) Previous allergic history

Adverse reactions to drugs are more likely to occur in patients with a history of previous reaction to other drugs. In some surveys of adverse reactions, up to 25% of patients had previously demonstrated an adverse reaction to drug therapy.

(c) Effect of disease

The disease for which the drug is given may alter the patient's response. A potentially toxic drug whose use may be acceptable in the management of a life threatening situation, should not be used for a relatively trivial indication, e.g. the use of chloramphenicol for the treatment of typhoid fever is indicated but not its use for urinary tract infections.

(d) Pregnancy

Pregnancy alters the response of the mother to certain drugs as well as exposing the fetus to potentially harmful agents. For example large doses of tetracycline have been implicated as a cause of hepatic damage in pregnancy, but not at other times. Tetracycline also damages bone and teeth in the fetus. Further, the normal pattern of drug handling may be distorted in pregnancy when both the rate of gastric emptying (leading to delay in drug absorption) and the rate of drug metabolism are slower. Most drugs given to the mother are readily transferred via the placenta to the fetus. This depends largely on the relative lipid solubility of the drug. Once drugs have reached the fetus, they may accumulate because of the poorly developed ability of the fetus to eliminate them by metabolism. Special problems of toxicity thus arise when drugs are given in earlly stages of pregnancy and may cause developmental defects. For this reason drug administration in pregnancy should be kept to a minimum.

Two other aspects of pregnancy merit special mention. During labour, sedatives and analgesics that are given to the mother may pass to the fetus and may interfere with the onset of spontaneous respiration after birth. After birth, drugs may be excreted in breast milk in sufficient concentrations to cause toxic effects in the neonate. Such drugs include carbimazole, phenobarbitone, diazepam, and some laxatives. Interestingly, the oral anticoagulant, warfarin, is not excreted in breast milk and may be used safely in mothers who are breast feeding. In general the concentration of most drugs being excreted in the breast milk of mothers taking standard therapeutic dose is small and unlikely to have any therapeutic effect on the child. A specific reference manual should be consulted for detailed information.

(e) Drug dose

Iodiosyncratic drug reactions are not related to dose, but many others, which are related to alterations in drug handling by the body, clearly are related to dose.

(f) Timing of reactions

Adverse reactions to drug therapy can occur at any stage during a course of treatment or after its completion. Anaphylactoid reactions characteristically occur with the first administration of a drug to which the patient had been previously exposed. Others may not be observed for months after the drug has been withdrawn, e.g. peritonitis which can occur with use of practolol.

(g) Multiple drug therapy

In general, with an increase in the number of drugs administered, a higher incidence of adverse reactions will occur since the number of drug interactions will be greater.

1.6.3 Types of adverse drug reactions

Adverse reactions to drugs can be divided into two main types. First, there are those which arise from an exaggerated but otherwise normal pharmacological action of the drug concerned. In most studies about 80% of adverse drug reactions reported have been of this type. Examples include bleeding with anticoagulant therapy, postural hypotension with antihypertensive therapy, and drowsinesss following the use of sedatives. These are termed type A adverse effects and are usually predictable and dose-dependent. Although their incidence is high, the resulting mortality is usually low. Since most of these reactions are merely an extension of the pharmacological action of the drug, it follows that factors affecting these reactions are those that modify the therapeutic effect of the drug. These are conventionally divided into pharmacokinetic factors and pharmacodynamic factors. The responsible pharmacokinetic and pharmacodynamic factors involved have been described in section 1.1.2.

In contrast to type A reactions, type B adverse effects represent a totally aberrant, novel and unpredictable action of the drug. Examples of this type of reaction include agranulocytosis due to drugs such as chloramphenicol and phenylbutazone, and malignant hyperthermia from anaesthetic agents. Although these are less common than type A reactions, they carry a higher mortality.

The cause of type B reactions may be due to the drug or related to the patient. For example, out-of-date tetracycline may degenerate particularly in warmer climates, to anhydrotetracycline and epiandrotetracycline and may produce a Fanconi-like syndrome in the patient. Paraldehyde older than six months may contain acetaldehyde which forms acetic acid and is highly toxic when injected. Drug preparations contain many

substances other than the drug itself, e.g. stabilizers, colouring and wetting agents, and other excipients designed to produce an identifiable tablet of suitable size. Sensitivity to tartrazine, an orange-yellow dye used to colour drugs and soft drinks has been reported frequently. The prevalence may be as high as 12 in 10 000. The most usual manifestations are urticaria, acute asthma and non-thrombocytopenic purpura, but anaphylactic shock has also occurred. Cross-reaction to tartrazine occurs with aspirin; probably 10% of patients with aspirin sensitivity are also sensitive to tartrazine.

A novel metabolite of a commonly given drug may produce a type B adverse effect. This was seen in two members of a Swiss family who developed methaemoglobinaemia when phenacetin was given. This was due to the idiosyncratic formation of a minor metabolite, 2-hydroxyphenetedin. Glucose 6-phosphate dehydrogenase (G6PD) deficiency, which is inherited as a sex-linked dominant characteristic, leads to lack of reduced glutathione in red cells, which, on exposure to certain drugs (see Table 1.9), haemolyse. Males and homozygous females are particularly at risk of developing haemolysis because the deficiency of G6PD is likely to be greater. Other genetic traits carrying with them an increased likelihood of developing adverse drug reactions have been discussed in the section on pharmacogenetics(1, 2, 7).

Type B adverse drug reactions are sometimes described as being "allergic" in nature, but this word is often not used in its proper sense. An allergic mechanism depends on an interaction between the drug or drug-protein complex and host antibodies or sensitized lymphocytes. Frequently it is not the parent drug which acts as an allergen but a metabolite which becomes tightly bound to larger macromolecules. The antigen reacts with T lymphocytes to initiate the immune response, and this is followed by activation of B lymphocytes to form antidrug antibodies. The antigen must have the capability of forming a bridge between cell bound antibody molecules thus producing conformation changes in the cell membrane. Reaction with complement and release of various vasoactive peptides ensue. Two manifestations of type B adverse reactions are of special importance. They are:

(a) Anaphylactic reactions

These are mediated by IgE antibodies and occur very quickly after drug administration. The reaction may be in the skin (acute urticaria), in the respiratory tract (asthma), or in the gastrointestinal tract (abdominal pain and vomiting). A generalized anaphylactic reaction may be life threatening. It usually occurs at the start of a treatment to which the patient has previously been exposed. Penicillin is a common cause of this type of reaction which tends to occur more frequently in atopic individuals.

(b) Serum sickness

This is a less acute form of reaction and results from damage by circulating immune complexes. The current theory is that it results when antigen remains in the circulation for a long period. When antibody,. usually IgG or IgM, is first formed, circulating antigen reacts with it forming antigen-antibody complexes. If antibody is in relative excess, the complexes are small and may lodge in blood vessels causing local inflammation and a general systemic response.

Allergic drug reactions may take many forms and may involve mechanisms other than the two detailed above. The formed elements of the blood are frequently involved probably because the antigen (drug)-antibody complex is absorbed onto the cell surface, complement is activated, and haemolysis or cell damage occurs.

RELATED BIBLIOGRAPHY
TO CHAPTER 1

Breckenridge, A. (1975) Advanced medicine symposia: topics in therapeutics 1. London, Pitmman Medical.

Ciba Foundation Symposia (1980) Symposium 76. Environmental chemical enzyme function and human disease. Amsterdam, Excerpta Medica.

Davies, D.M., ed.(1981) Textbook of adverse drug reactions, 2nd ed. Oxford, Oxford University Press.

Davies, D.S. & Prichard, B.N.C., ed. (1973) Biological effects of drugs in relation to their plasma concentrations. London, Macmillan.

Goodman, L.S. & Gilman, A. (1985) Pharmacological basis of therapeutics, 7th ed. London, Macmillan.

Hill, A.B. (1960) Controlled clinical trials. Oxford, Blackwell Scientific Publications.

Prescott, L.F. & Nimmo, W.S. (1980) Drug absorption. Sydney, Adis Press.

Rowland, M. (1978) In: Melmon, K. & Morelli, H., ed. Clinical pharmacology, basic principles in therapeutics, 2nd ed. London, Macmillan, 25.

Sjoqvist, F., Borga, O. & Orme, M. L'E. (1980) Fundamentals of clinical pharmacology. In: Avery, G.S., ed. Drug treatment, 2nd ed. Sydney, Adis Press, p. 1.

Vessell, E.S. ed. (1971) Drug metabolism in man. Annals of the New York Academy of Sciences, 179: 1-772.

Williams, R.T. (1959) Detoxication mechanisms. New York, Wiley.

World Health Organization (1972) Clinical pharmacology: scope, organization, training. WHO technical report series, No. 446.

CHAPTER 2 : PHARMACOKINETICS

CONTENTS

This chapter is based on the presentations of Professors Musa Mohamed, V. Navaratnam and L.A. Salako and Drs. L. Fleckenstein and A. Jamaludin, as well as the discussions in plenary sessions and practical exercises.

2.1 BASIC PRINCIPLES

Administration of a given dose of a drug to different patients may produce a wide variety of responses. Some might exhibit an inadequate or subtherapeutic response, others might show the desired therapeutic effects. In both groups there might be individuals who show signs of toxicity. Such variations in response are, to a significant extent, due to interindividual variability in pharmacokinetics. Such individual variations may be due to exogenous or endogenous factors. Examples of exogenous factors are dietary intake, consumption of other chemicals such as alcohol etc. Examples of endogenous factors include age, weight and genetic traits.

Variations in responses might also be related to the drug (chemical entity) itself such as its physicochemical properties, e.g. degree of ionization, solubility, molecular size or to the individual whose physiological status affects biochemical kinetic processes e.g. enzyme-regulating activity, binding as well as drug receptor activity.

These factors are the main determinants of both the pharmacokinetic and pharmacodynamic properties of a drug.

2.1.1 Concepts

The onset, the duration, as well as the intensity of action of a drug are controlled by the rate at which the drug reaches its site of action and by the concentration of the drug at the receptor.

The physiological behaviour of a drug is controlled by three important processes - absorption, distribution and elimination. Pharmacokinetics are the mathematical consideration of these processes which relate the dose given, the concentration in the blood, and the pharmacological response.

To produce an effect, a systemic drug must first enter the blood stream and be carried to the target organ. For this to occur, with the exception of intravenous administration, the first process is that of absorption, be it from the gastrointestinal tract, from an injection site or from the respiratory system. The route of administration is a key factor in determining the rate and extent of absorption.

The traditional way of describing drug absorption is by the pH-partition hypothesis. According to this hypothesis, the passage of a drug into the blood may be effected by the diffusion of nonionized molecules across the lipid barrier of the cells of the gut lining. Obviously this hypothesis is an over-simplification since the gastrointestinal wall is not a single lipid membrane but a layer of cells.

According to this hypothesis aspirin (pKa 3.5) is absorbed in the stomach at about pHZ when the drug is nonionized. The drug diffuses passively across a single membrane into the blood vessels at pH 7.4. There it becomes ionized and unable to return to the stomach because there is a net concentration gradient of 25, 000 to 1, stomach to plasma of the nonionized species. In reality aspirin is not completely absorbed due to poor blood supply and small surface area. Moreover, contrary to the hypothesis the absorption of ionised species also occurs.

In general, absorption depends mainly on the large difference in drug concentration between the gastrointestinal tract and the blood. Factors such as physicochemical characteristics of the drug, extent of blood flow to the different parts off the gastrointestinal tract, pH, and available surface area for absorption are also important.

Other factors also affecting absorption are related to the release of a drug from a pharmaceutical preparation. For example, when a drug is administered orally, as tablets or capsules, these must disintegrate, deaggregate and dissolve in the gastrointestinal fluid before the absorption process can begin. A poorly forrmulated pharmaceutical preparation may disintegrate or deaggregate too slowly, causing a drug to be lost in the stools.

Bioavailability (F) is defined as the rate and extent of absorption following nonvascular administration. It is calculated by comparing the area under a serum/plasma drug concentration-time curve (AUC) following nonvascular administration with the AUC following intravenous administration after correcting for any differences between doses.

$$F = \frac{\text{AUC (extravascular)}}{\text{AUC (intravenous)}}$$

Differences in the bioavailabilities of various pharmaceutical formulations of a given drug, i.e. lack of bioequivalence, may have clinical significance and explain differences in therapeutic or toxic effects.

Drugs and metabolites which are eliminated from the liver into bile subsequently pass into the gastrointestinal tract and may then be reabsorbed either directly or indirectly following further metabolism. These substances are usually of high molecular weight and contain hydrophilic residue in the molecule. This recycling of a drug is known as enterohepatic recycling.

After absorption of a drug into the systemic circulation, it is then distributed throughout the body. Drug distribution depends on several factors including the blood flow to the tissues, the physicochemical properties of the drug and the degree of protein binding, both to tissues and plasma. In general, the rate of distribution is controlled by factors such as diffusion rate and tissue perfusion rate as well as extent of binding.

Diffusion-rate limitation occurs when the rate of distribution is controlled by drug diffusion through a lipid barrier and is a function of the partition coefficient, the molecular weight and the degree of ionization.

The binding of drugs to plasma and tissue proteins is a key factor. Plasma protein binding, however, is a reversible process and the drug molecules constantly shift between the drug-protein complex and the free drug form whilst remaining in overall equilibrium.

The apparent volume of distribution (V_d) is an important concept. It denotes the hypothetical volume of fluid into which a drug distributes. It can be thought of as the volume required for a specific amount of drug in the body to produce a specific plasma or serum concentration. V_d is a hypothetical volume with no physiological basis. It is calculated as the total amount of drug in the body divided by the plasma drug concentration. This will be discussed in more detail in section 2.1.2.1 of this chapter.

Most drugs are eliminated from the body by metabolism in the liver and/or by excretion of the drug and its metabolites by the kidneys. Elimination may also occur through other organs such as the lung and the skin.

The elimination of drugs from the body can be described quantitatively using the concept of clearance. Clearance is not the amount of drug eliminated over time, but it is the volume of body fluid from which the drug is removed over a period of time. Hence the total body clearance is the sum total of clearances from the various drug metabolizing and eliminating organs. It is expressed as follows:

$$Cl_{total} = Cl_{hepatic} + Cl_{renal} + Cl_{pulmonary} + Cl_{others}$$

In the liver, certain drugs are biotransformed. Biotransformation involves the chemical alteration of a drug and can produce inactive metabolites which may be readily excreted in the urine or it may yield pharmacologically active metabolites with pharmacodynamic properties.

To summarize, pharmacokinetics involve the study of the kinetics of absorption, distribution and elimination (metabolism and excretion) of a drug in the body. Pharmacological response and toxic effects are usually determined by the concentration of the drug or its active metabolite at the receptor sites. Measuring the plasma levels of the drug or active metabolite is one procedure that can be utilized to monitor the course of therapy.

2.1.2. Pharmacokinetic models

In order to obtain information on the pharmacokinetics of a compound, it has to be administered and its concentration measured with time in the plasma.

Figure 2.1 shows a typical plasma concentration-time profile for a compound that has been administered orally.

The absorption of a drug is usually more rapid than its elimination. Following administration, the drug is absorbed, distributed and eliminated from the body.

C_{max} (maximum concentration) represents the peak plasma concentration which appears at time t_{max} (time of peak plasma concentration). t_{max} gives a rough indication of the rate of absorption of the drug. A drug which has C_{max} at 10 minutes has a faster absorption rate than one with a C_{max} at 20 minutes. C_{max} is related to the dose and the rate of absorption and elimination of the drug. AUC is related to the amount of drug absorbed into the systemic circulation.

Pharmacokinetic models are used to provide a more accurate interpretation of the relationships between plasma levels and therapeutic or toxic response. Pharmacokinetic information also provides a means to plan dosage regimens and doses that increase therapeutic responses without producing toxic side effects. A model or hypothesis is formed using mathematical terms to express concisely quantitative relationships. Various mathematical models can be devised to simulate the rate processes of drug absorption, distribution and elimination. These mathematical models permit the development of mathematical equations to describe drug concentrations in the body with time.

Since drug concentrations are dependent on time, drug concentration is the dependent variable while time is the independent variable. The plasma concentrations are then plotted with time to produce a concentration-time profile (Fig. 2.1).

The number of parameters needed to describe a model depends on the route of administration and the complexity of the pharmacokinetic model. Each concentration value at a particular time is a data point and to estimate accurately the parameters, the number of data points collected must exceed the number of parameters.

The pharmacokinetic models permit a calculation of pharmacokinetic parameters that will be useful in the selection, monitoring and evaluation of drug therapy, in interpreting and evaluating drug literature as well as in developing and evaluating drug dosage requirements for therapeutic purposes. The most common routes of drug administration are the intravenous, intramuscular, subcutaneous and oral routes. In some cases, suppositories may also be used where the large number of blood vessels in the rectal area permit an efficient absorption of drug. For a study of the pharmacokinetic principles, only the relatively rapid intravenous and oral route of administration will be considered here.

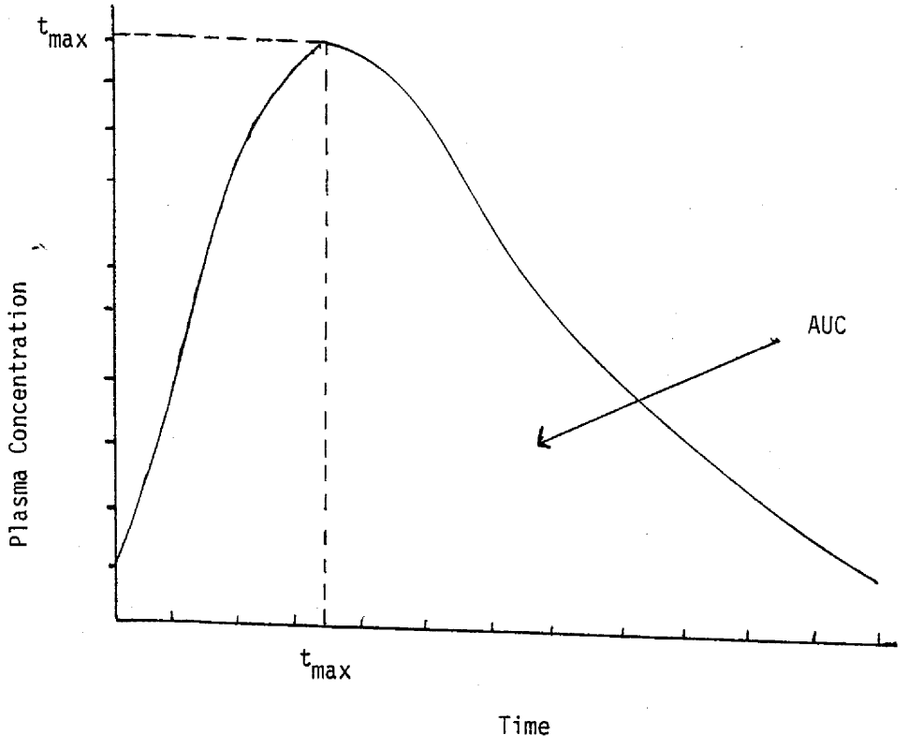


Fig. 2.1 A typical plasma concentration-time curve after oral adminstration.

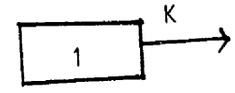
C_{max} = maximum concentration

t_{max} = time when C_{max} occurs

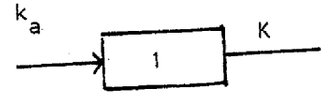
AUC = area under the curve

a = absorption phase

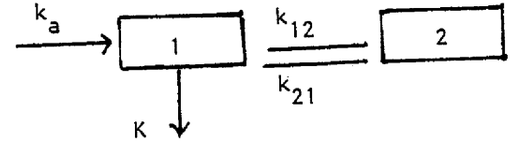
b = elimination phase



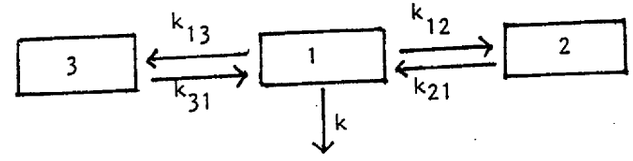
MODEL 1. One-compartment open model, IV injection.



MODEL 2. One-compartment open model with first-order absorption



MODEL 3. Two-compartment open model with first-order absorption.



MODEL 4. Three compartment open model, IV injection.

Figure 2.2 Different types of compartment models

Compartment 1 = Central compartment

Compartment 2 = Peripheral compartment

Compartment 3 = Deep tissue compartment

2.1.2.1 One-compartment model

The pharmacokinetic models for a drug following rapid intravenous or oral administration are shown below.

Where the drug is given rapidly by the intravenous route, the entire dose enters the body instantaneously and the rate of absorption (k_a) can be neglected in all calculations.

For the oral administration, the rate of absorption needs to be measured as it determines the appearance of the drug in the body.

The one-compartment model for a drug following intravenous administration is shown as follows:

$$iv \xrightarrow{\qquad} D_b,\ V_d \xrightarrow{K} De$$

The one-compartment model for the drug following oral administration is shown below:.

$$D_{GI} \xrightarrow{ka} D_b,\ V_d \xrightarrow{K} D_e$$

where iv= intravenous route,
D_b = amount of drug in the body,
V_d = apparent volume of distribution or the apparent volume in the body in which the drug is dissolved,
K = rate of elimination of drug from this compartment,
k_a = rate of absorption into the body
D_e = amount excreted and
D_{GI} = amount in the gasstrointestinal tract.

In most cases, following absorption, the drug then distributes via the circulatory system to all the tissues and equilibrates in the body fairly rapidly. The apparent volume in which the drug dissolves is known as the apparent volume of distribution (V_d).

V_d relates the concentration of drug in the plasma (C_p) and the amount of drug in the body (D_b) as follows:

$$D_b = V_d C_p$$

Most drugs have a V_d smaller than or equal to the body mass but the V_d may be several times larger than the body mass for some drugs.

(a) Intravenous administration

In a one-compartment open model with intravenous administration the drug is administered instantaneously and there are no absorption processes involved. The model is called an open model as excretion occurs.

(i) Apparent volume of distribution (Vd)

The drug is assumed to distribute into an apparent volume known as V_d.

$$V_d = \frac{\text{dose administered}}{C_p^{\,o}} = \frac{D_b^{\,o}}{C_p^{\,o}}$$

where $D_b^{\,o}$ = amount of drug in the body following administration of intravenous dose.

$C_p^{\,o}$ = initial plasma concentration at t = 0.

(ii) Plasma concentration with time

Figure 2.3 shows the plot of plasma concentration against time.

The change of drug concentration with time :

$$\frac{dC_p}{dt} = -KC_p$$

if the rate of elimination follows first-order process.

Integration of the above equation results in

$$\log C_p = \frac{-Kt}{2.303} + \log Cp^{o}$$

Further transformation of the above equation results in

$$C_p = C_p^{\,o} e^{-Kt}$$

Hence a plot of plasma concentration on the log scale against time on the linear scale will produce a straight line and extrapolation of the regression line to the y axis will produce the value for $C_p^{\,o}$.

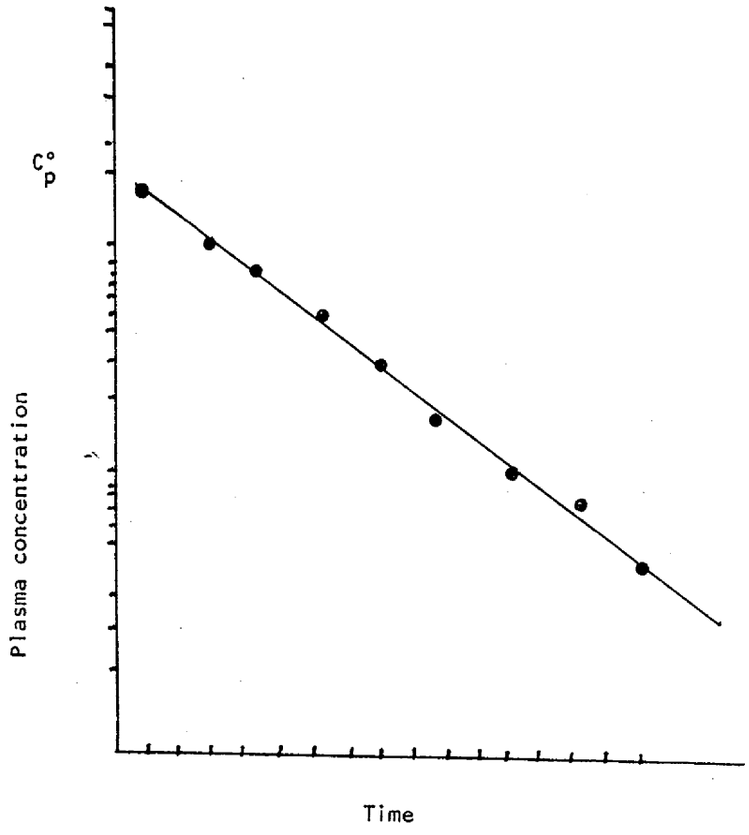


Fig. 2.3 Semilog graph of the plasma concentration with time following intravenous injection with a one compartment model

(iii) Elimination half-life ($t_{1/2}$)

The elimination of the drug can also be represented by $t_{1/2}$, the half-life of elimination of the drug.

An elimination $t_{1/2}$ that obeys first-order kinetics will be independent of the concentration and can be calculated as shown below:

$t_{1/2}$ = time for the concentration to decrease by half

$$t_{1/2} = \frac{\ln 2}{K} = \frac{0.693}{K}$$

(iv) Elimination rate constant (K)

For most drugs, the rate of elimination (K) from the body is a first order process. During sampling of the central compartment or systemic compartment, the parent or active drug is measured. However K is affected by both metabolism and excretion of the drug. Therefore,

$$K = k_m + k_e$$

where k_m = rate of metabolism of the parent drug and
k_e = rate of excretion of the parent drug.

K normally has units of hr^{-1}. Both k_m and k_e are assumed to be first-order rate constants.

(v) Area under the concentration-time curve(AUC)

The integral $\int_0 C_p dt$ represents the summation of the area under the curve from $t = 0$ to $t = \infty$ i.e. $AUC_{0\to\infty}$ For simplicity $AUC_{0\to\infty}$ is denoted by AUC

$$\text{dose} = KV_d\ AUC$$

To calculate V_d, the following equation is used:

$$V_d = \frac{\text{dose}}{K\ AUC}$$

(vi) Relationship between V_d and plasma concentration at time 0, (C_p^o)

For intravenous administration, the V_d of the drug depends on the C_p^o. A small C_p^o will result in a large V_d. This normally occurs when the drug tends to distribute spontaneously more into peripheral tissues and organs, with less being available in the systemic compartment.

Drugs that are highly bound to plasma proteins will tend to stay in the systemic compartment and will have a smaller V_d and a larger C_p^o.

V_d is normally expressed in units of L/kg and is a constant for each drug. Therefore if the V_d of a drug is known, it is possible to determine the total amount of that drug in the body from the plasma concentration at any time following the distribution phase.

In computer programmes that fit the best curve to a concentration-time profile, it is necessary to provide known values of V_d.

(vii) Clearance (Cl)

Another important parameter to describe elimination is the total body clearance of a drug. Here an assumption is made that the drug is completely metabolized by one organ e.g. the liver, and that Cl is equal to the blood flow through that organ.

$$Cl = KV_d$$

(viii) Relationship between clearance, plasma half life and apparent volume of distribution

$$Cl = KV_d$$

$$\text{Since } K = \frac{0.693}{t\ 1/2}$$

$$Cl = \frac{0.693\ V_d}{t\ 1/2}$$

$$t_{1/2} = \frac{0.693\ V_d}{Cl}$$

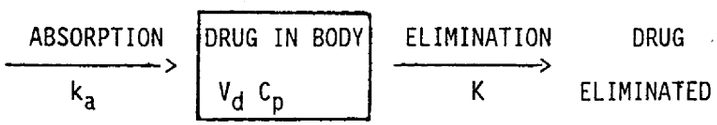
$$\text{Hence, } Cl = KV_d$$

(b) Oral administration

Oral administration as well as intramuscular or subcutaneous injection, topical, buccal, rectal and inhalational administrations can also be described by the one-compartment first-order absorption model.

(i) First-order rate of absorption (k_a)

Most pharmacokinetic models assume that the rate of absorption of a drug into the body may be described by a first-order process.



(ii) Rate of change of drug with time

As shown above, the rate of appearance of the drug depends on how fast it is absorbed and also on how quickly the absorbed drug is eliminated.

Therefore,

$$\frac{dD_b}{dt} = \frac{dD_{GI}}{dt} - \frac{dD_e}{dt}$$

and $$\frac{dD_{GI}}{dt} > \frac{dD_e}{dt}$$

During the absorption phase of the plasma concentration- time curve and t_{max} (time of appearance of C_{max}) the following applies:

$$\frac{dD_{GI}}{dt} = \frac{dD_e}{dt}$$

(iii) Elimination rate constant (K), first-order rate of absorption (k_a) and fraction of drug absorbed into the systemic circulation (F)

At the time when the drug is depleted at the absorption site, the elimination phase will dominate. The elimination phase is then described as a first-order process.

If the drug is absorbed with a first-order rate and also eliminated with a first order process, the first-order absorption model can be used to describe its behaviour in the body.

Orally administered doses are generally not completely available to the body and F, or the fraction of the drug absorbed is included in the following equation to describe the rate of drug change in the body:-

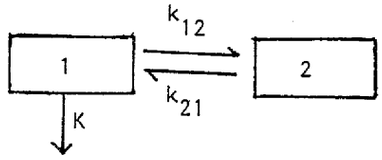
$$\frac{dD_b}{dt} = F\ k_a D_{GI} - KD_b$$

where k_a = rate of absorption, D_{GI} = amount of drug in the gastrointestinal tract.

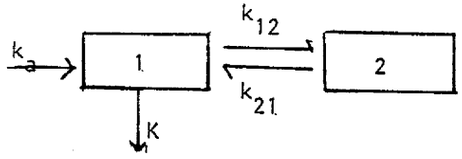
(iv) Drug amount or concentration with time in the plasma

As the depletion of the drug in the gastrointestinal tract also follows a first-order process D_{GI} may be expressed as

$$D_o e^{-kat} \text{ and}$$



MODEL 1. Two-compartment open model with rapid intravenous administration.



MODEL 2. Two-compartment open model with first-order absorption.

Fig. 2.5 Two-compartment open models following intravenous and oral administration

Drugs that are highly lipid soluble may tend to accumulate in the fat tissues and some drugs can bind to tissue proteins and other macromolecules of the tissue. These fat tissues and the tissue proteins and macromolecules form part of the deep tissue compartment. Drugs showing this behaviour are best described by a three-compartment model behaviour. (Figs. 2.6 and 2.7). For each new compartment added to the model, further first-order rate constants are added to the equation that describes the relationship between concentration and time.

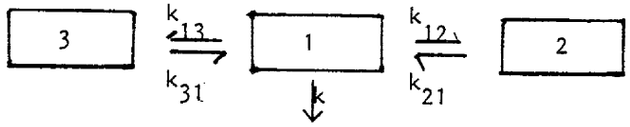


Fig. 2.6 Three-compartment open model following rapid intravenous administration

Compartment 1 = Central compartment
Compartment 2 = Peripheral compartment
Compartment 3 = Deep tissue compartment

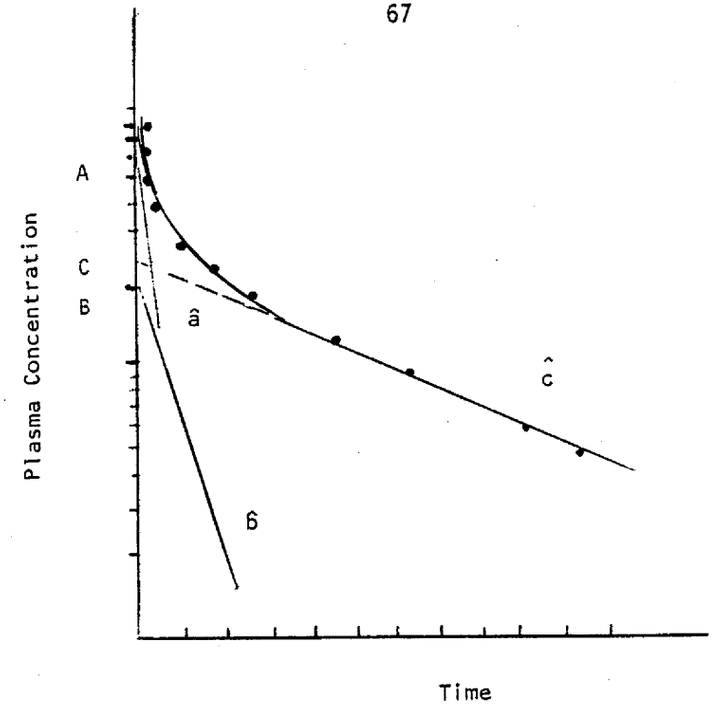


Fig. 2.7 Plasma concentration-time curve for a three-compartment open model following rapid intravenous administration

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\delta t}$$

The rate constants α , β and intercepts A and B were calculated by the method of residuals.

2.1.2.3 Two-compartment open model

(a) Intravenous administration

The concentration-time profile of a drug administered intravenously that is best described by a two compartment model will show a distribution phase when the drug equilibrates in the central compartment and an elimination phase when the drug eliminates from the central compartment (Figs. 2.4 and 2.5). The models used in pharmacokinetic studies normally assume that the drug is eliminated from the central compartment.

Primaquine diphosphate is an example of a drug that shows two-compartment model behaviour following intravenous administration to the monkey.

The drug normally distributes fairly rapidly and the distribution phase is usually represented by a steeper slope as compared to the elimination phase.

(i) The rate of drug change in the tissues with time

The rate of drug change in the tissues is represented by

$$\frac{dC_t}{dt} = k_{12}C_p - k_{21}C_t$$

where k_{12} and k_{21} are first-order rate constants and C_t and C_p are the drug concentrations in the tissues and plasma, respectively. They are also known as transfer constants or microconstants as they relate to the amount of drug transferred between the two compartments. These constants cannot be determined directly as it is not feasible to sample the concentration in the tissue compartments.

(ii) The rate of drug change in plasma with time

The rate of drug change in plasma is represented by

$$\frac{dC_p}{dt} = k_{21}C_t - k_{12}C_p - KC_p$$

Simplification and transformation of the above equation yields

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where $\alpha + \beta = k_{12} + k_{21} + K$ and

$xp = k_{21}K$

The constants α and β are rate constants for the distribution and elimination phase, respectively. The constants, A and B are intercepts on the y axis for each exponential section of the concentration-time curve. A and B are normally determined by the method of residuals or by the use of a computer programme.

(iii) Apparent volumes of distribution, V_d

For a multicompartment model, the following V_d parameters can be estimated: the apparent volume of the central compartment, the apparent volume at steady state of the drug, the extrapolated apparent volume of distribution and the apparent volume of distribution by area.

(iv) Apparent volume of the central compartment. (V_p).

During a pharmacokinetic study, blood samples are normally taken. This means sampling from the central compartment. The apparent volume of distribution of the central compartment is related to dose, the rate of elimination (K) and the area under the curve (AUC) as follows :- K and AUC as follows:-

$$V_p = \frac{D_o}{K\ AUC} = \text{clearance}$$

$$\text{as } \frac{D_o}{AUC} = \text{clearance}$$

(vi) Apparent volume of distribution at steady state (Vd_{ss})

At steady state the rate of entry of the drug into the tissue compartment from the central compartment is equal to the rate of loss of the drug from the tissue compartment to the central compartment. The apparent volume of distribution determined at this state is called the apparent volume of distribution at steady state i.e. Vd_{ss}.

$$Vd_{ss} = V_p + \frac{k_{12}}{k_{21}} V_p$$

(vii) Extrapolated apparent volume of distribution, Vd_{exp}

$$Vd_{exp} = \frac{D_0}{B}$$

where B is the y intercept obtained by extrapolation of the plasma concentration-time curve.

(viii) Apparent Volume of Distribution by Area (Vd_{area})

$$Vd_{area} = \frac{D_o}{AUC}$$

The total body clearance of the drug is

$$= \frac{D_0}{AUC}$$

and substituting this into the above equation yields

$$Vd_{area} = \frac{\text{clearance}}{\beta}$$

$$= \frac{KV_p}{\beta}$$

Vd area therefore alters with changes in rate of elimination and total body clearance.

For a drug that obeys two-compartment model kinetics, it is preferable to calculate Vd_{ss} as this parameter is not affected by changes in drug elimination. It, therefore, reflects true distributional volume changes and is not affected by changes due to renal function.

Special precautions must be taken in fitting data to a two-compartment model as compared to a one-compartment model since the microconstants, k_{12} and k_{21}, may vary greatly as a result of minor differences in fitting and experimental variation.

(ix) Drug concentration in the tissue

The drug concentration in the tissue may be important in relation to the pharmacological activity. It can be calculated by using the following equation:

$$D_t = \frac{k_{12}D_o}{\alpha - \beta} (e^{-\beta t} - e^{-\alpha t})$$

(b) Oral administration

For a drug following two-compartment model kinetics after oral administration, the equation describing its behaviour has three exponentials as shown below:-

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} - (A + B)\, e^{-kat}$$

A and B are intercepts on the y axis following extrapolation of the lines of the phase and phase, respectively, while â and b̂ are the slopes for the distribution and elimination phases, respectively.

2.1.2.4. Multiple dose administration

Most drugs are given repeatedly for therapeutic purposes. Dose repetition will usually cause an accumulation of the drug in the body.

However, careful control of the intervals between dosing can produce the therapeutic concentration required without the occurrence of toxicity.

(a) Intravenous administration

(i) Dosing intervals

The intervals between dosing are important in multiple dose therapy. If the dosing interval is too long and the drug from the earlier dose has been completely eliminated before the replenishment by a new dose, the second dose acts in no way differently from a single dose and there is no time for accumulation of the drug.

Figures 2.8 and 2.9 show two dosing intervals, one where the dosing interval is too wide and another which is good enough to allow a steady accumulation of drug until steady-state is reached.

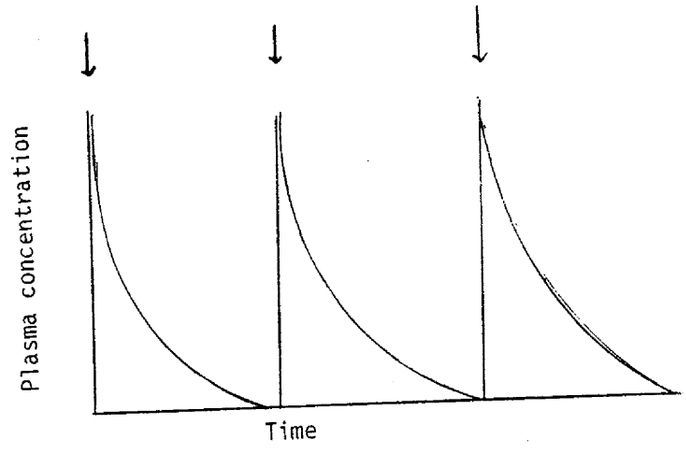


Fig. 2.8 Linear plot of plasma concentration with time after multiple intravenous bolus doses. No accumulation is shown as dosing interval is too wide.

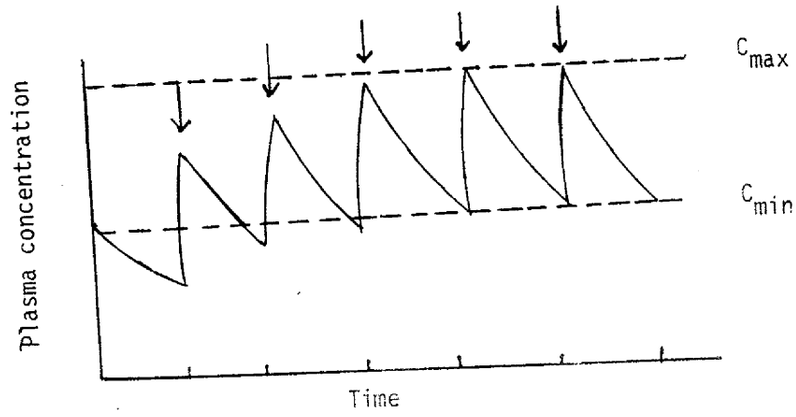


Fig. 2.9 Linear plot of plasma concentration with time after frequent multiple intravenous bolus doses.

Plasma levels accumulate until steady state where C_{min} is at the end of each dosing interval and C_{max} is at the start of each dosing interval + t_{max}.

The arrows indicate times of dosing.

(ii) Plasma concentration at the end of a dosing interval and at the time immediately after dosing

The concentration at the end of the first dosing interval is:

$$C_p = \frac{\text{Dose}}{V_d} e^{-KT}$$

where T = dosing interval.

If the dosing interval is equal to $t_{1/2}$ then

$$e^{KT} = 0.5.$$

When the dosing interval is not equal to $t_{1/2}$

then $C_{T,1} = \frac{\text{Dose}}{V_d} R$ at the end of

the first dosing interval.

Shortly after the second dose the concentration becomes

$$C_{o,2} = \frac{\text{Dose}}{V_d} R + \frac{\text{Dose}}{V_d}$$

and at the end of the second dosing interval

$$C_{T,2} = \frac{\text{Dose}}{V_d} R^2 \frac{\text{Dose}}{1 - V^d} R$$

$$\text{Thus } C_{o,n} = \frac{\text{Dose } [1 - R^n]}{V_d [1 - R]}$$

$$C_{T,n} = \frac{\text{Dose } [1 - R^n]}{V_d [1 - R]} R$$

(b) Oral administration

For multiple oral administration, the presence of the first order absorption process makes the equation to describe concentration with time rather complex. However the most commonly used equation in multiple oral dosing is:

$$C_{av} = \frac{F \text{ Dose}}{V_d K} = \frac{F \text{ Dose}}{Cl}$$

C_{av} is the average plasma concentration achieved at steady state. The above equation is valid if the drug obeys linear kinetics and K does not change with time.

It is also important to note that the same C_{av} is achieved if the dose and dosing interval are altered in the same proportion. However, the fluctuations between peak and trough concentrations will be larger.

2.2 COMPUTER MODELLING AND SIMULATION OF PHARMACOKINETICS*

2.2.1. Objectives of modelling

Pharmacokinetic models are used to describe real data and are composed of a structural model and a variance model. The structural model describes the physical structure which produces the data. The variance model describes the errors associated with the data observatioons which the structural model describes.

The major objectives of modelling are :

(a) Parameter estimation. Estimation of parameters of a pharmacokinetic model given a set of dependent and independent variables.

(b) Model comparison. Modelling can be used to find the best pharmacokinetic model. Generally, the more complex models will give a better fit of any given data set. However, this must be balanced against a simpler model that still describes the data adequately. Various criteria, discussed later, can help choose the best model. Comparison of different pharmacokinetic models can be used to test hypotheses which are expressed as different models.

(c) Simulation. Given a set of parameters and independent variables simulation makes it possible to produce plots of the structural and variance model predictions.

2.2.2 Regressions

Most scientists are familiar with "linear" models. The simplest example is the equation of a straight line:

$$Y = A + B.X$$

where Y is the dependent variable, X is the independent variable, A is the intercept and B is the slope.

---

* Practical exercises on pharmacokinetics are provided in ANNEX 4

In nonlinear models, at least one of the parameters appears other than as a coefficient, e.g.

$$Y = Ae^{-B.X}$$

This model can be linearized by taking the logarithm of both sides, but it is a nonlinear model as written.

Models which cannot be linearized by transformation are nonlinear models or nonlinear regression models. An example of such a model is the sum of two or more exponentials.

$$Y = A_1e^{-B_1.X} + A_2e^{-B_2.X}$$

The topic of general nonlinear modelling has been described by Draper & Smith (1981) and Beck & Arnold (1977). Nonlinear modelling for kinetic analysis and pharmacokinetics is described by Endrenyi (1981).

2.2.3. Fitting the model to the data

Given a data set from a pharmacokinetic experiment, a structural model of the data and certain assumptions about the error structure of the data, one can "fit" the model to the data using maximum likelihood or least squares procedures to obtain estimates of the model parameters (Jennrich & Moore, 1975).

In least squares fitting the best estimate is that with the lowest sum of the squares of the deviations between the observed values and the values predicted by the model. The methods for computing the least squares estimates in nonlinear models are iterative procedures. Thus, initial estimates of the parameters are made and then in some way these initial estimates are modified to give improved estimates. That is, estimates which result in a smaller sum of squared deviations. The iterations continue until the minimum or least sum of squares is reached. Since it is impossible to know exactly what the minimum is, some criteria must be given at which point it is assumed that the method has converged to the minimum sum of squares.

Pharmacokinetic systems are linear differential equations. While it is possible to fit the differential equations, the equations are usually solved and it is the integrated form of the equation which is parameterized and used for fitting data.

A number of computer programmes are available for fitting pharmacokinetic data. Two such microcomputer software packages are MKMODEL (Holford, 1988) and PCNONLIN (Metzler & Weiner, 1986).

2.2.4. Algorithms used in nonlinear estimations

The two most common algorithms used as minimization routines are the simplex procedure and least squares approximation using various modifications of the Gauss-Newton algorithm.

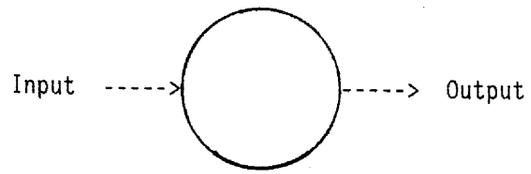
(a) Simplex procedure. The simplex algorithm is a very powerful and robust minimization routine. It is computationally intensive, and thus more suited to the more recent crop of powerful microcomputers. It is useful when good approximations of the initial parameter estimates are not available. Since the procedure may follow a tortuous pathway in approximating the minima from the initial estimates, least squares minimization should be used to refine simplex parameter estimates.

(b) Least squares minimization. The technique used for the minimization of the sum of the squared residuals is one used in numerous applications for the past two decades. It is a well-studied algorithm that uses a linear approximation to the model.

2.2.5. Parameter constraints

It is sometimes desirable to set limits on the admissible parameter space. The model may contain a parameter which must be non-negative. If the data set contains too much error the actual least squares estimate may be negative, yielding a physiologically unrealistic result. At other times setting reasonable constraints on the parameter estimates may prevent the algorithm from wandering off and getting lost. Many pharmacokinetic software programmes are flexible allowing for no constraints, absolute constraints or possibly a function of the mean and standard deviation of the parameter.

Structural models



The time course of drug concentration versus time is described by an input function and a disposition (output) function. The input function describes the rate and duration of drug input. The disposition function defines the distribution and elimination of the drug. The most commonly used input models are: bolus, zero-order and first-order; the most useful disposition models are one and two compartment open models.

2.2.5.1. Input model parameters

(a) Bolus input. Instantaneous input into the body following intravenous bolus drug administration.

(b) Zero-order input. Zero order input is drug administration at a constant rate. The input rate equals dose divided by the duration of the input.

$$\text{Input rate } (R_0) = \frac{\text{Dose}}{\text{Infusion time}}$$

If a drug is given by infusion, the infusion time will be constant. When drug absorption is a constant rate process, the infusion time is unknown and must be estimated as a model parameter.

(c) First order input. First-order drug input assumes that the rate of drug absorption is proportional to the amount of drug to be absorbed.

$$\text{Input rate} = K_a.A(t)$$

where A(t) is the amount of the dose remaining at the absorptive site at time t and K_a is the absorption rate constant. For most drugs the Ka is about .35 to 1.4 hours $^{-1}$. This corresponds to an absorption half life $\frac{(0.693)}{K_a}$ of 0.5 to 2 hours. However, for many antimalarials the absorption half life is substantially longer (e.g. for mefloquine the absorption half-life may be 3.5 to 12 hours).*

(d) Absorption lag time. For oral drugs there can be a substantial delay between the time of drug administration and the appearance in the blood stream. This can be caused by various factors, such as tablet disintegration and dissolution and stomach emptying. If the estimate of the lag time is 0.25 hours or less, one should compare the fit with a model setting lag time to zero.

---

*In pharmacokinetic analysis it may also be useful to consider "mean resident time", a concept based on moment theory. It can be defined as the time it takes for half of the drug molecules to be eliminated from the body. It is more appropriate in some situations for judging the duration of effective drug concentrations in the blood than the half-life which is a hybrid parameter dependent on volume of distribution and rate of elimination.

2.2.5.2 Disposition model parameters

(a) Volume of distribution. The distribution of a drug is described by the apparent volume of distribution. The volume of distribution is basically a scale factor between the amount of drug in the body and concentration of drug in the measured biological fluid. In the one compartment model, the body is conceived as a kinetically homogeneous unit, with a single definable space, V. For the two compartment model, there are two definable spaces, the central compartment and the peripheral compartment, Most kinetic packages parameterized the volume of the central compartment, V, a scale factor between amount of drug in the body and concentration at T = 0. The steady state volume of distribution, V_{ss} is generally more useful in comparing one drug with another and this volume term can be readily calculated.

V can be estimated by dividing the dose by the peak drug concentration. This initial estimate will be an overestimate, especially the later the peak occurs.

(b) Clearance. Clearance is a drug elimination term calculated by dividing dose by AUC. Clearance represents all processes of drug elimination, including metabolism and excretion of unchanged drug.

(c) Micro rate constants (k12, k21, k10). The micro rate constants are the first-order transfer constants between compartments in the two-or multicompartment models. Since it is difficult to determine the micro-rate constants and because they do not tend to be very stable, it is best to parameterize hybrid rate constants for initial data analysis.

(d) Hybrid rate constants ($\lambda 1, \lambda 2$). The hybrid rate constants known as the distribution and elimination rate constants are used in the two compartment open model. The distribution or rapid disposition constant, λ_1 , is also known as alpha or L1. The elimination or slow disposition constant, λ_2 is also known as beta or lambda z (LZ). Initial estimates of these parameters may obtained using curve stripping programmes, or graphically using the method of feathering.

2.2.6 Parameterization of pharmacokinetic models

Pharmacokinetic models may be parameterized in several ways. One approach is to use the "sum of exponentials" which estimates the coefficients and exponents of one or more exponential terms. The parameters of this model are then transformed into more meaningful physiological terms such as clearance and volume of distribution.

Another approach parameterizes clearance and steady state volume of distribution. This is basically a noncompartmental approach from which exponential rate constants can be calculated.

2.2.7 Variance model

A common variance model is :

$$\text{var}\ (Y) = SD^2 x (VO + Y^{PWR})$$

where

ar (Y) is the predicted variance
Y is the concentration predicted by the model
SD is the standard deviation scale parameter
VO is the expected variance when Y is 0 and PWR is non-zero
PWR is a power parameter.

PWR = 0. Constant variance. The variance is estimated by the square of the variance scale parameter, SD. This corresponds to unweighted least square regression.

PWR = 1. Poisson distribution of errors. This corresponds to weighted least square regression where the weight is inversely proportional to the predicted value.

PWR = 2. Constant coefficient of variation. This corresponds to weighted least squares regression where the weight is inversely proportional to the square of the predicted value.

PWR <> 0. This model allows the variance to depend upon a non-integer value of the PWR parameter. This variance model is available in MKMODEL, an extended least squares regression programme (Peck et al, 1984). The principal difference from the extended least squares estimation is the ability to estimate the parameters of an error model and thus use the information in each data point with an appropriate "weight." It is recommended for data analyses where the errors arise from a variety of sources during sampling and measurement.

2.2.8 Selecting the best model

Various criteria, e.g. the Akaike information criterion (Akaike, 1976), log likelihood (Sheiner, 1985) and Schwartz criterion (Schartz, 1978), can help choose the best model.

(a) The Akaike Information Criterion (AIC).

$$AIC = n.\ln \left(\sum_{t-1}^{n} w_i \ (Yobs_1 - Ycal_c^2) \right) + 2p$$

The AIC presents the "information content" of a given set of parameter estimates by relating the coefficient of determination to the number of parameters (or degrees of freedom) that were required to obtain the fit. Comparing two models with different members of parameters, this criterion places the most weight on the model with the greater number of parameters. This model will have a better coefficient of determination. The AIC permits also a quantitation judgement of the coefficient of determination and its adequacy, and thus indicates ways towards the improvement of models so that they become more appropriate. The most appropriate model will have the largest AIC because the "information content" of the model should be maximized.

(b) Objective function and log likelihood.

The objective function and log likelihood are used in MKMODEL to evaluate the goodness of fit.

The objective function used for parameter estimation is obtained from each observation and parameter by calculating:

$$O_i = \frac{(Yo_i - yp^2 i)}{V_i} + \ln (V_i)$$

where i is the "i"th observation or parameter, Yo is the observed value of the dependent variable or the current parameter estimate, Yp is the predicted value of the dependent variable or the expected parameter value, and V is the predicted variance of the observation or the expected variance of the parameter. The "objective" is to search for the set of parameter values that minimizes this function.

The log likelihood is computed from:

$$\log_{Likelihood} = \sum_{t-1}^{N} O_i - \frac{N}{2} \cdot Ln\ (2.p_1)$$

where N is the number of observations. The log likelihood indicates how well the parameter values in the model describe the data. The larger the number, the better the model in describing the data.

(c) The Schwartz criterion.

The Schwartz criterion can be used to test if one model is better than another in describing a particular set of data. It takes into account not only the log likelihood but also the number of parameters being estimated and the number of observations.

2.2.9 Constraints

It often occurs that computer advice is sought for the analysis of data, the collection of which was deficient. Computer modelling should not be used as a way out for badly collected data. This emphasizes the need for seeking computer advice when the study is being designed so as to ensure appropriate sampling both in timing and with regard to the number of data points. Sampling should continue for three times the putative half-life. Five samples should be taken at each phase of the concentration time curve, i.e. absorption, distribution, elimination.

There is no way as yet to produce computer simulations of the concentration-time profile of a drug from a very limited number of concentration-time points, but it is conceivable that the future may bring this possibility.

An as yet insufficiently explored field is the use of urine for pharmacokinetic analysis, although the feasibility of such an approach has been shown for drugs which have a urine clearace of at least 20%, provided that urine collection is complete and appropriately timed. Drug concentrations in the urine are generally higher than in plasma. Thus useful sampling may be continued over relatively long periods.

2.3 BIOSTATISTICAL APPLICATION TO THE INTERPRETATION OF PHARMACOKINETICS

2.3.1 Principles

As described above, pharmacokinetic studies use compartmental models to describe the behaviour of a drug in the body. The model chosen is the one that best represents the concentration-time data obtained.

In order to fit the data to the model, there are certain assumptions made. For example, in the two-compartment model which describes the concentration-time profile shown in Figure 2.4, it is assumed that the drug is rapidly distributed within each compartment and the concentration of the drug is uniform in each compartment. This may not occur in practice but the model used assumes that the equilibration in the compartment takes place at a ratio which is faster than the rate of loss from that compartment.

Another assumption is that output is unidirectional and solely dependent on the concentration in that compartment. It is also assumed that the rate constants and compartment volumes do not change with time. In practice this may not be true but as long as this assumption is valid during the actual experiment, any variation from the assumption can be neglected.

For most pharmacokinetic studies, the simplest model is chosen that can fit all the available experimental data. The process starts by selecting the parameters that fit the experimental data within reasonable limits of error. During this stage, there is also a need for finding whether a simpler model than that chosen may be good enough.

A plot of the concentration-time data on a log-linear scale will give a rough indication as to whether a one, two or even a three-compartment model will fit best. The appropriateness of the chosen model should be ascertained.

If numerical values are assigned to the rate constants and volumes, a concentration-time curve can be simulated. Most of the relevant computer programmes provide this capability. The simulated curve must pass within acceptable limits of error of the data points. However, and more importantly, the simulated curve chosen should not deviate from the trend obtained from the actual data points.

To solve the mathematical equations describing the model, the computer programme needs to perform certain functions. One approach is based on iterative estimation. The computer is useful in performing this function. This is illustrated in more detail in the section 2.3.2.1(b).

2.3.2 Model conformation or curve fitting of data

2.3.2.1 One compartment open model

(a) Linear regression method

The calculation of the pharmacokinetic parameters is rather simple for a drug rapidly administered by the intravenous route and which shows one-compartment model behaviour. Figure 2.10 shows the log-linear plot of plasma concentration with time following intravenous administration.

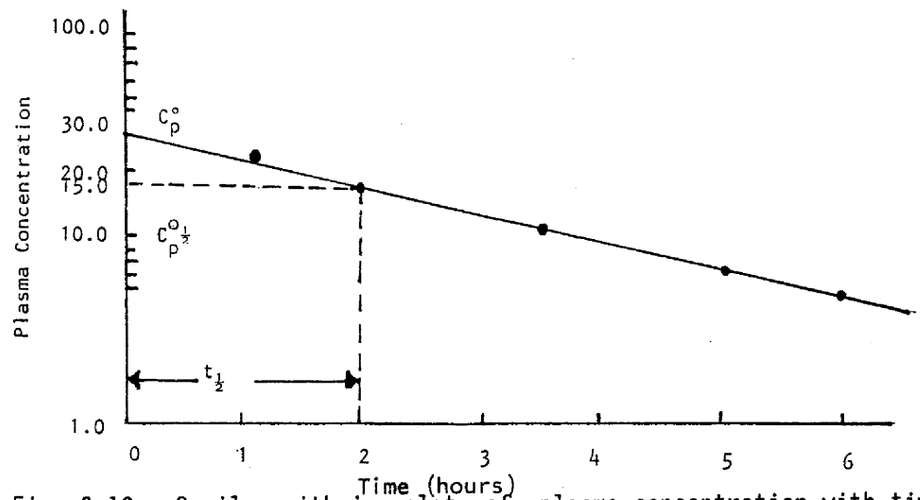


Fig. 2.10 Semilogarithmic plot of plasma concentration with time following intravenous administration.

This figure also illustrates the determination of C_p^o and $t_{1/2}$ of elimination

The plot in Figure 2.10 provides the following information:

(i) The one compartment model can be used to describe the drug's behaviour.

(ii) Extraapolation of the line to the y-axis yields C_p^o (concentration at time 0) which is 30 ug/ml.

(iii) Half-life of elimination = $t_{1/2}$ = time ffor concentration to decrease from 30 ug/ul to 15 ug/ml. From the graph the value of $t_{1/2}$ = 2.0 hours.

(iv) K, the rate of elimination can be calculated as follows:

$$K = \ln 2/t_{1/2} = 0.693/t_{1/2}$$

$$K = \frac{0.693}{2} = 0.3465 \text{ hr}^{-1}$$

(v) Since the dose administered is known (250 mg) the apparent volume of distribution (V_d) can be calculated:

$$V_d = \frac{\text{Dose}}{C_p^o} = \frac{250 \text{ mg}}{30 \text{ ug/ml}} = \frac{250\,000 \text{ ug}}{30 \text{ ug/ml}} = 8.33\text{L}$$

Since the dose has been given intravenously, it can be assumed that 100% of the dose is available.

The above parameters can also be calculated by means of a calculator or a personal computer programme. Before this can be done, the relationship between C_p (plasma concentration), C_p^o (plasma concentration at time 0), elimination rate constant, K and time, t, has to be known. Therefore, the following equation is used to describe this relationship.

$$\log C_p = \log C_p^o - Kt/2.303$$

The regression parameters of the line can be calculated by using 2 data points or by entering all available (usually 5+) points into a computer programme which can perform linear regression calculations. The detailed process is shown below:

(i) A table is made of the times of sampling, concentrations obtained and the logs of these concentrations (Table 2.1).

TABLE 2.1 THE TIMES OF SAMPLING, PLASMA CONCENTRATIONS OBTAINED AND THE LOG CONCENTRATIONS

Time (hours)	Plasma concentration (ug/ml)	Log concentration
1.125	25.0	1.3979
2.0	15.0	1.1761
3.5	8.0	0.9031
5.0	5.7	0.7560
6.0	4.0	0.6021

(ii) Since for a one-compartment model the log concentration with time is a linear function, the relationship between log concentration and time can be represented by the following equation:

$$y = a + bx$$

where y = log concentration
a = intercept at y axis = log C_p^o
b = slope of the line.

b x 2.303 = K, rate of elimination

(iii) Using a suitable pocket calculator or an appropriate computer programme the data under (i) are entered for processing under linear regression (Appendix 2 shows the example of such a programme for an HP calculator, ELIN; most of the modern scientific calculators have a logarithmic regression programme which permits a direct input of the concentration data against time).

The programme provides the following information:

- r = 0.9927 = coefficient of regression. A r of 1.000 or close to 1.000 shows that the data fit a straight line. This r value is an indication of how well the data fit a straight line. The closer to 1.000 the better the fit.

- The intercept a = 1.4086 = log C_p^o

  C_p^o = antilog 1.4086 = 25.62 ug/ml

  Although the coarse graphic approximation of the data gave a C_p^o of 30 ug/ml, linear regression calculation has provided a more accurate calculation of C_p^o. The visual graphic fit used 3 data points (C_p at t = 2, 5 and 6 hr), while all 5 points were used in the linear regression calculation. This is statistically more accurate.

- The slope b = - 0.1338

  b x 2.303 = 0.3081 hr^{-1} = K

  (N.B. 2.303 = ln (10), base e)

  Since K = -0.3081 hr^{-1}

$$t_{1/2} = \frac{0.693}{K} = \frac{0.693}{0.3081} = 2.25 \text{ hr}$$

As noted under remarks in Appendix 2, the least square method is used to fit the data to the equation. In least squares fitting, the best estimates are those which minimize the sum of the squares of the deviations between the observed values and the values predicted by the model.

Another advantage of the regression method is that each data point is given the same weight or emphasis. This is not the case, when data are plotted on the semilog graph paper, where the lower concentrations are normally given more emphasis than may be warranted. With most experimental data, the accuracy of the lower concentrations may be less than that of the higher concentrations due to a greater variation in the extraction and determination of low concentrations of drugs in biological fluids.

(b) Nonlinear regression method

The nonlinear regression method is another way to analyse data. The parameters of the following equation are adjusted to fit the untransformed equation to the data.

$$C_p = \frac{\text{Dose}}{V_d} e^{-Kt}$$

With nonlinear regression analysis, the data may be given equal weight (i.e. the magnitude of the error at each data value is assumed to be constant) or a weight proportional to the concentration value squared (i.e. the standard deviation of the error is proportional to the data), or any other weighting factor as dictated by the model chosen.

This method is quite complex and computers are necessary to perform the required functions. Examples of some of the computer programmes are NONLIN, NLIN and SAAM. However microcomputer or personal computer programmes like MULTI, CIPHER and PCNONLIN are now available to perform nonlinear regression analysis.

As already mentioned, the computer programmes perform a series of iterations. The programmes determine a "weighted best fit" to the data by iteratively altering the parameter values until the weight sum of squares (WSS) is minimized.

$$WSS = \sum_{i=1}^{i=n} [\text{weight}_i \times (\text{calc}_i - \text{Expt}_i)^2]$$

The WSS value is obtained by multiplying the weight given to each point by the square of the difference between the calculated concentration and the experimental concentration.

In order to fit the data, a weighting factor and initial estimates for all the parameters of the model must be provided.

Two weighting scales are compared below in predicting parameters. In one, equal weight is given to all data points and in the other, the weight given is proportional to the square of each concentration value. Initial estimates of K and V_d were given. The following data points were fitted (Table 2.2).

TABLE 2.2 PLASMA CONCENTRATION VERSUS TIME VALUES USED FOR NONLINEAR REGRESSION ANALYSIS

Time (hours)	Plasma concentration (ug/ml)
1	22.3
2	17.0
4	14.0
6	10.3
9	6.9
12	3.4

Using equal weighting, the values obtained were r^2 = 0.985; K = 0.152 hr^{-1}; and V_d = 4.00 L. Using weight proportional to the square of each concentration, the values obtained were r^2 = 1.00; K = 0.161 hr^{-1}; and V_d = 3.88 L.

Hence different weighting factors will produce different values for the parameters. Therefore when reporting pharmacokinetic data, there is a need to state what weighting factors were used.

(c) Area under the plasma concentration versus time curve (AUC)

AUC is normally used to determine the relative bioavailability of different dosage forms and also for calculating total body clearance (Cl).

The AUC obtained following intravenous administration is used as the baseline value for comparing with oral or other nonintravenous dosage forms.

Figure 2.11 shows the trapezooidal sections of the area under the concentration-time curve. Since there are 6 data points, the area can be divided into 5 trapezoidal sections. A sixth trapezoidal section is obtained when the extrapolated concentration at time 0 is obtained. The area of each trapezoid can be calculated as follows:

$$\text{Area} = \frac{(t_{i+1} - t_i) / (C_i + C_{i+1})}{2}$$

Each section is calculated and the areas of the five sections are summed. To calculate the trapezoidal section before the first data point, the best fit line is extrapolated to the y-axis to obtain $C_p{}_o$. The area of this section is then calculated as the other trapezoids.

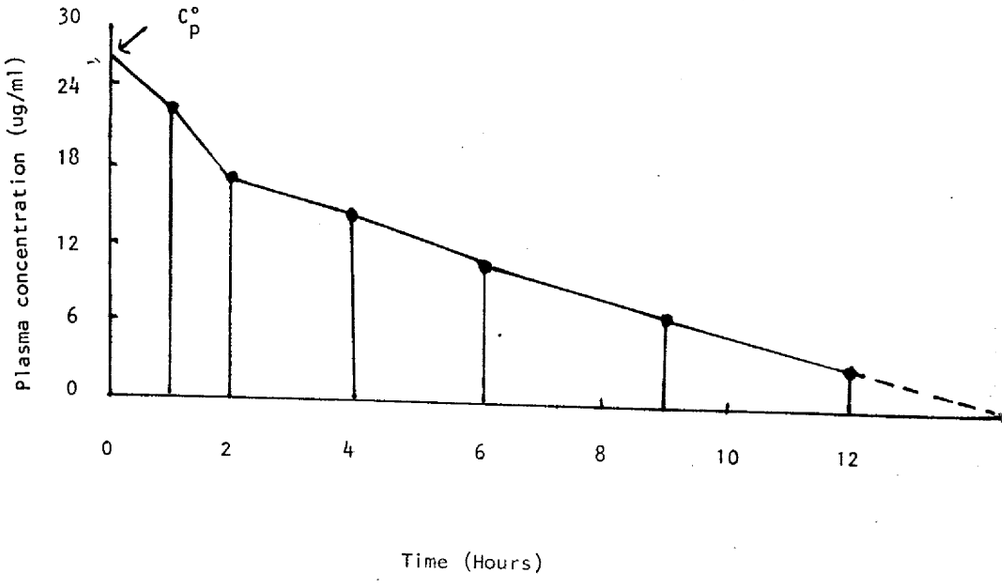


Fig. 2.11 Linear plot of plasma concentration with time illustrating the trapezoids used to calculate the area under the concentration-time curve.

To obtain the area from the last concentration point to infinite time, the following equation is used:

$$C_p = C_{last} e^{-K't}$$

where K' = rate constant for the monoexponential decline. For a single compartment model K' = K and the last area of the triangular segment is equal to

$$C_{last}/K$$

A simpler way to calculate the AUC of a one compartment model is to divide C_p^o by K.

(d) Total body clearance, (Cl)

$$Cl = Dose/AUC$$

The above equation is valid irrespective of the particular model as long as linear kinetics apply.

For a one-compartment model, Cl can be calculated as follows:

$$Cl = KV_d$$

This equation only applies for a one-compartment model and cannot be used for other compartment models.

(e) Intravenous infusion

To determine the pharmacokinetic parameters following an intravenous infusion, the rate of absorption can be ignored and only the values of dose and infusion rate need to be known.

When the infusion is terminated, a drug which obeys one-compartment modelling will decline mono-exponentially with time. The formula

$$C_p = \frac{ko}{V_d K}(1 - e^{-KT})\, e^{-K(t-T)}$$

explains the relationship between plasma concentration and infusion rate (k_o), T (duration of infusion), V_d, K and time (t).

To calculate V_d and K, a semilog regression analysis can be applied to the postinfusion plasma concentration with time data.

Alternatively a computer programme like PCNONLIN can be used to fit iteratively the data nonlinearly (Model 2 of the PCNONLIN Pharmacokinetic model library can be used) (see Fig. 2.12). Initial estimates of V_d and K are fed into the programme together with the values for dose and length of infusion. The programme can then provide values for AUC, K and C_{max}.

MODEL 2: One-compartment with constant IV input and first-order output.

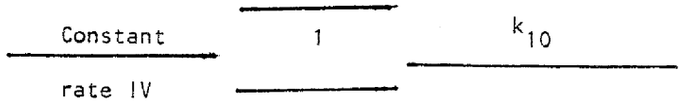


Fig. 2.12 Model 2 from the PCNONLIN Pharmacokinetic model library for one-compartment model with intravenous (IV) infusion and first-order elimination

K10 = K, elimination rate
V = V_d = apparent volume of distribution

C(T) = (D/TI)/V/K10*(EXP(-K10*TSTAR) - EXP(-K10*T)

where TSTAR = T - TI for T > TI
and TSTAR = 0 for T < TI

Estimated parameters: (1) V = volume
(2) K10 = elimination rate

Constants in input: (1) D = dose
(2) TI = length of infusion

Secondary parameters: (1) AUC = D/V/K10
(2) K10 half-life
(3) CMAX = C(TI)

The PCNONLIN programme can estimate "the least squares" estimates of the model parameters. This is done by having the option of three types of algorithms for minimizing the sum of squared residuals.

The algorithms used are:

- the Simplex algorithm;
- the Gauss-Newton algorithm and
- a Levenberg-type modification of the Gauss-Newton algorithm.

(f) Oral and extravascular administration

Extravascular administrations include intramuscular, subcutaneous and topical routes. For these routes and oral administration, there are four basic parameters that describe the drug behaviour in the body. They are:

(i) k_a, the rate of absorption of the drug

(ii) F, the fraction absorbed or bioavailable

(iii) K, the rate of elimination and

(iv) V_d, the apparent volume of distribution.

The relationship between these four parameters, plasma concentration at time 0 and time are shown below:-

$$C_p = (e^{-Kt} - e^{-k_a t})$$

and $$A = \frac{F \text{ dose } k_a}{V_d (k_a - K)}$$ and

where k_a = rate of absorption and K = rate of elimination.

(g) Methods of residuals

The method of residuals is one way to measure the rate of absorption. The data points are plotted on a semilog graph paper (Fig. 2.13). The terminal or elimination line is plotted and the data points that form the elimination or post absorption phase are identified. These data points are fitted into a linear regression to obtain values for K and the intercept, A. Table 2.3 shows the data needed to calculate k_a.

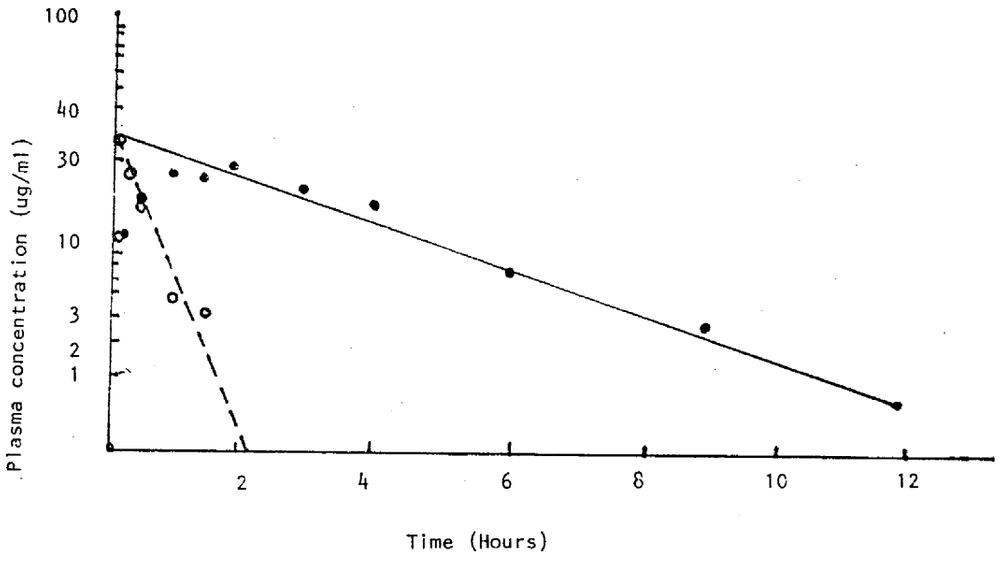


Fig. 2.13: Semilog plot of plasma concentration with time. The solidline through the terminal data points (solid circles) indicates the elimination phase. The broken line shows the straight line best fitting the calculated residuals (open circles) with time.

TABLE 2.3 DATA REQUIRED TO CALCULATE Ka

Time	Observed concentration (ug/ml)	Calculated concentration (ug/ml)	Calculated, observed, concentration (ug/ml)
0	0.0	31.5	31.5
0.25	9.6	29.6	20.0
0.5	13.8	27.9	14.1
1.0	19.9	24.7	4.8
1.5	17.7	21.9	4.2
2.0	19.8	19.4	-

The calculated concentration minus observed concentration values are called the residual values and these values are then plotted with time to give a value for the slope.

$$\text{slope} = -\frac{k_a}{2.303}$$

$$k_a = \text{slope} \times (-2.303)$$

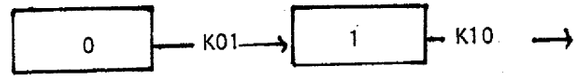
$$\text{since } A = \frac{F \text{ dose } k_a}{V_d (k_a - K)}$$

$$\frac{V_d}{F} = \frac{\text{Dose } k_a}{A (k_a - K)}$$

For computer analysis of the data, initial estimates for V_d, k_a and K are fitted into the model (Fig. 2.14) together with the value for dose.

From the nonlinear regression fit values for AUC, absorption half-life, elimination half-life, t_{max} and C_{max} may be determined .

MODEL 3 : One-compartment with first-order input, first-order output, and no lag time.



C(T) = D*K01/V/(K01 - K10)*(EXP(-K10*T) - EXP(-K10*T)

Estimated parameters: (1) V = volume
(2) K01 = absorption rate
(3) K10 = elimination rate

Constants in input: (1) D = dose

Secondary parameters: (1) AUC = D/V/K10
(2) K01 half-life
(3) K10 half-life
(4) T_{MAX} = time of maximum concentration
= ln(K01/K10)/(K01-K10)

(5) C_{MAX} = maximum concentration
= $C(T_{MAX})$

Fig. 2.14 Model 3 for a one-compartment with single oral administration where absorption and elimination are first-order

K10 = K, elimination rate
K01 = k_a, absorption rate
V = V_d, apparent volume of distribution

(h) Bioavailability, (F)

The extent of absorption or bioavailability is defined as the fraction of the administered dose in the general circulation.

The absolute bioavailability is calculated by dividing the AUC following oral administration by the AUC following intravenous administration.

The relative bioavailability is the comparison of AUC of one dosage form with that of another

$$F_{oral} = \frac{AUC_{oral}}{AUC_{iv}}$$

2.3.2.2 Two-compartment model, intravenous bolus

(a) Intravenous bolus

The concentration of a drug that conforms with a two-compartment model following rapid intravenous administration can be described by the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

Figure 2.15 shows the model used by PCNONLIN to fit the data for nonlinear analysis. Initial estimates for A, B, α and β are fitted into the programme together with a known dose. The pharmacokinetic parameters that can be calculated are AUC, $t_{1/2}$ elimination, α, β, K, k_{12}, k_{21}, V_d and C_{max}.

(b) Oral administration

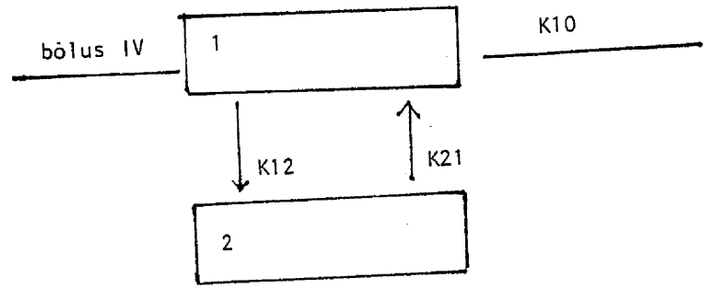
For a drug that shows two-compartment model characteristics and is orally administered, a three exponential equation describes the relationship between plasma concentration and time.

Figure 2.16 shows the model used by PCNONLIN to fit the data. Initial estimates used are A, B, k_a, and while the dose is the constant input. The values for K, k_{12}, k_{21}, AUC, $t_{1/2}$ elimination, $t_{1/2}$ absorption, K_{10}, C_{max} and V_d may be obtained using the computer programme.

2.3.2.3. Goodness of fit of the data points to the model

Appendix 3 presents an example of the computer output from the fit of data points to a pharmacokinetic model.

MODEL 8: Two-compartment with bolus input and first-order output; macro-constants as primary parameters.



C(T) = A*EXP(-ALPHA*T) + B*EXP(-BETA*T)

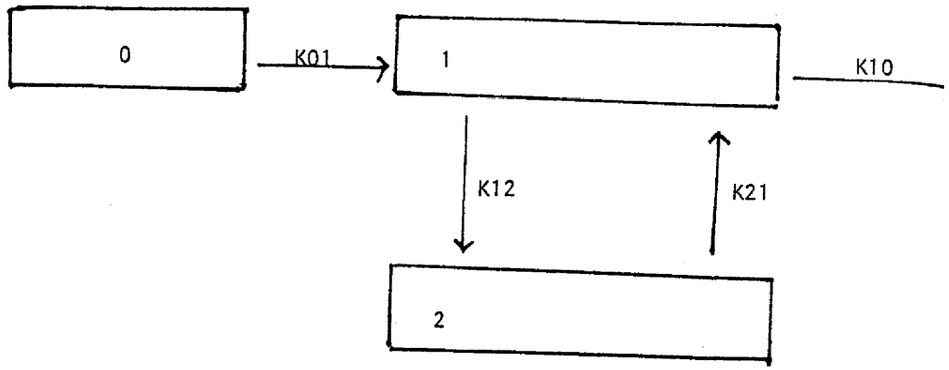
Estimated parameters:
(1) A
(2) B
(3) alpha
(4) beta

Constants in input:
(1) # doses
(2) dose 1
(3) time of dose 1
(4) dose 2
(5) time of dose 2 etc.

Secondary parameters:
(1) AUC = A/alpha + B/beta
(2) K10 half-life
(3) ALPHA half-life
(4) BETA half-life
(5) K10
(6) K12
(7) K21
(8) volume
(9) C_{MAX} = D/V

Fig 2.15 Model 8 from the PCNONLIN Pharmacokinetic Model library for a two-compartment model with intravenous bolus adminstration.

MODEL 13 : Two-compartment with first order input, first-order output, no lag time and macro-constants as primary parameters



C(T) = A*EXP(-ALPHA*T) + B*EXP(-BETA*T) + C*EXP(-K01*T).

Estimated parameters: (1) A
(2) B
(3) K01 = absorption rate
(4) alpha
(5) beta
Note: C= -(A + B)

Constants in input: (1) # doses
(2) doses 1
(3) time of dose 1
(4) dose 2
(5) time of dose 2 etc.

Secondary parameters: (1) K10
(2) K12
(3) K21
(4) AUC = D/V/K10
(5) K10 half-life
(6) K01 half-life
(7) ALPHA half-life
(8) BETA half-life
(9) volume

Fig. 2.16 Model 13 from the PCNONLIN Pharmacokinetic library for a two-compartment model following single and oral administration.

2.4 PHARMACOKINETICS OF ANTIMALARIAL DRUGS

The antimalarial drugs which are currently in use or have reached advanced stages of clinical trials can be classified as follows :

(a) 4-aminoquinolines, e.g. chloroquine, hydroxychloroquine and amodiaquine.

(b) 8-aminoquinolines, e.g. primaquine.

(c) cinchona alkaloids, e.g. quinine and quinidine.

(d) quinolinemethanols, e.g. mefloquine.

(e) antifols comprising :

biguanides, e.g. proguanil, chlorproguanil and cycloguanil.

sulfonamides, e.g. sulfadoxine and sulfalene.

sulfones, e.g. dapsone.

(f) the antibiotics, e.g. tetracycline, lincomycin and doxycycline.

(g) the phenanthrenemethanols, e.g. halofantrine.

(h) the sesquiterpene lactones, e.g. artemisinin, artesunate, arteether and artemether.

2.4.1 4-Aminoquinolines

2.4.1.1 Chloroquine

Chloroquine was introduced into therapy in 1946 shortly after the end of the second world war, most of the preclinical and initial clinical evaluation having been undertaken during the war. It quickly became the most commonly used antimalarial and has retained that position until now although its universal application as the drug of choice for the treatment and prophylaxis of Plasmodium falciparum infections has been undermined in recent years by the development and spread of resistance. Chloroquine is not only the most widely used antimalarial drug, it is also probably the one whose pharmacokinetics have been the most extensively studied. Knowledge of the pharmacokinetics of chloroquine has expanded considerably in the past ten years as a result of studies in Africa, Asia and Europe. These studies have defined comprehensively the disposition of chloroquine in health and disease and have led to a more rational use of the drug.

Absorption

Chloroquine is rapidly and almost completely absorbed after oral administration. After a single oral dose of 10 mg/kg, therapeutic blood levels are reached within 30 minutes. Peak plasma concentrations of about 250 ng/ml are attained with a t_{max} of 2-3 hours and an absorption half-life of 0.6 h (Adelusi, et al., 1982). In one study, tablets and solutions were absorbed equally with a bioavailability of 75% (Gustafsson et al., 1983b). Peak plasma concentrations rise with repeated daily or weekly administration (Adelusi et al., 1982; Brohult et al., 1979).

Chloroquine is also rapidly absorbed after subcutaneous and intramuscular administration. Peak blood levels are reached in 5-15 minutes with both routes in healthy adult volunteers (Salako et al., 1987) and in patients with acute severe falciparum malaria (White et al., 1987a). The bioavailability after intramuscular, subcutaneous and oral administration is almost complete (Gustafsson et al., 1987; Aderounmu et al., 1986).

Distribution

After absorption, chloroquine is widely distributed throughout the body. It is extensively bound to tissues particularly liver, spleen, kidney, lung and heart where the tissue concentrations can be as much as 300-500 times the plasma concentration (Adelusi & Salako, 1982a, b). As a result of this extensive tissue binding, chloroquine has an exceptionally large apparent volume of distribution of between 70 and 200 times the total body fluid volume (Gustafsson et al., 1983b). Chloroquine has a particularly striking affinity for melanin-containing tissue and its toxic effects on the skin and eye may, in part, be due to its high degree of binding to tissues rich in melanin (Lindquist, 1973).

In spite of its extensive binding to tissues, chloroquine is only moderately (about 60%) bound to plasma protein (Adelusi & Salako, 1982c; Walker et al., 1983a, b). The binding protein is mainly plasma albumin to which a little over 50% of the bound fraction is attached. Other binding proteins are alpha-1 acid glycoprotein and gamma globulin (Walker et al., 1983a). The binding increases proportionately to the plasma protein concentration; the percentage binding is not affected by the concentration of chloroquine. Aspirin does not affect binding (Adelusi & Salako, 1982c).

Since the blood stages of the malaria parasite are intraerythrocytic, there is interest in the concentration of the drug in the red blood cells. Chloroquine is concentrated about five fold in red cells compared with plasma, and the concentration is further increased in parasitized red blood cells (Adelusi et al., 1981; Ajayi et al., 1988). Red blood cells parasitized by chloroquine-resistant parasites concentrate the drug to a lesser extent than those parasitized by chloroquine-sensitive parasites (Warhurst and Hockley, 1967). Other formed elements of the blood also concentrate chloroquine (Bergqvist & Domeij-Nyberg, 1983). There is considerable individual variation in the distribution and concentration-time profiles and other pharmacokinetic parameters following equivalent chloroquine doses.

Metabolism

Chloroquine is 7-chloro-4-(4^1-diethylamino-1-methylbutyl-amino) quinoline, a tertiary amine. It is metabolized by side chain deethylation leading successively to desethyl- and then bisdesethylchloroquine. This compound can undergo deamination to form an alcohol, the 4^1-hydroxy compound, which can then be oxidised to the 4^1-carboxylic acid derivative. Successive dealkylation of this compound ultimately leads to the compound, 7-chloro-4-aminoquinoline (McChesney et al., 1966; Kuroda, 1962). The quinoline nucleus is resistant to degradation. Metabolism of chloroquine occurs slowly, predominantly in the liver and the main metabolites vary in different species. In humans, the main metabolites are desethyl- and bisdesethylchloroquine which constitute 25 and 5% respectively of the total quinoline (McChesney et al., 1966). The desethyl metabolite has the same pharmacokinetic profile and tissue distribution as the parent compound (Gustafsson et al., 1983b; Fletcher et al., 1975; Salako & Ajayi, 1987).

Elimination

Chloroquine is eliminated from the body very slowly with the result that after a single oral dose, the drug and its metabolites can be detected in the plasma for up to 56 days (Gustafsson et al., 1983b). Most estimates of the elimination half-life of chloroquine have varied between 2 1/2 days and 13 days (Alving et al., 1948; Gustafsson et al., 1983b). This wide variation is probably a reflection of the multiexponential nature of the chloroquine concentration-time curve (Aderounmu & Fleckenstein, 1983), the half-life in any study depending on how terminal the "terminal" slope really is (Adelusi & Salako, 1982b). Half-life values of several weeks estimated from some recent studies (Frisk-Holmberg et al., 1984, 1985) are probably of no therapeutic or pharmacokinetic significance since the mean residence time of chloroquine is only 20 days (Gustafsson et al., 1987). In the same way a half-life of about 30 hours reported in another recent study is not the terminal half-life because the sampling period was only 24 hours. The multiexponential nature of the chloroquine plasma concentration-time curve can also explain the different half lives obtained for different doses by Frisk-Holmberg et al. (1979) since duration of sampling was shorter with the lower doses than with the higher ones. Studies in which possible dose-dependence of the chloroquine kinetics was examined by comparing AUCs at different doses showed a linear relationship between the two which is inconsistent with dose-dependent kinetics (Gustafsson et al., 1983a).

The total plasma clearance of chloroquine varies between 750 and 1050 ml/minutes of which renal clearance is between 400 and 450 ml/minutes (Gustafsson et al., 1983b). This high renal clearance suggests that chloroquine is excreted by both glomerular filtration and tubular secretion. Chloroquine can be detected in the urine for up to 120 days after a single therapeutic dose. The graph of daily urinary excretion against time is exponential. The total urinary recovery of quinoline is about 60%, and about 10% of the administered dose is recovered in the first 24 hours. Chloroquine can be detected in the urine after repeated weekly doses for more than one year after the last drug administration (Gustafsson et al., 1987).

Chloroquine is excreted in saliva and in breast milk but the amount excreted in milk is not sufficient to constitute any danger to a breast-fed baby (Ogunbona et al., 1986, 1987). The concentration in milk is also below the suppressive threshold. The chloroquine concentration in the saliva does not correlate well with that in plasma, the ratio is generally between 0.1-0.2 (saliva/plasma).

Effect of malaria

Chloroquine given orally is absorbed as rapidly and as completely in patients with mild uncomplicated malaria as in normal subjects (Adelusi et al., 1983). Similarly, in severe malaria chloroquine given subcutaneously or intramuscularly is absorbed as rapidly and as completely as in healthy subjects (White et al., 1987a). The ratio of the concentration of chloroquine in red blood cells to that in plasma is higher in parasitized compared with unparasitized red blood cells (Ajayi et al., 1988), possibly due to its concentration by the intraerythrocytic parasites. The concentration of chloroquine in the cerebral spinal fluid in patients with severe malaria is usually very low with a mean cerebral spinal fluid to whole blood concentration ratio of less than 3% (White et al., 1987). The metabolism of chloroquine to desethylchloroquine is possibly a little less in malaria patients than in normal subjects, the metabolite concentration stabilizing at about 13.5% of the parent compound in malaria patients (White et al., 1987a) compared with 25-40% in normal subjects (Walker et al., 1987a). Desethylchloroquine is not detectable in the cerebral spinal fluid (White et al., 1987a).

Effect of hepatic and renal disease

The rate of decline of plasma concentration with time in patients with chronic renal failure is slower than in normal subjects giving rise to a longer $t_{1/2}$ (Salako et al., 1984). The total clearance is less than in controls and the percentage of the total clearance due to renal clearance is also less than in controls. By contrast, the disposition of chloroquine in hepatic insufficiency due to liver cirrhosis is not significantly different from healthy controls (Walker et al., unpublished data).

Effect of malnutrition

In kwashiorkor, there is a decrease in the intestinal absorption of chloroquine leading to a lower C_{max} and AUC. There is also a reduction in the concentration of the main metabolite, the mean C_{max} of the metabolite being 15% of the chloroquine C_{max} in kwashiorkor compared with 37% in normal children (Walker et al., 1987b). In another study, children with kwashiorkor excreted less desethylchloroquine (as a percentage of excreted chloroquine) before treatment than after (Wharton & McChesney, 1970). These findings suggest that chloroquine is absorbed and metabolised to a lesser extent in kwashiorkor than in persons with normal nutritional status.

2.4.1.2 Hydroxychloroquine

A recent study of the pharmacokinetics of hydroxychloroquine showed that the main pharmacokinetic parameters of this drug are similar to those of chloroquine (Tett et al., 1988).

2.4.1.3 Amodiaquine

Amodiaquine is a 4-aminoquinoline with a Mannich base in the side chain which probably confers on it properties different from those of chloroquine.

Amodiaquine is rapidly absorbed after oral administration, peak plasma concentration being reached within one hour. Concentrations in plasma are similar to those in whole blood and red blood cells. The terminal half-life is about 4 hours and the drug is not identifiable in blood after 8 hours (i.e. concentration <5ng/ml) (Winstanley et al., 1987a). Amodiaquine is rapidly and extensively metabolized to desethylamodiaquine (Churchill et al., 1985), the concentration of this metabolite reaching up to 20 times that of the parent drug. The amodiaquine metabolite can be detected in blood for up to two weeks after a single oral dose (Salako & Idowu, 1985). The drug is excreted in the urine as desethylamodiaquine which can be detected in the urine for up to 5 months after a single dose; the total quinoline recovery in urine is about 10% of the administered dose and of this 95% is the metabolite (Winstanley et al., 1987b). Like chloroquine, the AUC for amodiaquine varies linearly with dose (Winstanley et al., 1986). Metabolism occurs in the liver (Maggs et al., 1986).

The disposition of amodiaquine has also been studied after intravenous administration in healthy volunteers and patients with malaria (White et al., 1987b). The kinetics of the drug appear to be affected by both the route of administration and the presence of malaria. The intravenous study also confirmed the short elimination half-life obtained in the oral studies. The steady state apparent volume of distribution was less than that of chloroquine and was

significantly greater in malaria patients than in normal controls. In the intravenous study, the total clearance of amodiaquine was less in malaria patients than in healthy subjects. The metabolite was not detectable in the blood while the parent compound was detected for more than 24 h in these patients - a reversal of the observation in the oral studies.

There thus seem to be inconsistencies in the reported studies on amodiaquine which cannot be fully explained on the basis of route of administration or the presence of malaria infection. Further studies are needed to clarify the inconsistencies. Meanwhile, studies in rats have shown that 90% of administered radioactive amodiaquine was excreted in the faeces and 10% in the urine but not as the parent drug or desethylamodiaquine. Radioactivity was concentrated mostly in the liver and haemopoietic tissues (Winstanley et al., 1988).

2.4.2 8-Aminoquinidines : primaquine

Primaquine is rapidly absorbed after oral administration, with an absorption half-life of 0.5 hours a time to peak concentration (T_{max}) of 2-3 hours and a bioavailability of about 95% (Mihaly et al., 1987, Nora et al., 1987). After absorption, the concentration declines monoexponentially with an elimination half-life of about 6 hours (Nora et al., 1987; Mihaly et al., 1987). It is distributed into tissues but not to the same extent as chloroquine, the apparent volume of distribution being only about 3 l/kg. Primaquine exhibits linear pharmacokinetics. There is no significant difference in the pharmacokinetic parameters ($t_{1/2}$ elimination, $t_{1/2}$ absorption, V_d) at different doses and the AUCs are linearly related to dose (Nora et al., 1987). Also, in spite of being an inhibitor of hepatic oxidative metabolic enzymes (Back et al., 1983a) primaquine does not appear to inhibit its own metabolism. The kinetics after one-week repeated administration do not differ from the single-dose kinetics. However, carboxyprimaquine accumulates in plasma after protracted primaquine administration (Ward et al., 1985). Primaquine inhibits antipyrine metabolism (Back et al., 1983b).

The main metabolite of primaquine in humans is carboxy-primaquine obtained by oxidation of the terminal amino-group of the side chain. Metabolism is rapid with a substantial hepatic first-pass metabolism. Consequently carboxyprimaquine appears early in the blood and reaches a peak in 6-8 hours, the peak concentration being more than 10 times the peak concentration of the parent drug. The metabolite is detectable in the blood for more than 96 hours after dosing - much longer than the parent compound. Primaquine is detected in the urine for more than 24 hours after the dose. The amount of primaquine recovered in the urine is usually less than 4% of the total dose administered. Carboxyprimaquine is not detectable in urine in spite of its presence in high concentration for 72-96 hours in the blood. This suggests that carboxyprimaquine is further converted to another metabolite before urinary

excretion. Its persistently high plasma concentration at a time when the parent drug is no longer detectable suggests not only that it is less tissue bound than the parent drug but also that it probably also undergoes a measure of enterohepatic recirculation.

Studies with ^{14}C-labelled primaquine have shown that only about 2% of the plasma radioactivity is due to the parent compound, 55% is due to carboxyprimaquine and the remainder to unidentified metabolites. About 64% of an administered dose is excreted in the urine in one week mainly as metabolites. Only 3.6% is recovered as the parent compound.

Primaquine is a low clearance drug (total plasma clearance 24.2 l/h). It is not selectively concentrated in red blood cells, the whole blood to plasma concentration ratio being approximately 1.

2.4.3 Cinchona alkaloids

2.4.3.1 Quinine

The pharmacokinetics of quinine in normal volunteers and malaria patients have been extensively studied and reviewed (White et al., 1982, 1983a, b; Silamut et al., 1985; White 1985, 1987). Below is a summary of the available information. Details can be obtained in the publications referred to above.

Quinine is rapidly and almost completely absorbed after oral administration. Peak plasma concentrations are reached 1-3 hours after a single oral dose. After absorption, it is distributed throughout most of the body fluid and is concentrated to a small extent in tissues giving an apparent volume of distribution of about 2 l/kg in normal adults. The concentration in red blood cells is one-quarter to one-third that in plasma. About 92% of the plasma concentration is protein-bound.

Quinine concentration declines rapidly in the blood after reaching peak concentration and is not detectable in the blood for more than 72 hours after the final drug administration. The elimination half-life is 10-12 hours. After a slow intravenous "push" injection, the decline in drug concentration is biexponential with an initial distribution phase of 1.9 minutes. A similar biexponential decline is seen after oral dosing. With a slow intravenous infusion at rates of 5mg/kg/hour or less, distribution is virtually complete by the end of the infusion and the decline phase is then monoexponential.

Quinine is metabolized in the liver and is excreted partly unchanged but mainly as the hydroxylated metabolite. The metabolite appears in the urine within one hour of administering the parent drug and little remains in the body after 48 hours. The systemic clearance is about 200 ml/minute and this is mainly by hepatic metabolism. Renal clearance of unchanged drug accounts for only 20% of the total clearance in

normal subjects. Renal excretion of quinine is probably by both glomerular filtration and tubular secretion. Quinine is excreted in saliva, the concentration in this fluid, parallelling that of plasma but being about 33% that of plasma (L.A. Salako, personal communication).

Effect of malaria

The pharmacokinetics of quinine are altered significantly in cerebral malaria. Quinine concentration in red blood cells is about half the plasma concentration in cerebral malaria compared with one-fourth to one-third normal subject. In cerebral malaria the central spinal fluid concentration is 7% that of plasma. The apparent volume of distribution is reduced to about half the normal value in severe malaria, and the systemic clearance to about one-third. The reductions are proportional to the severity of the infection and are probably responsible for the higher quinine concentrations observed in patients with severe malaria.

Effect of hepatic and renal disease

Metabolism of quinine is reduced in hepatic insufficiency, and this probably accounts, in part, for the reduced clearance in severe malaria. Renal insufficiency does not alter significantly the disposition of quinine.

Effect of pregnancy

The pharmacokinetics of quinine are significantly altered in pregnant women with severe malaria as compared to non-pregnant women with malaria of comparable severity (Phillips et al., 1986). The elimination half-life is shorter and the apparent volume of distribution smaller in pregnant women but the total clearance is not different. The placental cord plasma quinine concentration is around one-third that of maternal plasma. A similar fraction was found in the heart blood of a fetus aborted at term. The ratio of breast milk to plasma quinine concentration in lactating women averages 0.31 during oral and 0.21 during intravenous treatment.

Plasma protein binding is less in pregnant women than in non-pregnant and less still in fetal umbilical cord plasma (Mihaly et al., 1987).

Effect of malnutrition

Severe protein-energy malnutrition otherwise known as kwashiorkor in African children, has been shown to decrease the rate of absorption and elimination of quinine after a single oral dose. Peak plasma concentration is lower, systemic clearance of the orally administered dose is less and the AUC is greater in kwashiorkor than in normal children (L. A. Salako et al., unpublished data).

Protein binding

In normal volunteers, quinine is about 92% bound to plasma and this fraction is the same in males and females. The binding is more to alpha-1 acid glycoprotein than to albumin. Percentage binding increases with increasing protein concentration and decreases with increasing drug concentration (Mihaly et al., 1987).

2.4.3.2 Quinidine

The pharmacokinetics of quinidine have been studied more in relation to its use in cardiology than in malaria. In healthy subjects, the reported values of the volume of distribution and systemic clearance of quinidine are approximately twice those of quinine. Consequently plasma concentrations tend to be lower for quinidine than for quinine given at identical doses. Like quinine, quinidine is bound more to alpha-1 acid glycoprotein than to albumin. The total plasma protein binding of quinidine is about 87.5% which is less than that of quinine. The higher percentage of unbound quinidine offsets the lower concentration due to the greater volume of distribution and systemic clearance. Similar to quinine, the protein binding of quinidine is drug concentration dependent. The binding is less during pregnancy and less still in umbilical cord plasma (Mihaly et al., 1987).

2.4.4 Quinolinemethanols:mefloquine

The pharmacokinetics of mefloquine have been studied in different racial groups, in normal volunteers and in malaria patients in the past ten years. The first of such studies (Desjardins et al., 1979) described the slow absorption and elimination of the drug, and the long persistence of therapeutic drug levels after a single oral dose. These findings have been confirmed in subsequent studies. After a single oral dose, peak concentration is reached in 2-12 hours with an absorption half-life of 1-8 hours (Desjardins et al., 1979; Schwartz et al., 1982). The long $t_{1/2}$ and T_{max} are due to a biphasic absorption in which a relatively rapid increase in concentration is followed by a relatively slow phase. However, therapeutic blood levels of 500 ug/ml are reached within 3 hours (Karbwang et al., 1987a; Looareesuwan et al., 1987). After absorption, concentration in the blood remains at over 50% of the peak level for up to seven days with a long elimination half-life of up to 35 days. Mefloquine is widely distributed in tissues with an apparent volume of distribution of 27-50 L/kg (Schwartz et al., 1980, 1982; Riviere et al., 1985). It is extensively bound to protein (98%) (Schwartz et al., 1980; Looareesuwan et al., 1987). It is highly concentrated in red blood cells *in vitro* (San George et al., 1984). However, *in vivo*, there is no difference between the plasma and whole blood concentrations of mefloquine (Karbwang et al., 1987a). The difference between the *in vivo* and *in vitro* observations may be related to differences in the study methods. In the *in vitro* study the red blood cells were

suspended in buffered saline and the high red cell-saline concentration ratio may be due to binding of mefloquine to the cell membrane which has been shown to possess high-affinity binding sites for the drug (Fitch et al., 1979). In-vivo, the high protein binding of mefloquine greatly increases the plasma concentration (compared with buffered saline) and cancels out the six-fold concentration gradient observed in vitro.

Mefloquine is eliminated slowly from the body, the total clearance being about 1.5-5.1 l/h in normal subjects (Schwartz et al., 1980; Riviere et al., 1985; Looareesuwan et al., 1987). Mefloquine is eliminated from the body mainly by biliary excretion and its long persistence in the body may be due, in part, to enterohepatic recirculation. About 5-13% of an administered dose is excreted in the urine unchanged or as the acid metabolite (Schwartz et al., 1982).

Effect of other drugs

Mefloquine is also available in a fixed-dosage combination with pyrimethamine and sulfadoxine. Its pharmacokinetics have therefore been studied when administered with these drugs. The three compounds are characterized by a long elimination half-life (pyrimethamine 100 h; sulfadoxine 200 h; mefloquine 400 h). The time to peak concentration is 4 hours for sulfadoxine and pyrimethamine and 2-12 hours (means 8h) with mefloquine. The apparent volume of distribution is least with sulfadoxine (0.13 l/kg), about 15 times higher with pyrimethamine (2.1 l/kg) but very much higher with mefloquine. The red cell/plasma partition coefficient with sulfadoxine and pyrimethamine is 0.5 compared with approximate unity for mefloquine. The systemic clearance value for sulfadoxine is very low (0.52ml/h/kg) and is responsible for its long $t_{1/2}$. By contrast, pyrimethamine and mefloquine have large clearance values (pyrimethamine = 18 ml/h/kg; mefloquine = 20 ml/h/kg) and their long elimination half-lives would therefore be due to their large volumes of distribution. More than 90% of an oral dose of sulfadoxine is recovered in the urine. The corresponding figures for pyrimethamine and mefloquine are 33% and 5-13%, respectively.

Although in one study the mean resident time and elimination half-life were significantly longer when mefloquine was given as the triple combination than when given alone (Karbwang et al., 1987b), in general, the pharmacokinetic parameters and protein binding of mefloquine are not materially influenced by the presence of the other two drugs (Karbwang et al., 1987b; Schwartz & Weidekamm, 1982).

Mefloquine, like primaquine but unlike chloroquine inhibits hepatic microsomal metabolism (Riviere and Back, 1985). However, unlike primaquine, but like chloroquine, mefloquine has no effect on antipyrine metabolism and kinetics in human (Riviere et al., 1985).

Effect of malaria

Mefloquine is well absorbed from the gut in patients with malaria, the absorption half-life being the same as in normal subjects. However, the pharmacokinetics of mefloquine seem to be affected in some important respects by malaria. Peak mefloquine concentration is higher in malaria patients than in normal subjects (Karbwang et al., 1987a; Looareesuwan et al., 1987). Oral clearance of mefloquine is significantly lower and the elimination half-life is significantly shorter in malaria patients than in normal subjects (Looareesuwan et al., 1987). Neither the protein binding nor the red cell/plasma concentration ratio is affected by the presence of malaria infection (Karbwang et al., 1987a; Looareesuwan et al., 1987).

Effect of race

There is no systematically significant difference in the pharmacokinetic parameters of mefloquine reported in Caucasians, Africans and Malaysians (Desjardins et al., 1979; Schwartz et al., 1982; V. Navaratnam, personal communication). In Thais, however, plasma and whole blood concentrations of mefloquine are significantly higher than in Caucasians given an identical dose of the drug (Karbwang et al., 1987a; Looareesuwan et al., 1987).

2.4.5 Antifols : proguanil

Proguanil (chloroguanide) is rapidly absorbed from the intestine. The absorption half-life is about 1 h and the time to peak concentration is 2-4 hours. It is concentrated in red blood cells giving a whole blood to plasma concentration ratio of approximately 5. It is also widely distributed in tissues with a V_d of 25 l/kg.

It is metabolized in the body to a dihydrotriazine metabolite (cycloguanil) which is thought to be the active compound, and 4-chloro-phenylbiguanide which is inactive. Plasma and whole blood concentrations of proguanil and its two metabolites decline in parallel with a terminal elimination half-life of about 16 hours. Of the two metabolites, cycloguanil has the higher concentration in plasma but is only one-fourth of the concentration of the parent compound. The ratio of the AUCs of proguanil, cycloguanil and chlorophenylbiguanide is 100:22:8. Like the parent compound, chlorophenybiguanide is concentrated 4-5 fold in red cells but the concentration of cycloguanil in the plasma and red cells is practically the same. Excretion takes place largely through the liver. The oral clearance is about 20 ml/kg/minute more than half of which is accounted for by renal clearance of the parent drug and its metabolites (Wattanagoon et al., 1987; V. Navaratnam personal communication).

2.4.6 Phenanthrenemethanols : halofantrine

Studies of the pharmacokinetics of halofantrine are still rudimentary and there may still be some problem with the

bioavailability of the dosage forms currently marketed. The data so far available on the pharmacokinetics of halofantrine in healthy volunteers have been reviewed by Broom (1988). Absorption is apparently slow, the the t_{max} being reached in approximately 6 hours. The Cmax after an oral- dose of 500 mg does not differ from that after 1000 mg. Similarly the AUC after 500 mg is the same as that after 1000 mg. These facts suggest that there may be no advantage in giving single doses greater than 500 mg. The elimination half-life after a single oral dose is variable, being between 12 and 48 hours. The longer half-lives are associated with the higher doses. The pharmacokinetics after repeated doses (3 x 500mg 6 hourly) differ markedly from the single dose kinetics, the t_{max}, C_{max}, $t_{1/2}$ and the AUC being greater after multiple dosing. The main metabolite of halofantrine is N-desbutyl-halofantrine which also has antimalarial activity. After a single oral dose of 500 mg halofantrine, the C_{max} and t_{max} of the metabolite are four times and twice respectively those of the parent compound. The concentration of the metabolite declines more slowly than that of the parent drug thus giving a $t_{1/2}$ three times and AUC twice the magnitude of the parent drug. After repeated dosing the t_{max} of the metabolite is three times that of the parent drug, but the C_{max} is only one-tenth that of the parent drug, suggesting a reduced metabolism of the parent compound to its metabolite. The $t_{1/2}$ of the metabolite after repeated dosing is three times and the AUC one and a half-times those of the parent compound.

Newly developed high-performance liquid chromatography (HPLC) techniques for the detection of halofantrine are likely to facilitate pharmacokinetic investigations in the future. These may also elucidate the suspected "absorption window" and the correlation between food intake or type of food and absorption of halofantrine.

2.4.7 Sesquiterpere lactones: urtemisinin and its derivatives.

Artemisinin and its derivatives, artesunate, artemeter and arteether have been submitted to only very limited pharmacokinetic studies, partly because the analytical methods currently available for the drugs are not sensitive or specific enough for pharmacokinetic studies. In addition, the drugs undergo rapid structural changes on account of enzymatic action, a process that may necessitate the use of enzyme blockers immediately after sample collection. A radioimmunoassay (RIA) method has been developed for artesunate but it does not discriminate between artesunate and its (active) metabolite, dihydroartemisinin. The compounds undergo pyrolysis in gas chromatography (GC) and GC-Mass Spectrometry (MS) is too expensive and time-consuming. The best results have so far been obtained with an HPLC method which has been used to examine the concentration-time profile of artesunate in animals and humans.

After the intravenous administration of 100 mg artesunate to rabbits, the parent drug disappeared rapidly from the blood with an elimination half-life of 1.7 minute. However, dihydroartemisinin was formed rapidly and its concentration in whole blood could be followed for up to 2 hours using an HPLC-electrochemical (EC) method. The concentration ranged from 66 ug/ml at 1 minute to 293 ug/ml at 150 minutes. Half-lives of 3.0 ± 0.4 and 29.0 ± 2 minutes were calculated for phase 1 and phase 2 respectively of the bi-exponential decay curve (Zhou et al., 1987).

In another study in rabbits given 5 mg artesunate and using GC-MS, a similar result to the one above was obtained with mean dihydroartemisinin $t_{1/2}$ of 4.3 minutes (Phase 1) and 18.4 minutes (Phase 2) (Theoharides et al., 1987).

In a study in dogs given 6 mg artesunate intravenously , the plasma concentration-time profile obtained using the radioimmunoassay for artesunate fitted a one-compartment model with a half-life of 27 minutes (Song Zhen-Yu, personal communication). In another study in dogs, 5 mg artesunate was given intravenously and GC-MS was used for drug analysis. This latter study suggested that dihydroartemisinin formation and disappearance in the dog was similar to that in the rabbit, but the terminal half-life of the active metabolite was longer in the dog with a mean value of 2.6 hours (Theoharides et al., 1987).

Some human studies have been performed with artesunate given intravenously at doses of 3.3-4.4 mg/kg. The drug concentration-time profile fitted a one-compartment model with a mean $t_{1/2}$ of 31 minutes. When an HPLC-EC method was used for drug analysis, it was possible to separate artesunate from its metabolite dihydroartemisinin. The data showed rapid disappearance of artesunate from the blood. Dihydroartemisinin appeared rapidly and disappeared more slowly with a bi-exponential decay curve showing a mean terminal half-life of 40 minutes (Song Zhen-Yu, personal communication).

The pharmacokinetics of artemether have also been studied in rabbits and monkeys given the drug intravenously as an oil emulsion. The data fitted a two-compartment open model with terminal half-lives of 0.9 hours in rabbits and 9.8 hours in monkeys. Only about 30% of the artemether was converted to dihydroartemisinin in 24 hours compared with artesunate which is quantitatively transformed into dihydroartemisinin within minutes (UNDP/World Bank/WHO, 1986). Oily preparations of artemether were also shown to be absorbed from the skin of rabbits and monkeys.

The time course of appearance of dihydroartemisinin and disappearance of artesunate was studied in experiments in which artesunate was incubated with heparinized blood from various species. The experiments showed that rabbit, dog and human blood (in that descending order) rapidly hydrolysed artesunate

to dihydroartemisinin. The reaction was completely abolished by di-isopropyl-fluorophosphonate suggesting that it was catalysed by blood esterases (Zhou et al., 1987).

Experiments with radio-labelled artemeter in mice and rats showed that the drug is probably demethylated in the liver. Excretion was rapid with 56% and 39% of the radio-activity being excreted in the urine and faeces respectively within 72 hours. In another study, artemether was also shown to be highly bound to plasma protein and to be rapidly transferred to human and monkey red blood cells in vitro. Artemether readily crosses the blood-brain barrier. The highest concentration after an intravenous injection of the drug in rabbits was found in the brain where the peak level was reached within 15 minutes (UNDP/World Bank/WHO, 1986).

2.4.8 Conclusion

The development of highly sensitive and specific methods for the analysis of drugs and their metabolites in body fluids has made possible studies of detailed pharmacokinetics of new antimalarial drugs and the re-evaluation of old ones. The results of these studies have led to a more rational use of antimalarial drugs, e.g. chloroquine, quinine, quinidine and proguanil, and have therefore enhanced the place of drugs in the control of malaria.

REFERENCES
TO CHAPTER 2

Adelusi, S.A. & Salako, L.A. (1982a) Tissue and blood concentrations of chloroquine following chronic administration in the rat. Journal of pharmacy and pharmacology, 34: 733-735.

Adelusi, S.A. & Salako, L.A. (1982b) Kinetics of the distribution and elimination of chloroquine in the rat. General pharmacology, 13: 433-437.

Adelusi, S.A. & Salako, L.A. (1982c) Protein binding of chloroquine in the presence of aspirin. British journal of pharmacology, 13: 451-452

Adelusi, S.A., Dawodu, A.H. & Salako, L.A. (1982) Kinetics of the uptake and elimination of chloroquine in children with malaria. British journal of clincal pharmacology, 14: 483-487.

Aderounmu, A.F. & Fleckenstein, L. (1983) Pharmacokinetics of chloroquine diphosphate in the dog. Journal of pharmacology and experimental therapeutics, 226: 633-639.

Aderounmu, A.F., Salako, L.A., Lindstrom, B., Walker, O. & Ekman, L. (1986) Comparison of the pharmacokinetics of chloroquine after single intravenous and intramuscular administration in healthy Africans. British journal of clinical pharmacology, 22: 559-564.

Ajayi, F.A., Salako, L.A. & Kuye, J.O. (1988) Comparison of the partitioning in vitro of chloroquine and its desethyl metabolites between the erythrocytes and plasma of healthy subjects and those with falciparum malaria. African journal of medical science (In press).

Akaike, H. (1976) An information criterion (AIC). Mathematical sciences, 14: 5-9.

Alving, A.S., Eichelberger, L., Craige, B. Jr. Jones, R. Jr., Whorten, C.M. & Pullman, T. (1948) Studies on the chronic toxicity of chloroquine. Journal of clinical investigation, 27: 60-65.

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

Back, D.J., Purba, H.S., Park, B.K., Ward, S.A. & Orme, M..L'E. (1983b) Effect of chloroquine and primaquine on antipyrine metabolism. British journal of clinical pharmacology, 16: 497-502.

Beck, J.V. & Arnold K.J. (1977) Parameter estimation in engineering and science. New York, John Wiley & Sons.

Bergqvist, Y. & Domeij-Nyberg, B. (1983) 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. Journal of chromatography, 272: 137-148.

Brohult, J., Rombo, L., Sirleaf, V. & Bengtsson, E. (1979) The concentration of chloroquine in serum during short and long term malaria prophylaxis with standard and "double" dosage in non-immunes : clinical implications. Annals of tropical medicine and parasitology, 73: 401-405

Broom, C. (1988) Human pharmacokinetics of halofantrine hydrochloride. In: Halofantrine the treatment of multidrug resistance malaria. Swarhurst, D. & Schofield, J., ed. Parasitology today (special issue): 15-20.

Churchill, F.C., Patchen, L.C., Campbell, C.C., Schwartz, T.K. Nguyen-Dinh, P. & Dickinson, C.M. (1985) Amodiaquine as a pro-drug. Importance of metabolite(s) in the antimalarial effects of amodiaquine in humans. Life sciences, 36: 53-62.

Desjardins, R.E., Pamplin, C.L., Von Bredow, J., Barry, K.G. & Canfield, C.J. (1979) Kinetics of a new antimalarial, mefloquine. Clinical pharmacology and therapeutics, 26: 372-379.

Draper, N.R. & Smith, H. (1981) Applied regression analysis, 2nd ed. New York, John Wiley & Sons.

Endrenyi, L., ed. (1981) Kinetic data analysis design and analysis of enzyme and pharmacokinetic experiments. New York, Plenum Press.

Fitch, C.D., Chan, R.L. & Chevli, R. (1979) Chloroquine resistance in malaria. Antimicrobial chemotherapy, 15: 258-262.

Fletcher, K.A., Baty, J.D., Evans, D.A.P. & Gilles, H.M. (1975) Studies on the metabolism of chloroquine in rhesus monkeys and human subjects. Transactions of the Royal Society of Tropical Medicine and Hygiene, 69: 6.

Frisk-Holmberg, M. Bergqvist, Y., Demeij-Nyberg, B., Hellstrom, L. & Jansson, F. (1979) Chloroquine serum concentration and side effects; evidence for dose-dependent kinetics. Clinical pharmacology and therapeutics, 25: 345-350.

Frisk-Holmberg, M., Berqvist, Y., Termond, E. & Demeij-Nyberg, B. (1984) The single dose kinetics of chloroquine and its major metabolite, desethylchloroquine in healthy subjects. European journal of clinical pharmacology, 26: 421-430.

Frisk-Holmberg, M., Bergqvist, Y. & Termond, E. (1985) Further support for changes in chloroquine disposition and metabolism between a low and a high dose. European journal of clinical pharmacology,, 28: 721-722.

Gustafsson, L., Rombo, L., Alvan, G., Bjorkman, A., Lind, M. & Walker, O. (1983a) On the question of dose-dependent chloroquine elimination of a single oral dose. Clinical pharmacology and therapeutics, 4: 383-386.

Gustafsson, L., Walker, G., Alvan, G., Beerman, B., Estevez, F., Gleisner, L., Lindstrom, B. & Sjoqvist, F. (1983b) Disposition of chloroquine in man after single intravenous and oral doses. British journal of clinical pharmacology, 15: 471-479..

Gustafsson, L., Lindstrom, B., Grahnen, A. & Alvan, G. (1987) Chloroquine excretion following malaria prophylaxis. British journal of clinical pharmacology, 24: 221-224.

Holford, N. (1988) MKMODEL, an extended least squares modelling program. Cambridge, Elsevier-Biosoft.

Jennrich, R.I. & Moore, R.H. (1975) Maximum likelihood estimation means of nonlinear least squares. American Statistical Association: Proceedings of the Statistical Computing Section, pp. 57-65.

Karbwang, J., Looareesuwan, S., Phillips, R.E., Wattanagoon, Y., Molyneux, M.E., Nagachinta, S., Back, D.J. & Warrell, D.A. (1987a) Plasma and whole blood mefloquine concentrate during treatment of chloroquine-resistant falciparum malaria with the combination mefloquine-sulphadoxine-pyrimethamine. British journal of clinical pharmacology, 23: 477-481.

Karbwang, J., Bunnag, D., Breckenridge, A.M. & Back, D.J. (1987b) The pharmacokinetics of mefloquine when given alone or in combination with sulphadoxine and pyrimethamine in Thai male and female subjects. European journal of clinical pharmacology, 22: 173-177.

Kuroda, K. (1962) Detection and distribution of chloroquine metabolites in human tissues. Journal of pharmacology and experimental therapeutics, 137: 156-161.

Lindquist, N.G. (1973) Accumulation of drug on melanin. Acta radiological, 325: (suppl.) 1-92.

Looareesuwan, S., White, N.J., Warrell, D.A., Forgo, I., Dubach, U.G., Renalder, U.B. & Schwartz, D.E. (1987) Studies of mefloquine bioavailability and kinetics using a stable isotope technique. A comparison of Thai patients with falciparum malaria and healthy caucasian volunteers. British journal of clinical pharmacology, 24: 37-42.

Maggs, J.L., Colbert, J., Winstanley, P.A., Orme, M.L'E. & Park, B.K. (1986) Irreversible binding of amodiaquine to human liver microsomes; chemical and metabolic factors. British journal of clinical pharmacology, 23: 649P.

McChesney, E.W., Conway, W.D., Banks, W.F. Jr. Rogers, J.E. & Shekosky, J.M. (1966) Studies of the metabolism of some compounds of the 4-amino-7-chloroquinoline series. Journal of pharmacology and experimental therapeutics, 151: 482-493.

Metzler, C.H. & Weiner, D.L. (1986) PCNONLIN, Edgewood, Kentucky, Statistical Consultants.

Mihaly, G.W., Ching, M.S., Klein, M.B., Paul, J. & Smallwood, R.A. (1987) Differences in the binding of quinine and quinidine to plasma proteins. British journal of clinical pharmacology, 24: 769-774.

Nora, M.V., Thomas, R.W., Parkhurst, G.W., Ptak. L., Kennedy, E.J., Hohl, R., Garcia, B., Trenholme, G.M., MacLeod, C.M., Frischer, H. & Carson, P.E. (1987) Pharmacokinetics of primaquine in man after single oral doses. In: Wernsdorfer, W.H. & Trigg, P.I., ed. Primaquine: Pharmacokinetics, metabolism, toxicity and activity. New York, John Wiley and Sons, pp. 77-89.

Ogunbona, F.A., Lewal, A.A. & Onyeji, C.O. (1986) Saliva secretion of chloroquine in man. Journal of pharmacy and pharmacoogy, 38: 535-537.

Ogunbona, F.A., Onyeji, C.O., Bolaji, O.O. & Torimiro, E.A. (1987) Excretion of chloroquine and desethylchloroquine in human milk. British journal of clinical pharmacology, 23: 473-476.

Peck, C.C., Beal, S.L., Sheiner, L.B. & Nichols, A.I. (1984) Extended least squares nonlinear regression: a possible solution to the "choice of weights" problem in analysis of individual pharmacokinetics data. Journal of pharmacokinetics and biopharmaceutics, 12: 545-558.

Phillips, R.E., Looareesuwan, S., White, N.J., Silamat, K., Kietrinun, S. & Warrell, D.A. (1986) Quinine pharmacokinetics and toxicity in pregnant and lactating woman with falciparum malaria. British journal of clinical pharmacology, 21: 677-683.

Riviere, J.H. & Back, D.J. (1985) Effect of mefloquine on hepatic drug metabolism in the rat: comparative study with primaquine. Biochemical pharmacology, 34: 567-571.

Riviere, J.H., Back, D.J. Breckenridge, A.M. & Howells, R.E. (1985) The pharmacokinetics of mefloquine in man: lack of effect of mefloquine on antipyrine metabolism. British journal of clinical pharmacology, 20: 469-474.

Salako, L.A. & Idowu, O.R. (1985) Failure to detect amodiaquine in the blood after a single therapeutic dose orally. British journal of clinical pharmacology, 20: 307-311.

Salako, L.A. & Ajayi, F.O. (1987) Distribution and urinary excretion of the desethyl metabolites of chloroquine in the rat. Journal of pharmacy and pharmacology, 39: 859-860.

Salako, L.A., Walker, O. & Iyun, A.O. (1984) Pharmacokinetics of chloroquine in renal failure. African journal of medical sciences, 13: 177-182.

Salako, L.A., Aderounmu, A.F. & Walker, O. (1987) Influence of route of administration on the pharmacokinetics of chloroquine and desethylchloroquine. Bulletin of the World Health Organisation, 65: 47-50.

San George, R.C., Nagal, R.L. & Farby, M.E. (1984) On the mechanism of red cell accumulation of mefloquine, an antimalarial drug. Biochemica biophysica acta, 803: 174-181.

Schwartz, D.E. & Weidekamm, E. (1982) Pharmacokinetics of mefloquine, sulfadoxine and pyrimethamine combination in animals and man (Unpublished document WHO/MAL/82.980).

Schwartz, D.E., Weber, W., Richard-Lenoble, D. & Gentilini, M. (1980) Kinetic studies of mefloquine and one of its metabolites, RO-21-5104, in the dog and in man. Acta tropica, 37: 238-242.

Schwartz, D.E., Eckert, G., Hartmann, D., Weber, O., Richard -Lenoble, D., Ekue, J.M.K. & Gentilini, M. (1982) Single dose kinetics of mefloquine in man. Chemotherapy, 28: 70-84.

Schwartz, G. (1978) Estimating the dimension of a model. Annals of statistics, 6: 461-464.

Sheiner, L.B. (1985) Analysis of pharmacokinetic data using parameter models. II. Point estimates of an individual's parameters. Journal of pharmacokinetics and biopharmaceutics, 13: 15-540.

Silamut, K, White, N.J., Looareesuman, S. & Warrell, D.A. (1985) Binding of quinine to plasma proteins in falciparum malaria. American journal of tropical medicine and hygiene, 34: 681-686.

Tett, D.E., Cutler, D.J., Day, R.O. & Brown, K.F. (1988) A dose ranging study of the pharmacokinetics of hydroxy chloroquine following intravenous administration to healthy volunteers. British journal of clinical pharmacology, 26: 303-313.

Theoharides, A.D., Smyth, M.E., Ashmore, R.W., Halverson, J.M., Zhou, Z.M., Ridder, W.E. & Lin, A.J. (1987) Determination of dihydro-qinghaosu in blood. A major metabolite of the antimalarial drug artesunic acid by pyrolysis gas chromatography-mass spectrometry. Analytical chemistry, 60: 115-120.

Walker, O., Birkett, D.J., Alvan, G., Gustafsson, L.L. & Sjoqvist, F. (1983a) Characterisation of chloroquine plasma protein binding in man. British journal of clinical pharmacology, 15: 375-377.

Walker, O., Dawodu, A.H., Adeyokunmu, A.A., Salako, L.A. & Alvan, G. (1983b) Plasma chloroquine and desethyl-chloroquine concentrations in children during and after chloroquine treatment for malaria. British journal of clinical pharmacology, 16: 701-705.

Walker, O., Salako, L.A., Alvan, G., Ericsson, O. & Sjoqvist, F. (1987a) The disposition of chloroquine in healthy Nigerians after single intravenous and oral doses. British journal of clinical pharmacology, 23: 295-301.

Walker, O., Dawodu, A.H., Salako, L.A., Alvan, G. & Johnson, A.P.K. (1987b) Single dose disposition of chloroquine in kwashiorkor and normal children - evidence for decreased absorption in kwashiorkor. British journal of clinical pharmacology, 23: 407-472.

Ward, S.A., Mihaly, G.W., Edwards, G., Looaraeesuwan, S., Phillips, R.E., Chantavanich, S., Warrell, D.A., Orme, M.L'E. & Breckenridge, A.M. (1985) Pharmacokinetics of primaquine in man II. Comparison of acute vs chronic dosage in Thai subjects. British journal of clinical pharmacology, 19: 751-755.

Warhurst, D.C. & Hockley, D.J. (1967) Mode of action of chloroquine on Plasmodium berghei and P. cynomolgi. Nature, 214: 935-936.

Wattanagoon, Y., Taylor, R.B., Moody, R.R., Ochekpe, N.A., Looareesuwan, S. & White, N.J. (1987) Single dose pharmacokinetic of proguanil and its metabolites in healthy subjects. British journal of clinical pharmacology, 24: 775-780.

Wharton, B.A. & McChesney, E.W. (1970) Chloroquine metabolism in kwashiorkor. Journal of tropical paediatrics, 16: 130-132.

White, N.J. (1985) Clinical pharmacokinetics of antimalarial drugs. Clinical pharmacokinetics, 10: 187-215.

White, N.J. (1987) The pharmacokinetics of quinine and quinidine in malaria. Acta Leidensia, 55: 65-76.

White, N.J., Looareesuwan, S., Warrell, D.A., Warrell, M.J., Bunnag, D. & Harinasuta, T. (1982) Quinine pharmacokinetics and toxicity in cerebral and uncomplicated malaria. American journal of medicine, 73: 564-572.

White, N.J., Chanthavanich, P., Krishna, S., Bunch, C. & Silamut, K. (1983a) Quinine disposition kinetics. British journal of clinical pharmacology, 16: 399-404.

White, N.J., Looareesuwan, S. & Silamut, K. (1983b) Red cell quinine concentration in falciparum malaria. American journal of tropical medicine and hygiene, 32: 456-460.

White, N.J., Watt, G., Bergqvist, Y. & Njelesani, E. (1987a) Parenteral chloroquine for treating falciparum malaria. Journal of infectious diseases, 155: 192-201.

White, N.J., Looareesuwan, S., Sovenchai, Edwards, G., Phillips, R.E., Karbwang, J., Nicholl, D. D., Bunch, C. & Warrell, D.A. (1987b) Pharmacokinetics of intravenous amodiaquine. British journal of clinical pharmacology, 23: 27-135.

Winstanley, P.A., Edwards, G., Orme, M. L'E. & Breckenridge, A.M. (1986) The detection of amodiaquine in blood after oral administration. British journal of clinical pharmacology, 21: 552.

Winstanley, P.A., Rodick, C., Edwards, G., Orme, M. L'E. & Breckenridge, A.M. (1987a) Pharmacokinetics of amodiaquine after oral administration : effect of the dose size. British journal of clinical pharmacology, 23: 648P.

Winstanley, P., Edwards, G., Orme, M.L'E & Breckenridge A.M. (1987b) The disposition of amodiaquine in man after oral administration. British journal of clinical pharmacology, 23: 1-7.

Winstanley, P.A., Edwards, G., Curtis, C.G., Orme, M.L'E. Powell, G.M. & Breckenridge, A.M. (1988) Tissue distribution and excretion of amodiaquine in the rat. Journal of pharmacy and pharmacology, 40: 343-349.

UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (1986)) The development of artemisinin and its derivatives: Report of a meeting of the Scientific Working Group on the chemotherapy of malaria 6-7 October 1986. TDR/CHEMAL/ACTA/863, WHO/TDR.

Zhou, Z.M., Anders, J.C., Chung, H. & Theoharides, A.D. (1987) Analysis of artesunic acid and dihydroqinghaosu in blood by high performance liquid chromatography with reductive electro-chemical detection. Journal of chromatography, 414: 77-90.

CHAPTER 3 : DRUG ASSAYS AND THEIR APPLICATION

CONTENTS

---

* This chapter is based on the presentations of Professor W.H. Wernsdorfer and Dr. F.C. Churchill and on the relevant plenary discussions and laboratory demonstrations by the staff of the National Drug Research Centre, Universiti Sains Malaysia, Penang.

3.1 PRINCIPLES

3.1.1 Introduction

The assessment of the pharmacokinetics of a drug depends on the availability of sufficiently sensitive and reliable methods for the measurement of the concentrations of the parent compound and its major metabolites in biological material. With respect to antimalarials and their use in humans, the biological material will usually consist of blood plasma, whole blood or urine, occasionally also of serum, saliva or separated cellular elements of blood, but quite rarely of tissues (drug concentrations in the latter are, however, an important part of preclinical studies in animals).

Unlike pharmacognostic and quality control tests, assays of drugs in biological material need to be highly sensitive since feasible and effective plasma concentrations of some drugs may be as low as 10^{-10} mol/l. On the other hand the drugs and their metabolites are accompanied by a large number of other chemical substances most of which will be physiological components of the biological material, others may be patho-physiological components of human origin and yet others foreign substances originating from the intake of food and/or medicaments.

With the exception of enzyme-linked immunosorbent assays (ELISA) and bioassays, it is necessary to extract the drug and/or its metabolites from the biological material prior to the qualitative or quantitative assay in order to eliminate coreactive substances. In many instances, a simple extraction by one or several solvents may not be sufficient for achieving the required purity. In such instances, chromatographic separation will be needed, e.g. by thin layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), gas chromatography (GC) or high performance/high pressure liquid chromatography (HPLC).

In some cases the physicochemical characteristics of the compounds are such that they render a chromatographic separation difficult or impossible especially by GC and HPLC . In these cases derivatization may overcome the problem and yield derivatives which can be chromatographically processed.

Since every extraction and derivatization step is invariably associated with a loss of the drug and/or its metabolites, it is important to keep the number of such steps to the minimum. This can usually be achieved by the selection of the most appropriate extractant(s) and derivatizing agent.

The detection and measurement of the drug and its metabolites is finally effected by the use of a suitable detection system such as:

densitometry
absorption spectrophotometry (visible light)
ultraviolet spectrophotometry
fluorometry
electrochemical detection
electron capture detection
mass spectrography
infrared spectrrophotometry

The reliability and accuracy of the assay systems, starting from the collection of the biological samples until the final reading, should be assured by adopting rigidly standardized procedures for every step (this includes also the use of standardized equipment and reagents) and by the use of internal standard for calibration and monitoring of variation.

In the context of the human pharmacokinetics of antimalarial drugs, blood plasma, whole blood and urine are the usual biological materials to be examined for drugs. Therefore the following considerations will be confined to these materials and to the major assay methods used for antimalarials with the exception of the ELISA systems which are covered in section 3.2.3 of this chapter.

3.1.2 Drug extraction

The drug is extracted by the use of a suitable solvent or a mixture of solvents. In some cases adequate purity may be obtained only after the successive use of several solvents and column chromatography. In this context the extraction efficiency is related to the polarity of the drug and the polarity of the solvent. Some common solvents are given in Table 3.1.

TABLE 3.1 COMMON SOLVENTS AND THEIR POLARITY, 1975

Solvent	Dielectricity constant (at 25°C)	o
Hexane	1.89	0.01
Heptane	1.92	

TABLE 3.1 (CONTD.)

Solvent	Dielectricity Constant (at 25°)C	Σ^{o}
Cyclohexane	2.02	0.04
Benzene	2.28	-
Toluene	2.38	0.29
Acetonitrile	3.88	0.65
Diethyl ether	4.34	0.38
Chloroform	4.87	0.40
Formic acid	5.0	-
2 Methylbutan-2-ol	5.82	-
Ethyl acetate	6.02	0.58
Acetic acid anhydride	6.15	-
Tetrahydrofuran	7.58	0.45
Dichloromethane	9.14	-
2-Methylpropan-2-ol	10.9	-
Pyridine	12.3	-
2-Butanol	15.8	-
2-Methylpropan-1-ol	17.7	-
1-Butanol	17.8	-
2-Propanol	18.3	0.82
1-Propanol	20.1	-
Acetone	20.7	0.56
Ethanol	24.3	0.88
Methanol	33.6	0.95

Moreover, the pH of the sample preparation prior to and at intermediate steps of extraction has a major bearing on the efficiency of extraction. The optimum pH is therefore obtained by the use of appropriate buffers. The acids and bases most commonly used for the preparation of buffers in biochemical work (including drug assays) and their pKa are listed in Table 3.2.

Examples of extraction procedures specific for antimalarial compounds are given in Appendix 4.

Table 3.2 ACIDS AND BASES COMMONLY USED IN DRUG EXTRACTION PROCEDURES 1975
(Selected from Williams & Wilson, 1975)

Acid or base	pKa at 25°C
Acetic acid	4.75
Barbituric acid	3.98
Carbonic acid	6.10; 10.22
Citric acid	3.10; 4.76; 5.40
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid[a]	7.50
Phosphoric acid	1.96; 6.70; 12.30
Piperazine-N-N'-bis(2-ethanesulfonic acid)[b]	6.80
Phthalic acid	2.90; 5.51
Succinic acid	4.18; 5.56
Tartaric acid	2.96; 4.16
2-Amino-2-hydroxymethylpropan-1,3-diol[c]	8.14

a HEPES
b PIPES
c TRIS

The pH dependence of a given assay method applies as long as the drug is contained in a liquid phase, i.e. in the case of HPLC until and inclusive of the mobile phase.

Often the degree of purity achieved by extraction, especially that obtained by column chromatography, is adequate for using the extract directly in absorption spectrophotometry in visible or ultraviolet light or in fluorospectrometry techniques. However, if higher resolution/sensitivity is required it is necessary to use a highly efficient chromatographic procedure (TLC, HPTLC, GC, HPLC) for a further separation/purification of the drug (or metabolite) concerned.

3.1.3 Chromatographic separation techniques

Chromatography is based on the distribution of a specific compound between two non-miscible phases. At equilibrium the distribution of such a compound between two such liquid phases or solvents can be described at a given temperature by the distribution coefficient(K) :-

$$\text{Distribution coefficient(K)} = \frac{\text{concentration in solvent 1}}{\text{concentration in solvent 2}}$$

The two phases however, may also be solid/liquid or liquid/gas, when the distribution coefficient(k) is defined as

$$K = \frac{\text{concentration in the mobile phase}}{\text{concentration in the stationary phase}}$$

In contrast, the effective distribution coefficient (Ke) is defined as

$$Ke = \frac{\text{total quantity of compound in Phase 1}}{\text{total quantity of compound in Phase 2}}$$

and thus as the product of distribution coefficient and the ratio between the volumes of Phase 1 and Phase 2.

In order to separate the compounds, the stationary and the mobile phases are selected in such a way that the compounds have different distribution coefficients. In practice this may be achieved by an adsorption equilibrium between a stationary solid phase and a mobile liquid phase (adsorption chromatography) or a distribution equilibrium between a stationary liquid and mobile gas phase (gas/liquid chromatography). There are also various other equilibria which play a part but these are of lesser importance in drug assays.

The principle of separation is best illustrated by a glass column which is packed with the stationary phase to a height of 10 cm and where 1 cm height corresponds to 1 ml. When the effective distribution coefficient of the compound is 1 (as in Fig. 3.1), and the column is dosed with 1 ml of the test solution, followed in successively by 1 ml quantities of solvent, then 50% of the compound will be bound to the stationary phase at each step. After 10 additions of 1 ml of solvent, distribution of the compound in the column will be such that the maximum concentration will be at the centre of the column. Compounds with an effective distribution coefficient of <1 will show more than 50% adsorption to the stationary phase at each step and the maximum concentration will be situated in the upper half of the column while compounds with an effective distribution coefficient of > 1 will have the maximum concentration in the lower half (see Fig. 3.2).

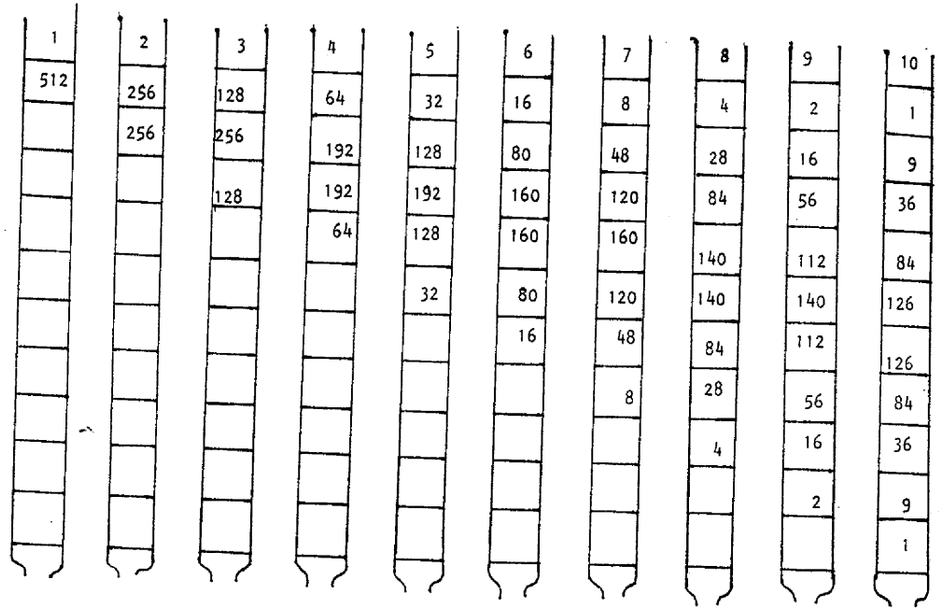


Fig. 3.1 Principle of column-chromatographic separation.

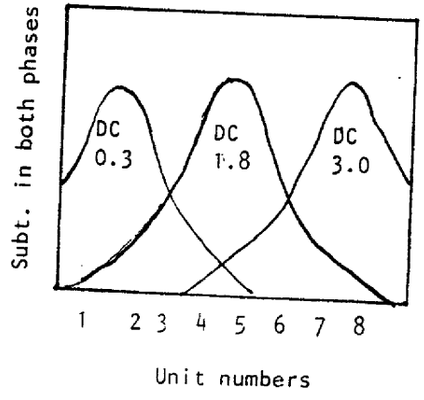


Fig. 3.2 Distribution of substances with different distribution coefficients [DC].

The concentration in a particular part of the column will be the higher the greater the number of equilibrium stages in the column. In practice, the flow of solvent in the column is continuous, resulting in thousands of equilibrium stages ("theoretical platforms") and the flow rate of a column is dependent on the compound's effective distribution coefficient. The resolution of the compound in a column is dependent on the number of equilibrium stages to which it has been exposed while passing through the column (see Fig. 3.3).

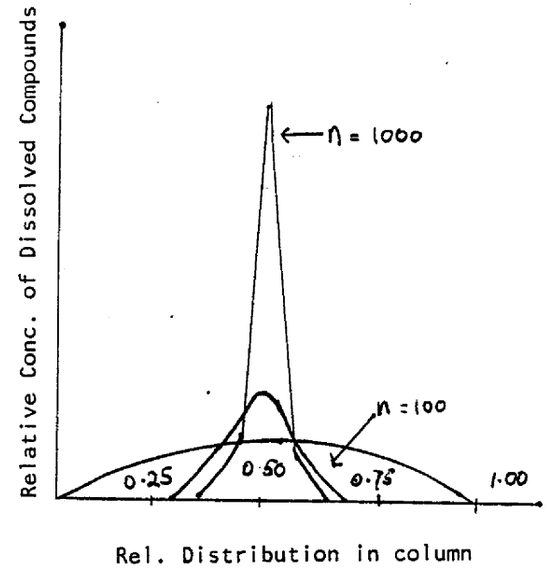


Fig. 3.3 Correlation between number of platforms ("plates") and band pattern.

This principle is applicable to liquid/liquid phase, liquid/solid phase and gas/liquid phase chromatography. With the exception of the gas/liquid, these systems do not essentially use columns but may involve paper with liquid/liquid phase chromatography and thin-layer plates with liquid/solid phase chromatography.

3.1.3.1 Thin-layer chromatography (TLC)

Thin layer chromatography (TLC) can be used on a micro-scale for the separation of very small quantities of material as in drug assays, but it may also be employed at the preparative stage for the isolation of drug metabolites from biological material.

TLC is, in principle, a form of adsorption chromatography. The carrier is a glass plate, aluminium foil on a plastic sheet (see Fig. 3.4), coated with the solid phase (silica gel, aluminium oxide, Kieselguhr, magnesium silicate or cellulose) the adherence of which is usually assured by mixing the solid phase with a "binding agent" such as calcium sulfate.

Ready-made TLC plates, precoated with various carrier and solid phase materials, are commercially available.

The choice of the mobile phase depends on the compound(s) to be separated. The mobile phase may consist of a single solvent or a mixture of solvents (see Table 3.1 in section 3.1.2). The solvents should be pure (chromatography grade), stable in air, non-toxic and easily removable from the plate after the separation is completed. It is clear that the solvents should not react with the compound(s) to be separated. After coating with the carrier and drying (superfluous if ready-made plates are used) the plates are marked with a line parallel to and 2 cm distant from the plate's lower edge. Sample preparations are applied along this line at 1 cm distances from each other. A normal plate can thus hold from 10 to 20 samples. The sample preparations (solutions) are applied in quantities of 1-10 ul, ensuring that the spot will not exceed 4 mm in diameter (otherwise resolution may be lost). The micropipettes used for this purpose should be reliably calibrated (max ± 2% of specified volume), the solvent volatile and of low polarity. The spots should be dried before placing the plate into the chromatographic chamber.

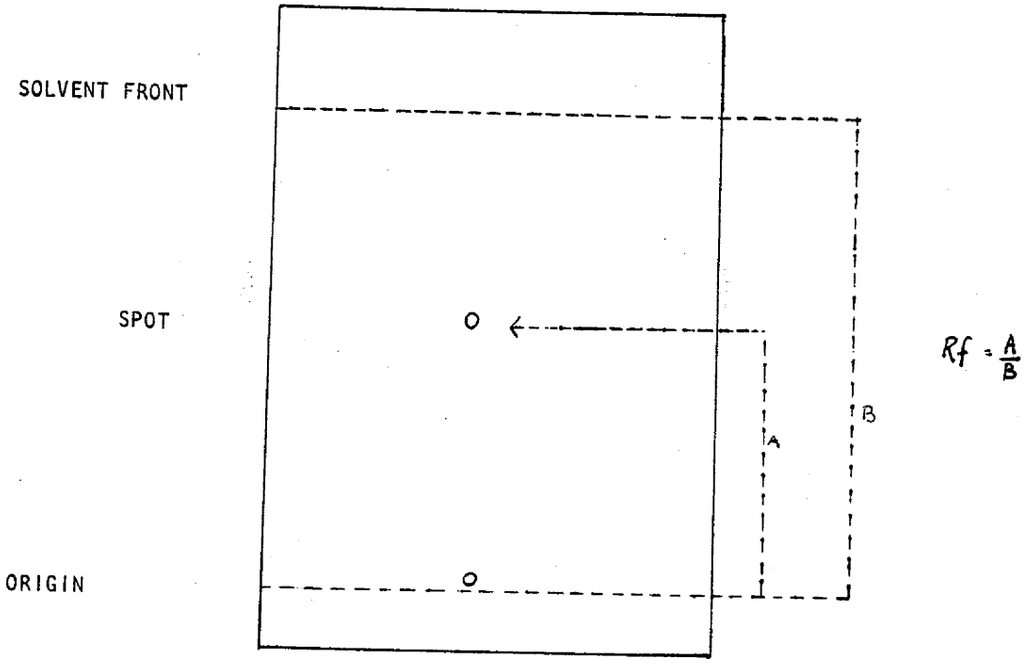


Fig. 3.4 Thin-layer chromatography (TLC)

The chromatographic chamber generally consists of a rectangular glass tank of 25 cm height which has a sealed lid and is filled with the mobile phase up to a height of 10-15 mm. It is a wise precaution to flush the chamber with nitrogen prior to its saturation with the vapour of the mobile phase (this helps avoiding the oxidation of compounds during the process). Saturation of the chamber's atmosphere is normally achieved in 30 minutes. at normal ambient temperatures but it is safer to flush the chamber for 60 minutes. The plate is then placed vertically in the chamber with the sample spots just above the mobile phase. The chamber is then closed and the plates are left until the solvent rises 10-15 cm up the plate. The time required for this to occur depends on the solvent. "Runs" of 10-15 cm taking usually 45-120 minutes, produce the best resolution, but short "runs" of 5 cm are faster and have the advantage of higher sensitivity.

When the "run" to the predetermined distance has been completed, the plate is removed from the tank, the distance run by the mobile phase is noted and the plate is dried either naturally or by a hot air dryer. The compounds are detected on the plate by spraying it with an appropriate staining solution.

A specific compound has a specific Rf value in TLC.

$$Rf = \frac{\text{Distance of compound from the origin}}{\text{Distance solvent front travelled from origin}} \times 100\%$$

The distance the compound has travelled from the origin is measured from the centre of the original dot to the centre of the substance spot after the "run". If the spot is "tailed", the distance is measured to the densest part of the spot. The Rf values may be subject to considerable variation due to the quality of the plates (stationary phase), the mobile phase, the condition of the chromatography vessel, the temperature, the amount of sample applied and the time allowed for the separation. TLC is therefore more suitable for qualitative than for quantitative work. Whatever the use an internal standard should be run in parallel with the samples.

Compounds may be separated in two-dimensions by TLC. For this purpose only one sample is put on the plate in the lower left corner (2.5 cm from the left and 2.5 cm from the lower edge). The plate is developed in one solvent vertically, taken out and dried. Then it is developed again (standing on the left side) in another solvent. This technique produces a 2-dimensional resolution. It is particularly helpful in the separation of parent drugs and metabolites.

3.1.3.2 High-performance thin-layer chromatography [HPTLC]

High performance thin layer chromatography (HPTLC) is superior to ordinary TLC since it employs a smaller particle size (2-7 um) and a narrower particle size distribution in the stationary phase, and smaller carrier plates. The typical HPTLC plate measures 100 x 100 mm and has a thickness of 150-200 um. The sample volume is <0.1 ul and the sample spot diameter before development < 1.5 mm. The developed spot does not usually exceed 5 mm in diameter. The running distance is 3-6 cm, shortening the running time to 5-15 minutes. For strongly absorbing compounds the detection limit is between at 0.5 and 5 ng compared to 10-100 ng for normal TLC and at 0.01-0.1 ng compared to 0.1-1 ng for normal TLC of strongly fluorescing substances. The Rf values and the quantification is more reproducible. Other identification techniques cannot be used after eluting a separated sample as the absolute quantities of the compound(s) are very small.

3.1.3.3 Gas chromatography (GC)

Gas chromatography (GC) separates the compounds on a column containing the stationary phase as a liquid. The stationary liquid phase covers an inert granular carrier material and is contained in a relatively thin glass or metal column maintained at a defined temperature in an oven. The mobile phase consists of an inert carrier gas, e.g. N_2orAr, which is pumped through the column. At a given temperature the test compound evaporates and the carrier gas will transport it through the column. The distribution between the liquid and the gas phase will depend on the distribution coefficients of the compounds. Those with a high distribution coefficient will stay longer in the column while those with a low distribution coefficient will come out faster. After the compound has come out of the column it passes an appropriate detector (see section 3.1.4) which is fitted with a recorder.

The support material for the liquid phase usually consists of various Chromosorb materials based on specially treated diatomaceous earth ground to a specific particle size. Prior to use, the support material must be deactivated in order to remove impurities which could interfere with the separation process. Silanizing agents are employed for reducing adsorptive effects caused by the presence of surface OH-groups. Deactivated support material for packing the columns is commercially available. Some support materials have the liquid phase already bonded to them.

There is a large choice of liquid phase materials which can be classified according to their McReynolds Constants which reflect polarity and can be used to compare the ability of given stationary phases to separate different classes of compounds.

On the whole, two types of columns, namely SE-30 (Max OT 300° and Reynolds Constant of 217) and OV-17 (Max OT 350° and Reynolds Constant of 886) are adequate for most drug assays. The liquid phase of the SE-30 column is a dimethyl silicone polymer, whereas that of the OV-17 column is phenylmethyl silicone. The OV-17 column is more oxygen-sensitive than other silicone liquids.

Also capillary columns are available. They are mostly made of silica (natural quartz), soda glass, borosilicate glass, or stainless steel, and have an internal diameter of 0.2 - 0.4 mm and a length of 10-50 m (coiled). These capillary columns produce a very high resolution and purity of the compounds after separation. The high efficiency of these colunns is evident from the narrow, tall peaks and a high signal/background noise ratio which enhances the assay sensitivity. The walls of the capillary columns are coated either with the liquid phase or with a support (such as microcrystals of NaCl or $BaCl_2$) bonded on the glass walls.

After the installation of the column between the injector inlet and the detector end, the column is conditioned in order to eliminate volatile impurities and purge oxygen from the system. For this purpose the detector is disconnected and a flow of carrier gas of 10-20 ml/minute maintained for 60 minutes in normal GC columns. Thereafter the temperature of the oven is raised (1°C/minute with automatic programmer or 25°C/30 min with manual operation) until it reaches 20°C above the operating temperature. This is maintained for 12 hours with careful monitoring so that the maximum operating temperature is not exceeded.

The samples are injected by means of a syringe and a needle through a silicone-rubber septum. The sample may be injected into a heated zone in the gas stream anterior to the column or directly into the column, especially in the case of labile substances. A high degree of purity of the solvent is mandatory in order to avoid contamination of the column.

It is sufficient to operate the oven at one specific temperature, whereas the separation of complex mixtures may require a variation of the column temperature during the analysis; most GC instruments have devices for programming the desired temperature changes.

A constant carrier gas flow is an essential prerequisite for consistent and reproducible results. Automatic flow controllers will facilitate the maintenance of the specified gas flow.

The performance of a column is judged from the narrowness of the peak and its symmetry. Ideally it should show full resolution, with a narrow base and no "tailing" or "fronting". There are formulae for expressing the quality of the GC peak (Leach & Ramsey, 1986). One of these allows a comparison to be made between different laboratories. This is the retention index (RI) which is based on the retention time of the test compound in relation to the retention time of paraffins with two different chain lengths according to the formula:

$$RI = 100\ (P_{z+n} - P_z) \left[\frac{\log t_R(x) - \log t_R(P_z)}{\log t_R(P_{z+n}) - \log t_R(P_z)} \right] + 100\ P_z$$

Where P_z = number of carbon atoms in the shorter chain paraffin

P_{z+n} = number of carbon atoms in the longer chain paraffin

t_R = retention time

x = test compound (unknown)

A variety of factors affect the reproducibility and accuracy of the method. It is preferable to use the lowest oven temperature yielding an analysis within a reasonable time. An acceleration is better achieved by reducing the stationary phase loading than by increasing the column temperature. The particle size of the column packing should not exceed 1/28 of the column's diameter. The particle size distribution should be as narrow as possible. Inert non-polar solvents are preferable as the stationary phase like others may derivatize the test compound. The carrier gas flow (mobile phase) should be adjusted to both the type of column and gas used.

3.1.3.4 High-performance liquid chromatography (HPLC)

This method of separation is based on the use of stainless steel columns packed with a stationary solid phase (particle size < 10 um) and elution by a liquid phase under high pressure. The apparatus consists of a reservoir for the eluant, a high pressure pump, an injector for introducing the test sample, the stainless steel column, a detector (see section 3.1.4) and a recorder.

The columns usually have an inner diameter of 4.5 - 5.0 mm and a length of 10-30 cm. The packing is retained by mesh disks at both ends of the column.

Since the HPLC apparatus is operated under high positive pressure a valve injector is required which introduces the sample under pressure into the system, ensuring that the system remains filled at all times. The flow rate in the HPLC column is usually between 1 and 3 ml/minute, at a pressure between 3.4and 27.6MPa(34-276 kg/cm^2). There are constant pressure or constant flow systems. The latter are preferred as they compensate for changes in column resistance and eluant viscosity, thus ensuring the highest stability of the detector response. The most common HPLC pumps are multiple piston electrical reciprocating pumps which produce a very regular flow from relatively small piston volumes (~ 100 ul) since the 2 or 3 pistons are working at 180^0 or 120^0 phase shift. The flow produced by these pumps can be regulated by adjustment of the piston speed.

HPLC systems for drug assays usually employ adsorption or partition chromatography. In adsorption chromatography the stationary phase is solid and the mobile phase liquid, the solid phase being more polar than the mobile ("normal phase"). In partition chromatography the stationary phase is liquid, coated on an inert solid support and immiscible with the mobile phase eluant. The system can be "normal phase" if the stationary phase is more polar than the mobile phase or "reversed phase" if the mobile phase is more polar than the stationary phase.

The columns are packed with rigid, round, porous particles with a diameter of 3-10 um. Silica is the most widely used material; commercial products have generally a pore size in the range of 4-33 nm and a surface area of 100-800 m^2/g. This is a polar material which is particularly suitable for "normal phase" HPLC. Reaction of silica with organochlorosilanes or organoalkoxysilanes produces a non-polar surface which is suitable for "reversed phase" HPLC. For this purpose octadecyl-silica (ODS-silica) is most widely used.

There are also polymer-based packing materials which are particularly useful for reversed-phase HPLC and more stable over wide pH ranges than ODS-silica which does not tolerate a pH below 3. Since prepacked columns are quite expensive, packing is generally done in the laboratory.

The purity of solvents, stationary and mobile phase, is of utmost importance in HPLC in order to avoid spurious peaks and high background noise. The purity of the solvents extends to the absence of particulate matter and the absence of dissolved air which could lead to bubbles in pump, column and detector and thus to serious technical complications.

There is a greater latitude in choosing solvents for use in TLC than in HPLC. In the latter case the solvents must have properties that will not degrade the performance of the column with continued use and must be compatible with the detector

system employed. For example, a solvent which strongly absorbs ultraviolet light cannot be used with an ultraviolet spectrophotometric detection system for HPLC and the electrochemical detector used in HPLC requires electrolytes in the mobile phase to permit operation.

The samples are usually dissolved in the mobile phase before injection. Where this is not possible and a different solvent is employed, the injected volume should be small to keep detector disturbances to a minimum.

3.1.4 Detection systems

There is a variety of detection systems applicable either directly to the biological material (ELISA or bioassays), or after extraction of the compound without further separation/purification (spectrophotometry and fluoroscopy) or following high-grade separation by TLC, GC or HPLC (spectrophotometry, fluoroscopy, mass spectrography and other physicochemical systems).

3.1.4.1 Ultraviolet (UV) and visible light spectrophotometry

Changes of light absorption/extinction caused by a compound constitute the underlying principle of spectrophotometry. A uniform, transparent medium will absorb light uniformly. The fraction of light (radiation) passing the medium is called transmission (T) expressed as:

$$T = \frac{I}{I_o}$$

Where I_o = intensity of incident radiation

I = intensity of transmitted radiation

The absorption (A) or extinction (E) equals $\log \frac{1}{T}$ or $\log \frac{I_o}{I}$

The law of Lambert-Beer indicates that the extinction is proportional to the concentration of the absorbing substance and the thickness of the layer

$$E = \varepsilon cd$$

where ε = molar extinction coefficient for the absorbing material at wave length

c = molar concentration of the absorbing solution

d = path-length of light beam in the absorbing material (in cm)

T is usually expressed in the range of 0-100% but extinction may vary between 0 and oo.

Since the molar extinction coefficient ( ) of a compound can be quite high, one may quote in such cases the extinction of a 1% aqueous solution at a light-path of 1 cm, denoted $E^{1\%}_{1cm}$.

The applicability of the law of Lambert Beer may be compromised by a variety of factors such as ionization, the polymerization of compounds at high concentrations (rarely with drug tests) or failure of the instruments to produce a sufficiently narrow band of wave lengths. Stray light is an important source of error/disturbance in spectrophotometry. Also solvents absorb radiation and, if their absorbance spectrum coincides with that of the test compound, a precise measurement of the test compound may become impossible. A change of solvent will then be necessary. Test and solvent absorption/extinction can be compensated by running a solvent control in parallel to the test.

For spectrophotometry in visible (400-700 nm) and UV (200-400 nm) light one employs usually a double-beam instrument which permits the simultaneous reading of the sample and the reference (control) cell. After passing through the sample and the control, the split beam is recombined at the detector which records signals 10-20 times per second. The instrument's monochromator is driven by a scan motor in order to effect a set and constant change in wave length per time unit. This move is synchronized with a recorder to produce the spectrum. Usually a scan speed below 2 nm/s is employed, but modern computer-controlled equipment (with a high frequency of signals) permits scanning speeds of up to 20 nm/s without compromising resolution.

Rapid-scan spectrophotometers with a linear array of several hundred photodiodes are able to record simultaneously a relatively large band-width, e.g. 200 nm, within a fraction of a second at a resolution of 0.5 nm.

For the visible light range, matched pairs of glass cuvettes are used, but UV-spectrophotometry requires the use of quartz or fused silica cells which have high transmittance for light in the 190-1000 nm range. The light path of the cuvette is usually 1 cm.

Fig. 3.5 shows the composition of a dual-beam spectrophotometer and Fig. 3.6 a typical extinction spectrum.

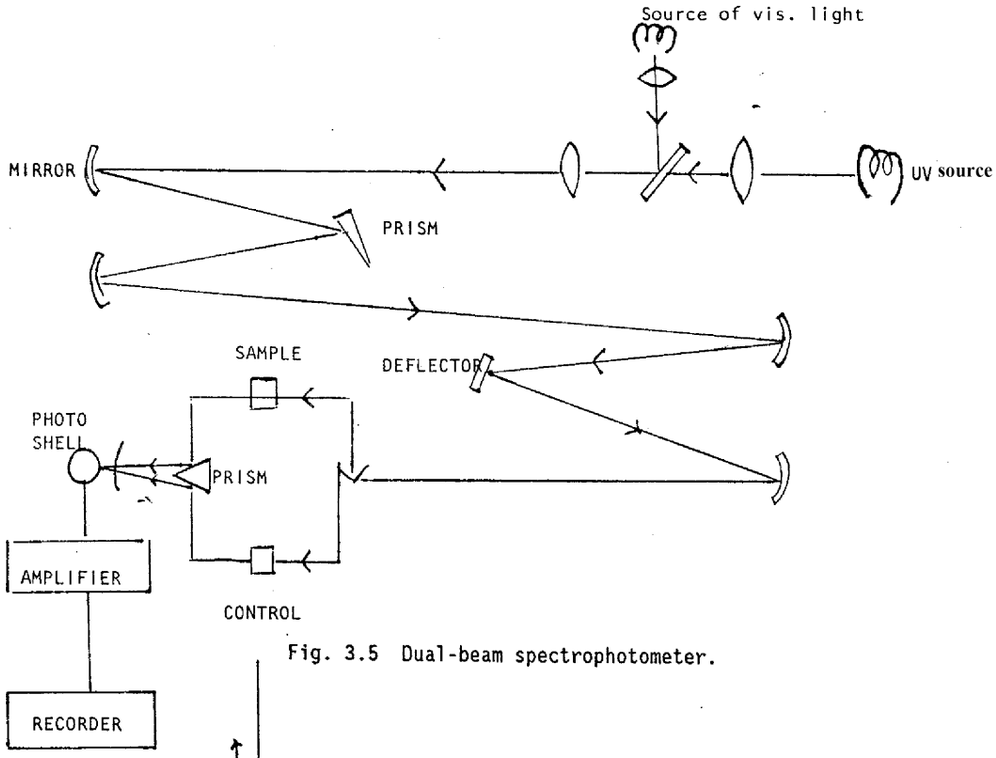


Fig. 3.5 Dual-beam spectrophotometer.

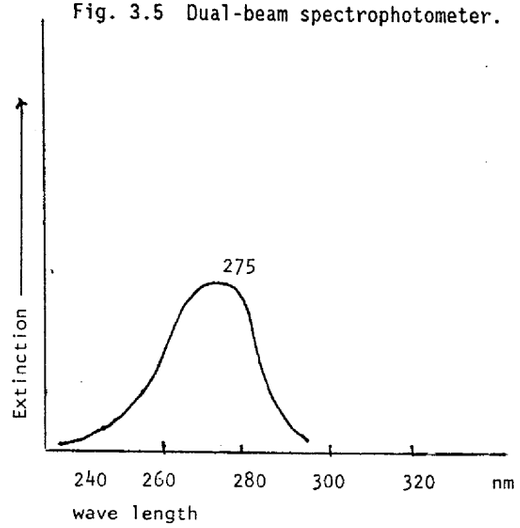


Fig. 3.6 UV extinction spectrum of ubiquinone.

The quantitation of light and UV spectroscopic readings is effected according to the law of Lambert-BBeer.

By special adaptation, UV and visible light spectrophotometry can also be used in association with HPLC (continuous flow analysis with special cuvettes).

3.1.4.2 Fluorescence spectrophotometry

Fluorescence denotes the ability of a molecule to emit radiation of a longer wave length after the absorption of radiation of a shorter wave length. Thus, light in the UV range may be absorbed and light be emitted in the visible range. In practice, a given excitation wave length (λ_{ex}) is used and the fluorescence emission wave length (λ_f) recorded. The difference between λ_{ex} and λ_f is described as the Stokes Shift.

The samples can be analysed as dilute solutions, gases, suspensions and solid surfaces. Fluorescence spectrophotometry can thus be combined with a large range of chromatographic procedures, especially TLC and HPLC.

The instrument consists, in principle of an excitation light source, an excitation mono-chromator, the quartz sample cell, an emission monochromator, a tube detector, an amplifier and a recorder (see Fig. 3.7). Modern fluorescence spectrophotometers have independently motor-driven scanning monochromators for λ_{ex} and λ_f which permit the rapid production of a fluorescence spectrum (Fig. 3.8). The latter should, in principle, match the absorption spectrogram.

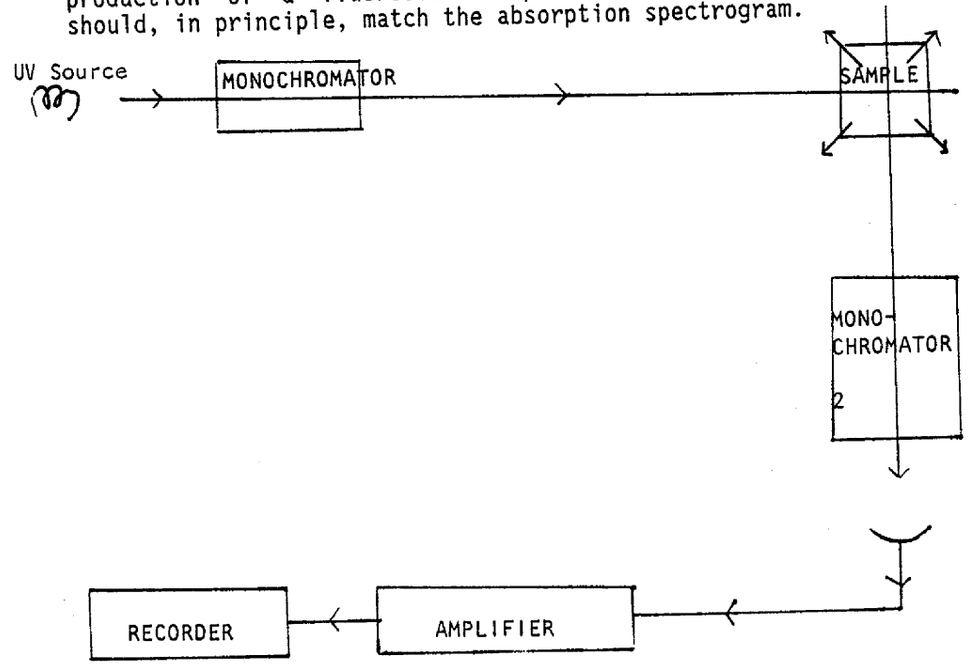


Fig. 3.7 Fluorospectrophotometer

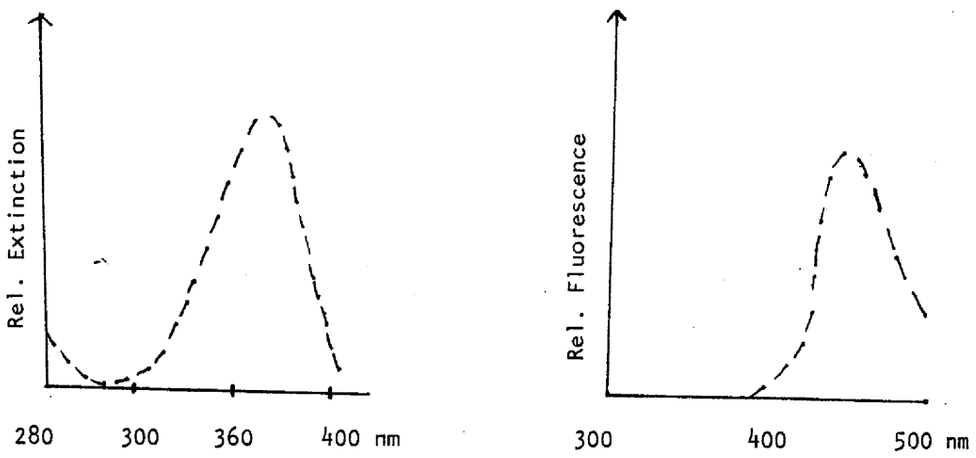


Fig. 3.8 Absorption and fluoroscence spectra of methylumbelliferon-anion.

Fluorescence spectrophotometry is considerably more sensitive than absorption spectrophotometry.

3.1.4.3 Electron capture detection (ECD)

This form of detection is particularly suitable for use with GC. It is based on the principle that substances with a high affinity for electrons, e.g. compounds containing a halogen, nitro group or carbonyl group, are able to reduce the electron flow in an otherwise constant electron flow field by capturing electrons.

The detector consists typically of a chamber with two parallel electrodes under 2-30 volts tension and a radioactive source such as ^{63}Ni near the cathode in order to ionize the carrier gas. Thus, a constant electron flow is established which is only reduced in the presence of substances that capture electrons. This entails a loss/reduction of signal. The problem of the nonlinearity of signal suppression has been overcome in modern electron capture detectors by the use of automatic (electronic) linearization devices, and the addition of small amounts of a quench gas e.g. methane to the carrier.

Electron capture detection is highly selective and sensitive. It can detect 1 pg of substance. Compounds which are primarily unsuitable for ECD due to the lack of electron-capturing atoms or groups may be detected by this method following appropriate derivatization, e.g. with hepta-fluorobutyric anhydride which would add reactive halogen atoms to the compound.

3.1.4.4 Electrochemical detection

In an electrochemical detector the compounds undergo electrolytic oxidation or reduction at the surface of an electrode resulting in the generation of an electric current which can be measured. Two electrochemical detection devices are available. The coulometric detector operates with a large electrode surface at which the whole compound can be reduced or oxidised. This results in the generation of larger current responses than the amperometric detector which has small electrodes and an electrochemical conversion of only about 10% of the substance. However, in practice the sensitivity of the two systems is not substantially different.

The most widely used electrode material is glassy carbon. This is subject to contamination which may interfere with operation and reliability of the electrode. Mercury electrodes can also be used. They have the advantage of a continuous renewal of the electrode surface but they can only be used in the reduction mode.

In order to be suitable for electrochemical detection (e.g. in connection with HPLC) the eluant, or mobile phase must be electrically conducive. This is achieved by adding chemically inert electrolytes at concentrations of 0.05 - 0.1 M, e.g. potassium nitrate with aqueous eluants and tetra-alkylammonium perchlorates with organic eluants.

3.1.4.5 Mass spectrometry (MS)

This technique can be used for the structural analysis of compounds and the confirmation of identity of specific molecules. Apart from these qualitative uses, it can also be employed for quantitative analysis where it offers the advantage of very high sensitivity. MS may yield a complete structural analysis from 50 pg of material and once the characteristics of a particular substance are known the limits of detection may come down to the femtogram range.

The principle of MS consists of the conversion of a molecule into the parent molecular ion and ionized fragments. These are all positively charged ions which can be separated according to their different mass/charge (m/z) ratios. The way a molecule fragments during the ionization process is typical for the particular compound, i.e. "cracking pattern" analogous to the "fingerprint" in infrared spectrophotometry analysis.

Mass spectra show a series of peaks or lines which correspond to the m/z values of the parent molecular ion and the fragment ions.

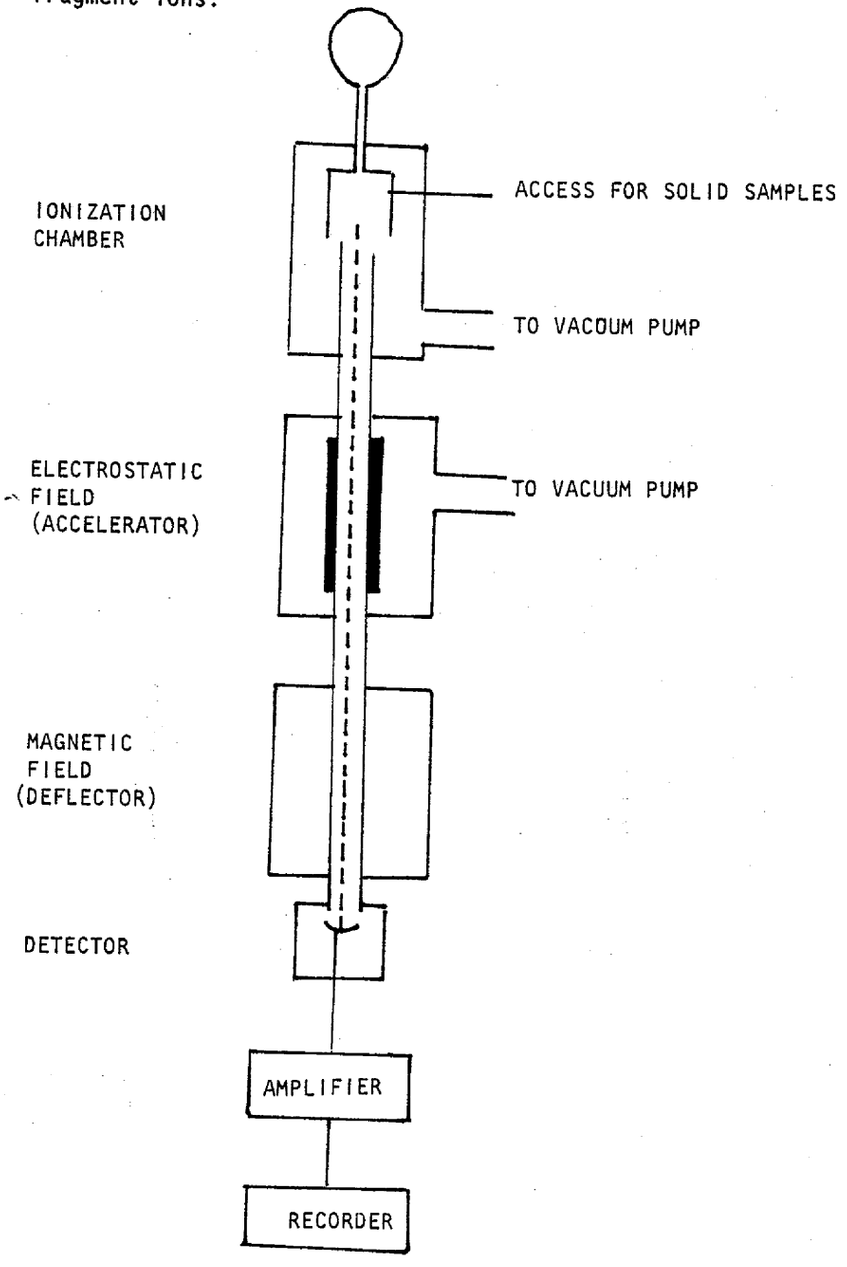


Fig. 3.9 Composition of a mass spectrometer.

The MS analysis is carried out in a very high vacuum (10^{-5}Pa). The MS apparatus (Fig. 3.9) consists of a reservoir of the sample gas which feeds an ionization chamber (this may also accommodate solid samples). Ionization is effected by bombardment with electrons, strong electric fields or UV light. The ionized sample passes into an electrostatic accelerator (vacuum) where the ions are accelerated to a constant speed. They then pass a strong magnetic field where they are deflected from their original path according to their mass. Ions with the lowest mass are subject to the highest degree of deflection. The detector is a simple electrode (Faraday cage) or an electron-multiplier. The ion current is subsequently amplified and recorded. The number of ions of a given mass arriving in the detector is a measure of the frequency of this ion (permitting quantitative analysis).

Modern analytic MS equipment permits simultaneous wide-spectrum recording. However, for quantitative analysis only a few typical peaks are selected and recorded and then correlated with those obtained from internal standards.

MS can be theoretically interfaced with any chromatographic method. However, this is rarely done for material originating from TLC since the purity of the eluate is usually inadequate. GC and HPLC produce compounds of the desired purity. Capillary GC can be directly linked to the MS since the gas flow can be accomodated without undue disturbance of the vacuum. For GC with packed columns, the carrier gas flow is too strong for a direct link-up with the mass spectrometer. Therefore an interface is used which eliminates most of the carrier gas before the compound arrives in the ionization chamber. The greatest difficulty is experienced with interfacing HPLC and MS since the evaporated solvents would cause a breakdown of the vacuum in the mass spectrometer. Devices for the evaporation and selective withdrawal of the mobile phase, i.e. "moving belt interface", resolve this problem.

3.1.4.6 Infrared spectrophotometry (IRS)

The infrared range of the electromagnetic spectrum extends from 1 to 250 um, but only the range of 2.5 - 20 um is normally used for infrared spectrophotometry.

For an asymmetrical molecule with n atoms the theory of molecular oscillation predicts 3n-6 basic oscillations. The infrared spectrum shows those oscillations which cause a move of the charge. The specific absorption spectrum is characteristic (and unique) for a given compound, i.e. a "fingerprint".

All commercial IR spectrophotometers are dual beam instruments, for simultaneous reading of the sample and the control. They work with NiCr burners since these produce the

desirable range of wavelengths. The light passes through the sample and the control and a wedge or comb attenuator to equalize absorption in both beams. The light then passes via a mirror to the detector which is linked to an amplifier and a recorder. The detector may be a thermocouple or a Goloy cell. The recorders are usually programmed to linearize the readings.

IRS can be interfaced with GC and HPLC but the application to TLC samples entails an elution process which does not always yield the compound(s) at an adequate purity.

3.1.4.7 Densitometry

This is applicable to TLC spots which have been visualized. Initially, the wavelength which yields maximum instrument response is determined. Coloured spots are best scanned at a complementary wavelength. Once the best wavelength has been determined, the sample can be examined, preferably in a double-beam mode, determining the control/reference background at the same time.

Transmission scanning is generally preferable but not applicable to aluminium foil matrices where reflectance has to be used. Reflectance yields a lesser background noise and may therefore be advantageous also with glass and transparent plastic plates.

The limit of detection of a particular compound is at the point where the densitometric peak height is double the background noise. For reliable quantitative measurement the peak height/noise ratio should be >20:1.

The measurement of fluorescence in the reflectance mode is by far the most sensitive and accurate method of reading TLC spots but applicable only to a limited range of compounds.

For a typical densitometeric record see Fig. 3.10.

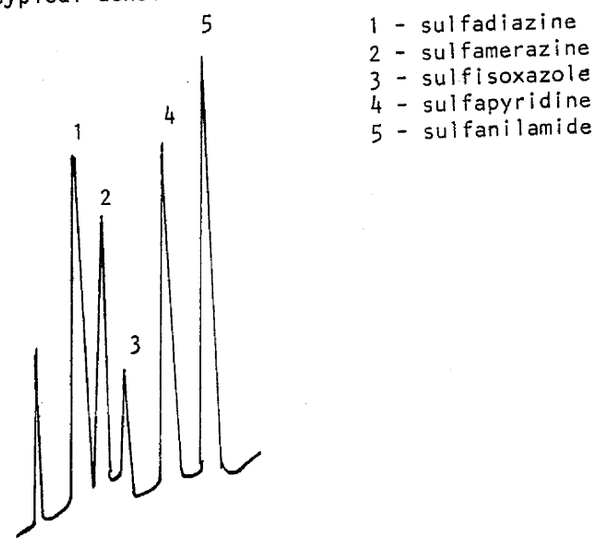


Fig. 3.10: Densitometric record from TLC

3.1.4.8 Enzyme-linked immunosorbent assay (ELISA) techniques

These are discussed in section 3.2.3.

3.1.4.9 Bioassays

Bioassays were widely used in the quantitative determination of antimicrobial agents prior to the advent of highly sensitive and accurate physicochemical assay methods. GC and HPLC with suitable detector systems have reduced the application of bioassays to a small number of highly active compounds for which determination of this bioactive concentrations is not yet feasible with the currently available physicochemical methods.

The principle of bioassays with antibacterial compounds is the comparison of the inhibitory activity of the biological sample and of known drug concentrations on standard clones of bacteria.

Bioassays of antimalarial compounds in biological material are so far limited to cultured blood stages of Plasmodium falciparum. They are applicable to compounds and drug metabolites which are bioavailable and active at concentrations below the detection threshold of the current physicochemical assay methods, e.g. chlor-cycloguanil. For the purpose of the bioassay it will be advantageous to use a clone with high sensitivity to the particular drug/metabolite and an assay system which measures the metabolic function of the malaria parasites in culture, e.g. by the determination of the incorporation of radiolabelled hypoxanthine. The bioactivity of the given plasma/serum sample (and if applicable its dilutions) is assessed in comparison to known concentrations of the drug/metabolite and normal controls. Bioassays have a relatively large margin of error, but this may be reduced by running several replicates. (see also chapter 4, section 4.3.2).

3.1.4.10 Use of radiolabelled compounds

The use of radiolabelled compounds in human clinical pharmacology is very limited for ethical reasons. Ethical clearance for this type of work may only be expected if the study is to resolve a problem of vital nature, if it cannot be conducted without the use of radiolabelled material, if the label causes only low radiation, and if the drug is known to be almost completely eliminated within a short time-span. However, radiolabelled compounds are used extensively in preclinical work in animals and in in vitro systems, especially for studies of drug distribution and fate, and in the elucidation of the mechanism of action in the malaria parasite.

3.1.4.11 Use of compounds labelled with stable isotopes

Antimalarial drugs labelled with stable isotopes, e.g. deuterium, can be usefully applied to pharmacokinetic studies in humans since they are not subject to the constraints associated with radiolabelled compounds. The usual assay methods for drugs labelled with stable isotopes are based on GC-MS.

3.1.5 Derivatization

A number of compounds have characteristics which render them primarily unsuitable for high-grade separation and/or highly sensitive detection systems. In the case of chromatographic separation, these characteristics may be too long a retention time, unsatisfactory resolution, fronting or tailing of peaks. These problems may be overcome by derivatization which changes the polarity of the compound and thus its chromatographic characteristics. Similarly halogenation may render compounds detectable through ECD. It is therefore customary to explore derivatization if a compound shows problems with separation and detection. Often, this may already be predicted from a compound's molecular structure.

3.1.6 Internal standards

The variability of assay conditions (atmosphere, temperature, barometric pressure, reagents, physical parameters of instrument operation, timefactors, operator) precludes the non-comparative quantitation of drugs. Therefore internal standards are used with all quantitative and most of the qualitative drug assays. In test systems offering the possibility of parallel processing and reading, e.g. TLC, the same compound can be used as the internal standard at a given concentration/quantity which provides the reference standard for the quantification of the compound in the sample.

With test systems which do not allow the simultaneous parallel processing, e.g. GC and HPLC, the internal standard consists usually of a similar substance which produces a well resolved peak near, but distinct from, the test compound's peak, e.g. pyrimethamine for proguanil and cycloguanil. Internal standards are always used at a defined concentration/quantity and added to the biological sample before extraction so that compensation is made for errors due to manipulation loss.

The chemical identity of the internal standard should be confirmed prior to its routine use, e.g. by MS or IRS.

3.1.7 Accuracy of assay results

In spite of careful standardization and quality control, there is often an unavoidable variability in the results. The

degree of this variability needs to be monitored in order to take remedial action when the variability exceeds the acceptable limits. For the precision of the assay system, it is customary to monitor within-day and day-to-day variation through the routine evaluation of the internal standard readings which are theoretically uniform throughout. Coefficients of variation consistently under 5% are desirable. A coefficient of variation greater than 10% should give rise to an investigation of the cause(s) of the imprecision.

3.1.8 Sample collection and storage

Although the collection and storage of biological material for drug assays seems to be a straightforward matter without major problems, it should be realized that "normal" routine practice may interfere with the analytic results.

When blood is collected by fingertip puncture into heparinized capillaries or onto filter paper it should be realized that these materials may exert physicochemical effects which may alter the sample's behaviour and drug content. Non-reversable adsorption/binding to glass (a capillary tube has a very large glass surface relative to its volume) of certain compounds and interference of the anticoagulant with one or more steps of the assay procedure may unduly influence the assay results when capillary-stored blood plasma is used. Filter paper, on the other hand, may behave like a stationary phase in chromatography when the elution process should be adjusted to achieving maximum efficiency.

With blood obtained through venepuncture, there is an even greater number of potentially deleterious factors. First, the choice of anticoagulants is greater, usually heparin, sodium oxalate or EDTA, all of which (especially EDTA) may cause interference with certain test systems. Second, glass or plastic vials may be used for storage of the sample. Both may exert non-reversible binding for certain compounds; plastic often releases low-molecular substances which may be particularly disturbing during extraction and separation, and which may be detected by MS or IRS.

When using serum for drug assays, thrombocytes are destroyed during coagulation and will release the drugs they may have selectively taken up previously. This accounts, for instance, for the consistently higher serum concentrations of chloroquine as compared to the plasma concentrations.

Since physiological enzymes may alter the behaviour of drugs in the assay if the sample is left too long at ambient temperature, it is recommended that the samples be frozen as soon as possible after collection. A storage temperature of -20°C is generally acceptable but freeze at -70°C is

essential with certain drugs. Storage at refrigerator temperature (4°C) will rapidly render the sample unsuitable for drug assays.

Certain compounds may be rapidly altered by blood enzymes, e.g. artemisinin and artemisinin derivatives, or by peroxidases, and a substantial loss may be incurred prior to freezing and on thawing. In these circumstances it may be necessary to add an enzyme blocker immediately upon sample collection; the blocker should be compatible with the drug to be assayed.

Urine is often stored and frozen in plastic bags and plastic containers. These may exert drug-specific binding and give off low molecular substances which may interfere with the drug assay.

Potential sources of error occurring during sampling and storage of samples are important enough to require thorough investigation before specific procedures can be adopted. Once such procedures are adopted they should be rigorously adhered to; deviations in terms of materials or reagents used for sample collection and storage or alterations in time scale are to be avoided.

3.1.9 Standardization of operational procedure

There are also numerous sources of error during the processing of the sample which may reduce the drug concentrations and therefore give faulty readings. Binding of drugs or their metabolites to laboratory glassware or plasticware is the most important source of such errors. Since the binding is not only affected by the surface material but also by time, standardization of the procedure must also take the time element into account. A sound evaluation of spiked samples of the biological material concerned, in association with internal standard, will provide the necessary calibration. Once a particular procedure has been adopted, there should be no change of materials, reagents and time schedule.

3.1.10 Assays for antimalarial drugs

A number of different assay systems exist for some antimalarials, e.g. chloroquine and mefloquine, while there is a paucity of such systems for others, e.g. chlorproguanil and chlorcycloguanil. In the one case, there may be no choice of methodology, where, in the other, selection of the most appropriate technology will be required, i.e. the method of analysis will have to be fitted to the task in hand.

The determination of antimalarial drugs in dosage forms (tablets, syrup, injectables, etc.) will rarely require more than straightforward spectroscopic methods; however, the precise quantitative determination of the drug concentrations in biological fluids will require not only extraction but often also derivatization, as well as purification and concentration, and finally highly sensitive and specific detection systems. HPLC and GC with UV, fluorimetric, electrochemical, electtron capture detection or MS and IRS are therefore the mainstay of analytic technology in the field of clinical pharmacology.

A number of assay methods for the common antimalarial drugs are summarized in Table 3.3.

TABLE 3.3 SELECTED QUANTITATIVE ASSAY (NON-FIELD) SYSTEMS FOR ANTIMALARIAL DRUGS AND THEIR METABOLITES

Drug/Metabolite	ASSAY	Biological Material	Limit of Detection	Reference
Chloroquine	HPTLC	P,WB,U	10NMOL/L	Betschart & Steiger 1986
Chloroquine and Desethylchloroquine [separate simult.]	HPLC	P,WB,U	10nmol/L(UVD) 0.5nmol/l(FD)	Bergqvist & Frisk Holmberg (1980) Bergqvist & Frisk Holmberg (1980)
Chloroquine and 2 main metabolites [separate simult.]	GC	P,WB,U		Bergqvist & Eckerbom (1984)
Amodiquine and main metabolite [separate simult.]	HPLC	P,WB,U	3nmol/l (ECD)	Mount et al., (1986)
Chloroquine + amodiaquine and their main metabolites [separate simult.]	HPLC	P,WB,U	3nmol/l (UVD)	Pussard et al., (1985)
Quinine + main metabolite [separate simult.]	HPLC	P,WB,U	100nmol/l (FD)	Edstein et al., (1983)
Quinine + Quinidine [separate simult.]	HPLC	P,WB,U	30nmol/l (FD)	Mihaly et al., (1987)
Mefloquine	GC	P,WB,U	2.6nmol/l (MS) 26nmol/l (ECD)	Schwartz & Ranalder (1981) Heizmann & Geschke (1984)
Mefloquine + main Metabolite [separate simult.]	HPLC	P,WB	25nmol/l	Arnold & Stetten (1986)
	TLC	P,WB	100nmol/l 25nmol/l	Bergqvist et al., (1988) Schwartz (1980)
Pyrimethamine sulfadoxine and acetyl-sulfadoxine [separate simult.]	HPLC	P,WB,U	Pyrimethamine 50nmol/l Sulfadoxine 5 nmol/l	Bergqvist & Eriksson(1985)
Proguanil and cycloguanil [separate simult.]	HPLC	P,WB,U	4nmol/l	Taylor et al., (1987)

Key: (i) Assays

HPTLC = high-performance thin-layer chromatography.

HPLC = high performance liquid chromatography.

GC = gas chromatography.

(ii) Biological material

P = plasma.
WB = wholle blood.
U = urine.

(iii) Methods of detection

UVD = ultra-violet detection.
FD = fluorescence detection.
ECD = electrochemical detection.
MS = mass spectroscopy.

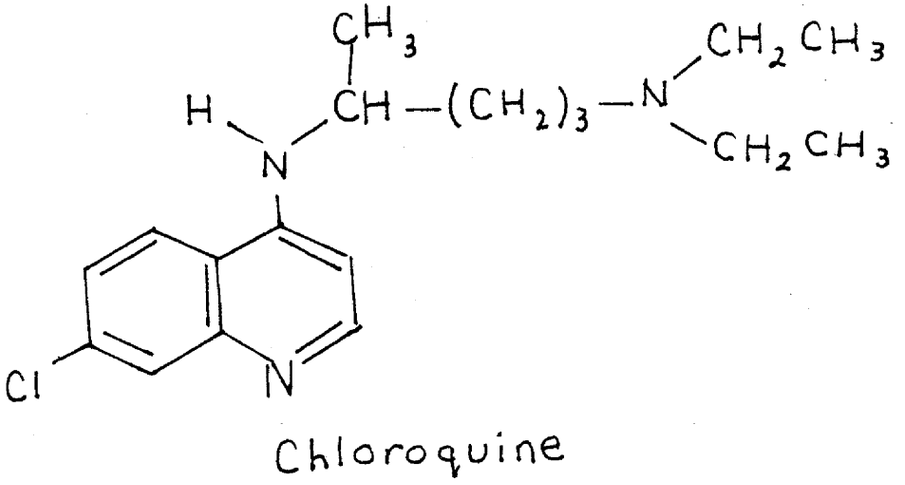
Simult. = Simultaneously

3.2 FIELD TECHNIQUES FOR THE ASSESSMENT OF ANTIMALARIAL DRUGS USING THE EXAMPLE OF CHLOROQUINE

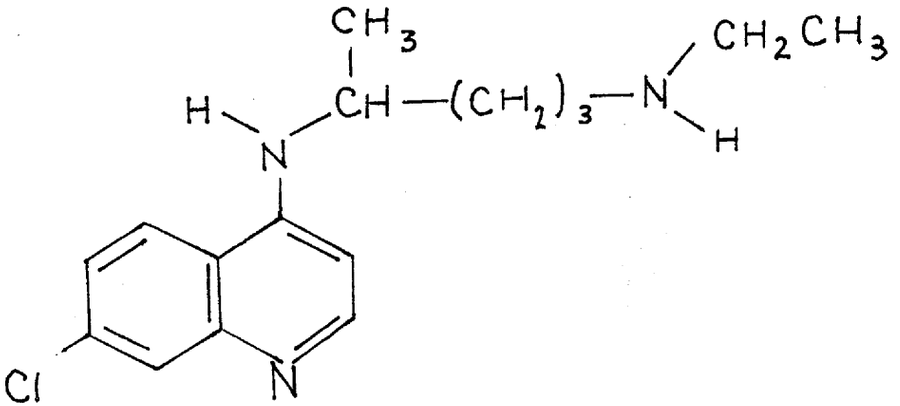
Many malaria field studies require methods for measuring antimalarial drugs and their metabolites in body fluids. Results of these assays are used to screen patients for in vivo drug sensitivity studies and to verify ingestion and absorption of drugs. For in vitro field tests, researchers need to know if blood samples contain drugs that contribute to the antiparasitic effect of an added drug and produce false results. Assays may be used to monitor chemoprophylaxis and treatment compliance. Assays are also useful when exploring the relationship between drug use practices and incidence of disease or development of resistance in a community.

Many laboratory assays of chloroquine and its major metabolite desethylchloroquine (Fig. 3.11) have been developed over the past 40 years. The most accepted reference method is high-performance liquid chromatography (HPLC) with fluorescence detection due to its great selectivity and sensitivity and the ability to automate the procedure. Methods using such sophisticated analytical instrumentation (Bergqvist & Frish-Holmberg et al, 1980; Alvan et al., 1982; Patchen et al, 1983) have been interfaced with field studies by preserving finger-stick blood samples on filter paper to permit transport of the samples to the laboratory without refrigeration (Patchen et al, 1983; Lindstrom et al, 1985).

It is often necessary or desirable to measure concentrations of chloroquine in urine or blood in the field. Compromises in selectivity (specificity), sensitivity, and/or precision are inevitable with field-adapted methods compared to laboratory methods. Historically, colorimetric assays have been used for qualitative determination of chloroquine in urine in the field (Wilson & Edeson, 1954; Haskins, 1958; Lelijveld & Kortmann, et al., 1970). A recent renaissance in colorimetric methodology for field-adapted detection of chloroquine and its metabolites (primarily desethylchloroquine) in urine has included quantitative ion-pair extraction methods that use bromothymol blue (Bergqvist et al., 1985) and methyl orange (Mount et al., 1987a), the Haskins test reagent. More recently, thin-layer chromatographic (TLC) methods (Betschart & Steiger, 1986; Mount et al., 1987b) and enzyme-linked immunosorbent assay (ELISA) methods (Shenton et al., 1988; Rowell et al., 1988) for measuring chloroquine or chloroquine together with its metabolites in urine have been developed. Additionaly, TLC (Betschart and Steiger, 1986; Mount et al., 1988a,b), radioimmunoassay (RIA) (Freier et al., 1986), and ELISA (Rowell et al, 1988) methods are available for determining chloroquine and its metabolites in blood or plasma. Some of these TLC and



Chloroquine



Desethylchloroquine

Fig. 3.11 Structure of chloroquine and desethylchloroquine

immunoanalytical methods have been field adapted (Mount et al., 1988a,b; Shenton et al., 1988; Rowell et al., 1988) and at least two have been assessed in field settings (Mount et al., 1987 a, b; Shenton et al., 1988).

The different approaches to field-adapted assays for chloroquine are discussed below. Tables 3.4 and 3.5 compare analytical characteristics of the available methods for chloroquine in urine and blood, respectively.

TABLE 3.4 CHARACTERISTICS OF FIELD-ADAPTED ASSAYS FOR CHLOROQUINE (CQ) IN URINE

Method	Detection limit (ug/ml)	Precision(%)	Selectivity (specificity)	Compounds detected	Reference
Wilson-Edeson	4	qualitative	fair	chloroquine, metabolites, quinine	Wilson & Edeson, (1954) Bergqvist et al. (1985) Rombo et al. (1986)
Haskins	2	qualitative	fair	chloroquine, metabolites,	Haskins, (1958) Bergqvist et al. (1985) Rombo et al. (1986)
Dill-Glazko	40-80 (variable)	qualitative	fair	chloroquine, amodiaquine, quinine	Lelijveld & Kortmann, (1970)a Bergqvist et al. (1985) Rombo et al. (1985, 1986)
Bromothymol blue	3	5-10	fair	chloroquine, metabolites, quinine, proguanil	Bergqvist et al. (1985)
Haskins MMII(MMI)	1 (0.3)	5-10	fair	chloroquine, metabolites, quinine, proguanil	Mount et al. 1987)a Steketee et al. (1987)a
Saker-Solomons CQI	1	qualitative	fair	chloroquine, metabolites, quinine, proguanil	Mount et al. (1989)
Saker-Solomons CQII	2	5-10	fair	chloroquine, metabolites, quinine, proguanil	Mount et al. (1989)
HPTLC-FQ[b]	0.25	semi-quantitative	excellent	specific for chloroquine desthyl chloroquine	Mount et al.(1987b)a

Method	Detection limit (ug/ml)	Precision(%)	Selectivity (specificity)	Compounds detected	Reference
HPTLC-FQ-SLM[c]	0.25	10-15	excellent	specific for chloroquine desthyl chloroquine	Mount et al. (1988)b
HPTLC-F[d]	0.025	semi-quant.	excellent	specific for chloroquine desthyl chloroquine	Mount et al. (1988)a
HPTLC-F-SLM[e]	0.025	10	excellent	specific for chloroquine desthyl chloroquine	Mount et al. (1988)b
ELISA[f]	0.001	not stated	good	detects chloroquine, amodiaquine, metabolites	Shenton et al. (1988)a
ELISA[f]	0.0003	5-10	excellent	specific for chloroquine	Rowell et al. (1988)

[a]Includes description of field application
[b]HPTLC with visual detection of background fluorescence quenching (FQ) by analyte spots under ultraviolet light (254 nm).
[c]HPTLC with detection by spot luminance meter (SLM) of background fluorescence quenching (FQ) by analyse spots under ultraviolet light 254 nm).
[d]HPTLC with visual detection of visible fluorescence (F) under ultraviolet light (254 nm).
[e]HPTLC with detection of spot luminance meter (SLM) of visible fluorescence (F) under ultraviolet light (254 nm).
[f] Later work showed both these methods to be unworkable. Further work was being carried out and the modification has been reported. (Nvaratnam & Payne, 1990).

TABLE 3.5 CHARACTERISTICS OF FIELD-ADAPTED ASSAYS FOR CHLOROQUINE IN BLOOD

Method	Detection limit (ug/ml)	Precision (%)	Selectivity (specificity)	Comments	Reference
HPTLC-F[a]	60	semi-quantitative	excellent	specific for chloroquine, desethyl chloroquine	Mount et al. (1988)a
HPTLC-F-SLM[b]	60	10	excellent	specific for chloroquine, desethyl chloroquine	Mount et al. (1988)b
ELISA-B	0.3	5-10	excellent	specific for chloroquine	Rowell et al. (1988)

[a]HPTLC with visual detection of visible fluorescence (F)under ultraviolet light (254 nm).
[b]HPTLC with detection by spot luminance meter (SLM) of visible fluorescence (F) under ultraviolet light (254 nm).

3.2.1 Colorimetric field assays

All methods for the field assay of chloroquine in urine developed before 1980 are qualitative. The Dill-Glazko test has been most used in malaria field studies because of its simplicity. However, recent studies indicate that this test is insufficiently sensitive, selective, or reliable for field use (Verdier et al., 1985; Rombo et al., 1985; Rombo et al., 1986). This test has a detection limit of 40 to 80 ug/ml depending on the pH of the urine, and haematuria can cause false positive results.

Three colorimetric methods - the bromothymol blue (BTB) method and two modified Haskins methods (Haskins MMI and Haskins MMII) - permit quantification of chloroquine and its metabolites in the field. Mount et al., (1987a) describe the use of a hand-held, battery-operated filter photometer (420-nm filter) in remote locations to facilitate colorimetric quantifications of urinary chloroquine (Fig. 3.12). Haskins MMI has a detection limit of 0.3 ug/ml and Haskins MMII 7 ug/ml. The BTB method can be modified to give a detection limit comparable to that of Haskins MMII (Y. Bergqvist, personal communication). Thus a negative result for any of these three tests corresponds to <1 ug/ml chloroquine plus its metabolites in the urine. It has been proposed that this corresponds to less than 100 ng/ml of chloroquine in the blood, the estimated minimal therapeutic

Fig. 3.12 Hand-held, battery-operated, filter photometer for colorimetric quantification.

concentration, so that these tests effectively screen patients with significant blood chloroquine levels to exclude them from drug sensitivity studies. The Haskins MMI assay requires more time and effort to run than the BTB or Haskins MMII procedures. Haskins MMII has been applied under field conditions to screen patients for an in vivo chloroquine sensitivity study and to document ingestion and absorption during the trial (Steketee et al., 1987, 1988).

The method of Saker & Solomons (1979) for assay of drugs of abuse in urine has been modified and adapted at the Centres for Disease Control laboratories in Atlanta to permit easy qualitative or quantitative determination of chloroquine and its metabolites in urine in the field (Mount et al., 1989). Two methods have been validated and successfully applied. The simpler method has a detection limit of 1 ug/ml and is superior in every respect to the Dill-Glazko test in malaria field studies.

3.2.2. Thin-layer chromatographic field assays for chloroquine

Betschart & Steiger (1986) used high-performance thin-layer chromatography (HPTLC) in the laboratory to quantify chloroquine and desethylchloroquine in urine and blood samples with a detection limit of 3ng/ml; adaptation of this method to the field would preclude use of the apparatus for sample spotting and use of the fluorescence densitometer.

Mount et al. (1987b, 1988a) reported field-adapted HPTLC methods for estimating chloroquine and desethylchloroquine in urine and in blood. The method for urine analysis separates the compounds on silica gel plates that contain an impregnated fluorophore; the investigator visually compares under ultra-violet irradiation at 254 nm the size of fluorescence-quenched spots from sample extracts with those from standard extracts. The method was applied in Ecuador where the results compared favorably with Haskins MMII values obtained on the same samples. Corresponding finger-stick blood samples preserved on filter-paper were analysed in the laboratory and tabulated for comparison with urine results.

In an HPTLC chromatogram of finger-stick blood extracts, chloroquine and desethylchloroquine fluoresce on silica gel following irradiation with 254-nm ultraviolet light which permits the visual estimation in eluted sample extracts when compared. Focusing of the chloroquine spots by the eluting solvent enhances detectability; the detection limit is 60 ng/ml for 100 ul blood samples.

It has recently been shown that a compact, battery-operated spot luminance meter (SLM) may be used to quantify chloroquine in extracts of 100 ul finger-stick blood spots on HPTLC plates with an average precision of 10% at the 240 ng/ml concentration (Mount et al., 1988b). The luminance meter is mounted on a platform to confer the stability necessary for repeatable measurements of light intensities as the TLC plate is moved to bring each sample and standard spot in turn within the optical field. The intensities of fluorescence emitted from the chloroquine spots for samples is compared with those plotted on a standard curve to yield quantitative results. The method was validated with HPLC assays of the same samples. The correlation between the results of the two methods was 0.949 (n=21) with a slope of 1.068. Alternatively, the spot luminance meter may be used to quantify chloroquine in urine extracts by measuring fluorescence quenching by chloroquine spots using fluorophore-impregnated HPTLC plates and ultraviolet irradiation at 254 nm. A larger volume of sample is used; sensitivity is substantially less and precision marginally poorer for quantification in the fluorescence quenching mode (see Table 3.4).

3.2.3. Immunoanalytical field assays for chloroquine

A radioimmunoassay for chloroquine in plasma that uses a monoclonal antibody has been developed with roughly equivalent sensitivities to chloroquine, amodiaquine and their deethylated metabolites (Freier et al, 1986). The detection limit is 10 ng/ml with a coefficient of variation of less than 10% between assays. The method is not adaptable to the field since it requires radioisotopic compounds and a liquid scintillation counter.

Two enzyme-linked immunosorbent assay (ELISA) methods have been described for the assay of chloroquine. Shenton et al (1988) describe two ELISA tests for urine analysis; one using polyclonal antibodies and the other monoclonal antibodies. Both types of antibodies were produced using a hydroxychloroquine-bovine serum albumin conjugate and are specific for the 7-chloro-4-aminoquinoline moiety. The antibodies react with chloroquine, amodiaquine and their deethylated metabolites; the assays are therefore not specific for chloroquine but may be used to screen for 7-chloro-4-aminoquinolines. The monoclonal ELISA is somewhat more sensitive than the polyclonal method, 30% inhibition of enzyme activity being produced at a 2 ng/ml chloroquine concentration. Both assays were used qualitatively in malaria studies in The Gambia by visually comparing colour; a 30% or above inhibition value was considered to show the presence of chloroquine.

The ELISA performed by Rowell et al. (1988) used a chloroquine analogue in which the 7-position substituant was a 2-aminoethylamino group instead of chlorine. Conjugation of this hapten with keyhole-limpet haemocyanin yielded an immunogen

that stimulated the formation, in sheep, of antibodies to the 4-(4-diethylamino-1-methylbutylamino) quinolinyl moiety, resulting in chloroquine specificity. The assay used a microtitre plate reader (Fig. 3.13) to quantify chloroquine in urine and in blood with detection limits of 0.3 ng/ml and 0.6 ng/ml, respectively. Between-assay coefficients of variation for both urine and blood samples were from 5 to 10%. Application of this ELISA method in the field has not yet been reported.

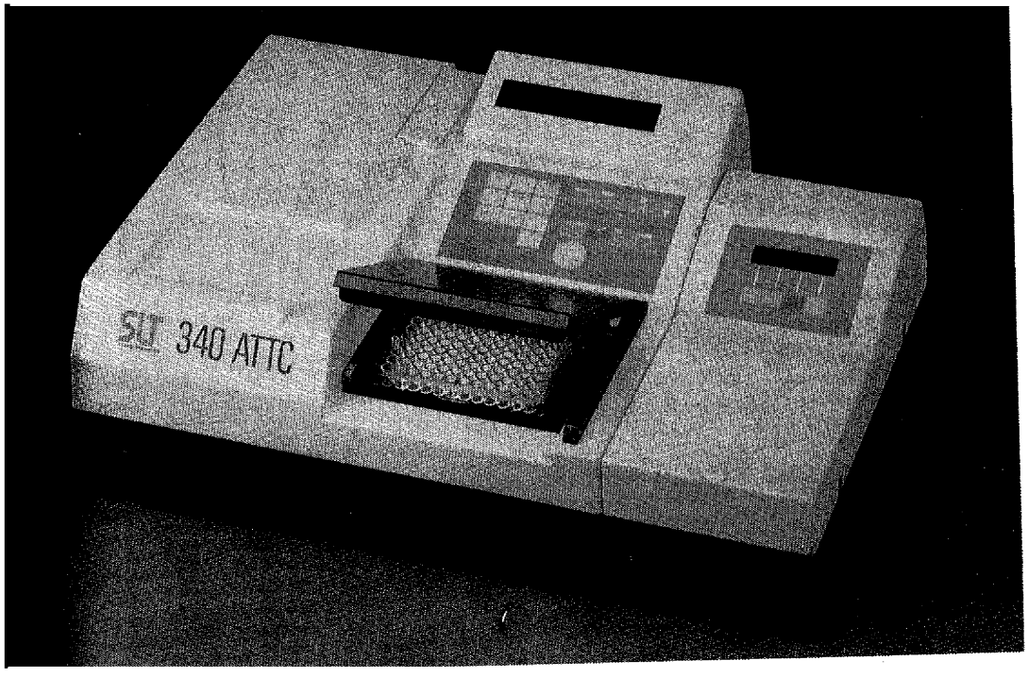


Fig. 3.13 Microtitre plate reader for ELISA quantification [photograph courtesy of Dynatech Laboratories Inc., Chantilly, V.A. (USA)]

3.2.4 Comparison of methods

Each of the three approaches described above permit either estimation of chloroquine levels visually or quantitatively using an appropriate sensing device to measure visible-light intensities. The method of choice depends on the requirements of the study and the available resources.

The new quantitative and semi-quantitative colorimetric methods for chloroquine and its metabolites in urine are well suited to field studies, for determining compliance to chemoprophylaxis and treatment, and to delineating practices of drug abuse. The modified Haskins assays satisfy the "Land Rover criterion" for field adaptability as shown in Fig. 3.14.

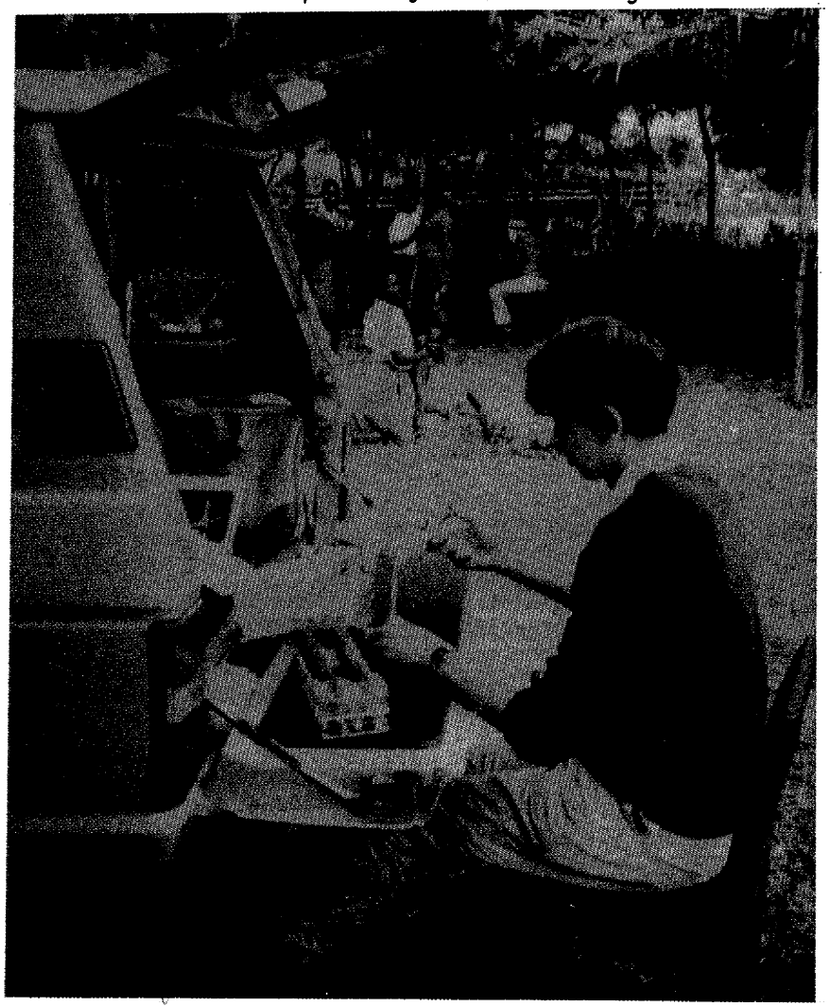


Fig. 3.14 Field application of modified Haskins assays in studies of chloroquine efficacy in malaria-infected gravid and nulligravid women in the Siaya District, Western Kenya.

Haskins MMI uses an aqueous defoamer and Haskins MMII a glass-wool swab to break emulsions without centrifugation. Both use a battery-operated filter photometer for measuring chloroquine so that no electricity is required. A concentration of chloroquine up to 1 ug/ml in urine indicates therapeutically negligible blood levels. Positive results for chloroquine in urine show only moderate correlation with blood levels, so that analysis of selected, paired finger-stick blood samples by an HPLC fluorescence method in the laboratory can provide valuable additional information.

The HPTLC-fluorescence quenching method for assay of chloroquine and desethylchloroquine in urine is marginally more sensitive and the HPTLC-fluorescence method much more sensitive than colorimetric assays. The HPTLC approach is specific for both chloroquine and desethylchloroquine. ELISA methods are the most sensitive of the three approaches and may be specific for chloroquine or selective for chloroquine, amodiaquine and their deethylated metabolites, depending on the antigenic conjugate used to develop the method. The precision of colorimetric, HPTLC, and ELISA methods depends on whether light intensities are compared visually or with instruments (see Table 3.4).

ELISA methods are sufficiently sensitive for a 1 ng/ml detection limit for chloroquine is to be possible with finger-stick blood samples. These assays are "pre-engineered" to permit easy analysis with high sample throughput. They use techniques similar to those that field laboratories may use now or in the future for diagnosis. However, setting up and running such assays requires appreciable sophistication; the reagents may degrade if not carefully stored, and quantitation requires a microtitre plate reader. It is likely that this technology will be transferred to field laboratories, but only after careful training of staff by persons experienced with the method. The method can be used only by those to whom the painstakingly produced immunological reagents are provided. The method measures concentration of chloroquine or of chloroquine and its metabolites. HPTLC-fluorescence provides separate values for chloroquine and desethylchloroquine but with a detection limit of 60 ug/ml for a 0.1ml sample. It is substantially less sensitive than ELISA, but perhaps sufficiently sensitive for most purposes. The solvents and reagents are generally available and anyone appreciably schooled in analytical techniques can apply the method. The evaporation step is somewhat tedious and requires a special but simply constructed apparatus. However, even the ELISA and HPTLC methods lack the combination of high sensitivity, specificity and precision characteristic of laboratory methods that used HPLC-fluorescence. It is therefore prudent to verify the results for critical samples by such a reference method.

3.2.5 Outlook

In the last four years, a wide variety of field-adapted methods, some of which have been field tested, has been developed for detecting and quantifying chloroquine. Additional field experience with the published assays is needed to learn the capabilities of the general approaches and specific methods. Analogous methods should be developed for other important anti-parasitic drugs. Such assays for proguanil, quinine and mefloquine would contribute to investigations of the most effective use of these drugs in malaria control. ELISA methodology with its great sensitivity would also be applicable for the detection of primaquine and/or metabolites. Prototypical chloroquine assays reviewed above and information available from their application demonstrates the value of immediately available analytical results to clinical field studies.

3.3 PRACTICAL ASPECTS IN THE SELECTION OF SPECIFIC ASSAY METHODS

When there is a choice between various assay methods, the selection of a particular technique will have to be made taking into consideration the task in hand (Fig. 3.15). The decision will have to be made in keeping with a variety of factors such as :

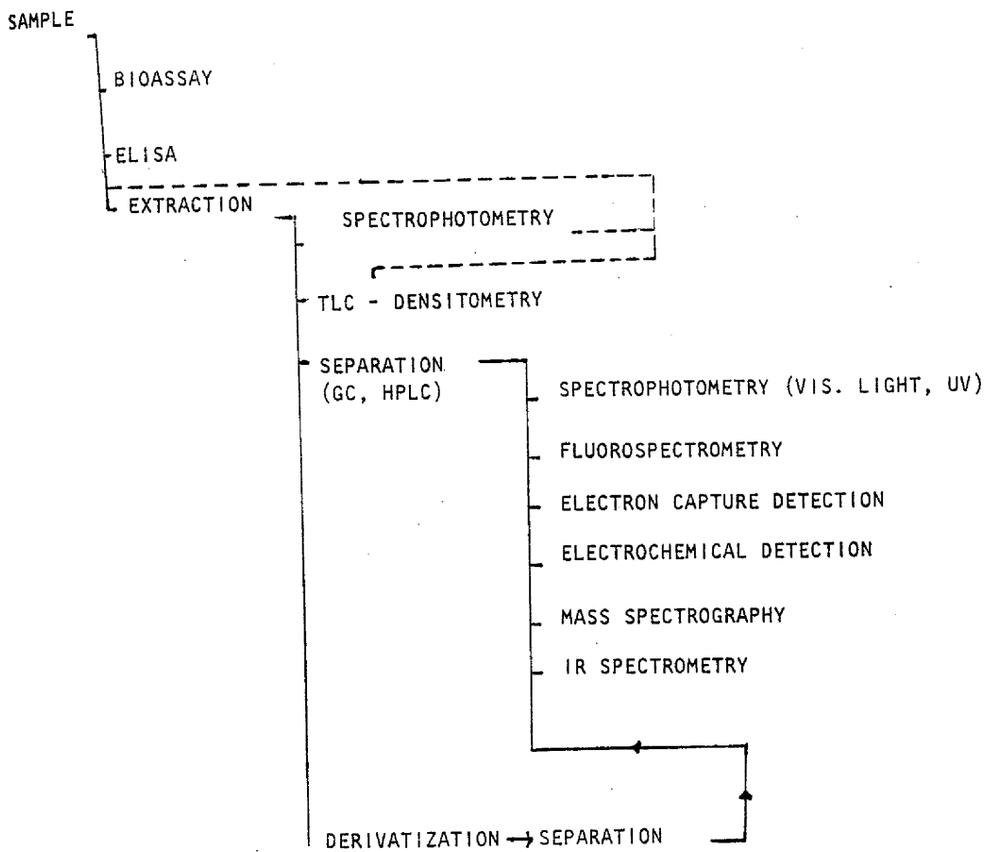


Fig. 3.15 Systematics of antimalarial drug assays in blood, plasma and urine.

(a) purpose of the study, e.g. pharmacokinetic profiles in severely ill malaria patients, longitudinal prophylactic study, drug profiles in outpatients, complementary drug assay in the context of drug response tests;

(b) site of the study, e.g. near central laboratory, near peripheral laboratory, remote field station without laboratory facilities;

(c) facilities of the laboratory doing the drug assays, including expertise of staff involved;

(d) time, e.g. how soon are the results needed?

(e) cost.

If high precision is required, as in pharmacokinetic/clinical pharmacological investigations, the most reliable and precise method available should be used. If local laboratory facilities do not permit the performance of the appropriate assay technique and if the locally available technology cannot provide the desirable precision, it will be preferable to do the clinical part of the study at a site when better laboratory facilities are available or, where this is not possible, to preserve the biological samples and to transfer them to a laboratory capable of running the originally specified assay.

If the assay results are needed within the shortest possible time, a rapid method will be required. Most of these rapid methods have limited sensitivity and precision, but they are generally adequate for providing guidance. There is always the possibility of taking duplicate samples and of preserving one for subsequent processing with a more precise assay technique.

The selection of a particular assay technique also requires common sense. In most studies involving drug level determination, clinical or field investigations, are the most difficult, demanding and costly, while the cost of the assays will be relatively modest. In these circumstances, it would be unethical and uneconomic to settle for an assay technique that may not provide the optimal information.

Bioassays have limited precision, but they are indispensable when there is no appropriate physicochemical method available.

ELISA, TLC and spectrophotometric determinations without or after minimum extraction have limited sensitivity and precision, but they are relatively rapid methods and therefore particularly suitable for monitoring. Moreover, they do not require highly sophisticated laboratory facilities.

GC and HPLC with appropriate detector systems are needed for work demanding high sensitivity and precision.

Field-adapted methods often involve compromises in sensitivity, selectivity and precision in return for low cost and high rate of sample analysis under primitive conditions.

It is often difficult to use venepuncture or to centrifuge samples in the field. Blood collection on filter paper using heparinized capillaries, is therefore the most appropriate method of collecting samples for drug assays in the field. It has been shown that the chloroquine concentrations measured in eluted filter paper samples are practically identical with those obtained by immediate processing of finger-tip blood. Moreover paired samples of blood obtained by venepuncture and finger tip puncture showed the same chloroquine concentrations. Thus the filter paper method can be regarded as reliable. Sample storage does not present a problem even at ambient temperature as long as the samples are kept dry and protected against contamination.

Accidental contamination with chloroquine and/or other antimalarial drugs is a distinct possibility if the samples are collected in health service establishments in malarious areas. When working in such facilities, it is therefore recommended to cover the work area with disposable covers preferably paper which are discarded after completion of the work. In non-contaminated premises it will suffice to keep the work area rigorously clean.

Filter paper samples yield essentially whole blood concentrations of drugs. While whole blood drug levels are generally acceptable, clinical pharmacologists prefer to work with plasma. Apart from some technical difficulties which may arise in assays with whole blood there should be no reason to opt for plasma concentrations if whole blood concentrations are perfectly acceptable. This issue deserves attention since some clinical pharmacologists feel that assay accuracy is higher with plasma than with whole blood. There are major differences between chloroquine concentrations in plasma, serum, whole blood and the various corpuscular elements. Whole blood levels would probably reflect better the chloroquine concentrations. Although the basic exploration of the kinetics and dynamics of drugs has to address the issue of drug partition between the various blood components, there should be no reason for singling out plasma as the only acceptable material for drug assays in blood.

Urine tests have become a useful tool for monitoring antimalarial drug intake and compliance.

It has been suggested that saliva may also be a suitable material in which to determine drug levels. However, apart from technical difficulties associated with drug assays in saliva, it has been found that the concentrations of antimalarial drugs in saliva are considerably lower than in plasma. There was also a poor correlation between the drug concentrations in saliva and

plasma. The use of saliva for the monitoring of drug intake/compliance is therefore difficult and unreliable.

3.4 TECHNICAL POINTS OF FUTURE INTEREST

Various points which deserve interest relate essentially to methodological aspects and standardization as well as the improvement of field tests.

3.4.1 Standardization and facilitation

The repository established by WHO and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases for the storage and distribution of metabolites of chloroquine and amodiaguine is an important resource for those involved in studies of the pharmacology and pharmacokinetics of these drugs. It is hoped that metabolites of other antimalarial drugs such as mefloquine and halofantrine, will be included in this repository. It is desirable also to have a similar resource for the storage and distribution of well characterized internal standard compounds for established assays and for the development of new assays.

3.4.2 Methodology

The current repertoire of methods for detecting antimalarial drugs may in the near future be increased by the addition of supercritical fluid chromatography (SFC), a new technique which has analytical capabilities bridging the gap between HPLC and GC. Applications of SFC, coupled with a variety of detection systems, are currently being studied with various antimalarial compounds, e.g. mefloquine and artemisinin.

3.4.3 Field tests

The increasing use of antimalarials other than chloroquine makes it desirable to extend the range of urine tests. The Haskins MMII test and Saker-Solomons CQI test are not specific for chloroquine. Both techniques show positive reactions also with several other antimalarial drugs. There is therefore a need for developing specific tests for all antimalarial drugs in therapeutic and prophylactic routine use.

The simplicity as well as the high specificity and sensitivity of the ELISA test would make it an eminently useful tool for drug assays in the field. However, problems due to the short shelf-life of the reagents, the irregular availability of certain crucial reagents and the poor logistics of supplies do still preclude the wider use of these tests. Efforts should be directed at producing and ensuring the availability of reagents which can be shipped and stored without major complications and whose shelf-life is long enough not to require frequent renewal.

REFERENCES TO CHAPTER 3

Alvan, G., Ekman, L. & Lindstrom, B. (1982) Determination of chloroquine and its desethyl metabolite in plasma, red blood cells and urine by liquid chromatography. Journal of chromatography, 229: 241-247.

Arnold, P.J. & Stetten, O.V. (1986) High-performance liquid chromatographic analysis of mefloquine and its main metabolite by direct plamsa infection with pre-column enrichment and column switching techniques. Journal of chromatography, 353: 193-200.

Bergqvist, Y. & Eckerbom, S., (1984) An improved gas chromatographic method for the simultaneous determination of chloroquine and two metabolites using capillary columns. Journal of chromatography, 306: 147-153.

Bergqvist, Y. & Eriksson, M. (1985) Simultaneous determination of pyrimethamine and sulfadoxine in human plasma by high-performance liquid chromatography. Transactions of the Royal Society of Tropical Medicine and Hygiene, 79: 297-301.

Bergqvist, Y. & Frisk-Holmmberg, M. (1980) Sensitive method for the determination of chloroquine and its metabolite desethylchloroquine in human plasma and urine by high-performance liquid chromatography. Journal of chromatography, 221: 119-127.

Bergqvist, Y., Hed, C., Funding, L. & Suther, A. (1985) Determination of chloroquine and its metabolites in urine: a field method based on ion-pair extraction. Bulletin of the World Health Organization, 63: 893-898.

Bergvist, Y., Churchill, F.C. & Mount, D.L. (1988) Determination of mefloquine by electron-capture gas-liquid chromatography after phosgene derivatization in biological samples and in capillary blood collected on filter-paper. Journal of chromatography, 428: 281-290.

Betschart, P. & Steiger, S. (1986) Quantitative determination of chloroquine and desethylchloroquine in biological fluids by high performance thin-layer chromatography. Acta tropica, 43: 125-130.

Edstein, M., Stace, J. & Shann, F. (1983) Quantification of quinine in human serum by high-performance liquid chromatography. Journal of chromatography, 278: 445-451.

Freier, C., Alberici, G., Turk, P., Baud, F. & Bohuon, C. (1986) A radioimmunoassay for the antimalarial drug chloroquine. Clinical chemistry, 32: 1742-1745 .

Haskins, W.T. (1958) A simple qualitative test for chloroquine in urine. American journal of tropical medicine and hygiene, 7: 199-200.

Heizmann, P. & GeschKe, R. (1984) Determination of the antimalarial mefloquine in human plasma by gas chromatography with electron-capture detection. Journal of chromatography, 311: 411-417.

Leach, H. & Ramsey, J.D. (1986) Gas chromatography. In: Moffat, A.C. ed. Clarke's isolation and identification of drugs. 2nd ed. London, The Pharmaceutical Press. pp. 178-200.

Lelijveld, J. & Kortmann, H. (1970) The eosin colour test of Dill and Glazko: a simple field test to determine chloroquine in urine. Bulletin of the World Health Organization, 42: 477-479.

Lindstrom, B., Ericsson, O., Alvan, G., Rombo, L., Ekman, L., Rais, M. & Sjogvist, F. (1985) Determination of chloroquine and its desethyl metabolite in blood: An application for samples collected in capillary tubes and dried on filter paper. Therapeutic drug monitoring, 7: 207-210.

Mihaly, G.W., Hymann, K.M., Smallwood, R.A. & Hardy, K.J. (1987) High-performance liquid chromatographic analysis of quinine and its diastereoisomer, quinidine. Journal of chromatography, 415: 177-182.

Mount, D.L., Pathcen, L.C., Nguyen-Dinh, P., Barber, A.M., Schwartz, I.K., & Churchill, F.C. (1986) Sensitive analysis of blood for amodiaquine and three metabolites by high-performance liquid chromatography with electro- chemical detection. Journal of chromatography, 383: 375-386.

Mount, D.L., Patchen, L.C., Williams, S.B. & Churchill F.C. (1987a) Colorimetric and thin-layer chromatographic methods for field assay of chloroquine and its metabolites in urine. Bulletin of the World Health Organization, 65: 615-623.

Mount, D.L., Nahlen, B.L., Patchen, L.C. & Churchill, F.C. (1987b) Field-adapted method for high-performance thin-layer chromatographic detection and estimation of chloroquine and desethylchloroquine in urine. Journal of chromatography, 423: 261-269.

Mount, D.L., Patchen, L.C. & Churchill, F.C. (1988a) Field-adapted method for high-performance thin-layer chromatographic detection and estimation of chloroquine in finger-stick blood. Journal of chromatography, 428: 196-202.

Mount, D.L., Williams, S.B. & Churchill, F.C. (1988b) A novel approach to quantification in thin-layer chromatography using a spot luminance meter: application to field-adapted assay of chloroquine in body fluids. Fortieth Southeastern Regional Meeting of the American Chemical Society, Atlanta, GA, November 9, 1988, Abstract No. 597.

Mount, D.L., Nahlen, B.L., Patchen, L.C. & Churchill, F.C. (1989) Adaptations of the Saker-Solomons test: simple, reliable colorimetric field assays for chloroquine and its metabolites in urine. Bulletin of the World Health Organization, 67: 295-300.

Navaratnam, V. & Payne, D. (1990) The Validation of Chemicals and Immunological Tests for Antimalarials in Body Fluids: Papers presented at a WHO/Universiti Sains Malaysia Workshop. International Monograph Series No. 3, p. 35 - 72.

Patchen, L.C., Mount, D.L., Schwartz, I.K. & Churchill, F.C. (1983) Analysis of filter-paper-absorbed, finger-stick blood samples for chloroquine and its major metabolite using high-performance liquid chromatography with fluorescence detection. Journal of chromatography, 278: 81-89 (1983).

Pussard, E., Verdier, F., Blayo, M.C. & Pocidalo, J.J. (1985) Biotransformation de l'amodiaquine et prophylaxie du paludisme a Plasmodium falciparum. Comptes rendus de l'academie des Sciences, Ser. III, 301: 383-385.

Rombo, L., Bjorkman, A., Sego, E., Lindstrom, B., Ericsson, O. & Gustafsson, L.L. (1985) Reliability of Dill-Glazko test, Lancet, i: 1509.

Rombo, L., Bjorkman, A., Sego, E., Lindstrom, B., Ericsson, O. & Gustafsson, L.L. (1986) Evaluation of three qualitative tests for detection of chloroquine in urine : agreement with plasma concentrations determined with liquid chromatography. Annals of tropical medicine aand parasitology, 80: 293-298.

Rowell, V., Rowell, F.J., Baker, A., Laurie, D. & Sidki, A.M. (1988) A specific ELISA method for determining chloroquine in urine or dried blood spots. Bulletin of the World Health Organization, 66: 211-217.

Saker, E.G. & Solomons, E.T. (1979) A rapid inexpensive presumptive test for phencyclidine and certain other cross-reacting substances. Journal of analytical toxicology, 3: 220-221.

Schwartz, D.E. (1980) Quantitative determination of the antimalarial drug mefloquine and its main metabolite in plasma by direct densitometric measurement on TLC-plates. In: Frigero, A. & McCamish, A., eds. Recent developments in chromatography and electrophoresis, Vol. 10. Amsterdam, Elsevier, pp, 69-74.

Schwartz, D.E. & Ranalder, U.S. (1981) Highly sensitive and specific determination of mefloquine in biological fluids using gas chromatography mass spectrometry with selected ion monitoring. Biomedical mass spectrometry, 8: 589-592.

Shenton, F.C., Bots, M., Menon, A., Eggelte, T.A., de Wit, M. & Greenwood, B.M. (1988) An ELISA test for detecting chloroquine in urine. Transactions of the Royal Society of Tropical Medicine and Hygiene, 82: 216-220.

Steketee, R.W., Brandling-Bennnett, A.D., Kaseje, D.C.O., Schwartz, I.K. & Churchill, F.C. (1987) In-vivo response of Plasmodium falciparum to chloroquine in gravid and nulligravid women, Siaya District, Kenya. Bulletin of the World Health Organization, 65: 885-890.

Steketee, R.W., Mount, D.L., Patchen, L.C., Williams, S.B., Churchill, F.C., Roberts, J.M., Kaseje, D.C.O. & Brandling-Bennett, A.D. (1988) Field application of a colorimetric method of assaying chloroquine and desethylchloroquine. Bulletin of the World Health Organization, 66: 485-490.

Taylor, R.B., Moody, R.R. & Ochekpe, N.A. (1987) Determination of proguanil and its metabolites cycloguanil and 4-chlorophenylbiguanide in plasma, whole blood and urine by high-performance liquid chromatography. Journal of chromatography, 416: 394-399.

Verdier, F., Ramanamirija, J.A., Pussard, E., Clavier, F., Biaud, J.M., Coulanges, P. & Lebras, J. (1985) Unreliability of Dill-Glazko test in detecting chloroquine in urine. Lancet, i: 1282-1283.

Williams, B.L., & Wilson, K. (1975) Praktische Biochemie. Thesis, Stuttgart.

Wilson, T. & Edeson, J.F.B. (1954) Studies on the chemotherapy of malaria. III. The treatment of acute malaria with chloroquine. Medical journal of Malaya, 9: 115-131.

CHAPTER 4 : DRUG DEVELOPMENT*

CONTENTS

---

* This chapter is based on the working papers and presentations of Drs. B.G. Schuster and P.I. Trigg and on the relevant plenary discussions.

4.1 DRUG DEVELOPMENT AND REGULATORY REQUIREMENTS

4.1.1 Introduction

The process of new drug development or pharmocogeny involves everything that occurs from the identification of an interesting chemical compound to its eventual use as a drug in clinical practice. Many regulations are intricately involved in this process and many countries have regulations that govern drug development and the use of drugs in that country. Some of the most detailed regulations belong to the United States of America and yet these also have worldwide influence. This chapter discusses mainly the Food and Drug Administration Regulations but with reference to other regulatory agencies.

Figure 4.1 shows the basic stages in drug development and where the regulatory process, as it occurs in the United States, impinges on this development. The collection of studies which precede the clinical studies and which support the safety and efficacy of the drug are defined as "preclinical". At a certain point in the preclinical development, a decision is made to introduce the new chemical compound into humans for the first time. In the United States, such clinical studies cannot take place without approval by the FDA through a review of the preclinical data which is contained in the Investigational New Drug Application or IND. At the end of the clinical phases of drug development, a new drug application or NDA is filled with the FDA. This is a petition to be allowed to market that drug. The NDA contains all the preclinical and clinical information that will support safety and effectiveness in humans.

After approval of the NDA, phase IV or post-marketing surveillance commences. The whole development process is slow and expensive, taking an average of 10 years and costing on average some 15 million US Dollars.

4.1.2 Preclinical development

Drug development is a dynamic multidisciplinary process which includes nearly every aspect of medical science. Promising compounds can come from several sources, e.g., from basic research, from different chemical synthesis programmes, or from chemical inventories. These compounds are selected for primary efficacy testing on the basis of their structures, of their known or suspected modes of action or some other testing screen that is designed to take large numbers of compounds and yield very tentative preliminary information about their efficacy. This efficacy screening also gives some preliminary information about toxicity and about the route of

administration. Thus, the primary screen also acts as a refining system and information from the primary efficacy testing can be used to identify other structures in the chemical inventory, or screening data can be used to direct the chemical synthesis programme in order to improve upon drugs that have efficacy but may have unacceptable toxicity. Compounds can also be selected to go on to advanced or secondary screens to quantitate efficacy in more definitive models. Usually the amount of a compound that is available for the primary efficacy screen is very limited and so it is often necessary to use a preparative laboratory to make the drug in sufficient quantity to carry out the studies necessary to build a preliminary data base from the advanced screening systems. Performance in all the testing systems is evaluated and at some point, the decision will be made to advance a drug to "preclinical development".

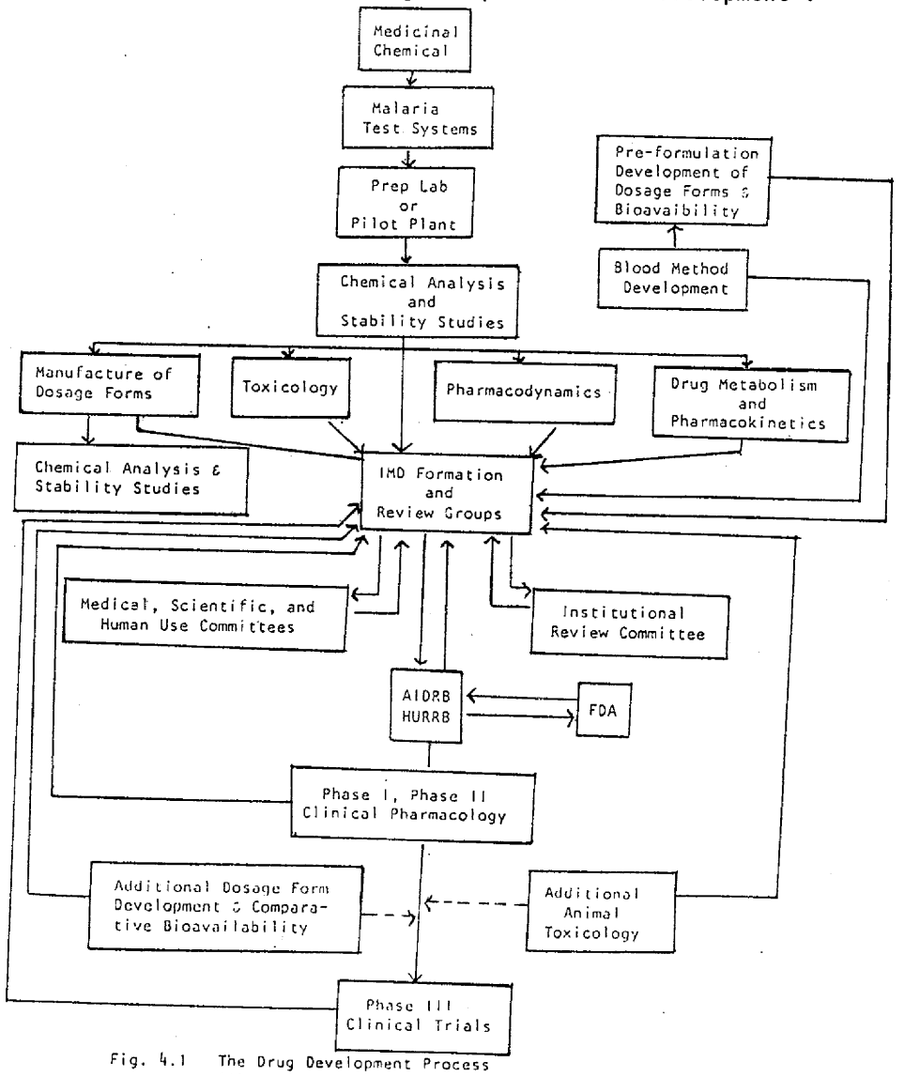


Fig. 4.1 The Drug Development Process
AIDRB - Army Investigational Drug Review Board
FDA - Food and Drug Administration
HURRB - Human Use Research Review Board
IDA - Investigational New Drug Application

The preclinical development plan must be designed to determine whether the drug is safe and effective for its indicated use in humans and, as a consequence, must also satisfy regulatory requirements. These preclinical studies include formulation development, analytical methods development, pharmacokinetics and drug metabolism and toxicology. The formulation studies should be designed to get as close as possible to the formulation that is intended for human use, taking into account the final route of administration. Often the compound has to be radiolabelled for initial metabolic studies because analytical methods at this stage are still under development.

4.1.3 Investigational new drug application (IND)

Eventually all the preclinical data are gathered and assembled into an IND application or its equivalent and submitted to the regulatory agency. This document will be reviewed, and, if it supports the safety and potential efficacy of the compound, permission will be given to study the drug in human beings. If, however any deficiences are noted, it may be necessary to do additional preclinical studies. The prime concern of the regulatory agency at this point in development is on the safety of the proposed drug.

The initial review of the IND by the FDA must be made within 30 days following submission. If the sponsor has not heard from the FDA after this period, he can proceed with the clinical study on the assumption that it has been approved.

The initials IND stand for Notice of Claimed Investigational New Drug Exemption. This is a report filed with the FDA in a prescribed form, FD 1571. The form FD 1571 is in fact a letter to the Secretary of Health, Education and Welfare for the Commissioner of Food and Drugs informing him of the planned investigation. Usually the sponsor of the IND is the pharmaceutical manufacturer of the product. However, frequently individual scientists at research institutions may wish to sponsor an IND.

The IND consists of sections covering details of the chemistry, manufacture and quality control of the drug, the preclinical data obtained with the drug details of the qualifications of the investigators and monitors of the clinical trials, a copy of the clinical trial brochure, and an outline of the clinical trials which are proposed. It also includes an agreement to notify the FDA if, when and why the investigation is discontinued and another that the FDA will notify the investigators if the investigation is to be discontinued or a NDA is aproved.

The progression from an original IND submission to all the material that is submitted in a NDA is a sequential event. Based on information submitted in an original IND the sponsor expects to begin early Phase I clinical trials in a limited number of human subjects. The sponsor's primary objective is therefore to provide evidence of safety.

In the original IND emphasis should be placed on controls of the raw material and primarily the new drug substance. This is done on the understanding that modifications of the method of preparation of a new drug substance and modifications and additions to the dosage forms are highly probable and that final specifications cannot be anticipated or expected. As the development of a new drug progresses and as more experience is gained in the laboratory and the clinic, the information in the IND is supplemented so that at the time the NDA is submitted the new drug is completely controlled.

The chemistry sections should provide a descriptive name of the drug including the dosage form as well as a complete list of the components present in the drug including the new drug substance and its chemical structure if known. The source and preparation of the drug should be described including the synthesis, extraction, isolation and purification of the new drug substance. It is understood that such details may be based on early knowledge of the drug and the sponsor is not bound to employ the same method in subsequent studies as better ones become available. The IND should, however, be updated accordingly. A description is also required of the methods, facilities and controls used for the manufacturing, processing and packaging of the new drug as well as establishing and manufacturing appropriate standards of identity, strength, quality and purity. This information is important since variations in the composition of the active ingredient or in the formulation from lot to lot may cause results to vary and thus affect the clinical information.

The information on preclinical efficacy, pharmacology, drug metabolism and toxicology should contain all the data to support the use of the drug in humans. In addition, any available clinical data for the drug which may have been used in another country or for another purpose may be included to support the sponsor's case. All the data should be summarized and the investigators' qualifications described. Any pertinent literature references should also be included.

The clinical trial brochure is basically the brochure which will be provided to the clinical investigators and should contain all the background on the chemistry of the drug, as well as on its efficacy in the laboratory, its toxicology pharmacology and metabolism. It should also draw attention to any special precautions or monitoring required during the trial.

Recently the FDA has changed the format of the IND as well as the way it reviews such applications. Now the FDA is more concerned with safety than with the details of the protocols and scientific methodology. Also the FDA is laying emphasis on the identification of competent clinical monitors of the proposed studies. In addition, the sponsor can now transfer his/her obligations to contracting agencies who carry out the clinical studies and these agencies will be held responsible by FDA for these studies.

The general investigation plan is not only an outline of the first clinical study for which the protocol will be given but of the overall clinical plan up until the submission of the NDA. The FDA will wish to determine whether this plan will provide the evidence to allow the sponsor to market the drug for the purpose envisaged. This usually promotes discussions between the sponsor and FDA so that a continuous dialogue between the two parties tends to develop.

Once an IND has been filed the sponsor is under obligation to observe the following requirements.

(a) Maintain adequate records of the distribution of the drug.

(b) Monitor the progress of the investigation.

(c) Report promptly to the Food and Drug Administration and to all investigators any significant hazards associated with the use of the drug.

(d) Discontinue the investigation if the hazards involved outweigh the potential benefits of the drug.

(e) Discontinue shipment to investigators who fail to maintain satisfactory records of their investigations.

(f) Submit progress reports of the clinical investigative programme at least annually.

In addition the Food and Drug regulations state that the investigations may not be unduly prolonged: the drug may not be commercially distributed or test marketed until an application is approved. Neither the sponsor nor any person acting on his/her behalf may disseminate any form of promotional material representing the drug to be safe or useful for the purposes under investigation.

Amendments are frequently submitted to INDs. Thus the FDA must be informed whenever new investigators are involved in the study, the clinical monitor is changed, the dosage form is modified, the protocol is revised, or changes are made in the manufacturing processes or quality control procedures.

At reasonable intervals, not exceeding on year, the sponsor must provide the Food and Drug Administration with a

summary of the preclinical data that have been obtained. As far as the clinical investigations are concerned the following specific information should be prepared for each clinical investigation:

(a) Number of subjects started on medication.

(b) Number of subjects for whom conclusions have been reached.

(c) Conditions treated.

(d) Dosages employed.

(e) Frequency of administration of the drug.

(f) Relevant clinical observations.

(g) Laboratory examinations made and results.

(h) Useful results observed.

(i) All adverse effects noted.

(j) Opinion as to whether the useful results or the adverse effects are indeed attributable to the drug under investigation.

Summaries should be prepared from the data of each clinical investigation. If data are not available from a particular investigation for the reporting period, an explanation should be provided. Possibly the onset of the study was delayed; the data may be in the process of analysis by an outside source; the study was discontinued prematurely or the investigation has been transferred to another investigator in the same institution or department.

The investigator must inform the monitor in writing whenever a study is discontinued or cancelled and request him to return all unused clinical supplies.

The progress report for each investigation should indicate whether or not the study is complete or still in progress. If the study has been completed, then the report should be designated as preliminary or final. Progress reports should also contain updated copies of the investigator's monographs whenever revisions have been made.

Aside from the regular progress reports, the following types of information should be reported to the FDA immediately upon receipt:

(a) Information concerning any error in the drug supply or its composition or in its labelling, such as a mixup with another article.

(b) Information concerning any bacteriological, or any significant chemical, physical, or other change or deterioration in the drug, or any failure of one or more distributed batches of the drug to meet the specifications established for it.

(c) Information concerning any unusual failure of the drug to exhibit its expected pharmacological activity.

The following types of information should be reported: information concerning any unexpected side effect, injury, toxicity, or sensitivity reaction or any unexpected incidence or severity thereof (whether or not determined to be attributable to the drug).

The completed IND provides a comprehensive summary of the investigational programme. The monitor, as medical representative of the sponsor, must review the IND objectively and assure himself that the IND contains suitable answers to the following questions:

(a) Are the animal toxicity studies adequate to justify trial in humans?

(b) Is there an adequate basis for the therapeutic benefits to humans?

(c) Do the investigators selected to carry out the clinical investigations have the proper professional qualifications and facilities?

(d) Have suitable precautions been defined to assure the safety of the experimental subject?

(e) Will the experimental design provide significant data that will prove the safety and efficacy of the new drug?

The paper work necessary for the preparation of an IND is extensive.

4.1.4 Clinical studies

The FDA classifies clinical studies into Phase I, II, III and IV. Phase I studies are the clinical pharmacology studies whose primary purpose is to define the safety and tolerance of the new drug in humans. These are done with small numbers of normal, healthy volunteers. Phase II studies are controlled studies whose primary purpose is to evaluate the efficacy of the new drug also in a small number of patients. Phase III clinical trials are usually wide-scale premarketing tests of a drug's utility, i.e. an attempt at gaining a "real world" look at a drug's performance. Although the emphasis in Phase II and III is on determining efficacy, safety and tolerance, data on the drug are also collected. Usually clinical studies proceed sequentially from Phase I to Phase IV, that is safety and

tolerance are established early on and then efficacy is substantiated. Since the Phase I studies are carried out on relatively small numbers of healthy subjects, it is important to remain vigilant for adverse effects as larger and often less healthy groups of people are exposed to a new drug. Exceptions to this usual scheme can occur. For example, many of the newer AIDS drugs are being evaluated under the provision of compassionate treatment in order to make a promising drug avaiable as quickly as possible for a disease for which there is no known cure. Thus, a drug which has shown some efficacy early in the development, might be used in a clinical treatment setting much sooner than it otherwise might have been.

The guiding principle of any clinical investigation is to safeguard adequately the welfare of the study partipants. The FDA thus has specific institutional reviews and informed consent guidelines for all clinical studies. The investigators also must have suitable credentials and qualifications for carrying out such trials. The investigations need to have clearly stated objectives, methodologies and valid satistical analysis of the results have to be applied. The volunteers/patients have to be carefully selected according to predetemined criteria, taking into consideration the status of the preclinical studies as well as, the risks and objectives of the trials. Investigators must adhere to the protocol. Any protocol modifications have to be documented and, if appropriate, the modifications have to be approved through the instittutional review process.

4.1.5 New drug application (NDA)

Once the clinical studies are completed and the drug has shown acceptable efficacy and tolarance in humans, a new drug application is submitted by the sponsor to the registration authority. This document is a compilation of all the manufacturing data, pharmacology, toxicology and clinical experience and constitutes the formal application to the registration authority to allow the drug to be licensed and marketed. The authority makes the decision on the basis of the review of the documents, utilizing experts in the various pertinent fields, such as medicine, biopharmaceutics, pharmacology, toxicology and statistics. A preliminary decision may be issued, allowing a period in which the sponsor can respond and correct any minor deficiences that may have been cited in the registration document. Final approval includes approval of labelling for the indicated use. After approval, supplements can later be submitted to the registration authority. These might contain new information about changes in the formulation or include information on adverse effects or noted changes in the efficacy profile, or perhaps other uses of the drug might be discussed. After a new drug is approved. the sponsor must continue to monitor and report to the authority on any adverse effects which might be attributable to the drug. This is the requirement for postmarketing surveillance.

The FDA, which has limited resources, establishes priorities for the review of INDs and NDAs using an official classification based on whether the drug is, for example, a new modecular entity, a new formulation of a drug already marketed or a new claim for a drug already on the market. It also classifies applications according to therapeutic value and importance. A new drug for AIDS would get very high priorly whereas a new medication for malaria which affects few people in the USA would have a much lower priority. Recently the Orphan Drug Act was brought into effect to assist the development of drugs which have a low potential market in the USA. Orphan drugs are those which would be useful for use in the USA by probably less 200 000 people a year. In the USA, antimalarial drugs clearly fall into this category. A system of tax incentives and licensing agreements is available which will allow a manufacturer, who would not have the economic incentive to develop such drugs, to proceed with the development and production.

The whole NDA review consists of several separate parts. The chemistry review evaluates the data on manufacturing, the control of the drug product labelling, stability and environmental impact. The pharmacology review evaluates all the nonclinical studies pertinent to the safety and efficacy of the drug. The biopharmaceutics review evaluates the bioavailability and the pharmacokinetic and metabolic studies, whilst the medical review evaluates the data including adverse reactions from the clinical trials which are pertinent to the effectiveness and safety of the drug for the proposed indications. There is also a statistical evaluation of all the data. The FDA publishes guidelines for the production of IND's and NDAs and provided these are closely followed the review process is made easier. It is, however, a long process. The review of the NDA for mefloqine in the USA has taken almost three years but the average is around two years, especially if the drug is a new chemical entity.

4.1.6 Good manufacturing, good laboratory and good clinical practices (GMP, GLP and GCP)

The FDA expect the studies and the data collection at all stages of drug development to be of a high standard. The laboratory studies, especially those related to the preclinical toxicity, must be performed according to good laboratory practice (GLP). The clinical studies must be performed under good clinical practice (GLP) and the manufacturer of the drug that was used throughout all the development stages must be produced according to good manufacturing practise (GMP).

Guidelines for all these Practices are produced by the FDA who also inspects the manufacturing plant or laboratory to ensure that these guidelines are followed, and to licence the facility to carry out the proposed work. The guidelines for GMP

lay down the specifications of all processes involved in the manufacture of the drug and production of the formulation from receiving the starting materials of known and specified quality through the documentation of all steps to accounting for every drug label that enters or leaves the facility. This review is intensive in manpower and detail. Moreover, since the FDA will not recognize a facility as satisfying GMP unless their inspectors visit it, this restricts the number of foreign manufacturing facilities which are acceptable to the FDA.

GLP is mainly a quality assurance for the toxicology studies and for the collection of any other data related to safety. It regulates the whole process of these studies covering the personnel, the facilities, the equipment, the documentation, quality control and the protocols. Again all the procedures have to be specified in detail and controlled. It is a very expensive procedure to comply with GLP because of the large amount of documentation and the separate quality assurance which has to be carried out to ensure that the studies were performed as stated.

GCP is a relatively new set of regulations. Basically they set out the responsibilities of the clinical investigator and the conditions for the trials facility to meet the needs of the proposed studies. The investigator has to ensure that the protocol is followed in detail, that the patients sign the consent form, the case report forms are completed, that adverse reactions are reported and account is made of all of the drug supplies. A number of inspections of clinical facilities have indicated that the most two common deficiencies are failure to complete the consent forms properly and failure to account for drug supplies. In general, adherence to the protocol is not a major problem, at least in the USA. The FDA will accept clinical studies carried out in countries other than the USA provided they meet FDA standards, i.e. the studies were adequately designed, well controlled and carried out by qualified investigators. The clinical facility must be adequate for carrying out the proposed study which has to meet ethical standards described in the Declaration of Helsinki or the laws of the country of oriigin whichever are the stricter.

4.1.7 Other regulatory agencies

In the USA, an IND must be approved before Phase I trials in humans are initiated, but this is not true of other countries. A Phase I tolerance and pharmacokinetic trial of a new drug can be carried out in the United Kingdom in normal healthy voluteers without approval of the Licencing Authority, i.e. the Committee on Safety of Medicines. This is now a matter of political debate, following two deaths in recent years during Phase I trials. However, before a clinical trial is carried out in patients, the supplier of the drug must obtain a Clinical Trial Certificate (CTX). The present law in the United Kingdom

does not prohibit the carrying out of unauthorized trials but it does prohibit the supply of drugs which have not been authorized.

The CTX is the equivalent of the IND and in general contains the same elements. Taken together the data supplied must give information on the therapeutic potential of a new drug and its likely margin of safety. Details on any clinical studies carried out abroad which are relevant should also be included. In the past, it often took 3 to 4 years to obtain a CTX, but now a decision must be given within 30 days of the receipt of the application by the Committee on Safety of Medicines.

Although drug regulations vary within Europe, it can be expected that by 1992 the countries of the European Economic Community (EEC) will have regulations which will satisfy all the countries. Submissions will be made to the EEC who will pass them to the relevant country's regulatory authorities for comment. At present, certain countries e.g. Switzerland, do not have regulations regarding the initiation of clinical trials. The responsibility in this case lies with the sponsoring company and the clinicians undertaking the trials. There is a tendency but, not a legal obligation, to form ethical committees at hospitals or similar institutions. Studies are carried out in Switzerland according to guidelines of the Swiss Academy of Medical Science which lay down the necessary ethical standards. Decisions on drug registration are carried by the Office of Control of Medicaments for which data similar to a NDA iis required and for which guidelines are issued.

4.2 PRECLINICAL EVALUATION

4.2.1 Introduction

Preclinical testing of a drug includes all laboratory investigations performed on a candidate drug before it is introduced into humans. Information obtained in well designed preclinical studies should exclude extreme toxicants, define organ toxicity, predict initial dosage for Phase I clinical pharmacology studies and form the basis for believing it will be safe to proceed to clinical trials.

Certain specific factors such as the chemical structure of the drug and the extent of its proposed use will influence the design of the preclinical studies, e.g. will the drug be given in single or multiple dose, will it be administered by the oral or intravenous route, what will be the target population for its use.

After carefully analysing efficacy data, a decision may be made to develop a medicinal chemical which appears to be superior to other drugs. In order to support all the planned preclinical and clinical investigations it is necessary to synthesize kilogramme quantitives. Large scale synthetic methods are developed in either a pilot plant or preparatory laboratory. A complete chemical analysis by an independent laboratory is performed on each bulk lot of drug and accelerated stability studies are performed on each unformulated lot.

Although the analysis of pure drug substances rarely poses problems, quantitative analysis of drug substances in the biological fluids is significantly more difficult. Therefore, while large scale syntheses are being carried out, sensitive and specific analytical methods for accurately estimating small amounts of the parent drug and its metabolites are usually being developed. In addition, the physical-chemical properties of the drug are studied at this time e.g. solubility, particle size, partition, coefficients, dissociation constants and protein-binding. The candidate drug is also prepared in a radiolabelled form to support pharmacokinetic and drug metabolism studies. When possible, pharmacokinetic estimates of blood levels are made in an animal model to ensure early achievement of steady-state levels in the subacute toxicity studies.

The design and extent of the toxicological studies are determined by the intended clinical use of the drug and by any information available on closely related compounds. In the initial stages, toxicological studies are conducted in sufficient depth and duration to support Phase I pharmacokinetic and tolerance trials in volunteers and limited Phase II studies in a small number of oligosymptomatic patients, usually male subjects. Later, stages of toxicological evaluation are designed to support repeated administration, or the use of the drug in large numbers or in other populations such as females and young children.

Each of these investigations outlined above culminates in a definite report which forms part of the IND or its equivalent.

Thus preclinical development is multidisciplinary and requires good coordination; preclinical and clinical studies also require close coordination.

4.2.2 Efficacy testing

Drug development programmes for antimalarials have a hierarchical network of efficacy testing. All compounds are assessed initially in one or more primary screening models. Ideally primary screens have optimum sensitivity, a high degree of reproducibility, a high capacity for screening, require a minimum quantity of test compound and have a low cost per test. A compound which is considered active in the primary screen is then evaluated further in successively more critical tests.

At the end of each successive stage of testing a decision is made to advance the compound to the next stage or to discontinue testing. These decisions are based upon review of all efficacy and tolerance data available at the time, and are influenced especially by a comparison of the properties of the compound concerned with those of its analogues. Secondary test systems and other advanced testing models are designed to verify and quantify the antimalarial activity of each candidate compound, to develop an understanding of its unique characteristics, to gain an appreciation of its limitations, to provide information to guide synthesis of analogues, and to provide information to guide development of a rational clinical protocol. Some pharmacological and toxicological information and clues to mode of action may be obtained in efficacy testing. However, definitive information in these areas generally requires special laboratory investigations outside of the province of routine testing. Relatively few compounds reach the final stages of efficacy testing.

4.2.3 Toxicological studies

The purpose of preclinical toxicological investigations is to define the potential risk associated with the proposed administration of a new drug, drug form, or drug combination to volunteers. Toxicological investigations are not static isolated entities; a dynamic and multidisciplinary approach is required.

Toxicological evaluation of each drug is individualized based on its structure characteristics, the results from efficacy, metabolic and pharmacokinetic studies as well as feed-back from clinical studies. It can be divided into three stages. The mutagenic potential of the drug is assessed first since the test is cheap and easy to perform and if the test is positive, one has a major problem. These studies are performed concurrently with the advanced efficacy testing so that the data

is available when the decision has to be made to develop the drug further. The second stage of toxicological evaluation generally consists of acute and 28-day subacute testing which provides the framework that supports the development of the drug through Phase I to Phase II clinical investigations. Once the candidate drug has been shown to be well tolerated and efficacious in the Phase I and II clinical studies, the drug enters the third or extended study phase of toxicological evaluation. During this phase, reproductive/teratological studies, chronic toxicology/carcinogenic studies, reversal studies and appropriate special studies are performed as required.

The system utilized for determining the mutagenic potential is the Salmonella/mammalian microsome mutagenicity test or Ames test. The candidate drug is tested against histidine-dependent strains of Salmonella typhimurium, TA 1535, TA 1538, TA 98 and TA 100, alone or in the presence of a metabolic activation system (S-9 fraction obtained from livers of rats treated with Aroclor 1254). The number of revertants to histidine prototrophy is determined and compared with the spontaneous reversion rate in simultaneously exposed control plates. If the ratio of drug-induced revertants to spontaneous revertants is less than two, with or without metabolic activation, and if simultaneous positive control tests are indeed positive, the candidate drug is not considered to possess significant mutagenic potential.

4.2.3.1 Acute toxicity

The acute toxicity studies include LD_{50} determinations in rats, mice and either guinea pigs or rabbits, plus an approximate lethal dose determination in beagles. These studies are designed to determine a medial lethal dose and to establish the dose-response relationship of the lethal action of the drug in different species and by different routes of administration. Acute LD_{50} experiments follow a standard protocol regardless of the drug, the animal species or the route of administration. This rigidity of format maximizes the usefulness of the LD_{50}s determinations since it facilitates direct comparison of the toxicities of structural analogues. In acute studies, animals are observed for at least 14 days after administration of the drug and mortality data are then evaluated using probit analysis. A single dose of the candidate drug is administered to different groups of animals by oral gavage and intraperitoneal injection or, occasionally, by intravenous injection. Comparison of oral and parenteral LD_{50}s may also provide an early approximation of the relative bioavailability of the drug.

The objective of the approximate lethal dose studies in beagles is to establish a lethality threshold or, at the minimum, a range of toxicity for the candidate drug when it is administered in a single oral dose. In addition, the data obtained from this study provide valuable insights into the

pharmacological responses associated with accidental poisoning. The basic procedure is to give the drug to two dogs, each at a different dosage level. The initial doses are selected based on previous experience with structurally similar drugs and the results of the LD_{50} studies in other species. The dose level selected for the second pair of dogs will be increased or decreased depending on the responses observed in the initial pair of dogs. Ideally, the dose(s) selected for the last pair of dogs would be used to confirm the approximation of the minimum lethal dose.

4.2.3.2 Sub-acute toxicity

The goal of subacute toxicity studies is to provide a detailed toxicological profile of the candidate drug in an attempt to anticipate human toxicity. This profile is then incorporated with other preclinical data into a Phase I clinical pharmacology protocol designed to minimize any adverse drug reactions. Four objectives have been established to assist in attaining this goal. These objectives are to document drug-induced functional and morphological pathology, to establish a "no observable effect" dose, to define those clinical measurements that presage drug-induced tissue dysfunctions and to educe a mechanism for the toxic action(s) of the drug. Data acquisition in support of the study objectives is markedly dependent upon the animal species and the drug doses selected.

In subacute toxicity studies, the candidate drug is administered for 28 consecutive days. These studies are intended to provide information on the effect of repeated exposure to sublethal doses of the candidate drug and to determine the relationship between effect and doses. The design of multiple-dose studies permits one to monitor progressive dysfunction with increasing duration of drug exposure and to assess drug-induced morphological changes.

Historically, the rat and beagle are the species which have been most often chosen for the subacute studies. These two species are readily available and a large toxicological data base has been generated for them. Furthermore, the rat and beagle are extremely sensitive to the effects of most antimalarial drugs. For example, repeated studies have indicated that the beagle is more sensitive than the rhesus monkey for subacute studies for all classes of aminoquinoline and aminocarbinol antimalarials, with the exception of the 8-aminoquinolines. Thus, during the subacute testing phase for 8-aminoquinolines, studies in the rhesus monkey have been performed in addition to studies in the beagle and the rat. Although the metabolism in these species has not always correlated with the metabolic profile derived from subsequent clinical studies, the inherent sensitivity of the rat and beagle to these classes of compounds justifies their use in the subacute toxicological evaluation.

Doses selected for multiple-dose studies should include a high dose that produces significant toxicity, a low dose that produces no observable toxic effects and an intermediate dose for dose-response estimates. The dosage regimens employed in the subacute studies are determined following a pharmacokinetic appraisal of the candidate drug in the appropriate species. The philosophy is that the animal should be presented with a steady-state concentration of the drug during most of the period of drug exposure. Since many antimalarials have long half-lives, this has necessitated the incorporation of a loading dose into the dosage regimen of some candidate drugs. Selection of actual dose levels is derived from the acute studies as well as from information obtained in efficacy studies and previous subacute studies with structurally similar compounds.

Young animals (6-8 month-old beagles and 5-6 week-old rats) are utilized for these toxicity studies. These animals are pubescent at the initiation of the study and during this period of rapid growth and development are more susceptible to the toxic manifestations of a candidate drug. The rate of weight gain has been found to be a sensitive index of drug toxicity in evaluating candidate antimalarials. In addition to body weight determinations, a variety of tests are conducted in each animal during the subacute studies to document drug-induced toxicity. These tests and observations include daily clinical signs, weekly food and water consumption, weekly urine and faecal analysis (baegle only), pre- and post-dosing ophthalmological examinations (beagle only), periodic haematological and clinical chemical tests, plus terminal organ weights and histopathological examination of all tissues.

4.2.3.3 Chronic toxicity

The third or extended phase of toxicological evaluation begins after the analysis of Phase II clinical pharmacological studies has clearly indicated promising therapeutics potential of the candidate drug. Information about the metabolic and pharmacokinetic characteristics of the candidate drug in several species, including humans, at this stage of development makes it possible to individualize the design of these advanced stages of toxicological investigation. This stage of evaluation generally includes carcinogenic, chronic toxicity, and reproductive/ tertatological studies. Often reversal studies or other special studies are necessary to define more completely the toxicity of the candidate drug.

The scope of chronic toxicity testing is dependent in part on the proposed clinical use of the candidate drug. A drug proposed for single-dose therapy may only require 3 - 6 months of toxicological testing while a proposed prophylactic agent may require studies of two years' duration or longer. The design of long-term toxicity studies is similar to that of the subacute studies described in section 4.2.3.2 except that dose levels must be adjusted approximately based on the results of the

subacute toxicity studies, pharmacokinetic and metabolic studies, and the proposed therapeutic dosing regimen. In addition, the number of animals is increased and the intervals between sampling of blood for haematological and clinical chemical determinations are lengthened.

Routinely, the chronic toxicity study in mice and rats is combined with carcinogenic studies as this combination provides for a more efficient and economical use of laboratory resources and personnel. During this two-year study, the drug is incorporated into the feed to provide a constant daily dose rate per kilogramme body weight. A minimum of 50 animals per sex is assigned to at least three drug dose levels to ensure an adequate ssample size in surviving groups at study termination. Currently, studies are initiated in pubescent animals rather than in utero as in a two-generation study. The current procedure was designed to assess the carcinogenicity of drugs being developed for a rather restricted target population, young adult males; however, future carcinogenic studies may require a multigenerational approach, as the target population will have a broader base.

A major aspect of the extended phase of toxicological evaluation is assessing the action of a candidate drug on the reproductive performance of test animals. These studies are routinely performed in three parts. Part 1 is an investigation of a drug's effect on general reproductive performance and fertility, part 2 is a determination of the teratogenic potential of a drug, and part 3 is an evaluation of the prenatal and postnatal toxicities of a drug. Although dose selection is critical for all toxicological studies, it is especially critical for reproductive studies because of the added complexity of parent-offspring interactions. Dose selection is further complicated by the long half-lives of many candidate antimalarials which cause a significant lag period before steady-state conditions are attained. The interpretation of results from parts 2 and 3 studies is especially difficult in these circumtances because these studies require relatively short-term dosing regimens. Consequently loading doses have been incorporated into these studies which ensure early attainment of steady-state conditions throughout the period of drug exposure. Currently, three dose levels of the candidate antimalarials are utilized in each part in an attempt to ensure that a maximally tolerated dose and no-effect dose are administered.

Often questions are raised during the toxicological evaluation of a candidate drug which cannot be answered with the standard battery of tests. A frequent problem is whether or not a lesion is reversible. This question may be answered by performing a reversal study in which an antidote is incorporated or in which the candidate drug is withdrawn during the later stages of the study. The observation of adverse effects on fertility by a candidate drug during part 1 reproductive testing in the male may necessitate performing a dominant lethal study to pinpoint the stage of spermatogenesis most sensitive to the

drug's actions. For those drugs being developed for intravenous administration, studies of their potential to induce venous toxicity are performed. These studies are modifications of the procedures utilized by the National Cancer Institute for antineoplastic drugs. In this procedure the lateral marginal ear vein of the rabbit is used to assess vasotoxicity. Other studies designed to answer specific questions concerning an individual drug's toxicity include blood compatibility studies, neurological studies in monkeys and special electron microscope studies. It must be emphasized that the toxicological evaluation is not a rigid screening of a compound but a systematic, individualized assessment of the toxicological potential of a new drug which is performed to gain a thorough knowledge concerning the relative risk of administering the drug to humans. This permits the design of safer, more efficient clinical protocols.

4.2.4 Pharmacodynamic investigations

The primary purpose of early pharmacodynamic screening of a candidate drug is to characterize its effects on the cardiovascular, respiratory and autonomic nervous systems. These studies are expanded considerably for those compounds selected for development as intravenous formulations. Any unusual or unexpected findings made during screening will necessitate additional studies to describe the mechanism of action or to determine whether the effects may be reversed or attenuated by standard clinical procedures.

Pharmacodynamic screening is divided into three phases, each of which utilizes 10-15 kg beagles, anaesthetized with sodium pentobarbital, as the experimental model. The first phase is the dose-response part of the screen in which standardized cardiorespiratory measurements are obtained during administration of a series of progressively increasing, bolus intravenous doses of the candidate drug. The second phase consists of a slow, intravenous infusion of the drug over a 45 to 60 minute period during which electrocardiographic intervals and arterial blood gas values are determined periodically in addition to the standard cardiorespiratory measurements. This second phase is conducted in an attempt to simulate, experimentally, what would occur clinically under conditions most favourable for rapid absorption of an orally administered drug. The third, or drug-interaction, phase of the screen is conducted to provide preliminary information on whether the candidate drug modifies the cardiorespiratory responses to a series of cardioactive compounds, e.g. noradrenaline, isoproterenol, serotonin, histamine, angiotensin and acetylcholine.

Following pharmacodynamic screening, a more intensive cardiovascular and respiratory assessment of the candidate antimalarial drug is performed prior to extensive clinical investigation or development as an intravenous preparation. This advanced pharmacodynamic evaluation includes measurement of the following cardiovascular indices: arterial and left ventricular pressures, left ventricular dP/dt, peripheral leads of the electrocardiogram, heart rate, pulmonary artery and wedge pressures, cardiac output, and pulmonary vascular resistance. Respiratory evaluations include tidal volume, dynamic airways resistance and compliance, as well as measurements of arterial and venous pO_2, pCO_2, pH values, and venous haematocrit. The candidate drug is infused into a pentobarbital-anaesthetized dog at one of three infusion rates over a 20-minute period and responses are monitored prior to, during and for 120 minutes following the infusion. Thus, these investigations provide a detailed prediction of the cardiovascular and respiratory effects which may be experienced by volunteers exposed to doses of the candiddate drug that range from non-toxic or minimally toxic to moderately or severely toxic.

During the preclinical testing of a compound unusual or unexpected results may be encountered which require additional investigation. These studies should define in more detail the pharmacological activity of a candidate drug and therefore aid in interpreting toxicological findings or provide a rationale for the management of potential side effects or adverse responses in humans. For example, WR 194965, when administered subacutely to beagles at lethal doses, produced a characterictic pattern of toxic signs but no observable changes in haematology, clinical chemistry and histopathology. The nature and duration of the toxic signs, which included emesis, salivation, diarrhoea, tremors, convulsions, lethargy and weakness, suggested that the lethal action of this compound was due in part to a profound but temporary augmentation of parasympathetic activity. Pharmacodynamic screening also suggested that WR 194965 enhanced parasympathetic tone. A more detailed investigation of the cardiovascular actions revealed that atropine pretreatment or bilateral vagotomy significantly attenuated the negative chronotropism produced by this drug, thereby supporting the hypothesis that enhancement of parasympathetic tone is a major component of the pharmacological response following administration of WR 194965.

Candidate antimalarial drugs possess the potential for considerable pharmacodynamic activity, especially on the cardiovascular, autonomic, and respiratory systems. Consequently, it is imperative that the preclinical assessment of a candidate drug should include pharmacodynamic studies. These studies provide the basis for the rational synthesis of compounds which will enhance the selective advantages of the candidate antimalarial versus its other pharmacological actions. and may also suggest a novel therapeutic indication.

4.3 PRECLINICAL DATA ON SOME ANTIMALARIAL DRUGS

4.3.1 Introduction

While preclinical studies are designed in part to satisfy the requirements of various regulatory agencies, their real purpose should be to develop rational and safe protocols for clinical studies. This complex series of studies has been described in section 4.2, the procedure for which was developed as a result of current drug restriction procedures which demand a very detailed documentation of candidate compounds. The same requirements did not exist during the period 1939 - mid-1960s when drugs such as chloroquine, amodiaquine, primaquine, proguanil and pyrimethamine were developed. As a result, one is often confronted with a paradoxical situation in which less preclinical data exist, on widely-used drugs, such as chloroquine and proguanil, compared to new compounds such as mefloquine. The same explanation also accounts for why the development of drug assays and galenical techniques may be more advanced for new drugs than for old ones, e.g., proguanil and chlorproguanil.

This review is not intended to provide preclinical data on antimalarial drugs in use and under development, but rather to highlight current studies and some of the problems which need to be solved in antimalarial drug development today.

4.3.2 Drug screening models

The complicated procedure of preclinical development is further compounded by the biological diversity of the parasite's life cycle, as well as by the parasite's ability to develop resistance to existing drugs. Many avian, rodent and simian models have been developed over the last fifty years to test the efficacy of potential drugs against the various stages of the life cycle. Today, few workers still use avian malaria models, although Gwadz et al. (1983) reported that a Plasmodium gallinaceum gametocyte screen was more sensitive than rodent models for detecting gametocytocidal activity.

Rodent and simian models continue to be used for determining the efficacy in vivo of drugs as blood and tissue schizontocides, as gametocytocides and as sporontocides (Ager, 1984; Rossan, 1984). In fact, the P. cynomolgi/rhesus monkey model is still the only available model for measuring hypnozoitocidal activity of drugs with the potential for treatment of the relapsing malarias due to P. vivax and P. ovale.

The adaptation of human malaria species to development in New World monkeys has been of great importance to research. Many different isolates of P. falciparum and P. vivax have been adapted over the last decade to develop in Aotus and Saimiri monkeys, now the two most commonly used hosts for such studies. However, unlike the simian parasites in macaques which infect their hosts on a predictable basis, the human malarias require long periods of adaptation if predictable results are to be obtained, may be difficult to transmit by mosquito passage and may not adapt to all karyotypes of the monkeys. These factors, coupled with the cost and difficulties in obtaining consistent supplies, limit the use of simian monkeys in antimalarial drug research.

Rodent malaria models are more widely used to determine preclinical efficacy of antimalarial drugs in vivo since it is easy to maintain both blood and sporozoite-induced infections in mice, the infections are reproducible, and only small drug quantities are required for screening. Moreover, a wide selection of well-characterized sensitive and drug-resistant parasite strains and lines are available and tests have been standardized to determine drug efficacy against all stages of the parasite life cycle. Such rodent screens were originally established over forty years ago, but are still the mainstay of preclinical efficacy testing of antimalarial drugs. However, it must not be forgotten that the pharmacokinetics and metabolism of many drugs differ radically between rodents, primates and humans.

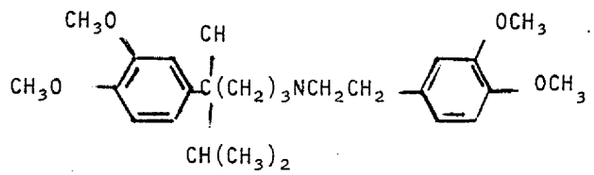
Following the developments of techniques for the continuous cultivation of P. falciparum in vitro (Trager & Jensen, 1976; Haynes et al., 1976), in vitro techniques have become an essential part of antimalarial drug development. Reliable methods for the isolation of single infected red cells have allowed the selection of characterized clones of P. falciparum (Oduola et al., 1988), two of which are routinely used by the Walter Reed Army Institute of Research (WRAIR) for screening by a modification of the in vitro test developed by Desjardins et al. (1979). The W-2 clone, derived from the CDC Indochina III isolate, is resistant to chloroquine, pyrimethamine, sulfadoxine and quinine but susceptible to mefloquine. The D-6 clone, derived from the CDC Sierra Leone isolate, is a naturally occurring mefloquine- resistant clone which is susceptible to other antimalarial drugs. The characteristics of these clones make them useful as simultaneous controls and the use of a growth medium which contains low concentrations of para-aminobenzoic and folic acids allows the evaluation of diverse chemical classes of drugs, including dihydropteroate synthase inhibitors and dihydrofolate reductase inhibitors. A large number of additional clones and isolates of P. falciparum of characterized drug susceptibility are also available for drug screening. In vitro tests for gametocytocidal activity against P. falciparum have also been developed. However, these tests generally assess the drug effect on the basis of morphological observations rather than parasite infectivity to mosquitos, the latter being cumbersome and labour intensive.

4.3.3 Blood schizontocides

4.3.3.1 4-Aminoquinolines

Chloroquine has been the most widely used antimalarial, both for treatment and prophylaxis, over the last forty years owing to its rapid action against the asexual blood stages of all sensitive human malaria parasites. It has no effect on the liver stages of any of the human parasites. Its use is, however, being increasingly compromised by the spread of chloroquine-resistant Plasmodium falciparum throughout the world. Recent studies have shown that resistance to chloroquine results from the active transport of the drug out of the parasitized red cell so that toxic levels do not build up in the parasite's cytoplasm (Martin et al., 1987; Krogstad et al., 1987).

Fig. 4.2



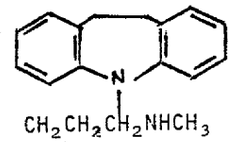
Verapamil

Verapamil (Fig. 4.2) and two other channel blockers, as well as vinblastine and daunomycin, each slowed the release and increased the accumulation of chloroquine by resistant (but not susceptible) P. falciparum in vitro. These results have stimulated several research groups to consider the possibility of using chloroquine in combination with calcium antagonists for the treatment of chloroquine-resistant malaria infections. A concentration of 1-2 x 10^{-6} molar of verapamil was required to "reverse" in vitro resistance in several clones of P. falciparum (Milhous et al., 1987). These studies were conducted with racemic verapamil. More recent investigations have shown that the R-(+)-isomers of verapamil, gallopamil and devapamil, which do not bind to cardiovascular calcium channels, also "reverse" resistance to chloroquine (Ye & Van Dyke, 1988).

"Reversal" of chloroquine resistance by such compounds has also been demonstrated in vivo. Preliminary results have shown that, in primary blood-induced infections with the multi drug-resistant Vietnam-Smith strain of P. falciparum in Aotus monkeys, treatment with nifedipine at 5, 10 or 20 mg/kg body weight plus chloroquine at 20 mg/kg administered orally

for three days initially suppressed parasitaemia. However, retreatment with nifedipine (20 mg/kg) plus chloroquine (20 or 40 mg/kg) was required before three out of four monkeys were cured; the fourth died of possible drug toxicity. Co-administration of SKF 21133A, a chloropromazine analogue without typical dopamine receptor side effects, with chloroquine resulted also in initial suppression of parasitaemia and parasite clearance upon retreatment, but the regimen was not curative. More recently, desipramine (Fig. 4.3), a tricyclic antidepressant, has been shown to have superior "reversal" properties to verapamil and to be effective in vitro at concentrations which are easily obtained with doses used in humans for the treatment of depression. When Aotus monkeys infected with chloroquine-resistant P. falciparum were treated with chloroquine plus desipramine, their parasitaemias were rapidly suppressed. Desipramine was found to be one of the most effective compounds yet described for the "reversal" of chloroquine resistance, both in vitro and in vivo (Bitonti et al., 1988).

Fig. 4.3



Desipramine

While these observations strengthen scientific evidence for a mechanism of resistance to chloroquine, the potential clinical application of calcium antagonists for the treatment of drug-resistant malaria infections should be viewed with cautious optimism since many questions remain to be answered before such combinations are tested in the clinic. For example, while several compounds with apparent lack of cardiotoxicity have been shown to "reverse" resistance to chloroquine in P. falciparum, detailed pharmacokinetic and toxicological studies must be conducted on these compounds alone as well as in combination with chloroquine before clinical evaluation can be considered.

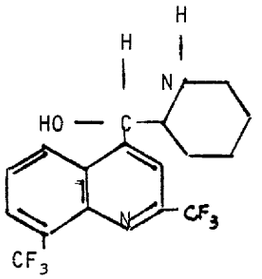
Desipramine produces arhythmias at doses above its therapeutic range as an antidepressant. As chloroquine shows a cardiotoxicity at high doses, a combination of the drugs might have increased toxicity, even at low doses. Such drug interactions may be important, particularly since chloroquine may accumulate in the host to a greater extent in the presence of such compounds therefore reducing the therapeutic index of chloroquine itself.

In contrast to the above, calcium agonists enhance efflux of calcium and actually increased "resistance" to chloroquine. These findings, combined with previous unconfirmed clinical malaria in patients undergoing chloroquine prophylaxis and dilantin therapy, suggest that this drug interaction may have significant clinical relevance (Kyle et al., 1987).

Verapamil, chlorpromazine and several other calcium antagonists which "reverse" resistance to chloroquine, do not "reverse" resistance to mefloquine. However, one calcium antagonist, WR 256473, was found to potentiate in vitro the action of mefloquine against mefloquine-resistant isolates and clones of P. falciparum without affecting the response of sensitive parasites (Kyle et al., 1988).

4.3.3.2 Mefloquine

Fig. 4.4



Mefloquine

Mefloquine (Fig. 4.4), a quinoline methanol, has been registered since 1984 as a single drug for both treatment and prophylaxis and as a fixed combination with pyrimethamine and sulfadoxine for treatment. It is probably one of the best studied antimalarials to date. It is a potent long-acting blood schizontocide active against multi-resistant falciparum malaria. It is effective in a single dose and so avoids patient compliance problems associated with quinine/tetracycline combinations which are also used for the treatment of multi-resistant falciparum infections. Mefloquine is only available as an oral tablet formulation since its irritant properties and its bitter taste have prevented so far the development of either a parenteral or paediatric formulation.

The above triple combination of mefloquine was developed in response to concern that reliance on mefloquine alone might give rise to the emergence of resistant parasites and in keeping with the principle of deploying drug mixtures to treat bacterial infections and prevent the development of resistance. Merkli & Richle (1980), using a serial technique, showed that resistance was developed rapidly by the N strain of P. berghei to pyrimethamine/mefloquine when used alone but more slowly to pyrimethamine/sulfadoxine and even more slowly to

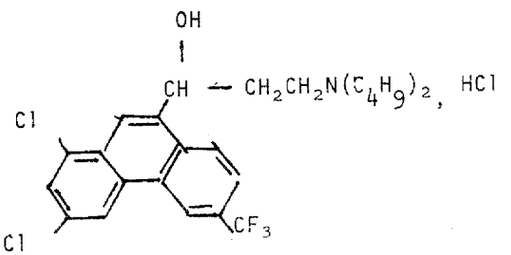
the triple combination of mefloquine/sulfadoxine/pyrimethamine. Similar results, obtained by Peters & Robinson (1984) using a relapse technique, also showed that the triple combination, as compared to mefloquine alone, considerably delayed the emergence of resistance in the N strain of P. berghei. There was also the suggestion that the triple combination limited the pre-existing increase of resistance to pyrimethamine/sulfadoxine. Both of these studies have now been criticized because they were performed with blood-induced infections which may not be relevant to the situation in the field. A laboratory model which incorporates the cyclic transmission of the parasite and the resultant genetical exchange during meiosis should be more applicable. So far, these above experiments have not been repeated with such a model.

4.3.3.3 Halofantrine

Halofantrine (Fig. 4.5), a phenanthrene methanol, was registered for the treatment of malaria infections in 1988 in France and certain francophone African countries and its registration is being sought world-wide. Like mefloquine, it was developed originally by the Walter Reed Army Institute of Research. This blood schizontocide shows an activity comparable to that of mefloquine in a variety of laboratory models (Canfield, 1980; Desjardins et al., 1979). It appears to be a safe and very well-tolerated drug.

Toxicological screening has provided no evidence of genotoxic or teratogenic activity, nor of adverse effects on fertility or fecundity (WHO, 1988b). It was found, however, to be embryotoxic at doses of 30 mg base/kg/day in rats and at 60 mg base/kg/day in rabbits over a period of 4 weeks. Dose-related reductions in growth rate and survival of pups occurred when they were exposed to milk from dams given 50 mg base/kg/day but such an effect was not observed at 25 mg base/kg/day. The use of the drug is therefore contraindicated in pregnant women and lactating mothers.

Fig. 4.5



Halofantrine hydrochloride

Halofantrine is largely insoluble in water. Its systemic absorption from the currently available tablet and suspension formulations varies unpredictably, but these variations are reduced when the dosage is divided. These observations suggest that these formulations are poorly bioavailable and continued efforts are being made to develop both an improved oral dosage form and an injectable solution.

The clinical and parasitological response to halofantrine has been evaluated in almost 1000 patients with acute falciparum or vivax infections. These studies have provided evidence that halofantrine can produce clinical and parasitological cures for infections resistant to chloroquine and to pyrimethamine/sulfadoxine combinations (Cosgriff et al., 1982; Boudreau et al., 1988). Early studies in vitro also indicated that halofantrine was active against isolates and clones of P. falciparum with reduced susceptibility to mefloquine (Cosgriff et al., 1982). However, more recent data indicate that this absence of cross-resistance may not be absolute. Webster et al. (1985) reported that mefloquine-resistant clones of P. falciparum isolated from a mefloquine treatment failure in Thailand were not susceptible to halofantrine.

In addition, the D-6 clone of P. falciparum has consistently shown over a period of three years a parallel low sensitivity to mefloquine and halofantrine (50% inhibitory concentration IC_{50}-halofantrine 3.94 ng/ml and IC_{50} mefloquine 7.26 ng/ml) compared to the W2 clone which is sensitive to both drugs (50% inhibitory concentration IC_{50}-for halofantrine 0.39 ng/ml and IC_{50} for mefloquine 1.31 ng/ml) (Oduola et al., 1987). Cross- resistance between halofantrine and mefloquine, as well as between halofantrine and chloroquine, quinine and primaquine, has been observed in rodent malaria models (Robinson & Peters, 1985; Robinson et al., 1986). The relevance of these laboratory findings to the clinical situation is unclear and cannot be determined until halofantrine is used in controlled clinical studies to treat infections which have failed to respond to mefloquine.

4.3.3.4 Artemisinin and its derivatives

Artemisinin (qinghaosu) (Fig. 4.6), is the antimalarial principle isolated in 1971 by Chinese scientists from the wormwood plant, Artemisia annua L. Following its isolation and characterization when it was shown to have the structure of a sesquiterpene lactone (Qinghaosu Antimalaria Coordinating Research Group, 1979), artemisinin and several derivatives have been widely studied by Chinese scientists with regard to their efficacy in laboratory malaria models, their pharmacology and pharmacokinetics and their toxicology. Artemisinin, its methyl ethyl derivative, artemether (Fig. 4.7), and a hemisuccinyl

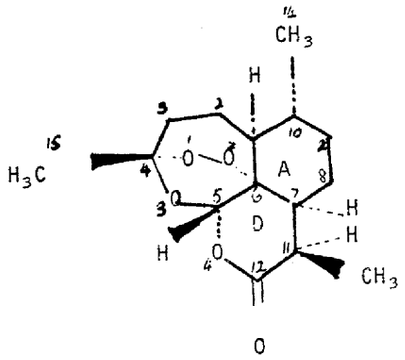
derivative, sodium artesunate (Fig. 4.8), have also been used in clinical trials conducted in China. Artemether has, in addition, been studied in the clinic in Burma and in Nigeria. Both artemether and sodium artesunate are registered for use as antimalarials in the People's Republic of China and an IND is currently being prepared by the Scientific Working Group on the Chemotherapy of Malaria on the ethyl ether derivative of artemisinin, arteether (Fig. 4.9) (Brossi et al., 1987). The apparent fetotoxic potential of these compounds may restrict their widespread use, but their rapid action makes them important candidates for the treatment of severe and complicated malaria.

Structure activity relationship studies indicate that the peroxide bridge is essential for antimalarial activity. Artemisinin is poorly soluble in water and in oils and this stimulated synthetic studies to increase its solubility in both types of solvents. Three classes of derivatives were synthesized by Chinese scientists. They differed in their relative antimalarial efficacy against blood infections of P. berghei as follows: carbonates > carboxylic esters > ethers > dihydroartemisinin > artemisinin (Li, Y. et al., 1981). Although the carbonates were found to be the most potent, Chinese scientists did not develop them further owing to difficulties in preparing them. Derivatives of artemisinin are more unstable than the parent compound. Both artemether and artesunate are susceptible to moist and acidic conditions. The usefulness of sodium artesunate is impaired by its poor stability in aqueous solutions (UNDP/World Bank/ WHO Special Programme for Research and Training in Tropical Diseases, 1986). Consequently, Lin et al. (1987) synthesized a new series of water soluble derivatives, of which artelinic acid (Fig. 9) was shown to have a higher activity in vivo against P. berghei than either artemisinin or sodium artesunate. Artelinic acid, therefore, has potential for further development, particularly as an intravenous formulation.

Artemisinin and its derivatives are potent blood schizontocides. They are ineffective against the exoerythrocytic forms of P. gallinaceum, P. cynomolgi and P. yoelii. Clinical studies conducted from 1973 demonstrated the activity of artemisinin, artemether and sodium artesunate against chloroquine-resistant P. falciparum. Such activity was subsequently confirmed by in vitro studies.

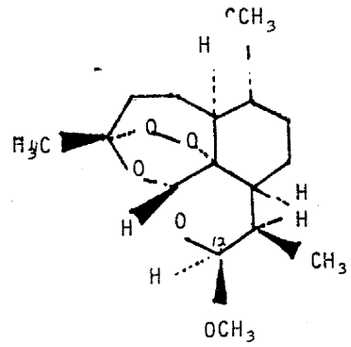
Initial studies conducted by Chinese scientists demonstrated the potent blood schizontocidal activity of artemisinin, artemether and sodium artesunate against two chloroquine-resistant isolates of P. falciparum from Hainan island, sodium artesunate being the most potent (Guan et al., 1982). Subsequently, scientists at WRAIR showed the activity in vitro of artemisinin against the Camp (chloroquine-susceptible) and Vietnam-Smith (chloroquine-resistant) isolates of P. falciparum to be comparable to that of mefloquine (Klayman et al., 1984).

Fig. 4.6



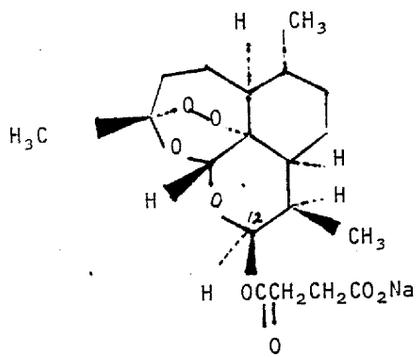
Artemisinin

Fig. 4.7



Artemether

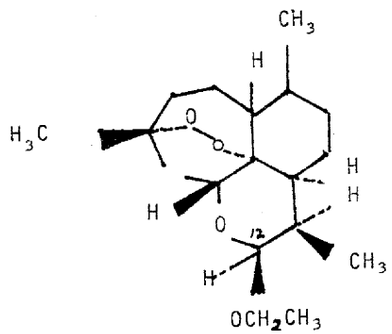
Fig. 4.8



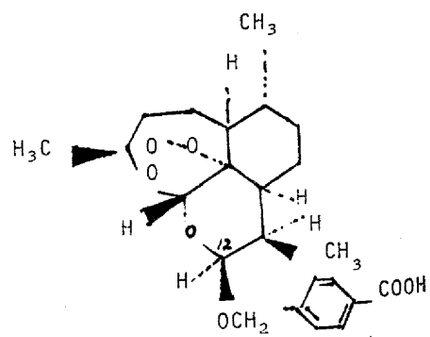
Sodium artesunate

Fig. 4.9

Fig. 4.10



Arteether



Artelinic acid

More recently, Milhous and his colleagues at the Walter Reed Army Institute of Research (WRAIR) have compared the activity of artemisinin and several of its derivatives against a variety of drug-resistant clones and isolates of P. falciparum in vitro. These studies suggested that there was no marked cross-resistance between artemisinin and chloroquine or sulfadoxine/pyrimethamine. However, among the parasites least susceptible to artemisinin, there were clones and isolates which were resistant to mefloquine. Milhous and his colleagues have also shown that both artemether and arteether were 2-3 times more active than artemisinin against both the Sierra Leone drug-sensitive D-6 and Indochina drug-resistant W-2 clones. In addition, there was no significant difference in the activities of arteether, its α-epimer or artemether. Dihydroartemisinin was the most active of all the compounds tested. (See UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 1986.) An absence of cross-resistance between artemisinin or artemether and chloroquine, pyrimethamine and pyrimethamine/ sulfadoxine has also been demonstrated in Thai isolates (Thaithong & Beale, 1985).

Arteether is the derivative which has been the most extensively tested in vivo in animal models. Both arteether and its α-epimer had similar activities against the drug-sensitive N-strain of P. berghei, both being 2-4 times more active than artemisinin. Arteether and its α-epimer also showed equivalent activities in vivo against lines of P. berghei resistant to primaquine, cycloguanil, pyrimethamine, sulfaphenazole and menoctone, similar to those against the drug-sensitive N-strain. Both arteether and its α-epimer were more active than artemisinin (Brossi et al., 1988).

Significant potentiation has been shown between artemisinin and mefloquine, both in vivo against P. berghei (Chawira et al., 1986a) and in vitro against P. falciparum in studies in London (Chawira & Warhurst, 1987) and at WRAIR (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 1986). The WRAIR studies indicated that mefloquine consistently potentiated the action of artemisinin against one naturally occurring mefloquine-resistant isolate from Sierra Leone but not against two other resistant isolates, i.e., the clinically resistant AFRIMS E12 and a laboratory induced mefloquine-resistant line.

Marked potentiation of the action of artemisinin against the drug-sensitive N-strain of P. berghei was also observed with tetracycline and spiramycin and with primaquine against a primaquine-resistant strain (Chawira & Warhurst 1987). The combination of artemisinin with primaquine was only synergistic against the N-strain of P. berghei. The authors suggest that observed potentiation may be related to the combined actions of these drugs on the parasite mitochondrion.

Artemisinin resistance has been induced under laboratory conditions in both rodent parasites and P. falciparum. An artemisinin-resistant line of P. yoelii produced by Chawira et al. (1986b) was cross-resistant to dihydro-artemisinin and artemether but surprisingly not to sodium artesunate. The line continued to be susceptible to primaquine, pyrimethamine, cycloguanil and sulfadoxine/pyrimethamine but was more resistant to chloroquine and markedly more resistant to quinine, mefloquine and amodiaquine. The patterns of cross-resistance to artemisinin-resistant clones, however, appeared to be variable. For example, there was a concomitant decrease in susceptibility to halofantrine and mefloquine with an increase in susceptibility to both chloroquine and quinine when artemisinin resistance was induced in a chloroquine-resistant Indochina clone. In contrast, changes in the susceptibility to halofantrine, chloroquine and quinine were not observed when artemisinin resistance was induced in a mefloquine-resistant clone of P. falciparum (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 1986).

Acute toxicity studies have shown that artemisinin, artemether, arteether and sodium artesunate have higher LD_{50} values and chemo-therapeutic indices than chloroquine. The major toxic effects of these compounds that have been reported so far relate to changes in the haemopoietic system and the myocardium, and to fetotoxicity. The drugs do not appear to be mutagenic or tetratogenic. In 14-day subacute toxicity studies in monkeys, the toxic effect of artemisinin was mainly manifested in the haemopoietic cells of the bone marrow, particularly those of the erythroid series. This was observed at the lowest dose tested of 24 mg/kg/day (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarial Drugs, 1982a). Similar changes have been observed in rats and dogs administered similar doses of arteether for 14 days.

Although dogs given 100 mg/kg of artemisinin orally per day for 5 successive days did not exhibit appreciable changes in respiration, cardiac rate or cardiac rhythm, such changes were observed after a single large dose of 800 mg/kg.

The murine bone marrow polychromatic erythrocyte micronucleus test and the Ames test have both failed to reveal any mutagenic activity of artemisinin. The Ames test has also failed to reveal any such activity of arteether.

However, teratogenic studies in mice and rats indicate that both artemisinin and artemether exhibit fetal toxicity at doses of 1/200 - 1/400 of their LD_{50}'s. Both death and and absorption of the fetus were observed and the effects were most marked during the middle and late periods of gestation (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982a; Chen et al., 1984).

Although artemisinin, artemether and artesunate have been used clinically, the regimens were not based on pharmacokinetic data mainly due to the absence of a satisfactory method for measuring the drug and its metabolites in body fluids. Various high pressure liquid chromatography (HPLC) and gas chromatography (GC) techniques have been reported but they are generally too insensitive for adequate studies, particularly in humans. GC-mass spectroscopy (GC-MS) with selective ion monitoring has formed the basis for an assay for dihydroartemisinin currently under development at WRAIR. Preliminary studies indicate that this method is capable of detecting amounts of dihydroartemisinin as low as 10 ng ml^{-1}. However, the quantitation is indirect and any compound giving pyrolysis products similar to dihydroartemisinin will also be measured (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 1986). More recently, Breckenridge and his colleagues (personal communication) have developed an HPLC technique based on the derivatization of arteether to a α, β-unsaturated decalone which is detected in the UV spectrum. Calibration curves were linear in the range of 0-250 ng.

In spite of the absence of highly sensitive detection methods, early studies on the metabolism of this group of compounds in animals showed that the drugs were rapidly metabolized by a first-pass effect in the liver. Following intravenous injection of artemisinin or artemether to rats and rabbits, the plasma drug concentrations fitted a two-compartmental open model, giving rise to short plasma half-lives of 30.1 minutes and 39.6 minutes for artemisinin and artemether respectively. More recent studies in rabbits and monkeys given artemether intravenously also show the pharmacokinetic data to fit an open two-compartmental model with phase 2 (beta) plasma half-lives of 0.9 hours and 9.8 hours respectively. Only approximately 30% of the artemether was transformed to dihydroartemisinin within 24 hours (Zeng et al., 1984; Zeng, Y.L., personal communication).

Artemisinin given to rats intramuscularly was absorbed somewhat more slowly than when given by the oral or intravenous route with a half-life at the injection site of 3.85-5.38 hours (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982b). When injected intramuscularly into dogs as a suspension in peanut oil, artemisinin was absorbed rapidly with a peak plasma level 2 hours after drug administration and a half-life of the elimination phase of 1.6 hours. Artemether administered by the same route as a solution in peanut oil was also easily absorbed. Peak drug concentrations were reached 4.0 hours after injection of 10 mg kg^{-1} and 1.9 hours after a dose of 30 mg kg^{-1}. The half-lives of the elimination phases were shown to be 4.0 hours and 6.5 hours at doses of 10 mg kg^{-1} and 30 mg kg^{-1} respectively.

The water soluble derivative, sodium artesunate, appears to have an even shorter plasma half-life and lower volume of distribution. Early studies in rats, using thin-layer chromatography (TLC) determinations, gave a plasma half-life of 15.6 minutes and a volume of distribution of 1.11 kg^{-1}. It was suggested that these data fitted a one-compartmental model (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982b). More recent studies confirm the rapid elimination of artesunate and its conversion to dihydroartemisinin. Measurement of blood levels in dogs by radioimmune assay following the intravenous administration of 6 mg kg^{-1} gave an elimination half-life of 0.45 hours and a volume of distribution of 0.151 kg^{-1} (Zhao et al., 1986). Using HPLC with reductive electrochemical detection to measure blood levels of artesunic acid and dihydroartemisinin in rabbits following the administration of artesunic acid, it was found that the parent compound disappeared so rapidly that it could not be measured. However, sufficiently rapid sampling was achieved in one experiment to suggest a plasma half-life for artesunic acid of 1.7 min (Zhou et al., 1987).

Dihydroartemisinin was formed rapidly following the administration of artesunic acid, the pharmacokinetics of which fitted a two-compartmental model. Plasma half-lives of dihydroartemisinin of 3.0 $\pm$ 0.4 minutes and 29.0 $\pm$ 2.0 minutes were calculated for phase 1 (α) and phase 2 (β) respectively. Similar kinetics of dihydroartemisinin have been observed following preliminary experiments in rabbits using gas-chromatography-selective-ion-monitoring (GC-SIM) techniques for the estimation of blood levels.

Artemisinin, dihydroartemisinin, artemether and sodium artesunate all bind to human serum proteins, their respective rates of binding being 64%, 43%, 76% and 59% (Li, W.H., 1982). It has also been reported that artesunate binds firmly to haemoglobin to form a complex (Edlund et al., 1984).

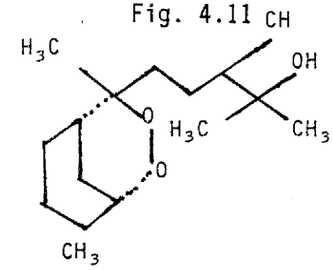
Appreciable levels of artemisinin, artemether and artesunate have been found in the brain and fetus of rodents following intravenous injection. These observations indicate that these drugs cross the blood-brain and blood-placenta barriers, a fact that may be relevant to the embryonic and central-nervous system (CNS) toxicities of the drugs and their antimalarial effects on cerebral malaria.

4.3.3.5 Trioxanes, tetraoxanes and peroxides

As the antimalarial activity of artemisinin and its derivatives appears to be related to the 1,2,4-trioxane ring, attempts have been made to synthesize simple trioxanes which may lead to potential antimalarial compounds. Over 150 such

trioxanes and tetraoxanes have been synthesized by Jefford and his colleagues, some of which have significant activity *in vitro* against the drug-resistant (Indochina W2) and drug-sensitive (Sierra Leone D6) clones of *P. falciparum* (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 1986; Jefford et al., 1988). Preliminary studies indicate that certain of these compounds have antimalarial activities *in vivo* against rodent malaria parasites similar to that of artemisinin (Jefford, C. & Peters, W., personal communication).

Fig. 4.11



Yinghaosu A

The demonstration of antimalarial activity of the sesquiterpene peroxide, yinghaosu A. (Fig. 4.11) from *Artabotrys uncinatus* (Liang, 1985), has also led to the synthesis of analogues of this compound, particularly since the parent compound is difficult to isolate. Hofheinz et al. (1988) have devised a method for the synthesis, starting from carvone, of derivatives of the 2,3-dioxabicyclic nonane ring structure which is the core structure of yinghaosu. Most of the compounds were active and three have been selected for preclinical development, i.e., Ro 40-6772, Ro 41-3823 and Ro 42-1611. These compounds have an extremely low acute toxicity in mice and rats with an LD_{50} of > 3000 mg/kg and show no mutagenic potential in the Ames test. Their activities *in vitro* against *P. falciparum* are comparable to those of mefloquine and quinine but 5-10 times less active than that of artemisinin. They are also active *in vivo* against rodent malaria models with higher activity being observed via the parenteral route as compared to oral administration.

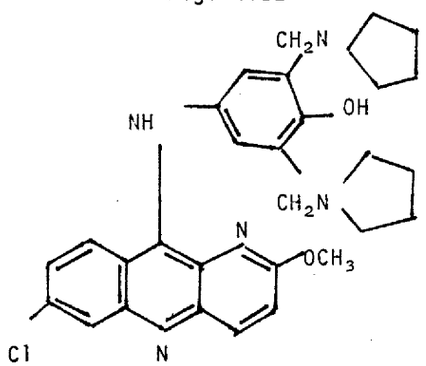
In vivo studies in rodent models indicate that Ro 42-1611 shows an apparent lack of cross-resistance with chloroquine, mefloquine and quinine and only a moderate level of cross-resistance with artemisinin. In contrast, Ro 40-6772 shows a high level of cross-resistance with chloroquine and Ro 41-3823 shows moderate cross-resistance with chloroquine and artemisinin. All three antagonize the action of chloroquine but are additive in their action when combined with mefloquine or artemisinin. These compounds clearly show potential for

clinical development and may be useful for the treatment of severe malaria since they appear to be fast-acting (Stahler et al., 1988).

4..3.3.6 Pyronaridine

Pyronaridine (Fig. 4.12), a mannich base, was synthesized in 1970 at the Institute for Parasitic Diseases, Shanghai. The compound exhibits marked blood schizontocidal activity against both rodent and simian malarias and has been used in the clinic for the treatment of naturally occurring malaria infections (New Drug Group of the Former Department of Malaria, 1980). It has been shown to be active against multidrug-resistant clones of P. falciparum in vitro, as well as against chloroquine-resistant P. berghei parasites in vivo. Its activity against a chloroquine-sensitive isolate of P. berghei was approximately 5 times that of chloroquine. It apparently has no action against the tissue stages (Zhang et al., 1986).

Fig. 4.12



Pyronaridine

Acute toxicity studies in mice yielded a LD_{50} of 1369 mg/kg after oral administration and 251 mg/kg following intramuscular administration. The corresponding values for chloroquine were 663 and 90 mg/kg respectively. Subacute toxicity studies have been conducted in rats, rabbits and dogs. Studies with rats given 20 mg/kg for 14 days failed to show any major side effects. Rabbits given 10 mg/kg for 7 days also tolerated the drug well. However, dogs given 12 mg/kg daily for one month died within 30 days after medication following loss of appetite, salivation, vomiting and trembling; one of these dogs showed serious heart failure before death.

Mutagenicity tests with the Salmonella typhimurium/ microsome system showed an induction of mutations without metabolic activation in strain TA 1537. Reversion was dose-dependent and similar to chloroquine. There was no induction of mutation in strains TA 98, TA 100, TA 1535 and TA 1538 (Ni et al., 1982a).

Teratogenicity tests in rats conducted with the equivalent of 8, 15, 30 or 33 times the clinical doses yielded no evidence of teratogenic effects, but the rate of fetal resorption was significantly increased (Ni et al., 1982b).

Resistance to pyronaridine could be induced through increasing subcurative doses of the drug in the P. berghei model. Within 23 passages, it reached a very high level with refractoriness to a dose of 2400 mg/kg. The virulence of the pyronaridine- resistant isolate was much reduced. The isolate showed also reduced sensitivity to mepacrine, 4-aminoquinolines and artemisinin. Without drug pressure, the pyronaridine-resistant line reverted to pyronaridine sensitivity within five passages (Shao et al., 1982).

Pharmacokinetic studies in rabbits indicated peak plasma concentrations of pyronaridine 15 minutes after intramuscular injection. The drug concentrations in plasma, erythrocytes and tissues decreased rapidly within three hours.

Pyronaridine was also independently tested by WRAIR. It effected cures against a chloroquine-resistant strain of P. berghei in mice in a schizontocidal test at doses of 20-640 mg/kg, with toxicity observed at the highest dose. It was effective in a suppressive test against both chloroquine-sensitive and -resistant strains of P. berghei, although the suppressive dose $(SD)_{90}$ values were slightly higher with the resistant strain. In the in vitro testing against P. falciparum, it showed similar activity against both the sensitive and resistant isolates. Thus, the drug does not appear to show cross-resistance with chloroquine.

The clinical use of pyronaridine has not been based on pharmacokinetic principles since sensitive methods for its detection in body fluids did not exist. Recently, a spectrofluorometric method which detects the compound in nanogram amounts has been described (Feng & Wang, 1986).

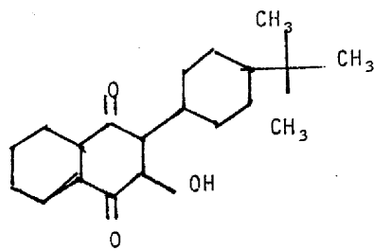
4.3.3.7 Hydroxynaphthoquinones

The antimalarial potential of the naphthoquinones was recognized in the mid-1940s when they were shown to have both suppressive and curative actions through their effect on the asexual tissue and blood cycles of avian and rodent malarias. Two compounds, M1916 and M285, showed disappointing activity against human vivax and falciparum malarias (Fieser et al., 1948) but Fawaz & Haddad (1951) successfully treated vivax malaria infections with intravenously administered lapinone (M2350).

Naphthoquinones were also investigated by workers at WRAIR who studied menoctone (WR 49,808) in patients infected with the Malaya (Camp) isolate of P. falciparum. This compound exerted only slight blood schizontocidal activity upon oral

administration of daily doses of 0.4-0.5 g for three days. With this regimen, the drug did not show any appreciable gametocytocidal or sporontocidal effect. It also had no causal prophylactic activity against the Malayan (Camp) or Uganda I isolates of P. falciparum. It was concluded that "poor absorption from the gastrointestinal tract is characteristic of compounds of this group and may explain the lack of demonstrable activity by WR 49,808". This brought further investigation of the naphthoquinones to a temporary halt. At the time, no attention seems to have been paid to the observations of Fawaz & Haddad (1951) which should have triggered studies on their bioavailability that could have explained the disappointingly low activity of the earlier fformulations in human malaria.

Fig. 4.13



BW 58C

Recently, interest in hydroxynaphthoquinones has been renewed. One compound, 2-(4-t-butylcyclohexyl)- 3-hydroxy-1, 4-naphthoquinone (BW 58C, Fig. 4.13), was shown to be highly active against P. falciparum in vitro and against P. berghei and P. cynomolgi in vivo (Hudson et al., 1985). It was also effective against Theileria parva, Theileria annulata and Eimeria tenella. In the P. yoelii nigeriensis/mouse system, BW 58C exhibited also causal prophylactic activity comparable to that of primaquine. Chloroquine-, mefloquine-, pyrimethamine-, sulfonamide- and primaquine-resistant lines of P. berghei were shown to be fully susceptible to BW 58C. The compound has a low mammalian toxicity, with an oral LD_{50} in rats of >2g/kg body weight. Unfortunately, clinical development of this BW 58C was discontinued after Phase I trials but research on this series of compounds continues.

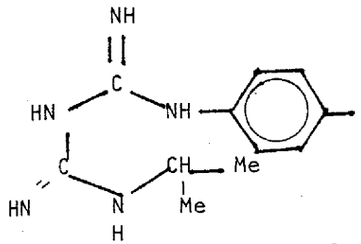
4.3.4 Causal prophylactic drugs

4.3.4.1 The biguanides

With the spread of chloroquine-resistant P. falciparum, there has been renewed interest in the biguanides, proguanil (Fig. 4.14) and chlorproguanil, particularly as chemo-prophylactic drugs. Both drugs are dihydrofolate-reductase inhibitors but, unlike pyrimethamine, they have a marked effect

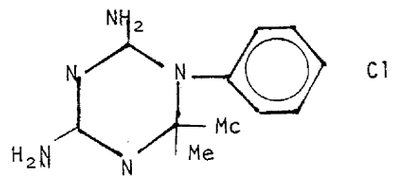
on the primary tissue stages of P. falciparum, P. vivax and P. ovale. Their effect on these stages of P. malariae is unknown. They may, therefore, have causal prophylactic activity, in contrast to the suppressive prophylactic activity of pyrimethamine. They are also effective against asexual stages although their action is slow. They do not have any activity against hypnozoites.

Fig. 4.14



Proguanil

Fig. 4.15



Cycloguanil

It appears that both drugs act as antimalarials through their primary metabolites, cycloguanil (Fig. 4.15) and chlorcycloguanil. Watkins et al. (1984) showed that cycloguanil was between 10 and 40 times more active as a blood schizontocide than proguanil and its p-chlorophenyl derivative which were only weakly active in vitro. Proguanil retained a similar activity against cycloguanil-resistant parasites, indicating that the activity was innate. However, it is unlikely that inhibitory blood levels of the parent compound or the p-chlorophenyl derivative are attained after a normal human dose of proguanil. Antagonism of the action of cycloguanil by both proguanil and its p-chlorophenyl derivative was demonstrated but the effect was weak and would not be expected to interfere with the antimalarial activity. Recent observations by Milhous (personal communication) indicate that chlorproguanil acts in a similar way. Studies on the action of either proguanil or chlorproguanil on the tissue stages have only recently been carried out following the availability of systems to grow these stages in vitro. Preliminary studies indicate that cycloguanil and chlorcycloguanil are also more active as tissue schizontocides than the parent drugs (Landau, personal communication).

These biguanide antimalarials were developed soon after the Second World War but were removed by WHO in 1982 from the list of recommended drugs for prophylaxis because resistance of P. falciparum to this class of drugs appeared to parallel that of pyrimethamine and was widespread. Reports of the usefulness of the biguanides continued to appear and, in view of the lack of drugs available, their use has again been recommended by WHO since 1984.

Cross-resistance between pyrimethamine and proguanil does not seem to be absolute. For example, the Camp strain of P. falciparum is resistant to pyrimethamine but not to cycloguanil while the FVO strain of this parasite is resistant to cycloguanil but not to pyrimethamine (Watkins et al., 1984). Milhous et al. (1988) have consistently found that cycloguanil is more potent in vitro than pyrimethamine against pyrimethamine-resistant clones and isolates of P. falciparum.

This incomplete picture of cross-resistance has led to recommendations for the use of proguanil in combination with chloroquine and to studies on its combination with short-acting sulfonamides for chemoprophylaxis (WHO, 1988a; Pang et al., 1988). The combination of proguanil with a short-acting sulfonamide such as sulfasoxazole or sulfamethoxazole is considered to be justified since there is evidence that sulfa drugs with short half-lives have less serious side effects compared with those with longer half-lives such as sulfadoxine which is thought to be the cause of some of the severe side effects seen during the long-term prophylactic use of Fansidar® (Anand, 1979; Scholer et al., 1984). As the biguanides and many of the short-acting sulfonamides have been registered for use in humans and have been used as ad hoc combinations, regulatory agencies and ethical committees have not required preclinical studies prior to the use of these combinations in humans. However, adequate preclinical studies have not been carried out; undoubtedly to safeguard the patient, toxicity studies in small animals, as well as pharmacokinetic and metabolic studies, should be carried out to determine any drug interaction, the safety and the optimal dose ratios and regimens of these combinations. These studies will not detect the severe skin reactions related to prolonged use of sulfonamides and so it will be necessary to monitor for these side effects during clinical and field trials.

The combination of proguanil with chloroquine is only additive in its effect against drug-sensitive parasites, while that of proguanil and sulfonamides is synergistic. Recently, Milhous et al. (1988) have consistently shown not only that cycloguanil is more active than pyrimethamine against pyrimethamine-resistant P. falciparum, but also that sulfamethoxazole is more potent than the long-acting sulfonamide, sulfadoxine, against sulfadoxine-resistant and -sensitive clones of the same parasite. The combination of cycloguanil with sulfamethoxazole also showed marked synergism against pyrimethamine- and sulfadoxine-resistant parasites, as well as sensitive ones. Chlorcycloguanil has also been shown to be synergistic with dapsone against pyrimethamine-sensitive and -resistant P. falciparum in vitro, maximum synergy occurring at lower concentrations than it did with pyrimethamine and sulfadoxine. These observations have led to clinical trials of the combination of chlorproguanil and dapsone for the treatment of P. falciparum infections (Watkins et al., 1988).

4.3.5 Tissue schizontocides

4.3.5.1 Primaquine

Primaquine, the most widely used tissue schizontocide, was introduced into operational use in the late 1940s. It is an 8-aminoquinoline, which not only is effective as a tissue schizontocide but also exhibits gametocytocidal and sporontocidal activity. This latter activity has led to its use as a single dose, in combination with blood schizontocides, in attempts to reduce transmission. It also has blood schizontocidal activity but this is too low to be of operational importance. The practical use of primaquine for radical treatment of vivax and ovale malarias, i.e., as an antirelapse drug, is associated with significant toxicity, especially haemolysis in persons deficient in glucose-6-phosphate dehydrogenase (G6PD).

Surprisingly little was known about primaquine until the 1980's when sensitive methods for its detection in body fluids and appropriate tests to determine efficacy and toxicity were developed.

The enantiomers of primaquine have been shown to differ in their antimalarial activity (Schmidt et al., 1977) but recent in vitro data indicate that (+)-and (-)-primaquine and the racemic mixture are equally active as tissue schizontocides, although (-)-primaquine appeared to be less toxic (Brossi et al., 1987). The reason for these differences is unknown; however, they may be related to different routes of metabolism of the enantiomers, to differences in their capacities for binding to receptors or to the possibility that the various metabolites of the enantiomers may possess different biological properties.

Haemolysis in subjects with G6PD deficiency, methaemoglobinaemia and abdominal pain are the clinically important toxic effects of primaquine. The basis for the toxic reaction of abdominal pain is unknown but its severity in certain population groups may be such as to make repeated dosing unacceptable. More is known on the other effects, although the role of the known metabolites of primaquine in causing side effects in vivo is still a matter of conjecture (Fig. 4.16). In vitro, many of the metabolites of primaquine (but not, for instance the carboxylic acid metabolite, which is the major metabolite in humans) have shown a greater propensity to cause methaemoglobin formation than primaquine itself. One structural requirement to cause methaemoglobinaemia appears to be the presence of an hydroxy group in the C5 position, irrespective of the nature of the side chain (Allahyari et al., 1984). This conclusion appears to agree with unpublished evidence on the ability of the various metabolites of primaquine to stimulate the pentose-phosphate pathway in vitro where the same structural requirement apparently exists. In vitro studies also suggest that the metabolites of primaquine at concentrations several times less than those of primaquine may produce methaemoglobin

and this may be one reason why it has been difficult to identify a toxic metabolite. Studies in humans have shown that methaemoglobinaemia produced by primaquine administration for 14 days disappears slowly over 7-10 days after drug withdrawal. This is in contrast to the known pharmacokinetics of primaquine in humans where the half-life of the parent drug is of the order of 6-8 hours. This suggests an effect due to metabolites which at a mechanistic level must impair normal methaemoglobin reductase or persist for several days to exert its oxidant effect or both. It has been suggested that both methaemoglobinaemia and haemolysis may be explained by the conversion of primaquine to a quinone or a quinoneimine. A unifying hypothesis has been suggested by Frischer (1987).

Adequate screens for in vivo causal prophylactic and blood schizontocidal activities have been available for years. However, in order to assess the spectrum of antimalarial activity of the putative metabolites of primaquine and other 8-aminoquinolines, suitable in vitro screens for tissue schizontocidal activity and gametocytocidal activity had to be established and standardized.

Tissue schizontocidal activity in vitro has been assessed in cultures of either mouse hepatocytes or hepatoma cells infected with P. berghei while gametocytocidal activity has been assessed against gametocyte producing clones of P. falciparum in vitro. However, this latter test does not detect the ability to kill mature gametocytes, neither does the former measure hypnozoitocidal activity for which a model is needed.

Primaquine exhibited tissue schizontocidal activity in all screens, both in vitro and in vivo whereas its major metabolite, a carboxylic acid, was inactive. Tissue schizontocidal activity was also observed with 5-hydroxy-6-demethyl primaquine and 5-hydroxyprimaquine, the former being over 10 times more active than the parent compound. Gametocytocidal activity equal to or greater than that of primaquine was observed with 5-hydroxy-6-demethyl primaquine, 5-hydroxyprimaquine and 6-demethyl primaquine. Surprisingly, tissue schizontocidal and gametocytocidal activities were also observed in vitro with certain new 8-aminoquinolines lacking the 8-N side chain.

4.3.5.2 Other tissue schizontocides

WR 238605, another 8-aminoquinoline, is being developed at WRAIR. It is 13 times more active as a hypnozoitocidal drug than primaquine as measured by the dose required to obtain a radical cure of P. cynomolgi infections in monkeys. In contrast to primaquine, it also has appreciable blood schizontocidal activity. Preclinical studies on this compound are nearly complete.

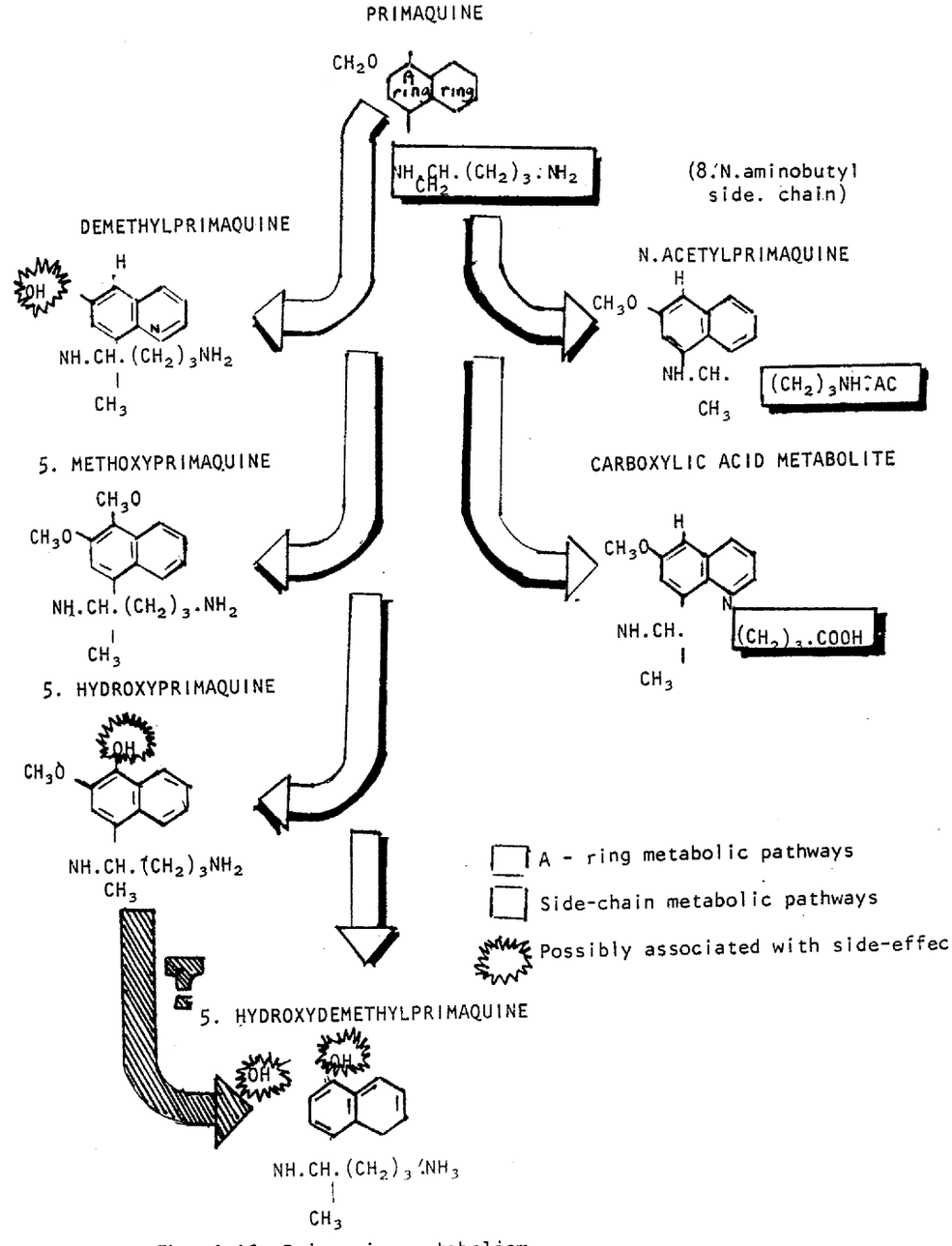


Fig. 4.16 Primaquine metabolism

4.3.6 Conclusion

Most of the drugs discussed in this section were produced from massive screening programmes. However, today, the advances in the understanding of the biochemistry of the malaria parasites and the ability to clone the genes of some of the parasite specific proteins allow a more rational approach to antimalarial drug development. With this knowledge, the drug development process could be improved, both by shortening the time needed to identify new compounds and by reducing the cost of the process. At present, it takes 9-10 years to market a drug after its initial synthesis, and unless such drugs are utilized with care, resistance to that drug may arise in less time than it took to market it.

REFERENCES TO CHAPTER 4

Ager, A.L. (1984) Rodent malaria models. In: Peters, W. & Richards, W.H.G., ed. Antimalarial drugs I. Heidelberg Springer-Verlag, pp. 224-264 (Handbook of experimental pharmacology, Vol. 68/I)

Allahyari, R., Strother, A., Fraser, I.M. & Vertiscar, A.J. (1984) Synthesis of certain hydroxy analogues of the antimalarial drug primaquine and their in vitro methemoglobin-producing and glutathione-depleting activities in human erythrocytes. Journal of medicinal chemistry, 27: 407-410.

Anand, N. (1979) Sulfonamides and sulfones. In: Wolff, M.E., ed. Burger's medicinal chemistry, 4th edition, Part II. Chichester, John Wiley & Sons, pp. 1-40.

Bitonti, A.J., Sjoerdsma, A., McCann, P.P., Kyle, D.E., Oduola, A.M.J., Rossan, R.N., Milhous, W.K. & Davidson, D.E. (1988) Reversal of chloroquine resistance in the malaria parasite Plasmodium falciparum by desipramine. Science, 242: 1301-1303

Boudreau, E.E., Pang, L.W., Dixon, K.E., Webster, H.K., Pavanand, K., Tosingha, L., Somutsakorn, P. & Canfield, C.J. (1988) Malaria: treatment efficacy of halofantrine (WR 171 669) in initial trials in Thailand. Bulletin of the World Health Organization, 66: 227-235.

Brossi, A., Millet, P., Landau, J., Bembener, M.E. & Abel, C.W. (1987) Antimalarial activity and inhibition of monoamine oxidases A and B by exoerythrocytic antimalarials. Optical isomers of primaquine, N-acylated congeners, primaquine metabolites and 5-phenoxy-substituted analogues. Federation of European Biochemical Societies (FEBS) Letters, 214: 291-294.

Brossi, A., Venugopalan, B., Dominguez Gerpet, L., Yeh, H.J.C., Flippen-Anderson, J.L., Buchs, P., Luo, X.D., Milhous W. & Peters, W. (1988) Arteether, a new antimalarial drug: Synthesis and antimalarial properties. Journal of medicinal chemistry, 31: 645-650.

Canfield, C.J. (1980) Antimalarial aminoalcohol alternatives to mefloquine. Acta tropica, 37: 232-237.

Chawira, A.N. & Warhurst, D.C. (1987) The effect of artemisinin combined with standard antimalarials against chloroquine-sensitive and chloroquine-resistant strains of Plasmodium falciparum in vitro. Journal of tropical medicine and hygiene, 90: 1-8.

Chawira, A.N., Warhurst, D.C. & Peters, W. (1986a) Drug combination studies with qinghaosu (artemisinin) against sensitive and resistant strains of rodent malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene: 80 334-335.

Chawira, A.N., Warhurst, D.C. & Peters, W. (1986b) Artemisinin (qinghaosu) combinations against chloroquine-sensitive and -resistant Plasmodium falciparum in vitro. Transactions of the Royal Society of Tropical Medicine and Hygiene, 80: 477.

Chen, L.J., Wang, M.Y., Sun, W.K. & Liu, M.Z. (1984) Embryotoxicity and teratogenicity studies on artemether in mice, rats and rabbits. Acta pharmacologica sinica, 5: 118-122.

China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarial Drugs (1982a) Studies on the toxicity of qinghaosu and its derivatives. Journal of traditional Chinese medicine, 2: 31-38.

China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarial Drugs (1982b) Metabolism and pharmacokinetics of qinghaosu and its derivatives. Journal of traditional Chinese medicine, 2: 25-30.

Cosgriff, T.M., Boudreau, E.E., Pamplin, C.I., Doberstyn, E.B., Desjardins, R.E. & Canfield, C.J. (1982) Evaluation of the antimalarial activity of the phenanthrene methanol halofantrine (WR 171.669). American journal of tropical medicine and hygiene, 31: 1075-1079.

Desjardins, R.E., Canfield, C.J., Haynes, J.D. & Chulay, J.D. (1979) Quantitative assessment of antimalarial activity in vitro by a semi-automated microdilution technique. Antimicrobial agents and chemotherapy, 16: 710-718.

Edlund, P.O., Westerlund, D., Carlqvist, J., Wu, B.L. & Jin, Y.H. (1984) Determination of artesunate and dihydroartemisinin in plasma by liquid chromatography with port-column derivatization and UV detection. Acta pharmaceutica suedica, 21: 223-234.

Fawaz, G. & Haddad, F.S. (1951) Effect of lapinone (M-2350) on P. vivax infection in man. American journal of tropical medicine, 31: 569-571.

Feng, Z. & Wang, C.Y. (1986) Spectrofluorometric determination of pyronaridine as an antimalarial. Acta pharmacologica sinica, 7: 354-357.

Fieser, L.F., Chang, F.C., Dauben, W.G., Heidelbergen, C., Heymann, H. & Seligman, A.M. (1948) Naphthoquinone antimalarials. XVIII. Metabolic oxidant products. Journal of pharmacology and experimental therapy, 94: 85-96.

Frischer, H. (1987) In: Wernsdorfer, W.H. & Trigg, P.I., ed., Primaquine: pharmacokinetics, metabolism, toxicity and activity. Chichester, Published on behalf of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases by John Wiley & Sons, p. 160.

Guan, W.B., Huang, W.J., Zhou, Y.C. & Gong, J.Z. (1982) Effect in vitro of artemisinin and its derivatives on Plasmodium falciparum. Acta pharmacologica sinica, 3: 139-141.

Gwadz, R.W., Koontz, L.C., Miller, L.H. & Davidson, D.E. (1983) Plasmodium gallinaceum: avian screen for drugs with radical curative properties. Experimental parasitology, 55: 188-196.

Haynes, J.D., Diggs, C.L., Hines, F.A. & Desjardins, R.E. (1976) Culture of human malaria parasites: Plasmodium falciparum. Nature (London), 263: 767-769.

Hofheinz, W., Jaquet, C., Masciadri, R., Schmid, G. & Stohler, H.R. (1988) Structure-activity relationship of novel bicyclic peroxide antimalarials related to yinghaosu. In: Kager, P.A. & Polderman, A.M., ed. Abstracts of the XIIth International Congress for Tropical Medicine and Malaria, Amsterdam, 18-23 September 1988. Excerpta Medica, International Congress Series 810, p. 128.

Hudson, A.T. Randall, A.W., Fry, M., Ginger, C.D., Hill, B., Latter, V.S., McHardy, N. & Williams, R.B. (1985) Novel antimalarial hydroxynaphthoquinone with potent broad spectrum antiprotozoal activity. Parasitology, 90: 45-55.

Jefford, C.W., Milhous, W. & Boukouvalas, J. (1988) 1,2,4-Trioxane derivatives as potential antimalarial agents. In: Kager, P.A. & Polderman, A.M., ed. Abstracts of the XIIth International Congress for Tropical Medicine and Malaria, Amsterdam, 18-23 September, 1988. Excerpta Medica, International Congress Series No. 810, p. 320.

Klayman, D.L., Lin, A.J., Acton, N., Scovill, J.P., Hoch, J.M., Milhous, W.K., Theoharides, A.D. & Dobeck, A.S. (1984) Isolation of artemisinin (qinghaosu) from Artemisia annua growing in the United States. Journal of natural products, 47: 715-717.

Krogstad, D.J., Gluzman, I.Y., Kyle, D.E., Oduola, A.M.J., Martin, S.K., Milhous, W.K. & Schlesinger, P.H. (1987) Efflux of chloroquine from Plasmodium falciparum: Mechanism of chloroquine resistance. Science, 238: 1283-1285.

Kyle, D.E., Oduola, A.M.J., Martin, S.K., Pierce, P.F. & Milhous, W.K. (1987) Anticonvulsants antagonize antimalarials. Abstracts of the 27th Inter-science Conference on Antimicrobial Agents, New York.

Kyle, D.E., Milhous, W.K. & Oduola, A.M.J. (1988) Reversal of mefloquine resistance in vitro. Abstract No. 318 presented at the meeting of the American Society of Tropical Medicine and Hygiene, 4-8 December, Washington, D.C.

Li, W.H., Shu, H.L., Xu, G.Y. & Zeng, Y.L. (1982) The binding of qinghaosu (artemisinin) and its derivatives to plasma protein. Acta pharmaceutica sinica, 17: 783-786.

Li, Y., Yu, P., Chen, Y., Li, L., Gai, Y., Wang, D. & Zheng, Y. (1981) Studies on analogues of artemisinin. 1. The synthesis of ethers, carboxylic esters and carbonates of dihydroartemisinin. Acta pharmaceutica sinica, 16: 429-439.

Liang, X.T. (1985) The chemistry of peroxidic antimalarials. In: Chang, H.M., Yeung, H.W., Tso, W.W. & Koo, A., ed. Advances in Chinese medicinal materials research. Singapore, World Scientific Publishing Company, pp. 427-432.

Lin, A.J., Klayman, D.L. & Milhous, W.K. (1987) Antimalarial activity of new water soluble dihydroartemisinin derivatives. Journal of medicinal chemistry, 30: 2147-2150.

Martin, S.K., Oduola, A.M.J. & Milhous, W.K. (1987) Reversal of chloroquine resistance in Plasmodium falciparum by verapamil. Science, 235: 899-901.

Merkli, B. & Richle, R.W. (1980) Studies on the resistance to single and combined antimalarials in the Plasmodium berghei mouse model. Acta tropica, 37: 228-231.

Milhous, W.K., Kyle, D.E., Oduola, A.M.J., Martin, S.K., Boudreau, E., Schuster, B.G. & Davidson, D.E. (1987) Calcium channel-blockers and malaria: The return of the spent magic bullet or a new dilemma in antimalarial drug development. Abstracts of the 3rd International Conference on Malaria and Babesiosis, 7-11 September 1987, Annecy, France, International Laveran Foundation, p. 35.

Milhous, W.K., Oduola, A.M.J., Kyle, D.E., Pierce, P.F., Cerena, L., Canfield, K., Shuster, B.G. & Canfield, C.J. (1988) Cycloguanil and sulfamethoxazole exhibit enhanced in vitro activity over sulfadoxine and pyrimethamine. Abstract No. 319 presented at the meeting of the American Society of Tropical Medicine and Hygiene, 4-8 December, Washington, D.C.

New Drug Group of the Former Department of Malaria (1980) Experimental studies on chemotherapeutic effects and toxicities of a new antimalarial drug 7351. Acta pharmaceutica sinica, 15: 630-632.

Ni, Y.C., Xu, Y.Q. & Shao, B.R. (1982a) Mutagenicity of a new antimalarial drug, pyronaridine, in the Salmonella/microsome system. Acta pharmacologica sinicaa, 3: 51-55.

Ni, Y.C., Zhan, C.Q., Ha, S.H. & Shao, B.R. (1982b) The embryotoxicity of a new antimalarial pyronaridine in rats. Acta pharmaceutica sinica, 17: 401-406.

Oduola, A.M.J., Kyle, D.E. & Milhous, W.K. (1987) Clones in malaria research. Abstract No. 104 presented at the meeting of the American Society of Tropical Medicine and Hygiene, 29 November-3 December 1987, Los Angeles, California.

Oduola, A.M.J., Weatherby, N.F., Bowdre, J.H. & Desjardins, R.E. (1988) Plasmodium falciparum: Cloning by single erythrocyte micromanipulation and heterogeneity in vitro. Experimental parasitology, 66: 86-95.

Pang,, L.W., Limsomwong, N., Singharaj, P. & Canfield, C.J. (1988) Malaria prophylaxis with proguanil and sulfasoxazole in children living in a malaria endemic area (Unpublished document WHO/MAL/88.1049).

Peters, W. & Robinson, B.L. (1984) The chemotherapy of rodent malaria. XXXV. Further studies on the retardation of drug resistance by the use of a triple combination of mefloquine, pyrimethamine and sulfadoxine in mice infected with P. berghei and "P. berghei NS". Annals of tropical medicine and parasitology, 78: 459-466.

Qinghaosu Antimalaria Coordinating Research Group (1979) Antimalaria studies on qinghaosu. Chinese medical journal, 92: 811-816.

Robinson, B.L. & Peters, W. (1985) Development of halofantrine resistance in Plasmodium berghei. Transactions of the Royal Society of Tropical Medicine and Hygiene, 79: 280.

Robinson, B.L., Peters, W. & West, A. (1986) Halofantrine resistance in Plasmodium berghei. Transactions of the Royal Society of Tropical Medicine and Hygiene, 80: 342.

Rossan, R.N. (1984) Malaria models in simian hosts. In: Peters, W. & Richards, W.H.G., ed., Antimalarial drugs I. Heidelberg, Springer-Verlag, pp 265-280 (Handbook of experimental pharmacology, Vol. 68/I).

Schmidt, L.H., Alexander, S., Allen, L. & Rasco, J. (1977) Comparison of the curative antimalarial activities and toxicities of primaquine and its d and l isomers. Antimicrobial agents and chemotherapy, 12: 51-60.

Scholer, H.J., Leimer, R. & Richle, R. (1984) Sulphonamides and sulphones, In: Peters, W. & Richards, W.H.G. Antimalarial drugs II. Heidelberg, Springer-Verlag, pp 124-206 (Handbook of Experimental Pharmacology, Vol. 68/II).

Shao, B.R., Ye, X.Y. & Zheng, H. (1982) Development of pyronaridine resistance in Plasmodium berghei. Acta pharmaceutica sinica, 17: 566-571.

Stohler, H.R., Jaquet, C. & Peters, W. (1988) Biological characterization of novel bicyclic peroxides as potential antimalarial agents. In: Kager, P.A. & Polderman, A.M., ed., Abstracts from the XIIth International Congress for Tropical Medicine and Malaria, Amsterdam, 18-23 September 1988. Excerpta Medica, International Congress Series 810, p. 320.

Thaithong, S. & Beale, G.H. (1985) Susceptibility of Thai isolates of Plasmodium falciparum to artemisinin (qinghaosu) and artemether. Bulletin of the World Health Organization, 63: 617-619.

Trager, W. & Jensen, J.B. (1976) Human malaria parasites in continuous culture. Science, 193: 673-675.

UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (1986) The development of artemisinin and its derivatives: report of a meeting of the Scientific Working Group on the Chemotherapy of Malaria (Unpublished WHO document TDR/CHEMAL/ART/86.3).

Watkins, W.M., Sixsmith, D.G. & Chulay, J.D. (1984) The activity of proguanil and its metabolites, cycloguanil and p-chlorophenylbiguanide against Plasmodium falciparum in vitro. Annals of tropical medicine and parasitology, 78: 273-278.

Watkins, W.M., Howells, R.E., Brandling-Bennett, A.D. & Koech, D.K. (1987) In vitro susceptibility of Plasmodium falciparum isolates from Jilore, Kenya to antimalarial drugs. American journal of tropical medicine and hygiene, 37: 445-451.

Watkins, W.M., Brandling-Bennett, A.D., Nevill, C.G., Carter, J.Y., Boriga, D.A., Howells, R.E. & Koech, D.K. (1988) Chlorproguanil/dapsone for the treatment of non-severe Plasmodium falciparum malaria in Kenya: A pilot study. Transactions of the Royal Society of Tropical Medicine and Hygiene, 82: 398-403.

Webster, H.K., Thaithong, S., Pavanand, K., Yongvanitchit, K., Pinswasdi, C. & Boudreau, E.F. (1985) Cloning of mefloquine-resistant Plasmodium falciparum from Thailand. American journal of tropical medicine and hygiene, 34: 1022-1027.

WHO (1988a) Development of recommendations for the protection of short-term travellers to malaria endemic areas: Memorandum from two WHO meetings. Bulletin of the World Health Organization, 66: 177-196.

WHO (1988b) Halofantrine in malaria. WHO drug information, 2: 58-60.

Ye, Z. & Van Dyke, K. (1988) Reversal of chloroquine resistance in falciparum malaria independent of calcium channels. Biochemical biophysical research communications, 155: 476-481.

Zeng, Y.L., Zhang, Y.D., Xu, G.Y., Wang, C.G. & Jiang, J.R. (1984) The pharmacokinetics and bioavailability of O-methyldihydroartemisinin in the rabbit. Acta pharmaceutica sinica, 19: 84-88.

Zhang, J.X., Lin, B.Y., Chen, K.Y. & Shao, B.R. (1986) On the residual activity of pyronaridine against Plasmodium cynomolgi in rhesus monkeys. Journal of parasitology and parasitic diseases, 4: 129-131.

Zhao, K., Liu, C., Liang, X., Yi, M. & Song, Z. (1986) Development of a radioimmunoassay for determination of artesunate, a derivative of the antimalarial qinghaosu. Proceedings of CAMS and PUMS, 1: 213-217.

Zhou, Z.M., Anders, J.C., Chung, H. & Theoharides, A.D. (1987) Analysis of artesunic acid and dihydroqinghaosu in blood by high performance liquid chromatography with reductive electrochemical detection. Journal of chromatography, 414: 77-90.

CHAPTER 5 : CLINICAL AND FIELD TRIALS OF ANTIMALARIAL DRUGS

CONTENTS

---

* This chapter is based on the presentations of Dr. D.M. Burley, Professor U.K. Sheth and Dr. D.B. Doberstyn and all the relevant plenary discussions and practical exercises. Section 5.1 is largely reproduced from Clinical Trials by Dr. D.M. Burley and A. Glynne, for which the publishers and first authors permission are gratefully acknowledged. Section 5.5 is largely based on the draft report of an EPID/TDR meeting held in Geneva in June 1988, entitled "Methods for field trials of interventions against tropical diseases", and on excerpts from Pocock, S.J., Clinical Trials: a practical approach, New York, Wiley, 1986, as well as from Abramson, J. H., Survey methods in community medicine, Edinburgh, Churchill Livingstone, 1984.

5.1 CLINICAL TRIALS

The most significant milestone in the development of a new drug is its first administration to a human being. It is an important and difficult decision but less onerous than might be thought since it is usually taken by a group in the light of accumulating preclinical data. Most drugs are first studied in non-patient volunteers. Limited studies are then undertaken in patients with the disease for which the drug is being developed. After this, clinical trials involving much larger numbers of patients are completed. Further trials may be required after marketing. Following American practice these stages have become known as Phases I-IV. In detail and scale they represent a more or less continual progression from depth to breadth. Although the scientific principles and underlying philosophy of clinical trials are the same throughout the process, there are important practical differences between the phases, so it will be convenient first to describe them in more detail.

Although others can make an important contribution to the clinical evaluation of a new medicine, trials must remain under the control of able and well-trained physicians. Only about 10% of new chemical entities reaching man are eventually marketed. Most of the remainder fail early in the trial programme.

5.1.1 Clinical trial phases

5.1.1.1 Phase I Studies

With the exception of cytotoxic compounds, initial studies in humans are carried out in healthy volunteers. Ideally the aim of these early studies should be to investigate all the pharmacodynamic and pharmacokinetic properties. However, many drugs are not designed to have pharmacological effects in healthy people e.g., anti-inflammatory drugs and antibiotics. There are two essential objectives to all initial Phase I studies. First, to assess the safety of gradually increasing doses and secondly, to obtain basic pharmacokinetic information. These are critical studies and observations made during this stage will influence the design of later phases. Of course, in many cases the finding of unacceptable adverse effects precludes any further development. The protocol for Phase I investigation is therefore vitally important.

(a) Design of a Phase I investigation

The design of the initial human evaluation will largely depend on the potential use of the drug, for instance, whether it is going to be used as an antimalarial, antibacterial or anticancer drug; therefore, it is only possible to make

generalizations about the design. Some of the more important factors influencing the design are set out below:

(i) The likely mechanism of action. If it has been possible to gain such information from the animal experiments it is important to check whether the same conclusions apply to humans. If the mechanism of action is known, it is possible to predict more accurately where and when the drug will be of clinical use, perhaps even to predict and avoid adverse reactions. Also the design of the investigation becomes much simpler.

(ii) The predicted effect is likely to occur only in diseased subjects. If no pharmacodynamic effects are expected, then it is essential to know whether the drug has been absorbed and to what extent from the gastrointestinal tract. In addition checks can be made to find out whether or not an adequate drug concentration is achieved in blood or urine, or at other appropriate sites.

(iii) Where a pharmacodynamic effect is expected it is not likely to occur after a single dose. Repeated administration may be necessary and these aspects may be more appropriate in Phase II when relevant patients are exposed.

(b) Selection of investigators and site

The first administration of a novel compound to human beings should be performed at a site where there are staff and facilities able to deal with any emergency situation. In most cases this means a special investigation room within a hospital.

The clinical investigator has to be both a physician and a pharmacologist. It is unimportant whether or not he/she chooses to call him/herself a clinical pharmacologist. He/she must have a good understanding of the details of animal pharmacology and toxicology and be able to assess the validity and level of confidence with which the preclinical pharmacological conclusions have been drawn. This is the only way in which the investigator can fully understand the risk to which the volunteers are being subjected. Fortunately experience has shown that in capable hands the early studies of drugs in humans are remarkably safe.

The most useful, the most valid and the safest studies are those which have been planned and implemented by collaboration between drug sponsors and an appropriate clinical pharmacologist or systems specialist within an academic or hospital institution. Pooling of knowledge, experience and expertise will ensure that the volunteers are subjected to the

absolute minimum of risk and that as much information as is practicable is obtained. It is therefore essential for the pharmaceutical physician to establish good working relationships with appropriate academic colleagues. Active "hands on" participation is valuable to obtain and maintain the commitment of others to the successful completion of the innvestigation.

(c) Selection of subjects

Volunteer subjects are usually recruited from industrial personnel and from the medical and scientific staff in academic units. They must always be genuine volunteers, i.e., they should never be in a position of subservience to the investigator. Many companies or institutes maintain registers of staff who have indicated their willingness to consider volunteering. A fair approach is to display a notice on a staff notice-board briefly indicating the nature of the study and the number and types of subjects who are required. Then the interested potential volunteers are invited to attend a meeting which is addressed by the clinical investigator who gives full details of the study to be carried out, explaining carefully the objectives and risks, however remote, and what is required from each volunteer. Following this any volunteers wishing to participate can communicate their willingness to the physician. Some payment for participation in such studies is now generally accepted, the level of payment being adequate to compensate for the degree of inconvenience and risk, but not large enough to be a financial inducement. Once a subject has volunteered, it is essential that he or she gives a complete medical history, and that clinical examination and appropriate screening tests should be carried out and interpreted by a doctor with a sound clinical background. Screening tests should include chest x-ray, electrocardiogram, full blood count and tests of hepatic and renal function. If the preclinical information indicates that the compound may have some effect on a specific organ, for example the thyroid gland, specific baseline tests such as testing of thyroid function should be carried out. If the subject is volunteering for the first time, then it is wise to inform the general practitioner as he may have pertinent information about the subject. It is essential that the subjects and the investigator be covered by adequate insurance. The ethics are fully dealt with elsewhere but written informed consent must be obtained in the presence of a witness. The protocol of the study and its procedures and the consent form should have been approved by an ethics committee.

In most Phase I studies subjects are usually between 18 and 60 years of age. Furthermore they are usually male. This is not an expression of male chauvinism. It is due to the fact that there is an increasing trend to delay full teratology studies until the Phase I studies have been successfully completed. Secondly, many potential female volunteers are on the contraceptive pill which in some instances may affect the pharmacokinetics of the study drug. It is undesirable to

restrict Phase I studies to men, as women may handle the drug somewhat differently, and this could be clinically important. Unless the disease for which the drug is intended is exclusive to males, it would seem sensible to find out whether the drug was teratogenic or not at an early stage even though the cost of such studies is considerable. It seems reasonable, at the earlier stage of clinical investigation, to restrict the age range; but, if the drug is intended for use in elderly patients or in children a full pharmacokinetic and pharmacodynamic investigation should be undertaken as soon as possible in such age groups. There are of course ethical problems, particularly with regard to studies in children. However, it is necessary that meaningful clinical data be obtained in all groups of subjects who are likely to receive the drug at the earliest opportunity and certainly before unrestricted sale.

(d) Drug administration

In the United Kingdom permission from the licensing authority is not required for the administration of a drug to non-patient volunteers. For this purpose a non-patient volunteer would be defined as a subject who is not expected to obtain therapeutic benefit. However, in the USA permission has to be obtained from the Food and Drug Administration before such studies can be carried out. As the predictive value of animal toxicity testing is often very questionable, guidelines on the amount of animal toxicity testing required before initial drug administration to humans are necessarily empirical (CIOMS, 1983).

In the United Kingdom it is now regarded as satisfactory for many compounds if the drug is administered for two weeks to two animal species, one of which is a non-rodent (Table 5.1). This permits a single dose or short series of doses on one occasion to be given to humans. Guidelines for initial dosage and subsequent increments are again empirical, but many investigators start with the equivalent of about 2% of the dose that is effective in animals and double this until either the expected dose is reached or the therapeutic effect or some toxic effect occurs. The route of administration must be the same in animals and humans. Although it is important to note that a minute dose of a radio-actively labelled drug will be all that is required for initial pharmacokinetic data, it is always better to correlate pharmacodynamic with pharmacokinetic changes. This can only be achieved with an effective dose. Wherever possible the dose of a drug should be titrated against its effect, and depending on the nature of the drug, this may have to be done in Phase II. Such dose titration studies will give more information than a single fixed dose study.

Repeated dosing in humans must be preceeded by longer toxicity testing in animals. The guidelines agreed between the United Kingdom Department of Health and Social Security (DHSS) and the Association of British Pharmaceutical Industry (ABPI) are set out in Table 5.1.

TABLE 5.1 DHSS AND ABPI GUIDELINES FOR TOXICITY TESTING

Administration to man	Toxicity programme
1 to 3 single doses	14 days in 2 species
Up to 7 days of treatment	28 days in 2 species
7 to 28 days of treatment	3 months in 2 species
1 to 3 months of treatment	6 months in 2 species

Animal toxicity testing should always be designed to give indications as to which organ systems to monitor, rather than attempt to show that the drug is completely non-toxic. Therefore, the physician will want to ensure that, subject to the pharmacological properties of the drug, a high enough dose has been given to the animals. The route or routes chosen for the investigation must be the ones by which it is intended that the drug will be given clinically. Variation in the route can result in important pharmacokinetic and pharmacodynamic differences.

(e) Measurements

These will depend mainly on the drug and the disease for which it was developed. It is important to be open-minded and not only to check whether the same properties are found in humans as in animals but also to investigate whether there are novel ones not detected in the animal experiments. Especially important are the tests which are difficult or impossible to perform in animals, e.g. psychological testing. However, it is important not to make the study too complicated. Well-validated, simple measurements are more useful than complex ones which may often be found to be unreliable. In appropriate circumstances electrocardiograms, electroencephalography and the use of radioisotopes can provide a great deal of information. The key to safety lies in good experimental design using techniques and facilities with which the investigators are happy and familiar.

In a single-dose study if the compound is given orally, it is always essential to know whether it has been absorbed. At this stage of the drug's development an assay method sensitive enough to detect predicted low levels of the compound or its metabolite may not have been developed. In such circumstances, the administration of a small dose of compound labelled with a radio-isotope should be incorporated into the investigation. In acute studies estimation of plasma concentration provides an indication of whether a drug or a metabolite is responsible for the pharmacological effect. It will also reveal whether the drug obeys first or zero order kinetics. Volume of distribution and clearance will be obtained for most drugs. Pharmacokinetic data obtained from repeated administration are necessary to check on initial impressions obtained from the acute

administration data. Clearly, if the dynamic data correlate well with the kinetic data then this is strong presumptive evidence that the drug itself rather than a metabolite is responsible for the pharmacological effect. Sometimes a metabolite which is more pharmacologically active is identified, raising the possibility of developing that compound instead.

In both single and repeated dose studies volunteers should be monitored closely with full blood count, hepatic and renal tests, which are repeated at appropriate times. If a serious reaction occurs which may or may not be due to the drug it is essential to investigate the subject and the circumstances as promptly and as thoroughly as possible. If this is not done future volunteers or even patients may be put at risk unnecessarily, or a potentially useful drug may be withdrawn. Important drug reactions can be looked for in Phase I studies, but these are usually observed only at a later stage after a good deal of patient information has been obtained.

5.1.1.2 Phase II studies

The primary object of these studies is to determine whether a drug shows promise in one or more clinical indications. Patients rather than healthy subjects are exposed for the first time. The early studies in Phase II are among the safest since they start with one or a few low doses of the new drug in a limited number of patients who are very closely monitored. Progression to multiple dose studies or expansion of the patient numbers is embarked upon after initial reassuring experience with the new drug.

It is quite legitimate for many of these early investigations to be carried out without controls and, provided they are properly conducted, they can contribute valuable data. They are best carried out by investigators who have a special interest in the treatment of the disease for which the drug is intended. Although such studies ar regarded as part of clinical pharmacology, clinical pharmacologists may not always have access to or experience with the required type of patients.

The trials are designed to define the most suitable dosage schedule to give an estimate of clinical efficacy in relation to concentration of the drug and its metabolites in body fluids and tissues, and to provide information on adverse effects. They should, if possible give enough information to allow a preliminary assessment of benefit/risk ratio. The pharmacokinetics of a drug should be investigated in patients because they may handle the drug differently from healthy people either due to the effect of their disease, or because patients with the relevant disease may be either much older or much younger than the healthy volunteers studied in Phase I. Vigilant observation by an open minded investigator may indicate even at this early stage some unforeseen property, beneficial or otherwise.

5.1.1.3. Phase III studies

Phase III trials are undertaken when there is evidence that :

(a) An adequate degree of efficacy exists.

(b) The risk profile of adverse reactions appears to be acceptable in terms of demonstrated efficacy.

Primary responsibility for this decision is obviously that of the clinical research department of drug sponsors. However, it is important that the feelings of the various hospital investigators who were involved in the Phase II studies be taken into account. Also in most countries including the United Kingdom and the United States of America the requirements of the regulatory authority must be met. In the United States it is quite common for an "end of Phase II" conference to be convened in which medical and scientific personnel from the Food and Drug Administration participate.

In Phase III studies the number of patients is gradually increased particularly as confidence in the drug grows. Several different types of trial design may be used and these will be discussed later. The objective of a Phase III clinical trial programme is not merely to obtain the approval of the government authorities so that a drug can be marketed. It is to generate information that enables the practising doctor to utilize the drug effectively and safely and put it to optimal use. Indications for use of the drug must be confirmed. Firm recommendations for dosage must be established. Those circumstances in which a different dosage should be used should be clearly defined to ensure that as precise prescribing information as possible is available.

5.1.1.4. Phase IV studies

These are trials undertaken after obtaining a marketing licence. Phase II and III studies suffer from three major limitations:

(a) Restricted patient populations.
(b) Limited duration of patient exposure.
(c) Limited patient numbers.

Consequently Phase IV trials should be constructed to show:

(i) Drug efficacy in prolonged use where perhaps the natural course of a disease may be modified over a period of several months or years.

(ii) Adverse reactions which only occur rarely or with long term use.

(iii) Long term comparative data.

(iv) The detailed examination of non-responders.

(v) The assessment of overdosage and misuse or abuse liability.

(vi) New dosage forms.

(vii) New indications.

(viii) Drug interactions.

With regard to drug interactions it should always be borne in mind that elimination can either be increased or decreased by the coadministration of another drug. Similarly, the excretion or absorption of a drug may be affected by another drug. Competitive antagonism at receptor sites may affect the efficacy of one or both drugs.

For most drugs which are given orally over long periods of time Phase IV trials will be organized with the cooperation of general practitioners. In the United Kingdom there are now firm guidelines for the conduct of trials of licensed medicinal products in general practice (Anon., 1983). These guidelines have been carefully drawn up to ensure legitimate ethical and scientific validity. Needless to say it still remains the ultimate responsibility of the pharmaceutical physician to ensure that these trials do meet the guidelines.

5.1.2 The experimental nature of clinical trials

Sir George Pickering in his Presidential Address to the Section of Experimental Medicine and Therapeutics of the Royal Society of Medicine said in 1949, "therapeutics is the branch of medicine that, by its very nature, should be experimental". This profound statement more than any other crystallizes the view that scientific method is the proper way to evaluate therapeutic intervention in sick people and places a "seal of approval" on the controlled clinical trial, which could be regarded as the centre-piece of pharmaceutical medicine.

There are of course examples of controlled trials dating back for centuries (Bull, 1959), the most familiar being James Lind's classical trial in 1747 in 12 sailors suffering from scurvy, in which two were given vitriol as a dietary supplement, two cider, two vinegar, two oranges and lemons and two a mixture prepared by the surgeon (? placebo). This experiment established the value of oranges and lemons as a cure for scurvy. Earlier in this century the Medical Research Council created in 1931 a Therapeutic Trials Committee which has been emulated more recently by the British Thoracic and Tuberculosis Association (now the British Thoracic Society) and for examples of well constructed randomized controlled clinical trials the early anti-tuberculosis chemotherapy studies with streptomycin, para-aminosalicylic acid and isoniazid are well worth studying (Anon., 1948, 1950; 1983).

Sir Austin Bradford Hill (1971), in his classic work Principles of medical statistics, which has run into numerous editions, set out nearly all the fundamental principles of clinical trials in his definition: "the clinical trial is a carefully and ethically designed experiment with the aim of answering some precisely framed question". Sir Austin can properly be regarded as the father of the medical branch of statistics, but his contributions to the literature on clinical trial design have ranged over such subjects as the use of controls, the allocation of treatments, the measurement and reporting of results and the ethical problems that have to be faced when a patient consents to take part in a clinical trial, or is randomized to a placebo treatment.

During the last 40 years the technique of the randomized controlled clinical trial has become accepted as the proper method for evaluating new therapeutic interventions and this has led to many valuable contributions to the literature on clinical trial design, the proper application of statistical methods, clarity in the presentation of data and appropriate decision making.

Clinical judgement and experience have been supported by the proper use of scientific method, and the pitfalls of relying solely on the former have been clearly demonstrated.

Some demonstrations of therapeutic activity have been so dramatic that both clinical experience and scientific assesssment would clearly have led to the same conclusions. The transformation of tuberculous meningitis from a fatal to a non-fatal disease by the advent of streptomycin and isoniazid, and the truncation of the classical phases of pneumococcal pneumonia by the advent of penicillin are familiar examples. But most therapeutic progress is brought about through a series of much smaller steps, or much less dramatic manifestations. It is in these situations that experience may be fallacious and judgement faulty or prejudiced so that the discipline of scientific method must be sought if one is not to be led along false trails or into blind alleys.

Since the Second World War there has been so much progress in the medicinal therapeutic field that there are few medical conditions that are not at least partially amenable to drug therapy. This in itself, however, has created ethical problems in recent years over such issues as consent to randomization and the use of placebos (Clayton, 1982). By their nature randomized trials tend to serve a collective rather than an individual ethic and as a result of 'experience' a practitioner must at least have some view as to the 'best' existing treatment. Hence it is argued that randomization which relegates the individual to the whim of the random number generator is unjustifiable and that the use of a placebo is a denial of treatment (Burkhardt & Kienle, 1978).

If these sentiments were to lead us away from scientific method and back along the path of unsupported belief, the prospect would be appalling for both doctor and patient. However, in as much as our ethical anxieties have led us towards considering new methods of using controls through adaptive designs (Weinstein, 1974) or reconsidering old methods (Cranberg, 1979), they have served a valuable function, and the recognition of the importance of the individual through proper consent procedures, aided by more recent methods proposed by Zelen (1979) has reduced the feeling that taking part in a clinical trial is simply a lottery.

Clearly in the future there will be much more discussion by informed laity and the legal profession (to say nothing of the media and various pressure groups) about medicinal treatment in general and the role of scientists and doctors. Some of the present views will need to be modified in the light of changing public opinion as to the role of the individual in society and the rights of the sick individual in relation to his medical attendant. Ethics are not written on tablets of stone.

5.1.3 Organizing a clinical trial

The essence of good clinical trial design is careful planning and the aim should be to maximize the benefit/cost ratio of any study, cost being measured not only in conventional terms but also in the use of patients. An inefficient trial utilizing human and technical resources, which by its very design can provide no information to guide future therapeutic decisions, is unethical from its inception.

The pressures engendered by the need to produce results from multiple Phase III clinical studies to satisfy health authorities and obtain marketing consent tend to lead to stereotyped thinking. The words of Sir Austin Bradford Hill (1960) when discussing the ethics of clinical trials "..... that one can make no generalization. One can lay down no general conditions. The problem must be faced afresh with every proposed trial," are equally true of clinical trial design. Guidelines are nevertheless valuable and this chapter attempts to set them out.

5.1.3.1 The protocol

The clinical trial protocol describes the procedure for carrying out a clinical trial starting with its aims and justification and ending with what is often forgotten, how the trial should be terminated. It is therefore useful to have a schedule or check list to ensure that nothing has been forgotten. Detailed check lists have been published (Anon., 1977) but the main headings are as follows:

Aims
Questions to be answered. Justification.

Material
Patients selected - exclusions. Consent.

Trial design
Group comparisons, crossovers. Numbers required.

Treatments
Allocation to patients - placebos.

Measurements
Validity.

Record keeping
Clerk and computer.

Drug supplies
Matching, labelling, code.

Handling adverse reactions
Reporting, code breaking.

Report writing
Results. Statistical considerations. Conclusions.

Closing the trial.

5.1.3.2. The team

The protocol must be written by a team who collectively will have at their finger-tips or in their cerebral computers all the accumulated information which will enable them to formulate a useful question (or questions) to be answered by the clinical trial and to decide whether that question can be answered with the resources and patients available (Burley, 1970). The protocol writing team will therefore include:

A physician who is responsible for the care and welfare of the patients taking part in the trial.

A recorder who is responsible for making patient observations and recording measurements.

A pharmaceutical physician who is the repository of the pedigree of the drug or drugs being used in the trial, their effects and side effects and their procurement.

A statistician to advise on type I and type II error in relation to the hypothesis to be tested and the number of patients available.

A pharmacist who will be responsible for the proper dispensing of drugs, often using a randomized procedure.

A monitor who will be responsible for the logistics of the trial: the completion of records, the continuity of drug supplies, to ensure Quality Control and the completion of the study.

In addition the cooperation of a number of others who will inevitably become involved in various aspects of the trial is vital to avoid obstruction and frustration. The ward or out-patient sister and her staff should be brought into the discussions, together with representatives of the departments of biochemistry, haematology, radiology, etc., who may be burdened with an increased workload and can often provide valuable advice on measurements, methodology and recording. All should be quite clear about the extent of their involvement.

In out-patient studies the family practitioner should be notified that his/her patient is taking part in a trial and his/her cooperation actively sought. He/she should also be notified when the trial has ended and, once the patient has been returned to his/her care, should be provided with advice on future management which the trial results may have suggested. It is difficult to think of everything, but if you don't somebody will be offended. We know from personal experience that a "happy" trial nearly always succeeds and an "unhappy" trial rarely produces anything of value.

The physician responsible for the trial does not necessarily have to take an active part in the interviewing of patients and the recording of observations, but the trial is his/her responsibility and he has to satisfy him/herself the credentials of all the other members of the team, are valid and adequate that the patients are being managed in an ethical manner and conventionally according to the Declaration of Helsinki, and that they have given valid consent to any procedure being carried out. As a reward the physician responsible is allowed to put his/her name at the end of the paper!

The recorder or recorders are responsible for the proper collection of the measurement data (objective and subjective) which will form the basis of decision making when the trial is complete. They need to be aware of all the problems associated with measurement such as bias, digit preference, intra- and inter-observer error and the inconvenience associated with missing data.

The pharmaceutical physician should regard it as his/her responsibility to be familiar with the literature not only of the drug in the trial, but the methodology and results of trials previously carried out with drugs of the same class. A literature search is an essential preliminary to any piece of scientific work and he/she should ask him/herself the following questions:

Is the question worth answering?

Has it already been answered?

Can it be answered by the proposed trial?
and perhaps most important,

Can the investigator answer it with the resources he/she has available?

It takes courage to shut down a trial when you know it will not yield useful information, let alone when you only suspect that it won't.

The contribution of the statistician is also in the planning stage of any trial, and not merely to make sense out of nonsense at the end of the trial.

The pharmacist in the past often became aware that a patient was participating in a clinical trial when he/she appeared with an empty bottle bearing the label "the trial tablets - take one every morning". This is now not permissible and it is essential for all hospital drug supplies to be dispensed from the pharmacy according to a prepared randomization list. A randomization code should also be kept there in case it needs to be broken. In fact many hospitals have now appointed a pharmacist with special responsibility for clinical trial supplies.

The monitor has to some extent been a response on the part of the pharmaceutical industry to the legal requirement that a pharmaceutical physician should be responsible (under signature) for the authenticity and accuracy of data submitted to health authorities. Such requirements are more stringent in the United States of America whose health authority (FDA) appears to have experienced, or at least anticipated, a greater degree of dishonesty in the reporting of clinical trial data for new drug applications (NDAs). Nevertheless, many firms in the United Kingdom now employ clinical research assistants who have as part of their responsibility the monitoring of data collection and ensuring that data transferred for computer processing is not given a spurious authenticity by being printed in standard format. There is no substitute for the careful inspection of the original hand written data on the original record form. If all the records are in the same handwriting with the same biro there are grounds for suspicion. It is most important that the record be accepted or rejected for analysis before the code is broken.

5.1.4 Trial aims

It is important not to attempt too much or to try to answer too many questions in a single trial. The number of patients available, the time constraints, the continued interest and dedication of the investigator will all argue in favour of answering a few questions at a time and ensuring they are answered well.

It is assumed that a comparative study will be carried out, although other forms of study do have a place and will be considered later. Early on it is necessary to decide whether the comparison will be against a positive control, usually the "best" of the other treatments available, or a negative control - a placebo, or against both. With serious or life-threatening conditions comparisons will have to be against a conventional treatment if one exists but for investigations involving simple illness or the management of symptoms placebo controls may be more appropriate.

Difficulties arise with trials in conditions such as hypertension or depression. It may be permissible to postpone effective treatment in order to carry out placebo controlled studies in patients with mild forms of the illness, but patients with severe hypertension could be at risk from stroke or severe depressives at risk from suicide, if effective treatment was withheld for a period. The problem with positive controls is that a finding of no difference between the new treatment and the standard may indicate that both are effective or both ineffective, and one needs to be sure that the standard treatment has been shown in the past to be consistently superior to a placebo to be able to dispense with the need for a negative control.

Having decided on the nature of the comparison the next consideration is what outcome measure is going to provide the best test of treatment effectiveness. The simplest objective measure which is not liable to give rise to dispute is mortality but most trials do not have this as a likely outcome. It seems logical to place strong emphasis on the patient's judgement concerning the benefit of treatment, particularly in conditions such as angina pectoris or even rheumatoid arthritis. In the latter condition it may be tempting to judge a response by measuring erythrocyte sedimentation rate (ESR) or the titre of rheumatoid factor, but in practice the patient's experience of morning stiffness, joint tenderness and his ability to carry out daily tasks are a much better guide to treatment response.

Finally, all treatments will be measured as a balance between risk and benefit so it is necessary to evaluate carefully the adverse effects of new and comparative treatments. In the nature of things a single trial involving relatively small numbers of patients will not reveal uncommon hazards. Subsequent trials will add information and special examinations of body systems will be required if there are any pointers from previous studies (human or animal) of damage to important target organs such as the liver, the kidney and the blood forming organs.

It is important in setting out the aims of the trial to indicate the nature of the hypothesis to be tested (usually the null hypothesis) together with the statistical limits for rejection and the power of the trial. Conventionally the null hypothesis is rejected when the probability of a demonstrated

difference being due to chance (type I error or α) is 5% (P=0.05) or in some trials 1% (P=0.01). The danger of not finding a difference which is present is called the type II error or β The power of a study ($1-\beta$) is therefore its ability to demonstrate the difference. β is often set at between 10% and 20% and therefore power between 90% and 80%. This is why expert statistical advice is so important; indeed consideration of these sources of error and of the minimum useful treatment benefit one hopes to detect, govern the number of patients that must be entered into the trial. (See also "Study design", section 5.1.6)

5.1.5 The patients

Since it is impossible to treat a whole population of patients with a given condition, only a typical sample can be treatedand herein lies a classical dilemma. Does one select a narrow homogeneous group who fulfill certain well defined criteria or does one accept a wide range of patients under a given diagnostic umbrella? The disadvantage of the first method is that it may be hard to find sufficient numbers of patients because of the numerous exclusions and the results of the trial may only be applicable to the chosen sub-group. On the other hand, by the choice of a wide range of patients positive results in an important subgroup may be so diluted that they are undetectable or do not reach statistical significance. It is perhaps best in most cases to choose a sample which is as broadly based as possible, yet define those patient characteristics which are most likely to affect response to treatment. By suitable randomization and a realistic choice of numbers it may be possible to have the best of both worlds and detect not only overall benefit but also benefit confined to certain sub-groups, provided always that the objectives are set out in advance.

The factors most commonly affecting response to treatment are:

Age
Sex
Disease severity
Disease duration
Previous or current therapy

These will form the basis for choosing response categories but other factors may be important in particular conditions, e.g. the presence or absence of cavitation in tuberculosis trials.

The criteria for patient inclusion and exclusion must be defined in the protocol in unambiguous terms and it may be desirable to provide a check list to ensure that ineligible patients are not included. Naturally, if more than one centre is involved there must be agreement between investigators on both the validity and determination of diagnostic categories.

Conventional exclusions are:

Elderly patients
Children
Women of child bearing age
Seriously ill or moribund patients
Those with other diagnoses
Those who are unlikely to cooperate with the trial regimen

Although exclusions are usually prudent, it is important not to extrapolate uncritically the results of the trial to such excluded patients at a later date. In addition, patients will need to be excluded if the new treatment under examination may cause a particular hazard, e.g. to patients with heart failure, bradycardia or asthma in trial of beta-adrenergic blockers.

5.1.6 Study design

If the clinical trial is the centrepiece of pharmaceutical medicine then the randomized comparative group study is the centrepiece of clinical trials and has become accepted generally as the method of choice for the study of therapeutic remedies. The comparative group study is therefore taken as the basis for considering the problems of design and the numbers of patients to be recruited, followed by consideration of other common types of design and more recent ideas which have been put forward to meet some ethical objections.

If it is not known which of two treatments is best and therefore drug administration is randomized between two groups, then, so far as can be judged on the evidence available, they are going to be equally efficacious. This state of ignorance is represented by the null hypothesis, i.e. there is no difference between the treatments. The aim of the study is to test this hypothesis and utilize a statistical test of significance to suggest whether it should be accepted or rejected. It is very much a clinical decision, however, what level of significance is chosen and what additional benefit is worth seeking and detecting, for clearly it will take a lot more work and a lot more patients to detect small differences in outcome than quite large ones.

To return to statistical significance, it must first be decided "how sure do we want to be that any difference we do detect has not arisen by chance". In the nature of things randomization imbalance and inherent variability in measurements of biological variables will throw up chance results which are misleading. How frequently are you prepared to be misled? As mentioned previously it is customary to choose a 0.05 (5%) risk, but since it is really a clinical decision there should be no rigidity about this. After all it would seem sensible to accept a smaller percentage risk of obtaining a false positive result when current treatment is satisfactory and acceptable than when

it is unsatisfactory or hazardous. Whatever you choose will again affect the numbers in the study. The smaller the probability (P) of obtaining a false positive result the larger the numbers. Therefore, as seen from the above, numbers are affected by:

(a) The size of the difference the clinical trial is hoping to detect.

(b) The P value.

There is, however, a third factor which is perhaps less easily understood and which often escapes consideration in clinical trials, both in design and in reporting:

(c) Power.

The power of the study is its ability to detect the worthwhile difference between treatments for which you are hoping. In other words it is no use looking for a 25% improvement in clinical effectiveness, asking for a 95% probability that a positive result is the correct one (5% due to chance) and randomizing 50 or 60 patients to the two treatments under study, if with these numbers there is only a faint chance of picking up the positive result. Yet this is how many studies are set up and the literature abounds with examples (Freiman et al., 1978; Ambroz et al., 1978).

The cynic might say that when pharmaceutical companies are testing a new remedy against a placebo they take more care to ensure the numbers are sufficient to pick up difference should they exist, than when for example they are comparing their new antidepressant with a standard agent. It may be useful to show that your new agent is as effective as say amitriptyline and this may well come about if the trial is not sufficiently powerful to pick up any difference one way or another. This is clearly improper because one can never "prove" no difference and one should not set up a trial with this objective. Temple (1982) has recently discussed these points in relation to clinical trial submissions in the USA.

Tables (Clarke & Downie, 1966) and nomograms(Altman, 1982) have been published to aid the calculation of numbers required in group comparative studies.

Finally the statistical test chosen has an important influence on the power to detect differences which do exist. A familiar example would be the use of the conventional "t test" with paired data when the "paired t test" is appropriate and more powerful.

5.1.6.1 Randomization

The purpose of randomization is to eliminate bias and to ensure that all patients have an equal chance of being allocated to either or any group. The clinical trial groups should, therefore, differ from each other only by chance. It does not of course eliminate imbalance but, since statistical tests are based on an estimate of random variation, it achieves its purpose. If it is seriously felt that some factor or factors which influence response to treatment may by chance be allocated to one treatment group in an unbalanced way, this can be limited by stratification.

Stratification is carried out by randomly allocating treatments to different classes of patients by the use of separate dispensing lists, e.g., male list/female list. In addition disease severity may be deemed a factor of importance in determining response to treatment, in which case the patients may be divided up into: severe disease/moderate disease/mild disease. This would now entail six dispensing lists as follows:

(i) Male: severe disease

(ii) Male: moderate disease

(iii) Male: mild disease

(iv) Female: severe disease

(v) Female: moderate disease

(vi) Female: mild disease

If further factors are stratified the number of dispensing lists will grow as a multiple of the number of levels for each stratifaction factor and this could become very cumbersome administratively and put a strain on the dispensary. Other techniques for achieving balance through the use of very large numbers of subgroups have been described by Taves (1974) using a minimization technique.

Randomization itself is carried out after the patient has been admitted to the trial and, in order to avoid any selection bias the randomization code attached to the dispensing list is usually constructed from a table of random numbers and is kept in the dispensary. The doctor admitting patients to the trial has no knowledge and cannot guess which patients have been allocated to each treatment. This is why randomization techniques involving the use of alternate patients for example, or the final digit of the patient's hospital number, should not be used since pre-knowledge or "pre-guesses" as to the likely treatment allocation may cause the physician to selectively reject an eligible patient.

Since the aim is usually to produce a balanced randomization, a preferred method of constructing a randomization list is simply to draw coloured balls from a bag, or marked pieces of paper from a hat, having put into the bag or hat originally an equal number of alternatives and a total representing the number of patients to be recruited. However, with stratification this is rather more difficult since it is not know at the outset how many patients in each sub-set are going to arrive. In theory, therefore, there should be a dispensing list for each sub-set which is as long as the anticipated patient total (since all the patients recruited could fall into the same group). A series of dispensing lists to cover the stratifications indicated above might start as follows:

Male :Severe disease

Name	Treatment code
1	X
2	Y
3	Y
4	X
5	etc

Male : Moderate disease

Name	Treatment code
1	Y
2	X
3	X
4	Y
5	etc

and similarly for groups 3-6

It is a wise precaution to balance the treatments every 4, 6 or 8 subjects when relatively small numbers of patients are likely to fall in one or the other stratification group, to avoid chance imbalance in treatment allocation. This has been done in the dispensing lists shown above after every 4 patients. If there are 3 treatments then the balance may be struck after 6, 9 or 12 subjects.

One other advantage of a stratified randomization is that it clearly indicates the patient subgroups which are going to be studied in the analysis. This will help to ward off accusations of "data dredging".

Having achieved a predictable degree of balance through randomization, it is important not to introduce bias in the management of patients. Complete blindness of the patient and the investigator to treatment allocation is the best way of achieving this, but clearly blindness is not always possible, for example, in dietary studies or comparisons of medical with surgical treatment. It is essential in such trials that management be standardized apart from the treatments under test. It is often difficult to ensure that this has been achieved.

5.1.6.2 Matched pairs

It might be thought that prematching of patients for relevant factors such as age, sex or disease severity, might be the best way to overcome imbalance in small trials. Each pair of well-matched patients could then have the two treatments randomly allocated. But then again one will not know exactly what to match for and the more complicated one makes the matching the greater will be the difficulty in securing a "pair". It tends to be a self-defeating exercise.

5.1.6.3 Crossover trials

The attraction of crossover trials is that, since all subjects receive both treatments during the study, the variance due to individual patient differences is thought to be cancelled out. However, the impact of time related differences is substituted and the patient is almost never the same in the second time period as in the first. In addition, treatment administered in the first time period may continue to influence responses in the second time period unless it is possible to incorporate a "wash-out" in between.

Crossover designs are necessarily restricted to those treatments which do not have a fundamental influence on the disease process as opposed to producing temporary relief of symptoms. An advantage is that only half the number of patients is required as compared with a parallel group study. On the other hand, the duration of the trial may be prolonged, particulary if there are more than two elements to the crossover.

Statisticians prefer to analyse parallel group studies because of the difficulty in disentangling carry over effects from one treatment to another, even if balance is achieved between the patients receiving the drugs in the first treatment period and the subsequent treatment periods. The problems inherent in the analysis of crossover designs have been examined by Hills & Armitage (1979) and by Cox(1968). An important conclusion reached by Hills & Armitage is that the use of a crossover design evolves the possibility of an interaction between treatments, dependence on the period of time between administration of the drugs that, to be safe, a crossover trial should be of sufficient size to permit its being analysed as a parallel group study. This of course sacrifices the advantage of smaller numbers, but, if numbers are limited, then it is important to have evidence that the basic assumptions concerning interactions have been considered and are properly presented when the study report is written.

There are a number of balanced designs which are of great value for studies in other scientific fields, such as engineering and agriculture, which are occasionally useful in medicine.

5.1.6.4 Latin square design

Where a number of treatments are being evaluated in a crossover study, it may be important to ensure that equal numbers of patients receive the drugs in all possible orders, or at least an equal number receive them as first agent, second agent, third agent, etc. For instance, with three treatments, ABC, there are six possible orders:

A B C
A C B
B A C
B C A
C A B
C B A

Therefore by randomizing patients into six equal groups, a complete balance can be obtained. However, it is usually sufficient to construct a Latin square as follows, using three groups:

A B C
B C A
C A B

so that A features as first treatment, second treatment and third treatment, as do the others. For four treatments a Latin square would be:

A B C D
B C D A
C D A B
D A B C

all the letters featuring equally in columns and rows. It is an easy matter to construct larger squares or consult Fisher & Yates (1974). Drop-out patients would need to be replaced to maintain balance in the analysis.

5.1.6.5 Factorial design

In this design, the main concern lies with evaluating more than one treatment, either alone or simultaneously, e.g. a beta-blocker and a diuretic in moderate hypertension in the elderly. Groups of patients could receive either no treatment,

the diuretic, the beta-blocker or both. This would permit a comparison of both treatments against control, as well as diuretic against no diuretic and beta-blocker against no beta-blocker. A classic experiment was reported by Mainland (1956), whereby a study of X-ray bone densitometry simultaneously investigated 7 variables - two kilovoltages, two positions, two processing methods, two strengths of developer, two fixation periods, two washing periods and two drying methods. This resulted in $2^7 = 128$ possibilities. Nevertheless, if the purpose was to study, say, the effect of the two drying techniques, there would be 64 films using one and 64 films using the other.

Such designs give a full play to analysis of variance - a powerful parametric statistical technique, by means of which the contribution of the individual factor can be assessed.

5.1.7 Long-term prevention studies

During the last 15 years a number of long-term studies have been performed with the aim of reducing morbidity and mortality from coronary artery disease. Earlier studies involved the investigation of agents such as stilboesterol, thyroxine and nicotinic acid and more recently a variety of beta-adrenergic receptor blockers, antithrombotic agents and diet. This has given rise to a spate of literature on what might be called the science of prevention studies.

First, a distinction has to be drawn between the "explicative" and the pragmatic approach. The explicative approach tries to investigate mechanisms of drug action by determining whether the application of a particular preventive measure reduces mortality in those patients who accept and adhere to the regimen. The pragmatic approach is to adopt a policy in a clinic whereby the results of intervention in all patients are analysed whether they comply with treatment or not. Since, in the practice of medicine, it is usual to offer a treatment to any patient with a particular disease state, before being able to take into account his compliance with the suggested treatment, it would appear sound to analyse results in comparison with any other treatment policy using all the patients admitted, even if after admission they never received even one tablet of the therapy or made the slightest effort to stick to an allocated diet. This was originally discussed by Peto et al., (1976) in relation to trials of anti-cancer drugs under the phrase "intention to treat", and subsequently developed by Lovell (1977) in respect of studies in coronary artery disease. Further contributions by Hampton (1981a, b) and Mitchell (1981) made it almost mandatory to set out the results of such prevention studies in a specific diagrammatic form.

The great merit of this procedure is that it clearly shows where the patients were drawn from, how they were randomized, how they complied with treatment and what mortality results were obtained for each subgroup and the comparative mortality for each treatment on an "intention to treat" basis.

Uncritical adherence to this formula has led to two side effects. First, a reluctance to accept any subgroup results, however significant and however clinically important. Secondly, it has inhibited consideration of whether in fact treatment policies are applied uncritically in the clinic. In clinical trials, selection bias has to be avoided, but in real life a physician may well withhold a treatment if he/she feels the patient will not comply properly, or alternatively the physician may take special steps to ensure that a critical treatment is administered regularly and correctly. Hence, the already published results of prevention studies may well underestimate, through dilution with unsuitable or non-compliant patients, the value of certain agents in coronary prevention.

Clearly sub-set analyses are less reliable than the main total patient analysis, both for statistical reasons - the more you analyse the more likely you are to discover chance benefits - and logistical reasons in that you are unlikely to be able to recruit enough patients overall to avoid gross reductions in your ability to detect real differences in sub-sets. Nevertheless sub-set analyses are valuable for generating hypotheses and occasionally the differences stick out like a sore thumb (sore thumb <0.01).

The enormous logistical problems attendant upon prevention studies are closely connected with the number of patients necessary to detect clinically important changes in mortality over quite long periods of time, when untreated mortality is itself quite low. Table 5.2 shows the total numbers required to demonstrate reductions of 10% up to 50% when the fatality rate in untreated patients during the trial period is either 5%, 10% or 15%. For this table alpha is set at 0.05 and beta at 0.1 (Hampton, 1981b). If these frightening numbers are not obtained, then there will be a sacrifice of power and a consequent reduction in the chance of detecting a worthwhile difference even if one exists. Further allowance will have to be made for the diluting effects of substantial numbers of dropouts.

TABLE 5.2. TOTAL NUMBERS REQUIRED TO DEMONSTRATE REDUCTIONS OF 10% - 50% FOR FATALITY RATES OF 5%, 10% OR 15% IN UNTREATED PATIENTS DURING TRIAL PERIOD

Percentage reduction in events	Event rate in untreated patients during trial period		
	5%	10%	15%
10	76000	36000	8800
20	18640	8600	5440
30	7600	3640	2300
40	4040	1840	1240
50	2420	1160	740

The organizational costs of such trials have run into several millions of dollars in some American studies and, even in the United Kingdom where financial constraints ensure that every effort made to simplify procedures where possible, costs of up to a million pounds have been incurred. In one American study it is reported that Pinkerton's detective agency was employed to trace dropouts!

In addition there are ethical problems concerning the use of placebo control groups now that a range of trials with beta-blockers has already demonstrated reductions in mortality. This ethical issue has given rise to strong emotions over the proposed placebo controlled trial of vitamin supplements in pregnancy to investigate their effect on the incidence of spina bifida (Wynn, 1982; Leck, 1983). Since there is already evidence that vitamin supplements may reduce the incidence, it is hard to establish this through a properly conducted placebo controlled study, especially as the hazards of giving vitamin supplements routinely to all pregnant women are probably small.

To overcome the objection that a potentially beneficial preventive treatment might be denied to a placebo group long after evidence of its value was available, it has become customary to examine the data at intervals, e.g., 6 monthly or annually. If the results are deemed to be significantly in favour of, or significantly adverse to a treatment, the trial could be stopped and acted upon accordingly. This has substantial implications with regard to type I error and "multiple looks" at the data have to be accompanied by a reduction in the P value attached to the decision making process (McPherson, 1974; Pocock, 1978). It is also necessary to have an independent monitoring committee look at the data at the appropriate intervals. This committee need not communicate with the trial team unless a decision is reached which will mean an interruption of the trial. This happened with a recent

beta-blocker prevention study, where it was decided half way through the trial that there was only a remote possibility of coming to a favourable conclusion with regard to drug therapy by the time all the patients had been recruited and carried through the trial; therefore further commitment of resources was unjustifiable.

It was concern over ethical issues of this kind that led to consideration of certain other adaptive trial designs.

5.1.8 Adaptive designs

The ultimate in "multiple looks" is to examine the results as each patient completes his assessment and to make a decision to curtail the trial when certain statistical criteria have been satisfied. Sequential charts can be constructed to facilitate the recording of results and the most familiar are the closed designs.

In closed design procedure patient results are obtained from matched pairs in a parallel group study or within patients in a crossover study. A preference for treatment A is recorded by placing a X vertically on the "leggings" and for treatment B by placing a X in the next box horizontally. In this design it is possible to reach a decision that A is superior to B (or vice versa) after a minimum of 8 patients or patient pairs, because the trial is closed immediately the next X crosses the boundary of the chart. With the closed design a "no important difference" result is registered when a X enters the space between the two legs. It is to be noted that 'tied pairs' or patients unable to express a preference between A and B do not contribute information and cannot be entered on the chart. It is also to be noted that a X must enter a boundary after a certain maximum of preferences has been recorded (58 on this chart), which helps to limit the size of the trial unless there are a large number of tied pairs. With open designs (Bross, 1952, 1958), the trial can be prolonged indefinitely because it only terminates when one of the outside boundaries is crossed. In this procedure usually called a "bat's wing" the result favouring treatment A is recorded as a line drawn as a north-east diagonal and a result favouring treatment B as a south-east diagonal. The construction of the charts will depend as usual on :

(a) the magnitude of the difference between the treatments that you hope to detect (theta) e.g. 80:20;

(b) the P value (alpha) e.g. 0.05;

(c) the power (1-beta) e.g. 95%.

However, since the results are inspected after each entry the 5% probability of a type I error (P=0.05) will require more patient results than for non-sequential studies. In Fig. 5.2(b) a line must reach a boundary after 40 usable results if not before.

Sequential studies help to overcome some of the ethical problems which arise with parallel group studies. For instance, the fear that many patients may receive an inferior treatment after unexpectedly favourable results have occurred with a new treatment or that many patients will continue to be put at risk when the new treatment is unexpectedly dangerous. However, a limiting factor in sequential studies is that the preferences (treatment A better than treatment B or vice versa) need to be either global or dependent upon some single important measure of outcome and this may pose difficulties. Those interested in studying sequential trials and their modifications in more detail should consult Armitage (1975).

In other adaptive designs, the proportion of patients allocated to treatments may be varied according to the results that have already accumulated. The first group of patients is allocated randomly on a 50-50 basis, but as results come in suggesting that one or the other treatment is superior the proportion is altered, with the objective of reducing the number of patients being allocated to a treatment which shows a trend towards inferiority (Day, 1969). As with sequential trials, it is important that the results should come in fairly quickly to maximize the benefit of these adaptive approaches and again it is necessary to have some single measure outcome which is of overriding importance.

Finally there are a number of non-randomized designs whose aim is to maximize the expected benefit to the individual patient. Such designs have been proposed by Lellouch & Schwartz (1971) and Zelen (1969).

5.1.9 Placebos and blindness

Placebos are composed of pharmacologically inactive substances which can be used for baseline comparisons against a new remedy provided there are no ethical objections. They are invariably made to resemble the active material so that both patient and investigator blindness can be preserved after randomization. Indeed, minimal specifications for the manufacture of matching materials have been set out by Joyce (1968). In addition Hill et al. (1976) have studied the qualities of matching samples and have made the additional point that active materials may age differently; the active white tablets become off-white after 6-12 months. The arguments for and against the use of placebos in clinical trials have been discussed by Joyce (1982).

Conventionally, a single blind trial is one in which the patient is kept in ignorance of which treatment is being employed although the doctor knows. This technique is not often used and it is customary to try and preserve blindness both for the doctor and the patient, to avoid bias in the assessments. In some countries health authorities will only consider seriously such double blind studies.

5.1.9.1 Problems when allocating patients to treatments

Preserving blindness when dispensing matching materials has already been mentioned but special difficulties arise if the treatments are dissimilar e.g. an injection and a tablet, or if dosage increments are to be allowed. The "double-dummy" technique allows each patient to receive a capsule plus a tablet, but in half the tablet is a dummy and in the other half the capsule is a dummy. Similarly active and dummy injections can be used, although the use of a mock injection for half the patients could be regarded as unethical deception.

Dosage increments can be organized by having the assessor blind to the drug dispensing. The assessor simply indicates that a dose needs to be increased (or decreased) and this is acted upon (or not) within the dose ranges allowed by the trial. It may be difficult to preserve blindness, however, since the patient may give away to the doctor what is happening to his treatment.

Another method is to have a complex packaging of active and dummy tablets (Scott & Huskisson, 1977). Every patient takes 3 tablets 3 times daily and hence it is possible to organize anything from once daily treatment (using 8 dummies) to a maximum of 3 treatment tablets three times daily (no dummies). As usual the increased complexity increases the danger of errors both in the preparation of the individual sachets and in the dispensing. Also poor patient compliance with the full programme of 9 tablets daily will have capricious effects on treatment response.

5.1.9.2 Seeing when blind

Double blind trials may fail to achieve blindness for a number of reasons:

(a) The "matched" treatments may be easily distinguised through initial differences, e.g. in colour, size, weight (Joyce, 1968).

(b) Well matched tablets may age differently. A white active preparation may become off-white after 6 months, whereas its matched placebo may remain pure white (Hill et al., 1976).

(c) Patients or doctors may make deliberate attempts to break the code (a sort of "defeat the clinical trial" game). In a beta-blocker trial patients bit into tablets which were supposed to be swallowed and found that some were sweet tasting and some were bitter tasting.

(d) One of the "blind" treatments may produce a physiological response or side effect which the matching placebo or alternative treatment does not, e.g. bradycardia and cold extremities with beta-blockers or dry mouth with certain antidepressants.

(e) One of the treatments may modify the course of the disease under assessment in such a way that outcome variables and end events are missed, e.g. a beta-blocker may suppress the pain of a cardiac ischaemic episode so that electrocardiogram enzymes are not measured.

5.1.10. Measurement in clinical trials

Since a decision about the value of a new treatment will depend on measuring changes in some variable or variables, the choice of a relevant indicator of improvement or deterioration is of the greatest importance.

Measurements can be subjective depending on a doctor's or patient's view of changes in symptoms, e.g. rheumatic stiffness, depression, headache, or can be an objective recording of parameters or events, e.g. blood pressure, haemoglobin, anginal attacks, death. It is generally considered that objective records are preferable where there is a choice, but this is not necessarily so. Treatment may well influence symptoms more than a disease process, e.g. rheumatoid arthritis, and from the patient's point of view his symptoms and survival often seem more important than clinical signs or the results of laboratory tests. Indeed, Scott & Huskisson (1977) have suggested that the most relevant outcome variable may be the one the patient chooses as the best description of the progress of his disease. Hence, measures should not be chosen simply because they are fashionable, complicated, cheap, expensive, interesting, or any other adjective. They simply have to be relevant and to have been validated through previous use as reliable predictors of outcome.

Outcome has two main components, the immediate effect on well being and the long term prognosis. In a clinical trial it may only be possible to test one of these. Indeed, if the aim is to postpone death in a beta-blocker intervention trial there may be no immediate benefit noticed by any of the patients and for the great majority of them the trial will end, say 2 years later, without the patient or the doctor being able to judge whether treatment has had any benefit at all.

It is preferable to study a limited number of variables, both for the sake of simplicity and to avoid spuriously significant results which are inevitable if multiple estimations are made. In any event the statistician will need to calculate the trial numbers based on a clinically relevant change in just a few measurements, or even one.

5.1.10.1 Types of measurements

There are essentially three types:

(a) Interval
Made by a recording instrument on a scale (ruler, blood pressure machine) in which there is a mathematical relationship between each observation.

(b) Ordinal
Where responses to treatment can be classified on a scale of response but not quantified.

(c) Nominal
Event recording. Alive: Dead
Better: Not better

Different statistical methods are applicable to these different types of measurement. Parametric statistics concern interval measurment and the most well known are Student's "t" test and analysis of variance. Non-parametric statistics handle ordinal and nominal data and the most familiar tests are the Wilcoxon rank tests and the Chi square tests (Siegel, 1965; Langley, 1970).

5.1.10.2 Rating scales

These were designed to give what some would say are spurious numerical values to subjective material. The Hamilton rating scales in depression were actually designed to help standardize the diagnosis of depression and to aid different skilled observers to reach a measure of agreement on the diagnosis and severity of depression. In order to use these scales properly, observers need tuition and practice. These scales were not intially designed for comparative purposes in drug trials although today they are often used in this way. Large numbers of rating scales have now been devised particularly in the area of psychiatry, neurology, mental handicap, etc. Their use needs to be tempered with caution since they have not always been validated as a reliable and relevant measure of outcome.

The analogue scale is very popular and it is a device for converting a subjective response to a number between 0 and 100, e.g.:

The patient is invited to place a X which represents how well he/she slept last night between the extremes at each end of the 10 cm line. A ruler is then used to measure the position of the X in millimetres. This figure is then used as if it were an interval parametric measure. The hazards and inaccuracies attendant upon this approach have been investigated by Seldrup & Beaumont (1975) and commented upon by Curson & Curson, (1983). It is beyond the scope of this chapter to discuss the statistical hazards of converting subjective ordinal information to an interval scale and then employing a number to parametric statistical tests. In the past this has been defended by insisting that the power of the test has not been illegally enhanced by rejecting non-parametric methods and that such methods if used lead to a similar P value.

5.1.10.3 Missing data and missing patients

Patients default from clinical trials for a number of reasons but these can be grouped into those associated with the trial and those not associated with the trial. The former are often related to the side effects of therapy but dislike of the hospital, the investigations or even the investigator are other possible reasons. Non-trial reasons might be moving from the district, or because they were killed by falling under a bus. It is possible, however, that patients leave an area to get away from the trial or they fall under a bus because of a sedative effect of one of the drugs!

Every effort has to be made to trace defaulting patients and ascribe a reason. Clearly drug related defaulters are highly relevant to the trial conclusions and must participate fully in the data analysis. The handling of incomplete data has been discussed by Chaput de Saintonge & Vere (1982).

Missing data from compliant patients is another matter and it may be due to inadvertence, lost or broken samples, and recording mistakes. If a small amount of data is missing in a random fashion this will probably not seriously affect the trial analysis. Alternatively good monitoring may enable missing data to be "recovered" albeit a day or two late. This becomes progressively unsafe the longer the interval between patient attendance and the discovery that an important assessment was not performed or recorded. Sometimes a missing value may be estimated from other variables and tested to see if analysis

gives similar results with and without it. However, if the amount of missing data is substantial the fact will have to be admitted and the conclusions of the study tempered with appropriate caution.

Finally, intermediate values are often not used in the analysis of trials, since the best comparisons are usually between the pre-trial and end-trial results. In crossover studies, where carry over drug responses are likely, the end of period value, after say 6 weeks, is likely to be more useful than values recorded immediately after the crossover. However, intermediate values can throw valuable light on the time course of drug action and will support the beginning and end comparisons if they show a steady trend to change. These issues have been discusssed in more detail by Evans (1982).

5.1.11 Adverse reactions

The handling of adverse reactions in clinical studies is often deficient because the main aim of a study is to evaluate efficacy. Also the significance of isolated events in small numbers of patients is hard to establish. Nevertheless, in the finality, the place of a new treatment in the therapeutic armamentarium is going to be assessed as a balance between efficacy and hazard. Across a number of clinical studies some impression of adverse effects is bound to emerge, hence careful recording of each event is vital, as is communication between one centre and another.

It is important to distinguish those reactions which are predictable from the pharmacology of the drug, such as dry mouth due to the anticholinergic effects of antidepressants, and those which are unexpected (idiosyncratic). Defaulting in clinical trials is often linked with adverse reactions and hence the need for the provision in the protocol for following up patients who do not report at the expected time.

It cannot be expected that rarer adverse effects (and these are often the most serious) will be picked up in clinical trials. As a rule of thumb, a 1% incidence is unlikely to be detected in less than 300 patients, and a 0.1% side effect in less than 3000 patients. Some form of postmarketing surveillance will be necessary to discover such uncommon happenings.

In clinical trials side effects are often classified as:

Trivial - nuisance value only.

Mild - impairing patient function, but not leading to discontinuance of treatment.

Serious - leading to discontinuance of therapy.

It is fairly easy to solicit a battery of trivial reactions in clinical trials by asking specific questions, e.g. have you had any headaches? Alternatively a patient may be asked to fill in a questionnaire about side effects at each visit. Such information may be useful to get an idea of 'background noise' before the trial starts, or when the patient is on a placebo, from which will emerge some reactions with a significantly higher incidence when the patient is on an active agent. The discovery of impotence on diuretic therapy in the Medical Research Council of United Kingdom mild hypertension study is a good example of a significant reaction which is often not mentioned by the patient and which has a background incidence from other causes (Medical Research Council Working Party, 1981). Serious reactions will clearly be classified as treatment failures in the analysis.

Finally, appropriate forms should be completed and sent to the health authority concerning all suspected adverse reactions in clinical trials.

5.1.12 Logistics

Since a clinical trial should be a carefully planned experiment, it is essential to have a coordinated plan and efficient documentation. Planning decisions range from simple local arrangements for recruiting small numbers of patients for a restricted pharmacodynamic study, to the almost military style operation required for an intervention trial on dietary changes or drugs (clofibrate or beta-adrenergic blockers) on coronary artery disease mortality. The vast problems raised by these latter studies have already been referred to but are beyond the scope of this chapter and have been dealt with at length elsewhere (Greenberg, 1982; Hampton, 1981a, b; Klimt, 1979). Nevertheless, some of the elements required for these big studies are worth listing since they have more general application:

(a) Main coordinating committee

This committee will have to decide and agree upon:

(i) the disease definition of the patients to be included

(ii) the exclusion criteria

(iii) the trial design

(iv) the trial end points and their definition

(v) the method of allocation of treatments to patients

(vi) the measurements to be made and when

(vii) how side effects are to be handlled

(viii) how drop outs are to be detected and followed up

(ix) how compliance with treatment is to be assessed

(x) how the patients are to be informed about the trials and communication with the patient's family physician is to be effected

(xi) submission to and liaison with independent ethics committees

(b) Monitoring sub-committees

These may be required to assess trial end-points independently of the trial organizers (e.g. assessing electrocardiograms for allocation to "definite" or "probable" myocardial infarction). They may also assess changes in laboratory and other tests (e.g. reading x-rays blind for extent of disease or cavitation in tuberculosis trials).

(c) Quality control group

This group assesses, by regular monitoring, the completeness and reliability of the recorded data. Such quality control of the data is particularly important before the results are processed on to computer files which may give bad data an air of respectability. Also legal requirements may demand that a medical adviser to certify and take responsibility for the data package. When using foreign data to support drug licensing applications, good quality control arrangements are even more necessary to reassure health authority assessors.

(d) Pharmacy drug monitoring group

Continuity and accurate dispensing of drug supplies is so important that major hospitals often employ clinical trial pharmacists. In addition they can advise on monitoring compliance. Although compliance with drug therapy is often better in clinical trials than in ordinary therapeutic practice, there may be problems if any of the treatments are unpleasant, if the patient feels well and does not see the need to continue therapy, and if the trial is long-term so that participants lose interest. Drug taking can be monitored by a variety of techniques: pill counting, direct drug assay in urine and blood, indirect assay using a marker (Ellard et. al., 1980). The most satisfactory is to supervise fully drug administration and this has even been possible in some out-patient trials of antituberculosis therapy.

5.1.13 Documentation

In the past, the production of good record forms has been very much an individual matter giving an investigator or pharmaceutical physician free rein for creativity. Now nearly all the data will be transferred to a computer file so it is necessary to enter information on standard coded forms. However, many of the old principles still apply, particularly the need to maximize simplicity and convenience. Most doctors cannot record data accurately even if they measure it accurately and often disobey simple instructions - like putting a cross where a tick is required and confusion about the use of some American terms like the word "check". The time factor is also vital. For instance, if it takes 20 minutes to measure and record all the observations on a patient at each visit and 1000 patients are to be recruited for the trial with an initial attendance two weeks before trial entry and subsequent follow up at 0,3,6,9 and 12 months, it will take one doctor working 8 hours a day, 5 days a week, to complete the work when the patient entry is at its maximum, and without taking any holiday! The motto must be: don't collect data if it is irrelevant, can't be used, can't be analysed or will simply clog up the computer or the statistician's office.

Essentials to be considered are:

- a protocol
- an eligibility form
- an initial assessment form
- a form for each follow-up visit
- forms for recording laboratory data
- end-point forms
- patient questionnaires
- instructions about treatment changes or use of non-trial drugs
- side effect forms and reason for patient withdrawal
- dispensing code and where it is held
- a concluding or exit form
- a timetable

Finally, an operating manual may be needed as a guide to the correct completion of the documentation, to remind physicians of the criteria being used for diagnosis, the determination of an endpoint and for the measurement of response variables. For very long trials regular newsletters have proved valuable to investigators, particularly in multicentre studies both to highlight problems that have occurred and to sustain interest and camaraderie.

5.1.14 Termination of the trial

Patients usually get what they perceive to be better treatment when they are taking part in a clinical trial. Certainly they may be allocated more doctor or investigator time and have a larger number of investigations carried out upon them. This increased attention often has a favourable influence on the patient's condition and it has been frequently observed that the morbidity and mortality in the placebo group in prevention studies is lower than historical data would suggest. Indeed, this is one of the reasons why historical controls from routine clinics make poor comparative groups compared with those being treated contemporaneously.

When the trial is over the patient may feel neglected and deteriorate. If he/she has done well on a new therapy, which has not been shown to be superior to the control drug for the majority, he/she may find the treatment withdrawn or become unobtainable. These issues have to be considered and patients must be eased out of a clinical trial in a humane and ethical manner. If they are returned to their general practitioner when they have been attending the hospital out-patient department for their assessments, it is essential to write a full letter to him about the future management of his/her patient and to offer further out-patient appointments if thing are not going well. Also remember to thank everyone who contributed to the trial's success and where appropriate send them a copy of the report.

5.1.15 Obtaining patient cooperation

The help of the patients is essential for clinical trials to be carried out at all. Fortunately most patients are extremely co-operative and in Great Britain often prefer to leave it to their medical attendant to look after their best interests. This confidence must not be abused or undermined through discourtesy or neglect. Patients participating in clinical trials often like to know the answers to the following questions:

What is the purpose of the study?

Who can participate?

Why should I be involved?

What will happen during the study?

How much time will I have to spend at the hospital/away from work?

How long is the trial going on for?

and the more sophisticated will want to know:

What are the potential benefits for me?

How does the medicine work?

What are the dangers and side effects?

Finally, the patient complies with treatment and the trial better if told:

That his/her participation is much appreciated.

That it is important he/she cooperate with every aspect of the trial.

That he/she keep all appointments at the specified time (and you should see the patient then).

That if he/she changes address or is going on holiday, he/she should notify you well in advance.

That if he/she takes any medicines outside the trial medication he/she should tell you.

5.1.16 Disadvantages of controlled clinical trials

The two main problems for the clinician are that clinical trials are inflexible and restricted. To obtain balance and control, a great deal of artificial rigidity must necessarily be built in, which is at odds with normal clinical practice. The patients participating in the trial may only represent a small subgroup suffering from the disease requiring treatment; alternatively the results from a broad group of patients may not be sensitive enough to pick up certain kinds of patients who do benefit.

Nearly all clinical trials, however negative, usually throw up individual patients who appear to obtain considerable benefit and who are most reluctant to discontinue when the trial is over. But it is rare for some common factor to be spotted in the "responders" that would suggest a further trial to be performed in that subgroup.

From the economic point of view clinical trials can be costly, cumbersome and time-consuming, particularly long-term studies. The cost benefit of the trial has therefore become an important item in the balance sheet.

A negative result ('it did not achieve statistical significance)

It is well worth quoting in full the final paragraph from the paper by Rose (1982) on bias:

"The power of controlled clinical trials is less than is generally supposed and it is still regrettably common to equate a failure to demonstrate an effect with the positive verdict that the treatment is useless. This danger would be avoided if authors and editors made it a practice in any trial with a seemingly negative outcome to give a confidence interval for the estimated effects. If this embraces what would be regarded as a clinically important result, then the only valid conclusion is that the question remains open and a more powerful trial is needed".

The inclusion of confidence interval data helps the reader to decide whether a result which is reported broadly as "not statistically significant" could have a concealed clinically important result or whether, even allowing for the vagaries of chance, it is hardly likely that anything of importance has been missed. It might equally be said of significant results that a confidence interval helps to prevent "over enthusiasm" when the lower end of the confidence interval embraces only a trivial improvement.

Causes of failure

Some of the causes of failure in clinical trials could be listed as follows:

(i) Lack of clarity of the questions to be asked with consequent incorrect trial designs being used. Trying to answer too many questions at once.

(ii) Failure to consult a statistician at an early enough stage.

(iii) Wrong investigator chosen; inadequate access to right patients, too busy and does not effectively delegate the work to competent assistants.

(iv) Inexperienced pharmaceutical physician working witthout adequate supervision.

(v) Delays in getting approval of ethical committees and regulatory authorities leading to the loss of interest of the investigator.

(vi) Inadequate follow-up by the physician and the clinical trials team. The frequency of follow-up visits will depend on the nature of the trial which in our opinion should never be less frequent than once a month. Telephone contact should be made no less frequently than every two weeks. Infrequent follow-up leads to a feeling of lack of commitment by the investigator.

(vii) Failure to produce the clinical trial materials on time leading to loss of interest by the investigator. Failure to keep up the supply of clinical trial materials leading to interruption or delay in recruitment.

(viii) Selecting the wrong patients, that is choosing patients with the wrong type of the disease, or those unable to comply with the requirements of the protocol.

(ix) Inadequate recruitment when the investigator seriously overestimated the number of patients who would be available and suitable.

(x) Lack of compliance by the patients because of a badly formulated drug, e.g. too large a capsule or tablet.

(xi) Poorly designed record card leading to omission of important information. Lack of supervision by the clinical trial team to ensure that good record cards are completed fully.

(xii) Failure to achieve an effective method for collection of record card and/or blood samples.

5.1.17 Uncontrolled studies

These are often in the nature of pilot studies with a new drug and can provide valuable information of practical importance for the later design of controlled trials. For example, such studies may enable the determination of suitable dosage intervals. Also pilot studies may be used to test a protocol, rather like "de-bugging" a computer programme.

Most uncontrolled Phase III and IV studies are performed in general practice which after all is the scene for most uncontrolled drug administration. The artificiality of clinical trials and the emphasis on group results rather than on individual response has already been commented upon. Knowledge of how the drug is handled in general practice and how well or badly it is working is valuable even if assessments cannot be controlled. Lasagna (1974) has pointed out that current methodology is deficient in estimating benefit and making sense of the cost/benefit equation. A drug's actual performance is what matters not what it ideally might do.

5.1.18 Clinical trials in general practice

The requirements for good clinical trial design are no different for studies under general practice conditions. Since 80% of drug therapy is prescribed by family practitioners in the United Kingdom and some medical conditions are hardly ever seen in hospital clinics there are positive reasons for preferring general practitioner (GP) trials. The objectives and mechanisms for such trials are often different because of limited laboratory facilities and time constraints. Most trials have to be conducted using a large group practice, or across many practices with different doctors or assessors. Controlled studies using matching materials need an efficient organization and monitoring service. Nevertheless, the rewards for conducting good studies are substantial, both in terms of realism and patient numbers. Some company medical departments run a very successful clinical trial group, providing opportunities for quite sophisticated clinical trial methodology.

The monitoring of adverse reactions and a proper assessment of the balance between effectiveness and harm broadens the base of knowledge concerning drug activity. It may also help to accumulate information on the general acceptability of a new drug formulation and patient compliance. Difficulties arise with the use of placebos in control subjects simply because patients attending a GP clinic expect to receive an accepted treatment, and, therefore, comparative trials against existing therapy are more usual. This leads to a second difficulty in that a patient in a trial may need to have an effective treatment withdrawn, if an alternative is to be substituted and this may be unacceptable. For example, it would probably not be ethical to take a patient with well controlled hypertension and put himself in a study with a new agent, unless there was substantial evidence that the new agent was at least as effective or might produce less adverse effects. It would also be imprudent to include a wash-out period during which the blood pressure might return to baseline levels.

In recent years uncontrolled GP trials have fallen into disrepute because they have been used as promotional exercises to obtain widespread usage; the scientific results being secondary to the major aim of encouraging the continued prescription of the drug after "the study" is completed. This has led in the United Kingdom to the publication of guidelines for GP trials drawn up as a result of a joint agreement between the British Medical Association, the Royal College of General Practitioners and the Association of the British Pharmaceutical Industry (Anon., 1983).

5.2. THE PLANNING OF CLINICAL TRIALS FOR ANTIMALARIAL DRUGS.

In principle, the planning of clinical trials for an antimalarial drug follows the general plan for clinical trials of any new drug. It includes Phase I, Phase II and Phase III trials and Phase IV experience and post-marketing surveillance in order to define the new drug's safety, tolerance and efficacy.

The plan has to be adapted to the evaluation for malaria according to the peculiar characteristics of the disease which is widespread and endemic in many parts of the tropical world. The plan involves not only hospital-based, controlled open or double blind studies, but also extensive evaluation in field studies.

The planning of clinical trials of antimalarial drugs is rendered complex because of the existence of four different species of human parasites, which are also physiologically different, Plasmodium falciparum and P.vivax being the most important, and because of the various developmental parasite stages at which antimalarials may be effective. Thus the concept for testing a causal prophylactic drug will be fundamentally different from that for a blood schizontocidal compound for clinical therapeutic use. On the other hand, one and the same candidate drug may have to be evaluated for therapeutic and suppressive activity. Some of the candidate compounds may be suitable for emergency treatment of severe and complicated falciparum malaria (e.g. artemisinin and its derivatives), others for the routine outpatient department (OPD) treatment of multiresistant falciparum malaria of lesser severity (e.g. mefloquine, halofantrine), others again for radical treatment of vivax malaria (e.g. phenoxyprimaquine derivatives) or exclusively for prophylaxis (e.g. biguanides). In addition to the conventional and specific trial objectives, there will also be a need for a continued evaluation of the effectiveness of any new antimalarial drug since the practical experience with the 4-aminoquinolines, sulfadoxine/pyrimethamine, proguanil and quinine has shown that P. falciparum has a strong potential for developing resistance to virtually any antimalarial compound.

Other factors of complexity are potential ethnic differences, variations in food habits, and the presence of specific genetic traits (e.g. glucose-6-phosphate dehydrogenase -G6PD-deficiency, thalassaemias etc.) which may modify the pharmacokinetic parameters, increase toxicity or reduce drug efficacy.

5.2.1. Perusal of preclinical information

The preclinical data provide important guidance to the planning of clinical trials. In vitro studies would indicate the activity of the candidate drug against P. falciparum and help elucidate the spectrum of activity in relation to other antimalarials. They would also yield information on the effective concentration levels. Rodent and simian models will provide information on antimalarial activity in vivo. The potential for developing drug resistance, by increasing drug pressure, can be assessed by in vitro studies and animal models. Toxicity studies at various dose and dose frequency levels and by various routes will help in determining the route of administration, the safety margin and the dosage calculations. Pharmacokinetic studies in animals would indicate absorption, metabolism, elimination half-life and excretion in various animal species. All these data should be taken into consideration for planning the various phases of clinical trials.

5.2.2 Clinical trials

5.2.2.1 Phase I studies

These are carried out in young healthy male adults in order to determine the range of tolerated doses, safety, and side effects and to conduct pharmacokinetic studies. The trial may also pertain to long term administration for prophylaxis. The results of these trials will help to plan Phase II.

To evaluate an antimalarial drug which will be used in endemic areas of various countries, Phase I studies are also carried out in the target population of particular areas, in subjects who are free of malaria, but are likely to have helminth infections, lower status of nutrition compared to subjects of early Phase I studies, low Hb values and higher eosinophilic counts. Clinical haematological and biochemical parameters used as indicators of safety, should be assessed in about 100 subjects from the target population who are free from malaria (but may have had malaria previously). This will provide baseline data for the evaluation of drug effects in the given population. For mefloquine such baseline data were obtained from Brazil (Belem), Thailand (Bangkok) and Zambia (Ndola).

Pharmacokinetic studies are also carried out in target populations. Apart from differences of drug absorption related to pathological conditions and diet, the serum protein levels and levels of protein fractions differ from those of healthy populations in developed

countries where the primary Phase I studies are likely to have been carried out. If a drug is significantly bound to protein, the pharmacokinetic data may show a difference. The number of subjects enlisted is 10 to 15 per study. In contrast to the classical (primary) Phase I trials some of these studies include for comparison a group receiving the antimalarial standard drug used in that particular area.

5.2.2.2 Phase II studies

These studies are carried out to assess the safety, tolerance and effectiveness of the drug. The studies are carried out only in adult male patients having symptomatic P. falciparum malaria without any complications, or any other serious disorder, e.g. hepatic or renal insufficiency, severe anaemia. The asexual malaria parasite count should not be higher than 100 000 per mm^3 of blood and not lower than $400/mm^3$.

Patients are hospitalized for the entire observation period of 28 to 42 days (or longer), depending on the half-life of the drug, in order to detect late RI type recrudescence. If the ward is not located in a malaria-free area, the patients are kept in a mosquito-proof ward to avoid reinfection, restricting outings to daytime.

The parameter of efficacy is the clearance of parasitaemia and fever. Parasite counts are made every 8 to 12 hours on blood obtained by finger prick, until the blood is parasite-free and then once daily until the end of the observation period. In some places where it is not possible to hospitalize the patient for 28 days or longer, counts are made daily till day 7. The patient is then called to the clinic every week for blood smear examination till Day 28 or the scheduled end of the follow-up period.

The response is classified as a type S RI, RII or RIII response according the WHO classification. Sometimes the patient leaves the trial before completing the scheduled end of follow-up and does not report for further evaluation. Then the response is graded as a type "S/RI" response.

In all cases of recrudescence, blood samples are collected at recrudescence and stored according to the instructions given in the protocol pending drug assay. This provides information as to whether adequate amounts of drug were present during the episode (drug failure), or whether the concentration was below the minimum inhibitory concentration (MIC) level indicating absorption failure, loss of drug due to vomiting or abnormally rapid disposition/elimination of the drug.

Blood may also be collected for in vitro testing in all cases of recrudescence in order to determine the response parameters of various antimalarial drugs including the test drug, on the patient's P. falciparum isolate.

Phase II trials are carried out at various dose schedules to determine the most effective dose and the frequency of administration. These studies are conducted simultaneously as double-blind randomized prospective trials. This helps to compare not only the efficacy but also side effects and safety. Such studies require a placebo identical to the test drug, randomization and double blind performance.

Haematological and biochemical investigations are carried out on day 0, before drug adminstration and then on day 2, 4, 7, 14, 21, 28, 35 and 42 unless the study protocol stipulates additional time points. The results are analysed for effectiveness, the best dosage and dosage schedule, tolerance and safety as seen by various clinical (Blood pressure, pulse, chest x-ray, Electrocardiogram) and laboratory parameters (haematological and biochemical). Drug levels in blood also could be compared if adequate samples of blood are collected during the study. The number of patients per group is usually 40 to 50 adult male patients.

Adverse drug effects are determined by carefully noting the subjective symptoms such as nausea, dizziness, loss of appetite, vomiting, diarrhoea, and objective symptoms such as skin rash, both by recording the patients' complaints, and by using a check list given in the protocol. The subjective symptoms are first checked before drug administration (base line symptoms related to disease) and then every day after drug administration. If vomiting occurred, the time is recorded and the amount vomited. The frequency and consistency of stools is also recorded. A judgement is made on the extent of "drug related" symptoms, distinct from those which are "disease related".

It may be useful to carry out Phase II dose finding studies at centres located in geographically different endemic areas, varying in the pattern and the degree of P. falciparum resistance to standard antimalarials. While conducting dose finding studies for a combination of mefloquine 250 mg per tablet with sulfadoxine and pyrimethamine (Fansimef), doses of 1 tablet, 2 tablets and 3 tablets as single oral dose were compared in Ndola Zambia where all doses gave a type 100% "S" response. In Bangkok (Thailand), the one tablet dose showed 37% type RI responses. The trial had to be altered early in its course, to compare only 2 tablets to 3 tablets. In Zambia

at that time P. falciparum was quite sensitive to chloroquine and sulfadoxine/pyrimethamine, whereas in Thailand resistance was present to both chloroquine and the sulfadoxine/pyrimethamine combination. Also the patients' immune status of the patients was different.

5.2.2.3 Phase III - studies

These are multicentre studies. An effective dose regimen has already been determined in Phase II. In these studies the new drug is compared to existing drugs, in a double blind, randomized design, using a double blind "double dummy" method (Placebos are matching both the test drug and standard drug). The basic method used is the same as in Phase II. The standard drug of comparison should be effective in the particular region e.g. mefloquine has been compared to chloroquine in Zambia, to sulfadoxine/ pyrimethamine in Belem (Brazil) and to quinine 600 mg three a times a day for X 7 days + tetracycline 250 mg four times a day for 7 days in Bangkok (Thailland).

Results are collected from different centres and analysed for efficacy, safety, and incidence and type of adverse effects.

5.2.2.4 Trials in special groups

(a) Paediatric trials

After having collected experience and data in adult male patients in Phase II and III, the new drug is studied in paediatric inpatients between 5 and 12 years old. Dose finding studies are carried out in two or more doses, proportionate to those in adults, and the results are analysed in terms of dose effectiveness in mg/kg of body weight. For example mefloquine was tried in doses of 1/2 tablet, 1 tablet, 1 1/2 tablets and 2 tablets in 4 different age subgroups. The design of the study is similar to phase III. Having decided the dose and dosage regimen required in children, similar studies can be carried out, comparing the new drug with the standard drug used in the area. Difficulties arise in trying to keep paediatric patients in hospital for a 42-day period. At times, the children are hospitalized for one or two weeks and then followed in the clinic every week, instructing the parents to bring the child to hospital immediately, if fever occurs during the study period. Pharmacokinetic studies also can be carried out in children with due attention to keeping blood sampling strictly to the minimum required for meaningful results.

(b). Trials in adult non pregnant women

Prior to extending the use of the new drug in the treatment of P. falciparum malaria in pregnancy, a trial is conducted in non-pregnant women to study if there are any sex related differences in the tolerability of the drug.

(c) Tolerance and pharmacokinetic studies in malaria patients with hepatic damage

These trials are carried out in patients with viral hepatitis, or liver cirrhosis to study the tolerance and handling of the drug in the presence of liver damage.

(d) Tolerance and pharmacokinetic trials in malaria patients with decreased renal function

The need for such trials in the presence of hepatic or renal dysfunction depends on the metabolic rate of the drug and its routes of excretion, since these may be affected in such patients. However these trials could be carried out after drug registration, unless there is reason to believe that either renal or hepatic damage would seriously affect the pharmacokinetics of the drug and its tolerability.

(e) Trials in pregnant women

P. falciparum malaria in pregnant women poses serious problems. At present the only drugs used are chloroquine or quinine. A new drug can be evaluated in the treatment of P. falciparum malaria during pregnancy if the drug is compared to the standard in a randomized double-blind fashion. The patient has to be followed till delivery. Effects on progress of pregnancy, uterine movements, foetus, types of abnormal pregnancies like abortions, premature labour/and the need for caesarian section are noted. The type of delivery, birth weight of newborn, Apgar score, detailed examination of new-born for development defects, etc., are recorded, and the infant is followed every month for 6 months to study his/her development and growth. The test drug and standard drugs are compared using these various parameters.

(f) Prophylactic trials

Prophylactic use of a new drug in travellers to endemic areas coming from non-endemic regions, in young children, and in pregnant women are the major types of prophylactic trials. They are often carried out after the drug has been registered.

(g) Evaluation in severe malaria

Evaluation for effectiveness and safety in severe malaria poses a difficult problem. With severe malaria, patients will be semiconscious or unconscious. Trials in such patients can only be conducted in a hospital where adequate facilites and laboratory services are available to cope with various emergency situations.

A detailed examination of central nervous system (CNS) functions must be made and repeated every two hours to note changes. Laboratory studies will include repeated parasite counts, blood sugar assays, tests for acid/basic status, renal function tests, liver function tests and determination of hydration status of the patient. Lungs need to be repeatedly examined as pulmonary oedema occurs as a severe complication. Besides giving the test drug, maintenance of electrolyte balance and hydration status, glucose replenishment, correction of acidosis and use of haemodialysis in cases of renal failure will be required. (See section 5.4 of this chapter).

In severe malaria, the drug will usually be given parenterally. This requires special studies in drug formulation, safety, dosage and pharmacokinetics.

5.2.2.5 Phase IV trials and post marketing surveillance

Phase IV studies and post marketing studies (PMS) are carried out after the drug is marketed. They serve to determine the incidence of adverse reactions to the drug when it is used in large populations involving thousands of patients, and to detect rare adverse drug reactions which seen in the relatively restricted population samples covered by Phase I-III studies. Phase IV trials and PMS pertain also to the status of the drug's efficacy.

These trials differ considerably from the experimental ones in hospital settings using selected patients and double-blind randomized trial designs. Field trials and PMS represent the study of a drug in a real life situation, and it is necessary to know how the drug performs in such situations.

As P. falciparum often develops resistance in the course of the continued use of an antimalarial drug, monitoring for efficacy is required as long as the drug is in use.

Drug interactions are likely to occur when the drug is used widely as some of the patients may be simultaneously receiving more than one drug (diabetics, heart conditions etc.). This requires drug interaction studies which may be geared to specific geographical areas and priorities.

These aspects are discussed in chapter 6.

5.3 EXAMPLE OF ANTIMALARIAL CLINICAL TRIAL ORGANIZATION: MEFLOQUINE

Malaria still remains one of the most important health problems today, particularly in tropical Africa, South-East Asia and South America. The emergence of drug resistance in P. falciparum has intensified search for new drugs which could be successfully used to treat resistant P. falciparum infections.

Mefloquine hydrochloride, a 4-quinolinemethanol, has been investigated clinically by WHO in the last few years. These studies are discussed to illustrate the organization of clinical trials for new antimalarial drugs.

5.3.1 Prerequisites for clinical trials

Before beginning a clinical trial with a new drug, certain prerequisites need to be fulfilled. These have already been covered in more detail in chapter 4 and will be only briefly enumerated here.

(a) Adequate preclinical data related to the effect of the drug in vitro and in animal models, pharmacological studies, acute and chronic animal toxicity studies, tests for mutagenicity, effect on reproduction and carcinogenic studies.

(b) Pharmacokinetic studies in animals.

(c) Clearance by the appropriate drug authority for clinical trials.

(d) Clearance by the ethical committees of the

  (i) organization sponsoring the trials (eg. WHO)

  (ii) country where the trial will be carried out.

  (iii) institution where the trial will be conducted.

(e) Adequate provision to meet liabilities of the investigator and safeguard the interest of the patients in the event of mishap during the trial, or adverse effects arising from the test drug.

(f) Experienced, reliable investigator and team.

(g) Adequate laboratory facilities.

(h) Voluntary consent of the patients to participate in the trial.

(i) Well designed and well written protocol, agreed upon by the investigator.

(j) Well planned patient record forms and adequate supply of trial drug.

(k) Adequate and meticulous recording, and prospect of completing the trial within the scheduled time span.

(l) Availability of suitable patients in adequate numbers over the stipulated period.

(m) Independent monitoring of the trial.

(n) Collection and analysis of patient records for evaluation of effectiveness, safety, tolerance and adverse effects.

5.3.2 Clinical trials of mefloquine

5.3.2.1 Earlier data on human use

Studies on the use of mefloquine in volunteers had been carried out between 1972 and 1978 before WHO trials were organized. These were carried out with a different formulation from that used in the WHO trials and were as follows:

(a) Tolerance and safety studies in healthy volunteers using a single oral dose in a double blind design with 19 dose levels, rising from 5 to 2000 mg. At each dose level 2 volunteers received the drug and 2 volunteers received placebo to evaluate the drug for safety. Physical examination, electrocardiogram and various haematological and biochemical tests were carried out. The drug was well tolerated. Some subjects complained of transient nausea and of dizziness within the first 48 hours after administration of 1750 mg and 2000 mg of the drug.

(b) Studies in nonimmune volunteers, infected with different resistant isolates of P. falciparum and P. vivax. The volunteers received various doses of the drug, between 2 and 10 days after the onset of parasitaemia (35 volunteers). Similar studies were carried out in a few partially immune subjects.

1000 mg to 1500 mg of mefloquine, given as a single oral dose was effective, well tolerated, and no recrudescence occurred during the observation period of 60 days. Radical cure of P. vivax infections required additional treatment with primaquine (Trenholme et al., 1975).

(c) Tolerance study with weekly oral doses of 500 mg over one year. There was no clinical or laboratory evidence of intolerance.

(d) A therapeutic field study in Thailand in patients who had naturally acquired P. falciparum malaria. A single oral dose of 1500 mg of mefloquine cured all 37 patients although those given mefloquine had a greater incidence of gastrointestinal side effects than those given sulfadoxine-pyrimethamine (Doberstyn et al., 1979).

(e) A sequential trial of quinine, in a short course of 4 doses equivalent to 2 g, followed by a single oral dose of 1500 mg mefloquine produced 100% cure in 35 patients (Hall et al., 1977).

(f) A single-dose trial with 1000 mg mefloquine and quinine as above against P. falciparum infections yielded a radical cure rate of at least 93%.

(g) A study of the suppressive prophylactic activity of the drug against P. falciparum and P. vivax. Weekly or biweekly administration of mefloquine produced practically complete protection. (Mefloquine HCl - Clinical Brochure. Walter Reed Army Institute of Research. Walter Reed Army Medical Center. Washington D.C., March 1978).

5.3.2.2 Clinical trials under the auspices of WHO

Clinical trials were carried out in Belem (Brazil) Ndola (Zambia), Bangkok and Chantaburi (Thailand).

All patients were hospitalized for the study period of 42 - 63 days. The wards were mosquito-proofed or the hospital was in a nontransmission area.

Baseline information was collected on all patients, before starting drug administration. The examination consisted of physical examination, electrocardiogram, chest x-ray, parasite count, haematological and biochemical investigations, urine and stool examination. Information was also recorded on symptoms as described by the patient prior to treatment. Only those patients who had not received any antimalarial drugs during the preceding four weeks were accepted in the trial. Urine was examined for the presence of 4-aminoquinolines and sulfonamides.

Most of the trials were prospective, randomized and double-blind. In the pregnancy trial at Chantaburi (Thailand), patients were admitted in the wards for one week during each episode of P. falciparum malaria and followed till delivery. Infants were controlled every month for 6 months. The results of laboratory tests were compared to those obtained from the target population of each centre which served as normal standard.

The patient record sheets were identical in all centres and the trials were periodically monitored.

(a) Phase I studies

Phase I studies in target populations were carried out at Belem in Brazil and Ndola in Zambia as follows :-

(i) Phase I clinical trial of mefloquine in Brazilian male subjects (Souza, 1983a). A double blind randomized Phase I clinical trial was carried out to compare mefloquine with sulfadoxine-pyrimethamine in 20 adult male volunteers. The observations started 2 days prior to drug administration and continued for 63 days after drug administration. Mefloquine was given as a single dose of 1000 mg (4 tablets X 250 mg) and sulfadoxine-pyrimethamine as a single dose of 2 tablets (each tablet containing 500 mg of sulfadoxine and 25 mg of pyrimethamine). Both drugs were well tolerated in adult male Brazilian volunteers from a malaria endemic area. No drug related adverse reactions were observed.

(ii) Single-dose kinetics of mefloquine in Brazilian male subjects (Souza et al., 1987). Ten male subjects from an endemic area in Brazil received a single oral dose of 1000 mg mefloquine. Blood samples were taken on day 0 prior to drug administration and then on days 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 35, 42, 49, 56 and 63. The plasma levels of the drug and its main metabolite were measured. The pharmacokinetic data obtained were well within the range of the values reported previously for Caucasians and Africans.

(iii) Mefloquine Phase I pharmacokinetics and tolerance study, double blind randomized study of mefloquine and chloroquine in 10 adult male subjects from a target population in Ndola, Zambia. Ten subjects received mefloquine as a single oral dose of 1000 mg. Mefloquine was well tolerated and no significant adverse reactions were observed (Schwartz et al., 1982).

(iv) Mefloquine Phase I multiple dose pharmacokinetic study with 500 mg mefloquine followed by 250 mg weekly for 8 weeks in 6 adult males (Schwartz et al., 1987). All 6 subjects tolerated the multiple dose regimen of mefloquine without any side effects or pathological changes in the various haematological and biochemical parameters.

(b) Phase II/III studies

(i) Dose-finding study

A phase II clinical trial of mefloquine in patients with chloroquine-resistant falciparum malaria in Thailand (Harinasuta et al., 1983).

A double-blind, randomized dose-finding Phase II mefloquine trial was carried out in 147 adult male patients suffering from acute, uncomplicated falciparum malaria, admitted to the Hospital for Tropical Diseases, Bangkok. Mefloquine was administered in single oral doses of 500, 750 or 1000 mg (base) in the form of the hydrochloride. The cure rates obtained with the 1000, 750 and 500 mg doses were 100%, 92.5% and 95% respectively during an observation period of 63 days. No significant changes were noted in haematological or biochemical parameters in any patient. Side effects were mild, including nausea, vomiting and diarrhoea. Behaviour disorder (reversible acute brain syndrome) was observed in one case on day 21 (1000 mg group). Mefloquine was well tolerated in one case of acute renal failure, 10 cases of moderately severe malaria with jaundice, 13 cases with G6PD deficiency and one case of thalassaemia. Clearance of parasitaemia and fever was more rapid in cases receiving the 1000 mg dose.

(ii) Comparative studies

A double-blind comparative clinical trial of mefloquine and chloroquine in symptomatic falciparum malaria (Kofi Ekue et al., 1983).

A total of 99 male Zambian patients with symptomatic malaria were treated in a double-blind randomized manner with either mefloquine (1000 mg single oral dose) or chloroquine (1500 mg given over 3 days). 100% cure was obtained in patients giving chloroquine and 98% in the mefloquine group. Both drugs were equally safe, effective, well tolerated. Side effects such as nausea, vomiting, dizziness, loose stools and pruritus were mild and transient. Pruritus was more common after chloroquine and dizziness more common after mefloquine.

A phase II clinical trial of mefloquine in Brazilian male subjects (Souza, 1983b).

A double-blind randomized trial was carried out in patients with symptomatic falciparum malaria (Souza, 1983b). Mefloquine 1000 mg (base) was administered to 49 patients as a single oral dose. Sulfadoxine 1500 mg plus pyrimethemine was administered to 48 patients 75 mg of pyrimethamine (i.e. 3 tablets Fansidar) in a single oral dose.

All patients receiving mefloquine were parasitologically cured. In the sulfadoxine + pyrimethamine group, 73% showed "S" responses, 16.6% RI, 6.3% RII and 4% RIII responses. Clearance of parasitaemia and fever was quicker with mefloquine. Side effects with both drugs were mild and transient and no adverse effects were observed in haematological and biochemical parameters with either drug.

An open randomized clinical trial of mefloquine compared to quinine plus sulfadoxine/pyrimethamine in the treatment of falciparum malaria in adult males was also conducted in Brazil. The patients were from Paragominas and were admitted to the Clinical Trial Centre in Belem. They received either 1000 mg of mefloquine (base) (49 patients) or 600 mg quinine orally 8 hourly for 3 days and 1500 mg of sulfadoxine + 75 mg pyrimethamine (3 tablets Fansidar) with the first dose of quinine on day 0 (50 patients).

All patients receiving mefloquine were cured of P. falciparum infections although there were 2 P. vivax relapses, one on day 29 and one on day 32.

The quinine + Fansidar group showed 92% "S" responses and 8% RI responses. P. vivax relapses occurred in 7 patients between day 16 and day 38.

Mild transient side effects, including nausea, vomiting, dizziness, diarrhoea, and abdominal pain, occurred in both groups. The incidence of nausea and vomiting was higher in the patients given quinine and Fansidar whereas the incidence of diarrhoea was higher in the mefloquine group. Tinnitus and transient hearing loss was observed only in the quinine + Fansidar group. None of the side effects required any specific treatment.

A comparative clinical trial of mefloquine and the combination of quinine with tetracycline in acute uncomplicated falciparum malaria in adult males in Thailand .

84 adult male patients admitted to the Hospital for Tropical Diseases, Bangkok, for the treatment of symptomatic P. falciparum malaria were treated in this trial. The period of observation was 42 days.

The patients received either 1000 mg base of mefloquine administered in an initial dose of 750 mg followed 6 hours later by 250 mg (42 patients), or quinine 600 mg 8 every hours plus tetracycline 250 mg four times a day for 7 days (42 patients).

There was one R-II rescrudescence following mefloquine administration; all other infections were cured. All patients in the quinine plus tetracycline groups were cured. The side effects were generally few and transient. Tinnitus and transient partial hearing loss were observed in the quinine + tetracycline group. One patient of the mefloquine group developed a transient behaviour disorder on day 10. No other adverse effects were observed.

The incidence of P. vivax relapses was 4.5% after mefloquine and 47.6% after quinine plus tetracycline.

(iii) Paediatric trial

A phase III clinical trial in children with chloroquine resistant falciparum malaria in Thailand. (Chongsuphajaisiddhi et al., 1987).

An open non-comparative trial of mefloquine was carried out in 82 patients aged 5 - 12 years suffering from P. falciparum malaria. All patients were hospitalised for 42 days. Mefloquine was given in doses of 18 - 29 mg ter kg body weight (1.5, 2, 2.5 and 3 tablets tf mefloquine hydrochloride each containing 250 mg mefloquine base). Radical cure ("S" response) was obtained in 97.5% of the patient and RI resp.tse was seen in 2.5% of cases (one on day 14 and one on day 21). P. vivax relapses occurred in 12 patients between day 23 and day 43. The drug was well tolerated in all patients including 11 with G6PD deficiency. Mean parasitaemia clearance time was 65 $\pm$ 17.9 hours. Side effects were mild and transient. The clinical-chemical parameters measured exhibited no drug-related changes.

(iv) Studies in women

- A Phase III clinical trial of mefloquine in non pregnant female Thai patients.

A total of 46 adult non pregnant female Thai patients with acute uncomplicated falciparum malaria were admitted to the trial. Mefloquine was given in a single oral dose of 750 mg followed by a further 250 mg after 6 hours. 34 patients completed the 42 days observation period. 12 patients "dropped out" for personal, non-drug-related reasons. However all patients were free of parasitaemia and fever by day 6. In the 34 patients who completed the study, the cure rate was 97%, one patient had an RI response on Day 28. Side effects were transient, and generally mild. Vomiting was observed in 34% of the patients. In one patient acute psychosis occurred on day 5. This was resolved by day 12. Clinical or laboratory examination showed no other drug-related changes.

Double-blind, randomized, comparative trial with mefloquine versus seven day course of quinine in pregnant women suffering from falciparum malaria in Chantaburi Thailand .

The study was conducted to assess the safety and efficacy of mefloquine, compared to quinine for the treatment of falciparum malaria in pregnancy, including the following:

- determining the effects of quinine and mefloquine on the uterus and their possible role in inducing abortion or premature labour;

- determining the effects both on the fetus and the subsequent development of the infant.

82 patients received mefloquine, and 72 patients quinine in a randomized double blind trial. A further 64 patients received quinine in an open non-randomized study.

As from the first admission, all patients were followed through the pregnancy, using the following parameters of assessment:

- clearance time for parasitaemia

- clearance time for fever

- laboratory parameters (haematological and biochemical) of safety;

- Side effects

As well as, special parameters related to pregnancy, delivery and outcome, namely

- maternal death;

- effects on pregnancy;

- effects on fetus;

- types of delivery;

- reasons for Caesarian sections;

- abortions;

- still-births;

- infant deaths;

- congenital malformations;

- uterine and fetal monitoring before drug administration and at intervals of one week following drug administration as well as at each subsequent antenatal visit;

- Apgar score of newborn and birth weight

- follow-up of infants once a month for the first 6 months;

- comparison between primigravidae and multiparae, for severity of illness and infant outcome;

- number of admissions for P. falciparum and P. vivax infections.

Both drugs were relatively well tolerated. Most of the side effects were mild to moderate. Vomiting was more common after mefloquine whereas quinine produced more tinnitus.

Mefloquine was more effective than multiple oral doses of quinine. The cure rate for mefloquine was 91%, that for quinine was 70%.

Quinine-treated patients had more admissions for acute attacks of P. falciparum than patients treated with mefloquine.

Quinine treatment was associated with significant hypoglycaemia. There were no differences in the incidence of uterine contractions, premature labour or fetal distress between the mefloquine and quinine treatment groups. Apgar score at birth was significantly better in patients treated with mefloquine.

Mean birth weight after mefloquine was not significantly different from that following quinine treatment (2808 g for mefloquine, 2660 g for quinine).

The frequency of still-births, infant deaths, congenital malformations and the size of infants for gestational age were similar in both treatment groups.

(v) Mefloquine in combination with primaquine

- At malaria clinics in Thailand, 287 outpatients with falciparum malaria received 750 mg or 1000 mg of mefloquine, each with 45 mg of primaquine given either on day 0 or day 3. The study design was double-blind and randomized for the two mefloquine dose levels. The combination was well tolerated, with no increase in adverse reactions to either drug.

- At the Tropical Diseases Hospital in Bangkok, 136 hospitalized patients suffering from symptomatic P. falciparum infection, received mefloquine together with 45 mg of primaquine given on either day 0 or day 3. The patients were observed for 42 days. There was no qualitative or quantitative increase in side effects due to addition of primaquine. The addition of primaquine prevented sporogeny in mosquitos, membrane fed on blood from gametocyte carriers, although the mosquitos did get infected on blood samples collected before giving the drug.

5.4 CLINICAL TRIALS IN PATIENTS WITH SEVERE AND COMPLICATED FALCIPARUM MALARIA

Severe and complicated malaria is a clinical emergency which warrants immediate diagnosis, implementation of specific treatment and ancillary management of the patient, and continuous monitoring of his/her condition. At present, specific treatment of severe and complicated malaria relies on the administration of intravenous (i.v.) infusions of quinine. However, artemisinin and especially some of its derivatives may offer an alternative since they appear to act faster than quinine and to be effective also in infections with quinine-resistant Plasmodium falciparum.

While a comparison between artemisinin or its derivatives and quinine in non-complicated falciparum malaria of low severity will provide valuable information, it is the performance of the drug(s) in patients with severe and complicated malaria that will define its use in such cases. Because artemisinin ethers can be formulated for intramuscular (i.m.) administration, they offer a distinct possibility of making availiable an effective therapeutic intervention to personnel of a qualified paramedical level and thus of reducing mortality from malaria, particularly in rural areas without easy access to higher levels of health care.

Clinical trials in patients with severe and complicated malaria require clinical facilities of a high standard which guarantees the best possible patient care and rigorous monitoring. Only then can they be ethically acceptable. The clinical establishment running such a trial must be familiar with the management of severe and complicated malaria.

Information on the definition, diagnosis and management of severe and complicated malaria can be found in a recent report on the subject (WHO/CTD, 1990).

5.4.1. Occurrence of severe and complicated falciparum malaria

There is an inverse correlation between acquired immunity and the occurrence of severe and complicated falciparum malaria. Atlhough very marked, this correlation is not absolute, and occasionally life-threatening manifestations of malaria are also seen in persons who had presumably built up a sound protective immunity before but whose protection had broken down for one reason or another.

The inverse correlation between immunity and life-threatening malaria explains the age pattern in the incidence of

severe and complicated falciparum malaria in tropical African lowlands where mostly infants over 5 months of age and young children are affected and only rarely adolescents and adults, or even pregnant women. In areas with lesser communal immunity, i.e. in areas where malaria transmission is seasonal or focal, this age pattern is lost and virtually all age groups are potentially at risk of developing severe and complicated falciparum malaria. This is most marked in nonimmune immigrants or visitors to malarious areas (e.g. tourists from nonmalarious countries), who usually develop life-threatening manifestations of falciparum malaria if their infections are not immediately treated.

Delayed and inadequate treatment is an additional factor affecting severe and complicated malaria. Although this factor is unlikely to play a major role in areas with easy access to diagnosis and treatment, it may affect special risk groups who live temporarily outside the reach of such facilties (e.g. forest workers, gem miners, persons engaged in illicit/clandestine activities). These groups consist mainly of young men and an age analysis of the incidence of and mortality from severe and complicated falciparum malaria in eastern Asia and South America show that it is this age and sex category that is the most seriously affected. In these groups self-medication is usually conducted with relatively cheap, easily available drugs, such as chloroquine or sulfadoxine/pyrimethamine, to which resistance is widespread. In addition, self-treatment is often underdosed or, in the case of multiple dose regimens, irregular and of too short a duration.

In clinical trials involving patients with severe and complicated malaria, the factor of pretreatment needs to be taken into account.

5.4.2. Constraints of clinical trials involving patients with severe malaria

As a rule, clinical trials of antibiotics and chemotherapeutic agents are limited to patients whose condition is not immediately life-threatening. In malaria, for instance, it is generally agreed that a new antimalarial agent is tested initially in patients without severe and complicated malaria since this is an exclusion criterion for these Phase I and II types of clinical trials. Moreover, pre treatment is an exclusion criterion for these trials. However, it would be very difficult in most areas to find sufficient patients for such a clinical trial if pretreated patients were to be excluded. In Africa, for instance, about 30-50% of the patients with severe malaria are pretreated, usually with chloroquine.

The type of pretreatment may have a bearing on inclusion or exclusion in trials of severe and complicated malaria. For instance, if a patient has already received quinine and one of the trial groups is to be treated with quinine given in an i.v. infusion, the patients should be excluded from the trial. Similarly, mefloquine pretreatment would also be an exclusion criterion since mefloquine-quinine interactions causing adverse reactions may occur in such patients.

The mode of administration of a trial drug may be an important factor with regard to its efficacy. A new drug which can be administered by the intravenous route (i.v. infusion/push injection/slow injection) is expected to reach the first compartment as fast or faster than quinine administered as an i.v. drip. However drugs administered by the intramuscular route may not be adequately or sufficiently rapidly mobilized in patients with circulatory disturbances. Such disturbances may occur especially in adults with severe and complicated malaria where they manifest themselves as circulatory collapse (shock and hypotension as part of "algid" malaria). These events are infrequently seen in children with cerebral malaria. Where hypotension stems from hypovolaemia, this problem can be quickly corrected by appropriate ancillary management and such patients may be entered in the trial, assigning them randomly to all trial groups.

The absorption of drugs given by nasogastric tube may be compromised by malaria-associated functional disturbances in the gastrointestinal tract.

A further constraint of clinical trials in patients with severe and complicated is that the patient flow tends to be more irregular compared to trials, involving patients with malaria of low or moderate severity.

Finally, minimum requirements for the monitoring of important clinical, parasitological, haematological and clinical-chemical parameters will have to be respected as in other clinical trials but in addition more frequent assessment will be imposed by the patients' condition, requiring a high degree of alertness and flexibility on the part of the treating physicians.

5.4.3 Capabilities/facilities required in a clinical trial centre undertaking trials in severe and complicated malaria

Clinical trials in patients with severe and complicated falciparum malaria should only be undertaken in clinical facilities which ensure a high standard of continuous patient care. This requires the availability of medical, nursing and laboratory services at all times and the capability to monitor all major clinical parameters and to provide virtually all patient management procedures (see WHO/CTD, 1990).

Before all else, parasitological and certain clinical -chemical tests need to be done almost immediately upon admission of the patient in order to enable the physician to decide upon inclusion in the clinical trial and to implement appropriate treatment independently of whether or not the patient is included in the trial.

The services should have the capacity to cope with several admissions at short intervals. Since the care for patients with severe and complicated malaria is, in fact, a specialized form of intensive care, appropriate adjustments have to be made in terms of staff.

The management of severe and complicated malaria requires a sound and long-standing specific experience on the part of the medical, nursing and laboratory services involved. It would therefore be unwise to graft clinical trials involving patients with severe and complicated malaria onto clinical centres without such specific experience, however familiar the centre and its staff might be with the performance of "normal" clinical trials.

A special case is that of trials to evaluate i.m. therapy given at peripheral health establishments, such dispensaries, health centres, auxiliary hospitals and other peripheral hospitals, from where patients are subsequently transferred to a clinical trial centre where they are followed up.

5.4.4 Features of artemisinin and its derivatives in the treatment of severe and complicated falciparum malaria

The main features of artemisinin are mentioned in a recent report on severe and complicated malaria (WHO/CTD, 1990). In clinical trials involving patients with severe and complicated malaria, the regulatory clearance of specific products, i.e. the issue of an IND (investigational new drug) document or its equivalent and their route of administration will be an important factor.

Artemether, as an injectable intramuscular formulation, and artesunate, as a water soluble intravenous formulation, have both been registered for antimalarial therapy in China. The registration documents are available in English which has recently led to the initiation of clinical trials of these formulations, particularly artemether, outside China based on compassionate grounds. Several other formulations such as artemisinin suppositories and oral formulations of artemether and artesunate have also been used in clinics in China for the treatment of symptomatic and severe malaria and limited trials of these compounds have likewise been carried out outside China. Moreover, IND for arteether, i.e. the ethyl ether derivative of artemisinin, is now available and clinical trials of this drug, as an injectable intramuscular formulation, will be carried out in 1991. Blinded i.v. as opposed to i.m. trials are, in principle, feasible since the arteether or artemether

treated patients will be on a maintenance i.v. drip as a managements procedure. The acceptance, or not, regarding the administration of i.m. injections of placebo to the i.v. quinine group will depend on the views of the relevant ethical committee(s). A similar problem exists with the management of the control groups in trials of artemisinin administered by the i.m. route or by nasogastric tube, or sodium-artesunate administered by nasogastric tube. The situation will be less complicated with sodium-artesunate by the i.v. route, but this formulation requires bolus injection in view of its very limited stability in aqueous solution. Here the i.v. bolus injection of the drug can be followed by an i.v. maintenance drip. A similar procedure may be adopted for the i.v. quinine group where the infusion of the medicament may be preceded by a bolus injection of physiological saline.

Artemisinin and its derivatives are reputed to have a very fast action against P. falciparum and the clinical manifestations of malaria. However, there is a significant recrudescence rate with some formulations. Follow-up treatment with a reliable blood schizontocide may therefore be indicated.

5.4.5 Development of clinical trials in patients with severe and complicated malaria

The following considerations are limited to arteether and it is assumed that the arteether formulation in question has an IND issued by a reputable regulatory authority. It is also assumed that it has undergone Phase I clinical studies for tolerability and the determination of its pharmacokinetics in healthy male adults, Phase II dose-finding studies in symptomatic, non-severe malaria, and Phase III studies in moderately ill patients with, nevertheless, non-severe malaria. If these trials have shown acceptable tolerability and an efficacy equal or superior to that of quinine, the formulation may be considered for trial use in severe and complicated malaria adopting the dose regimen that proved to be the most effective in the Phase II studies.

The first trials in serious forms of falciparum malaria should be conducted in adult males with high but not extreme parasitaemia (100 000-249 999 asexual parasites/ul) and/or hyperthemia (39-40^{o}C) and/or vomiting (preventing oral treatment). There should be no other major sign of complication, though cases with hypoglycaemia may be included. This is a group in which the case fatality rate provided the patients receive appropriate management, is low and which usually permits therapeutic modifications before there is a major threat to the patient's life. The ancillary clinical management of these patients follows the guidelines contained in WHO/CTD (1990). A comparison with the standard treatment of i.v. infusion quinine will show whether the arteether regimen is equivalent or superior to quinine, based mainly on parasite clearance, fever clearance and incidence of complications.

Subsequently trials may be conducted in adult male patients suffering from severe and complicated malaria except for cases of cerebral malaria, circulatory collapse and pulmonary oedema. Once these trials have produced satisfactory results with arteether, the last and probably most decisive groups may be included, i.e. patients with cerebral malaria and/or circulatory collapse. Since pulmonary oedema is mostly a post-therapeutic complication, there will be little cause of repeating antimalarial treatment trials in these cases, but it will be essential to determine the incidence of pulmonary oedema following treatment with quinine by i.v. drip or arteether so as to determine any predisposition caused by the medication.

Once the efficacy of arteether has been shown to be equal or superior to that of quinine (i.v. infusion) in adult males suffering from severe and complicated falciparum malaria, little time should be lost in expanding the experience to children, who are the main target group in Africa and to non-pregnant women. Pregnant women are to be excluded on account of the known fetotoxic effects of artemisinin and its derivatives. Severe and complicated malaria in pregnant women should continue to be treated with quinine (i.v. drip) and given the necessary ancillary management.

It could be argued that the evaluation of artemisinin and its derivatives , especially with regard to severe and complicated malaria could be accelerated by considerations of a compassionate nature. If a target patient group with high mortality from severe and complicated malaria could be identified, it would be plausible to proceed immediately to a double-blind trial comparing quinine i.v. with the appropriate derivatives. However, this requires:

(a) conclusive proof that the drug formulation concerned has performed at least as well as quinine in a preceding study involving non-complicated, clinically manifest falciparum malaria;

(b) the assurance that the dose regimen to be employed is close to the optimum;

(c) reasonable certainty that the frequency of serious adverse effects (particularly those affecting the bone marrow) is low.

Thus possibilities of shortcuts do exist, especially with regard to severe and complicated malaria, but not to the extent of forsaking essential information on clinical performance, dose-regimen and safety.

As pointed out in section 5.4.4 there are certain constraints in double-blinding trials of drugs with different modes of administration in patients with severe and complicated malaria since these patients should not be exposed to more than the strictly essential manipulations. The same applies to tests requiring massive blood sampling which is to be avoided, especially in patients with marked anaemia and/or hyper-parasitaemia since a substantial withdrawal of blood may increase the risk to the patient's life.

In situations where double blinding is impossible, group allocation should be done on a predetermined randomized basis, or at least in a strictly alternating fashion. General exclusion criteria for drug trials in severe and complicated malaria are complicating or associated infections other than malaria (e.g. bronchopneumonia, septicaemia) and pregnancy. Pretreatment with quinine within two days or mefloquine within three weeks prior to admission is also an exclusion criterion. These patient should be treated outside the study.

5.4.6 Clinical and laboratory parameters

The basic clinical and laboratory parameters should provide almost immediately a picture of the patient's condition upon admission so as not to delay the implementation of therapy. The initial examination would take account of the following:

Body temperature**
Parasitaemia**
Pulse**
Blood presssure**
Electrocardiogram (ECG)
Chest X-ray
Respiration**
Level of consciousness*
Vomiting*
Haemorrhages*
Complete blood status (including platelet and reticulocyte counts)***
Blood glucose**
Bilirubin***
Electrolytes***
Creatinine***
Antimalarials (blood/urine, initially only)

---

* Parameters that require continuous monitoring.

** Parameters that need to be assessed at 6-hourly intervals as long as the parasitaemia exceeds 10 000 asexual parasites/ul of blood. When parasitaemia drops below this level the interval may be extended to 12 hours.

*** Parameters that are to be monitored daily.

Monitoring of any of the above parameters at shorter intervals than those indicated by the asterisks may be required according to the patient's condition. The same applies to specific parameters (e.g. those related to liver function, blood clotting, and renal function). However, the blood tests should be kept to an essential minimum and performed with the most reliable microtechniques available in order to keep test-associated blood loss as low as possible. A soon as the patient's condition has been normalized and stabilized, usually within one week after admission, parasitological monitoring may be reduced to daily intervals; haematological and clinical-chemical parameters can then be checked twice weekly until the patient can be safely discharged. This will also provide the opportunity of assessing the efficacy of any "complementary" (radical) blood schizontocidal treatment (see 5.4.7 below).

5.4.7 Treatment

Administration of the trial drug and the comparison drug will be effected according to the appropriate protocol. With arteether or any other, artemisinin derivative it can be expected that the specific antimalarial treatment for the acute condition will be completed within 3 or 5 days from the start of specific medication, whereas with quinine at least a 7-day course is required. With quinine, the administration may be changed from i.v. infusion to the oral route once the patient is out of danger and able to swallow and retain the medicament.

Since neither quinine nor arteether is reliable for effecting a complete parasitological cure, treatment with these compounds must be followed by the administration of a reliable blood schizontocide for radical cure, according to the local drug sensitivity pattern of P.falciparum. In order to avoid drug interactions, this treatment should be given with an appropriate safety interval of at least 48 hours after the acute phase treatment as stipulated in the trial protocol.

The ancillary management of the patients follows the outlines given in WHO/CTD (1990). A careful record is to be taken of all such measures applied.

5.4.8 Ethical aspects

The ethical justification of clinical trials involving the treatment of severe and complicated falciparum malaria is based on two major considerations:

(a) the putative superiority of the trial drug (e.g. arteether or other artemisinin formulations scheduled for trial) over the current standard treatment with quinine by i.v. drip;

(b) the putative suitability of the trial drug to replace the standard treatment in areas afflicted by quinine resistance.

Trials under premise (a) may be carried out in any location where severe and complicated malaria occurs at a significant rate. Trials under premise (b) may be restricted to areas with a significant frequency of quinine resistance if the trial drug is likely to overcome this problem, even in the absence of a superiority over quinine in quinine-sensitive P.falciparum infections.

Ethical clearance of such trials is required in all and any circumstances. It should take into account the suitability of the clinical trial centre for this type of study (staff, relevant experience, facilities, organization, logistics, evaluation), the feasibility of completing the study within the set time frame, the availability of sound data from Phase I and II studies, and the suitability of the envisaged study population.

Other important considerations pertain to risk/benefit for the subjects themselves and eventual benefits for the population of the trial area, namely the feasibility of making the trial drug ultimately available (after registration) if it has proven to be superior to the standard therapy. Attention should be paid to supportive procedures beyond the normal standard of care since this may affect mortality in both the trial and control groups.

Informed consent is a major problem in clinical trials involving severe and complicated malaria since the lucidity of the patient is usually impaired (except for the first group mentioned in section 5.4.5). For such patients, informed consent will have to be obtained from the next of kin who, in most cases, accompany the patient to the clinical facility. After becoming lucid again adult patients should be informed of their inclusion in the clinical trial the purpose of which is to be fully explained. These persons are free to continue their participation in the clinical trial or to withdraw although this will have little bearing on the type of subsequent case management. In the case of minors, the parents should authorize the inclusion in the clinical trial and decide about an eventual withdrawal.

Specific monitoring mechanisms may be devised by the ethical committee concerned so as to ascertain that the consent of patients or of their guardians is truly informed and has been obtained without any form of coercion.

Apart from the withdrawal of patients, either on their own initiative or by decision of the next of kin, only two major factors may lead to an exclusion from the ongoing trial :

(a) The presence of an absolute exclusion criterion becoming known only after the initiation of therapy. This will be a rare occurrence. The records of these patients are not to be included in the final evaluation of the drugs, but the fact of an erroneous inclusion is to be mentioned in the final analysis since it may reflect shortcomings in the trial procedure.

(b) The manifestation of acute, severe, life-threatening adverse reactions to the medication. This will probably be a rare event, warranting the breaking of the code (if the trial is double-blind) and alternative management as appropriate under the circumstances.

5.4.9 Evaluation

The evaluation of treatment results may be based on a variety of parameters in keeping with the composition of the trial population, namely :

Case fatality rate
Occurrence of other complications (specify) after initiation of therapy
Time until regaining consciousness
Parasite clearance time
Fever clearannce time
Time until normalization of specific clinical and clinical-chemical parameters.

A predetermined group size is generally preferable as in clinical trials involving patients with non-severe/non-complicated malaria. This should be based on the outcome predictions with the standard regimen and the set confidence limits at which eventual differences may be detected. However, this concept may require flexibility in case major differences in the efficacy and safety between trial regimen and standard regimen should become evident already at a relatiavely modest number of patients. It is therefore usual to perform intermediate (independent) summary evaluations whenever a set number of observations (usually 10, 20 or 40) has been completed. Recently, this concept has been successfully applied to clinical trials of quinine and quinidine.

Similarly, an extension may be envisaged if this is likely to bring also a statistical confirmation of the highly suggestive differences between the regimeens under comparison.

It would not be necessary to enlarge the originally set group size if, upon reaching the scheduled number of patients, there is no indication of major difference in efficacy and safety since little would be gained by extending the trial under these conditions. However, a precise determination of the confidence limits of the efficacy parameters will be required also in this case.

In judging the efficacy of regimens for the management of severe and complicated falciparum malaria, the radically curative efficacy plays practically no role since the decisive criteria are survival and absence of sequelae (if required, radically curative treatment can be administered once the patient is out of danger).

With reference to section 5.4.8(a) and (b), it would be advisable to consider the response of P. falciparum to the trial drugs in evaluating therapeutic outcome.

If the standard treatment with quinine has proved to be superior, any further studies with the trial compound should be re-considered. If the trial compound has performed better, this will pave the way for more advanced or confirmatory trials.

5.4.10 Monitoring of trials

Clinical trials involving patients with severe and complicated malaria are a sensitive issue irrespective of the qualifications of the clinical centre conducting them. Such trials demand complete transparency and rigorous monitoring of trial procedures. Therefore, apart from the usual external (discontinuous) monitoring, they should have a mechanism of continuous independent monitoring, preferably by a local clinical trial monitor appointed by the ethical committee. The latter should meet at three-monthly intervals to review the progress and constraints of the clinical trial. Upon completion of the trial the committee should also scrutinize the final evaluation of the trial and append its observations to the report.

5.5 FIELD TRIALS OF ANTIMALARIAL DRUGS*

Field trials are necessary to determine how antimalarial drugs, both newly developed and older drugs may be best used in patients infected with or exposed to malaria parasites. Such trials require both substantial theoretical knowledge and practical field experience to plan and execute. Few investigators and few institutions exist in endemic countries that are adequately prepared for their conduct.

For drugs which are to be used for the treatment of established infections, case detection is the first important element, requiring an appropriate case definition and diagnostic method. Case-holding or follow-up of patients who have been treated is also an essential part of the process. For drugs to be used for the prevention of infection or of disease, different elements may be involved. Prophylaxis is usually aimed at preventing infection, particularly in individuals at high risk for a limited period of time. The value of such measures may be limited by the duration of action, by adverse reactions, and possibly by the induction of resistance.

The process of trying out new tools generally requires a series of studies that starts with basic synthetic and in vitro laboratory research , proceeds to animal testing in biochemical and toxicological studies, to human volunteers in a clinical setting, and finally to populations in endemic areas. After a drug has gone through the various phases of laboratory and animal testing, and has been through Phase I and Phase II studies in humans to demonstrate safety and at least some degree of efficacy, a randomized clinical trial normally must be carried out to provide detailed, objective assessment of its safety, acceptability and efficacy. It is after this that the field trials begin. The properly conducted randomized clinical trials are a prior sine qua non to field trials.

5.5.1 Trial approaches

5.5.1.1 Clinical trials as opposed to field trials

Clinical trials are experimental studies in which the participants are patients or volunteers. The trial is carried out under the controlled setting of a hospital or clinic where the subject can be carefully monitored.

Field trials are those in which the participants are home based and carrying out normal activities instead of being patients in a clinical setting. The size of the study population will be much larger than that of the usual clinical trials.

Generally a succession of trials is required, starting from those under carefully controlled conditions (to demonstrate safety and efficacy) and progressing to trials of increasing size to the point where they may be carried out as the procedure would be used under normal circumstances by malaria control programmes (to demonstrate public health effectiveness). Sometimes special problems arise in the field that require a return to the controlled conditions of the clinical setting and then back to the field.

In principle, the methodology of the randomized controlled trial can be applied to field trials. But the extension from the clinic to the field is not straightforward. In general, field trials provide further complications over and beyond those of the clinical trials. For example, in clinical trials the patients are usually quite ill, hospitalized, and relatively "captive", whereas in field trials, subjects are usually relatively less ill, home-based, and after the acute phase of the infection, usually pursuing their normal activity. Subjects in clinical trials often provide excellent cooperation; in the field, cooperation is usually problematical, and requires a great deal more supervision. Field study sizes are generally much greater than in clinical trials, and the follow-up period is often prolonged. In clinical trials, informed consent must be obtained, in the field, additionally, increased expectations of general help, increased expectations of health care and continuity of such care must often be dealt with.

The logistics of a double blind approach can be complicated even when possible in clinical trials and require many precautions, e.g., the ability to determine what a participant has been given if a serious adverse reaction occurs. Although double blinding in field trials of some drugs may be possible, it may often not be feasible especially when multi-dose combination regiments are used.

For trials carried out in a clinical setting the study population is relatively captive, good cooperation can usually be counted upon and follow-up for outcome assessment is relatively assured. For field trials it is necessary to persuade healthy individuals pursuing their normal activities not only to accept a study regimen in place of the drug expected, but also to participate in outcome assessment which often requires repeated examinations, which may be uncomfortable and time-consuming, interfering with normal activities.

Not only is it necessary to obtain ethical clearances from appropriate national and local authorities and informed consent from participating individuals, it may also be necessary to confer with and obtain the blessing of the political and community leaders before starting the study. For many field trials differential rates of participation among different segments of the population are likely to occur and may require special attention: adolescents and young men are often away

from the community for reasons of school or work; frequently people can only be found at home in the early morning or late evening, etc.

5.5.1.2 Alternatives to randomized clinical trials when randomization cannot be done

Sometimes it is not possible to carry out a randomized trial of a potentially useful treatment. In such circumstances there are alternative approaches which may allow valid assessment of the worth of a treatment.

The fundamental purpose of randomization is to reduce possible bias in the allocation of subjects to drugs being compared. The idea is to make the groups being compared the same in respect to all critical variables except for the drug/administration - and trust that randomization will roughly equalize the distribution of other unknown factors that might have some influence on the outcome. It is generally accepted that the randomized trial is the closest possible to the idealized laboratory study in which all relevant factors are controlled.

When randomization cannot be done, what are the next levels of approximation to ensure that differences in outcome are related to differences in the drug/administration received and not to extraneous variables and that if differences actually do occur as a result of the treatment that these differences are not obscured by extraneous factors? In certain circumstances, case-control studies may be possible; in others, studies involving a quasi-experimental design may serve to enable valid conclusions to be drawn.

(a) Case control approach

When a drug has already been in use in a population, then a case-control approach to assess the effectiveness of the drug may be applicable provided that some of the potentially eligible population have received the treatment and others have not. In contrast to case-control studies designed to determine treatment effectiveness, knowledge as to whether an individual has received the treatment or not can usually be fairly well determined. The critical question, then, is whether there was any difference between those who received the treatment as compared to those who did not, in terms of variables that may influence outcome. It is essential to obtain data which affect not only receipt of the treatment but also its outcome (e.g., education, income, exposure, health service access) and to test for comparability.

(b) Quasi-experimental approach

When a treatment has not yet been used, and when it must be used on a community-wide basis, and when randomization cannot be done, then under what circumstances is it possible to draw valid conclusions about the effect of a treatment?

Quasi-experimental designs have long been used for intervention trials, but their conduct has been so frequently seriously flawed by the inability to control for (or lack of understanding of) fundamental confounding factors and inherent biases that they have fallen into general disrepute. The basic quasi-experimental designs include: (i) "before and after" studies in the same population, and (ii) "one community versus another community" studies. In the "before and after" design, a baseline study is carried out to determine the status of the population (malaria incidence, death rates, etc., according to key descriptive variables of person, place and time) as well as all key variables that influence the occurrence and course of the disease. The intervention is applied and, at suitable time intervals thereafter, the health status and all key variables are again determined. The population under study serves as its own control, but at a different time period. If there are significant changes in the health status, the difficult part in interpreting results is to know whether changes can be attributed to the introduction of the intervention or whether they may be due to changes in other key variables that may have also changed with time (or may indeed have changed because of factors associated with the intervention introduction but not directly due to the intervention itself). To work this out requires not only knowing the key variables and their change during the time, but also the relationship of this change to the change in health status. (In a similar fashion, if there are not changes in the health status after the introduction of the intervention, it is important to know that there have not been countervail effects of the key variables during the time). The key issues concerning biases/confounding variables are those related to changes over time.

In the "one community versus another community" design, an intervention is applied to one community and the disease outcomes of interest among its residents are compared to those of a "control" community in which the intervention is not applied. Baseline studies of the kind mentioned in the "before and after" design are nearly always required to determine the comparability of the intervention and control communities in terms of the key variables that may influence the occurrence and course of the disease. Heterogeneity among communities is the rule rather than the exception; comparability must be demonstrated, not assumed. Because so many important variables are directly related to geographical area, the comparison of one community with another presents formidable challenges to the investigator to demonstrate the required comparability. In this case the key issues concerning biases/confounding variables are those related to differences in place.

In contrast to the case-control approach, in these quasi-experimental designs, it is clear why some persons received the intervention and others did not; the main issue is the comparability of the key variables that may influence outcome.

Just as in case-control studies of interventions one can never be absolutely certain that the very factor that led those to seek the intervention (thus by definition they are different from those who did not receive) did not affect the outcome, so too in quasi-experimental studies: one can never be sure that some factor(s) related to time or place did not affect the outcome. If, in fact, it is accepted that all key variables are comparable, then the outcome differences at the individual level reasonably may be attributed to the intervention.

In some circumstances the conclusions from quasi-experimental studies may be greatly strenghtened if there are independent means for measurinng the challenge of infection, transmission, or exposure. For example, in a primaquine trial in which an objective is to reduce transmission, entomological measures might be used to demonstrate a fall in transmission potential in the face of a comparable biting rate. Or in areas of relatively unstable transmission, entomological indicators might show that the fall in malaria cases associated with introduction of the drug could not be attributed to a fall in the vector mosquito densities.

5.5.2 Choice of study size

One of the most important factors to consider in the design of a treatment or prophylaxis trial is the choice of an appropriate study size. Studies that are too small may fail to detect important effects on the outcomes of interest, or may estimate those effects too imprecisely. Studies that are larger than necessary are a waste of resources, and may even lead to a loss in accuracy, as it is often more difficult to maintain data quality and high coverage rates in a large study than in a smaller one.

5.5.2.1 Criteria for determining study size

((a) Precision

One of the main objectives of most intervention trials is to estimate the effect of the intervention on the outcome of interest. Any such estimate is subject to error, and this error has two main components. The first component is bias. Possible sources of bias and ways of avoiding them have already been discussed. The second component is sampling error, which results from the fact that data come from only a sample of the population. Sampling error is reduced when the study size is increased whereas bias generally is not.

It is important to decide how much sampling error in the estimate is acceptable, and to fix the study size accordingly. When the data are analysed, the amount of sampling error is represented by the width of the confidence interval which is put on the estimate. The narrower the confidence interval, the greater the precision of the estimate, and the less the probable sampling error. When designing a study, it is necessary therefore to decide how wide a confidence interval is acceptable.

(b) Power of the study

An alternative approach is to choose a study size which gives adequate power. With this approach, the focus is on the result of the significance test which will be applied at the end of the study. The significance test assesses the evidence against the null hypothesis which states that there is no difference between the groups under comparison. A significant result means that the data conflict with the null hypothesis, and that there is therefore evidence of a real difference between study groups.

Because of the variations resulting from sampling error, it is impossible to ever be certain of a significant result at the end of a study, even if there is a real difference. Therefore, it is necessary to consider the probability of obtaining a significant result, and this probability is known as the power of the study. Thus a power of 80% means that if the study were to be conducted repeatedly, a significant result would be obtained four time out of five.

The power of a study depends on :

(i) the size of the true difference between the study groups; in other words, the true effect of the intervention. The greater the effect, the higher the power.

(ii) the study size; the larger the study size, the higher the power.

The power also depends on the required significance level, on whether a one-sided or two-sided test is to be performed, and on the underlying variability of the data.

(c) Choice of criterion

The choice between the above two criteria depends on the objectives of the study. If it is known unambiguously that the treatment has some effect, it hardly makes sense to test the null hypothesis, and the objective is clearly to estimate the effect, and to do this with acceptable precision. Studies of drug efficacy follow earlier (Phase II) clinical trials, so that testing against the null hypothesis would be inappropriate. On the other hand, trials of another type of intervention, e.g., testing the periodic application of primaquine to a population to reduce transmission, could well be considered in this way. In such a trial, it may not be known whether there will be any impact at all on the outcomes of interest. In such circumstances, it may be enough to ensure that there is a good chance of obtaining a significant result if there is indeed an effect of some magnitude. It must be stressed that the estimate obtained may be very imprecise. If it is important to obtain a precise estimate of the effect of the intervention, it is better to work in terms of setting the width of the confidence interval rather than to rely on power calculations.

(d) Studies with multiple outcomes

In most studies, several different outcomes are measured. For example, in a study of the efficacy of an antimalarial drug against Plasmodium falciparum infections, it may be useful to assess the effect of the drug on parasite and fever clearance, recrudescence rate, and , e.g., speed of return to work.

In order to decide on study size attention should first be focused on a few key outcomes, which the study should be able to evaluate adequately. Sample sizes would then be calculated for each of these, and ideally, the largest would then be selected as the actual study size.

Often, however one or more of the outcomes would require a study so large that it is clearly beyond the resources available. For example, detecting changes in mortality often requires very large studies. In such circumstances, the investigator may decide to be content to design the study to be able to detect an impact on morbidity, and accept that it will be impossible to say anything useful about the effect on mortality.

(e) Practical constraints

In practice, statistical considerations are of course not the only factors that must be taken into account. Resources in terms of staff, vehicles, laboratory capacity, time and money are all likely to be limited and it is usually necessary to compromise between the results of study size computations and what can reasonably be managed given the available resources. Trying to do a study that is beyond the capacity of the available resources is likely to be unfruitful, as data quality is likely to suffer, and the results may be subject to serious bias, or the study may even collapse completely, wasting all the effort and money that has already been put into it. On the other hand, if the calculations show that a study of manageable size will yield power and/or precision that is unacceptably low, it may be better to accept that the study should be abandoned, at least in that location.

5.5.2.2 Statistical method for a qualitative outcome

A relatively simple method for sample size computation for comparing cure rates of two drugs is presented below.

One must choose four items:

P_1 - percentage of successes expected using one treatment (usually the standard).

P_2 - percentage of successes on the other treatment which one desires to detect as being different from P_1.

a - the level of the x^2 significance test used for detecting a treatment difference (often set at p=0.05.)

$1-\beta$ = the degree of certainty that the difference $p_1 - p_2$, if present, would be detected (often set $1-\beta = 0.90$)

a, commonly called the "type I error", is the probability of detecting a "significant difference" when the treatments are really equally effective (i.e. it represents the risk of a false positive result).

β , commonly called the "type II error"", is the probability of not detecting a significant difference when there really is a difference of magnitude p_1-p_2 (i.e. it represents the risk of a false negative result).

$1-\beta$ is the power to detect a difference of magnitude p_1-p_2.

Here, p_1 and p_2 are the hypothetical percentage successes on the two treatments that might be achieved if each were given to a large population of patients. They merely reflect the realistic expectations or goals which one wishes to aim for when planning the trial and do not relate directly to the eventual results.

Example:

In a comparison of mefloquine versus quinine- tetracycline in southern Thailand the following values might be used:

p_1 = 90% cure rate on quinine-tetracycline
p_2 = 95% on mefloquine
a = 0.05
β = 0.01

Now, the required number of patients on each treatment n is given by the following formula:

$$n = \frac{p_1 \times (100-p_1) + p_2 \times (100-p_2)}{(p_2-p_1)^2} \times f(a,\beta)$$

where $f(a,\beta)$ is a function of a and β, the values of which are giiven in Table 5.3 below.

Therefore, for this trial,

$$n = \frac{90 \times 10 + 95 \times 5}{(95-90)^2} \times 10.5 = 578 \text{ patients for each treatment.}$$

TABLE 5.3 VALUES OF $f(a, \beta)$ TO BE USED IN THE FORMULA FOR REQUIRED NUMBER OF PATIENTS

		β (type II error)			
		0.05	0.1	0.2	0.5
a (type I error)	0.1	10.8	8.6	6.2	2.7
	0.05	13.0	10.5	7.9	3.8
	0.02	15.8	13.0	10.0	5.4
	0.01	1.8	14.9	11.7	6.6

As mentioned above, this statistical method is only a guideline to help determine how many patients are needed. It would be unlikely that a trial this large could feasibly be done in Southern Thailand. However, it is important now to realize that if the trial had fewer patients, this would automatically decrease the chances of finding a statistically significant difference in cure rates; that is, if the trial is made smaller, the power to detect important treatment differences is decreased and so the risk of a false-negative conclusion is increased.

5.5.2.3 A method for "negative" trials

The motivation behind most randomized trials is to hope for a "positive" result whereby one treatment is significantly better than another and the above methods have been based on that premise. However, there are trials in which one is more interested in showing the "negative" result that two treatments are equally effective. This usually arises in comparing a conservative treatment with a more intensive standard therapy, or in showing that a treatment known to have fewer serious side effects is as effective as one known to be associated with toxicity, e.g. mefloquine is as effective as the triple combination of mefloquine/sulfadoxine/pyrimethamine (MSP).

A suitable method for such trials is based on a qualitative measure of patient response, which works as follows. One first specifies p, the overall percentage of successes that one anticipates will occur. Then one chooses a value d such that if the two treatments really are equally effective the upper 100 (1-a)% confidence limit for the difference in percentage successes on the two treatments should not exceed d with probability 1-... Then the required number of patients on each treatment is given by :-

$$n = \frac{2p \times (100-p)}{d^2} \times f(a, \beta)$$

where the function f is as defined before.

For example, suppose MSP is expected to produce a favourable response(as measured by cure rate) in about 95% of patients with P.falciparum malaria. In a randomized trial one could specify that mefloquine will only be considered acceptable if it can be demonstrated with 95% confidence that it is at worst 10% inferior to MSP. Suppose one accepts a 20% risk that even if the drug is really equally effective one will fail to show it as acceptable in this sense. Then p = 95%, d = 10%, a = 0.05, and β = 0.2, so that

$$n = \frac{2 \times 95 \times 5}{10^2} \times 7.9 = 75 \text{ patients needed on each treatment.}$$

5.5.2.4 Allowance for interim analyses

It is sometimes desirable to incorporate interim analyses into the plan, involving review of the results at, for example, six-monthly or annual intervals. The idea is that if an interim analysis indicates that there is already strong evidence of the superiority of one of the interventions under study, the trial can be terminated, and one can thus avoid further subjecting individuals to a regime which is known to be inferior. The incorporation of interim analyses may be particularly valuable if the trial is planned to continue for a prolonged period, with gradual accumulation of cases, or if populations are to be entered into the trial sequentially.

There are also disadvantages in carrying out interim analyses. If the trial is terminated early because one regimen appears to be superior, there may be no opportunity of detecting any long-term sideeffects or other adverse consequences of the treatment.

If it is decided that interim analyses are necessary, these need to be planned carefully. If it is likely that early termination of the study will occur, it is necessary to increase the initial sample size in order to maintain the same overall level of significance. If there are to be two interim analyses, the sample size should be increased by a factor of approximately 15%. During the interim analyses, a stringent significance level of $p<0.01$ should be used to decide whether or not the trial should be terminated.

5.5.2.5 Losses to follow-up

Losses to follow-up occur in most longitudinal studies. Individuals may be lost because they move away from the study area, because they refuse to continue with the study, because they are away from home at the time of follow-up appointments, or for some other reason.

Such losses are of concern for two reasons. Firstly they are a possible source of bias, as the individuals who are lost may differ in important respects from those who remain in the study. Secondly, they decrease the size of the sample available for analysis and this decreases the power and precision of the study.

Losses can be minimized by selecting local trial supervisors with proven ability to elicit compliance. A primary health care worker or clinic chief who is held in esteem and trust by the community is a most valuable asset. Compensation for time lost by subjects while they are making themselves available for follow-up may be necessary, as may reimbursement for travel costs if they are required to report to a trial site.

5.5.2.6 Trials that are too small

The conduct of trials that are too small has consequences extending beyond the results of the specific trial. There is considerable evidence that studies showing large effects are more likely to be published than those showing little or no effect. Suppose a number of small trials of a specific drug are conducted. Because of the large sampling error implied by small sample sizes, a few of these trials will produce estimates of the effect of the drug that are much larger than the true effect. These trials are much more likely to be published, and the result is that the findings in the literature are likely to overestimate considerably the true efficacy of the drug. This "publication bias" is much smaller for larger trials because an adequate sample size means that such trials will give results that are much closer to the true effect.

5.5.3 Ethical considerations

The present consensus about ethical concerns in medical research is summarized in the Declaration of Helsinki (1975), also known as Helsinki II. The principles expressed in Helsinki II have been reviewed and expanded with special relevance to developing countries in Proposed international guidelines for biomedical research involving human subjects (CIOMS 1982). In regard to research in developing countries, these guidelines state:

> "14. Rural communities in developing countries may not be conversant with the concepts and techniques of experimental medicine. It is in these communities that diseases not endemic in developed countries exact a heavy toll of illness, incapacity and death. Research on the prophylaxis and treatment of such diseases is urgently required, and can be finally carried out only within the communities at risk."

5.5.3.1 Accepted ethical principles relating to research on human subjects.

The values that underlie the ethics of research on human subjects relate to the ultimate value of the individual and of his or her right to self-determination. The maintenance of ethical standards depends on informed consent and institutional review.

(a) Scientific merit

To be ethical, research must have scientific merit; the methods should be appropriate to the aims. The presence of any results from any relevant preceding and/or ongoing research should be established.

(b) Equitable selection of subjects

The burdens and benefits of research should be distributed equally. It is the economically and socially deprived who are most often at high risk. There is on the one hand an imperative to ensure that appropriate research is conducted in such communities and on the other an imperative to ensure that they are not exploited: that the research will in fact not benefit only the wealthy and privileged.

(c) Benefit and risk of harm

It is necessary to define the benefit and the beneficiaries. Risk should be similarly defined. One can consider the potential benefit of an intervention to:

- the individual,
- other individuals similarly at risk with the same disease
- the scientific community or even mankind in general, because of an addition to human knowledge.

Risk of harm can be graded relative to the risks an individual would encounter in his or her daily activities. Not only physical harm should be considered. Psychological harm may result from loss of privacy, intrusion of outsiders into a community; other harms may be social, political or environmental.

(d) Informed consent

The purpose of informed consent is to safeguard the right of an individual to determine the use of his or her own body independently of judgements about risk and benefit arrived at by others. The information provided to obtain informed consent in a trial in a developed country at the present time would be expected to include:

(i) a statment that the activity involves an experiment;

(ii) an explanation of the scope, aims, and purpose of the research.

(iii) a description of any reasonably foreseeable harm and the level of risk.

(iv) a description of likely or expected benefits;

(v) a disclosure of appropriate alternative procedures, causes of treatment, or preventive strategies that might be advantageous (in a field trial in a developing country this might be expected to include an explanation about medical treatment and its availability for the condition under study);

(vi) a statement that subjects will be notified of new information developing in the course of research that might affect their participation;

(vii) a statement about how confidentiality will be maintained (if appropriate)

(viii) an offer to answer questions;

(ix) an explanation about medical treatment and compensation for research-related injury;

(x) a statement that participation is voluntary and refusal to participate will involve no penalty; the subject can withdraw consent at any time.

The existence of a consent form and the presence of a witnessed signature are of doubtful protection to the subject, especially in a pre- or semi-literate society. What the consent form does do however, is to define the nature of the explanation that will be given. It is the investigator's responsibility to ensure that subjects are fully informed.

(e) Competence

Children are the most likely potential subjects of research not necessarily competent to give consent. A distinction has been drawn between consent and assent; the child's right to refuse should be respected. The child requires an advocate, whether parent or guardian; research should not be conducted on children unless, whenever appropriate, it has already taken place on animals and adults.

(f) Coercion

Where researchers are identified with state authorities, and this is likely if their point of entry is through a Ministry of Health, a considerable potential for coercion exists, often in subtle form. Research workers, especially those working in a foreign culture, should be aware of this.

(g) Deception and incomplete disclosure

There is no place for deception, incomplete disclosure of research aims or of hidden observers and secret observation procedures in community intervention studies.

5.5.3.2 Ethical considerations of special relevance to trials

Most of these issues relate to the need for scientific merit. Where risk of disease and death is high, this must affect assessment of the balance between potential benefit and the risk of harm.

(a) Randomization

Randomization is an essential component of most trials. Prejudice against randomization may relate to the feeling that one party has been unjustly preferred to another. If this is possible, then randomization should become an open procedure with, say, subjects drawing numbers from a "hat".

(b) Control and placebo groups

The need for control or placebo groups may create special problems. The basic ethical issues are the same as those in clinical trials. Important principles are that the control groups receive the best presently-established form of treatment, if one exists, and that if the new treatment procedure is demonstrated to be better then it should be provided for the control population as soon as possible.

(c) Stopping points

In field trials it is almost always necessary to establish the efficacy of the treatment not only in the population as a whole for the period of the trial but also in sub-groups. This may for example involve the establishment of efficacy in persons of certain ages or for persons with underlying or associated conditions such as malnutrition.

It is therefore argued that a trial should be stopped at the point when sufficient evidence has been collected to support the introduction of the intervention by the health services generally and not at the point when the difference in response of intervention and control groups is first established.

5.5.3.3 Level of review

Fundamental ethical principles are maintained by the processes of informed consent and institutional review. The level at which consent is given may be different; the research worker may need considerable insight and even specialist advice to ensure that the rights of individuals are respected. He may also have a responsibility to support the development of the processes of institutional review.

The research activities carried out under the auspices of WHO may involve four levels of review:

(i) The fundamental level of review is the individual person; the participant in a study who must provide informed consent to take part in the research. Illiteracy and differing cultural concepts of health and disease in no way alter the basic principles of informed consent. If informed consent has been achieved by virtue of a communal decision, the researchers and the review committees must asssure themselves that no coercion has occurred. The principle of assent, and the inherent right of refusal must be regarded as minimal rights.

(ii) The second level of review (where appropriate) is review by the community. Here, understanding is of fundamental importance. Whether or not a formal review committee can be established will vary with circumstances.

(iii) The third level is that of the responsible institutional or national level review.

(iv) The final level for WHO projects is SCRIHS which must have the approaches to the three prior levels documented before it considers a proposal for approval.

5.5.4 Randomization and coding

A randomized, blinded design is recommended to ensure that any observed difference in outcome between the intervention and control groups can be confidently attributed to a real effect of the intervention, and not explained by any differences in the initial composition of the groups, in the way they were handled or in the way they were assessed. Randomization eliminates the possibility of any subjective element influencing the assignment to the different trial groups. It is superior to any systematic method, such as alternate assignment or the use of date of birth or date of entry (with even days corresponding to the intervention group and odd days to the control), since in these it is possible for the investigator to know in advance to which group a participant will belong. The possibility cannot be ruled out that this knowledge may affect the decision regarding entry or not.

Standard randomization does not, however, guarantee that the initial compositions of the different treatment groups will be similar or even that equal numbers will be recruited to each group; the chance element can lead to an imbalance in either. For large trials, say of several hundred participants, any imbalance is likely to be small and can easily be taken into account in the analysis. This is not the case for small trials, and it is therefore recommended that the design be modified to ensure balance. Restricted or blocked randomization can be used to ensure balance in group sizes, while matched designs, either stratum or paired, ensure balance in composition of the groups with respect to those key variables chosen for the matching procedure.

5.5.4.1 Unrestricted randomization

Random allocation to the intervention and control groups is used to avoid any bias in selection (conscious or otherwise), either by the investigators or by the participants, that may lead to inherent differences between the groups. This is most conveniently carried out using a random number table, such as the one in Appendix 5, choosing an arbitrary starting point in the table and using a specified order of selection. Possible selection rules include proceeding row by row across the whole page, column by column down the whole page, row by row within a block and column by column within a block, with subsequent blocks selected either horizontally or vertically. Selection may take place backwards as well as forwards. The use of calculators is not recommended since they do not yield truly random numbers: it is not uncommon to obtain an unacceptably long run of allocations to one of the treatment groups. The same applies to some computer generated random numbers. These vary in quality depending on the particular algorithm used. When in doubt, a table should be used.

The simplest situation is that of unrestricted randomization with just two groups, intervention and control. One digit random numbers are selected. One possible allocation rule would be to assign odd numbers to the intervention group, and even numbers to the control group or vice versa. For larger numbers of groups, unrestricted randomization may be carried out according to the examples in Table 5.4.

TABLE 5.4 EXAMPLES OF ALLOCATION PROCEDURES FOR UNRESTRICTED RANDOMIZATION, FOR 2, 3 OR 4 INTERVENTION GROUPS

Number of intervention groups	Allocation procedures	Example of sequence of random numbers with corresponding allocation
2	A: 1,3,5,7,9	8 4 3 7 9 0 6 1 5 6
	B: 0,2,4,6,8	B B A A A B B A A B
	A: 0-4	8 4 3 7 9 0 6 1 5 6
	B: 5-9	B A A B B A B A B B
3	A: 1-3	8 4 3 7 9 0 6 1 5 6
	B: 4-6	C B A C C - B A B B
	C: 7-9 (0 ignored)	
4	A: 1-2	8 4 3 7 9 0 6 1 5 6
	B: 3-4	D B B D - - C A C C
	C: 5-6	
	D: 7-8 (9,0 ignored)	

The order of allocation of participants should be decided before the start of the trial, and should not be known to the investigator responsible for registration of trial participants. The usual method for treatment studies is to prepare a set of sealed envelopes containing the drugs or the allocations, which are opened in the order as each new person is accepted into the trial. It is important that the entry criteria are checked and eligibility satisfied before an envelope is opened, in order to exclude the possibility that the decision to accept a participant could be influenced by the knowledge of which group he or she would join.

5.5.4.2 Randomization systems

(a) Restricted randomization

Although on average an unrestricted randomization procedure should lead to equal numbers of participants in each group, this is by no means guaranteed for any particular sequence. For example there is more than a 5% chance that the imbalance in a series of 20 participants is of the order of 6 against 14 or worse. This problem can be overcome by using restricted randomization, also called blocked randomization or randomization with balance, which ensures equal numbers in each group after every so many allocations. The size of the blocks must, of course, be a multiple of the number of intervention groups. In order to minimize the possibility that an allocation can be guessed from previous allocations, the block size should not be too small (specifically, it should not be two) and when possible it should not be known to the investigator responsible for registration. Indeed as far as possible those registering participants should not even be aware that blocking has been carried out.

Two different procedures for carrying out restricted randomization will be described, one appropriate for small block sizes and one appropriate for relatively large block sizes, such as 10.

(i) Small block sizes With a small block size such as a size of 4 for two intervention groups, A and B, it is possible to list all combinations of four allocations that will yield two As and two Bs, as shown below, and to allocate a random number or a group of random numbers to each.

Allocation	Corresponding random number
AABB	1
BBAA	2
ABAB	3
BABA	4
ABBA	5
BAAB	6
Ignore	7, 8, 9, 10

(ii) Large block sizes Writing down all possible combinations of allocations within a block becomes less feasible the greater the block size. For example with a block size of 20, there are 190 different possible combinations each yielding 10 participants in each of two intervention groups, A and B. An alternative approach must be adopted. Consider the determination of this order within a block of size 20. Ten allocations are to be group A and 10 group B. The random number table is used to decide which 10 are to be allocated to group A by selecting 10 different two-digit random numbers between 01 and 20. For

example, starting at the top left of the middle block on the second page and reading down the page gives random numbers 67, 02, 79, 87, 34, 11, 52, 07, 04, 01. Continuing this yields the following ten numbers between 01 and 20 (those falling outside this range are simply ignored, as are duplicates):

02, 11, 07, 04, 01, 12, 10, 09, 05, 03

Thus the second, eleventh, seventh, fourth, first, twelfth, tenth, ninth, fifth and third participant within the block are allocated to group A and the other participants to group B. The complete sequence for the block of 20 is:

01	02	03	04	05	06	07	08	09	10
A	A	A	A	A	B	A	B	A	A
11	12	13	14	15	16	17	18	19	20
A	A	B	B	B	B	B	B	B	B

(b) Stratified randomization

Randomization may also be modified to ensure, for example, the same proportion of males and females in each group, by using separate random allocation series for each sex. This modification is called stratified randomization, since the population is stratified, in this case by sex, before allocation is carried out, and the groups are said to be stratum matched according to the stratifying variable. The rationale is to ensure against confounding bias, when looking at differences in outcome between the intervention groups, which could be caused by an imbalance in composition of the different groups. The alternative approach is to adjust for potential confounding variables in the analysis rather than the design. Stratification (either in the design or the analysis) should only be carried out for variables thought to be related to the outcome of interest, since variables other than these will not cause any such bias.

Stratified randomization can be carried out for more than one variable, for example simultaneously by age and by sex using a separate allocation series of each age-sex combination. Of course, the greater the number of variables, the greater the logistical complexity and thus the number of strata used should be kept to a minimum. Different coloured sets of envelopes or labels could be used for each series.

(c) Randomization with a matched pair design

An alternative to stratum matching is to use a matched pair design in which participants are matched into pairs according to their values for the main confounding variables. One member of each pair is then allocated at random to the intervention group and the other to the control group. A similar randomization procedure from that described for two groups can be used, modified as follows. The randomization

sequence determines to which group the first member of a matched pair is allocated; the other member then automatically goes to the other group. Consider the random number sequence given as an example at the top of the random number table supplied, namely;

8, 4, 3, 7, 9, 0, 6, 1, 5, 6

with odd numbers corresponding to group A and even numbers to group B. This would lead to the following distribution for the first ten matched pairs recruited:

BA, BA, AB, AB, AB, BA, BA, AB, AB, BA

This system can also be used for more than two groups. For example, with three groups, matched triads could be employed. The randomization procedure would then be based on the allocation of groups to each of the six possible combinations of order of the three groups (ABC, ACB, BAC, BCA, CAB, CBA) as described above.

(d) Coding systems

A double-blind design is achieved by the use of a coding system linking participants and their intervention groups. The coding system should be designed to minimize the effect on the loss of blindness in the circumstances when it may be necessary to break the code and to minimize the possibility of guessing. For these reasons the use of a single code for each intervention group is strongly discouraged. The ideal is rather to have a unique code for each participant and to have a separate list linking participant numbers with group allocation, or to have only a very small number of participants sharing the code number.

There are basically two different types of coding systems. The first is to have all the treatments labelled with ID numbers, and in no way identified as to the composition. A randomization sequence allocating individuals to treatment group as described above is first prepared. The treatments are then arranged in order according to this list and labelled with the ID numbers. The alternative approach is to use a fixed, but not too small, number of codes for the different interventions. For example 20 codes, 10 corresponding to the intervention and 10 to the control. The randomization sequence is produced using these 20 codes and the allocation of the designated treatment to an individual is done in the field.

With both approaches, it is helpful if the preparations contain removable sticky labels that can be affixed to the individual's form and to any samples taken, when the treatment is given, thus minimizing any recording errors. The second approach may be logistically easier to carry out when repeat dosings are involved as it is possible to be sure an individual

receives the same on each occasion by simply giving them a preparation with the same code as before. The first approach is only feasible in this situation if it is possible to prepare all dosings for an individual together at the onset. Otherwise the list will need to be decoded on each occasion, entailing a considerable amount of work. Thus it is not appropriate for trials of preparations with a short shelf life.

(e) Breaking the code

The randomization list should be prepared in advance of the trial and the codes assigned by someone other than the principal investigators. Although the manufacturer may play a useful role at this stage, the allocation procedure should be overseen and the code held throughout by a independent party.

Two different types of situation might occur in which it would be necessary to break the code. The first is that a circumstance may arise that makes it important to know to which intervention group an individual belongs. For example, an individual may become seriously ill and knowledge of the intervention received may be needed to decide on the appropriate treatment to give.

The second type of circumstance is when it becomes necessary to carry out a comparison between the intervention and control groups, but it is not necessary to know the group allocation on an individual basis. For example, consider a large field trial of mass distribution of ivermectin for the treatment of onchocerciasis. In such a trial it would be necessary to monitor for any adverse side effects, and if serious effects are seen to ascertain that these were not due to ivermectin. In this case it would be necessary to break the code to find out what proportion of those suffering severe reactions received ivermectin in order to compare this with the overall proportion in the trial who had received ivermectin.

In practice, the necessity to break the code is rare. It is important to decide before the start of the trial the conditions under which the code will be broken and the extent to which it will be broken. The decision should preferably rest with an independent party, such as the clinical trial monitors community and not with the investigators themselves.

5.5.5 Analysis

A natural sequel to the framing of clear-cut objectives is decision-taking on the main analyses to be undertaken. It is vital that this be done before the start of the study(and not after all the data have been collected), as it invariably promotes a clearer understanding of the basic questions to be answered.

Another benefit of deciding on analyses in advance is that it will serve as a check on whether the required outcome measures will be reliably obtained from the data collected - for instance, if the outcome measure is the development of new disease (incidence), what are the parameters by which disease is going to be diagnosed? Are all these amenable to accurate determination? What would be the sensitivity and specificity of the diagnostic procedure?

Advance analysis plans can also crystallize thought on analytical techniques to be employed, e.g. covariance, life-tables, regressions. It is then possible to verify whether appropriate software on the local computer system is readily available, and if not efforts can be initiated concurrently to evolve or purchase appropriate software. Large studies require much time for data processing and detailed advance plans can lead to precise estimation of computer time and appropriate budget requirements.

5.5.5.1 Confidence intervals

Any statistic, e.g. the mean, computed from a sample not only provides a summary measure for the particular sample but, more importantly, it offers scope for making some inferences about the population value (u). The latter is not in the nature of an exact statement, but a probabilistic statement; thus, it provides a range of values (1 to L) with a stipulated degree of confidence, usually 95%. The interval 1 to L is called the 95% confidence interval.

Another practical interpretation of the confidence interval is that any hypothesis stating that the population parameter u is equal to K, where K lies in the 95% confidence interval (1 to L) will not be rejected by a significance test at the 5% level. For example, supposing that the 95% confidence interval for the difference in average parasite density between an untreated group and a treated group is -0.6 to 1.4; this interval includes 0, and so it must be concluded that there is no real evidence that the treatment has had any effect on parasite density.

To compute the confidence interval, it is necessary to know the average value (a) of the characteristic (based on n observations), its sampling error ($sigma_A$) and the confidence level required; the latter is usually taken to be 95%. The 95% confidence limits are then given by (A-1.96 $sigma_A$) to (A+1.97 $sigma_A$). In practice, sigma $_A$ is seldom known and a sample estimate S_A will have to be used instead; in this case, the value of 1.96 must be replaced with the corresponding t value with appropriate degrees of freedom. The 95% confidence limits then become (A-ts_A) to (A+ts_A). If the sample size is large, 1.96 can be used instead of t.

A statistic, e.g. the mean, computed from a sample could also be used to test some hypothesis about the population. The procedure for this is referred to as a significance test.

Suppose, we have taken a random sample of n observations from a population, and wish to test whether the population has a mean = u. Let the sample mean value be denoted by x. We start with the assumption (null hypothesis) that the population mean is indeed u and then compute the probability of drawing a random sample of n observations with mean x from this population. If this probability is very small, we conclude that there is good reason to suppose that the population value is not u. (What constitutes a "very small" probability is a subjective matter, but conventionally a 5% significance level i.e. a probability of 0.05, is taken; sometimes though a 1% level is used, especially in large-scale epidemiological studies. If not, we conclude that there is little reason to suppose that the population mean is not u.

If we use a 5% significance level, there is a 1 in 20 chance that we may reject the null hypothesis when it is true (type I error) that is, we may conclude that the population mean is not u when it is indeed u, using a lower significance level e.g. 1%, would reduce the chances of such a wrong conclusion, but it would increase the risk of accepting the null hypothesis when it is false (type II error), i.e. it would increase the risk of overlooking a real difference. Therefore, we must decide on how important each of these errors is and choose our significance level accordingly.

5.5.5.2 Analysis of proportions

The sampling error of a proportion p, based on n subjects is given by $p(1-p)/n-1$. For example, if 0.4 (40%) is the prevalence of splenomegaly in 200 children in a survey, the sampling error (Sp) is approximately 0.035 (3.5%).

The 95% confidence interval for a proportion p is (p-1.96Sp) to (p+1.96Sp), where Sp is the sampling error of the proportion. In the example above, the 95% confidence limits work out to be 0.33 to 0.47, i.e. 33% to 47%.

To compare two proportions on different sets of subjects (is the difference between them likely to be zero? or is their ratio likely to be equal to one?), a 2 x 2 chi square test with Yate's correction for continuity may be employed. For example,

Intervention measure	Outcome measure Success	Failure	Total
Yes	a	b	a + b
No	c	d	c + d
Total	a + c	b + d	N

The formula for chi-square is given by:

$[N(|ad-bc|-N/2)^2 [(a+c) (b+d) (c+d) (a+b)]$

If this exceeds 3.84, we say that the success rates a/(a+b) and c/(c+d) are significantly different from one another at the 5% level. For example, if the proportions to be compared are 90/300 (30%) and 135/300 (45%), a= 90, b = 210, c = 135, d = 165; the difference between the two proportions is 15% and the ratio of the two proportions is 0.67. The value of chi-square turns out to be 13.77, which is significant at the 0.001 or 0.1% level. We conclude that the observed difference of 15% is significantly different from zero, and the observed ratio of 0.6 is significantly different from 1.

If any of the frequencies is very small, (0, 1, 2, 3, 4 or 5), an exact test due to Fisher may be employed.

The sampling error of the difference between two proportions p_1 and p_2, based on n_1 and n_2 observations, is - /[p(1-p) $(1/n_1+1/n_2)$] where $p = (n_1p_1 + n_2p_2)/(n_1 + n_2)$. In the example cited above, p = 0.375 and the standard error of the difference = /[0.375 x 0.625 (1/300 + 1/300)] = 0.04 approximately.

Since the proportions are based on large numbers, the 95% confidence interval for the difference between the proportions is $(p_1 - p_2) \pm$ (1.96 x sampling error of p_1-p_2), i.e. 0.15 $\pm$ 1.96 (0.04), i.e. 0.07 to 0.23, i.e. 7% to 23% (If the proportions p_1 and p_2 had been based on small denominators, the appropriate t value should be used instead of the value of 1.96).

For determining the confidence interval of the ratio of the two proportions (also referred to as "relative risk"), i.e. [a(c+d)]/[c(a+b)] the logarithm of the relative risk (to the base e) is first computed; its standard error (SE) is given by [1/a+1/(a+b) = 1/c+1/(c+d)] where a, b, c, d are the four cell frequencies. The 95% confidence limits are $\log_e$ RR $\pm$ 1.96 SE. By taking exponentials, the 95% confidence limits for the relative risk are obtained.

In the example quoted above, the relative risk (RR) is 0.67. The standard error of this logarithm is /[1/90+1/300+1/135+1/300], i.e. 0.1587; 95% confidence limits of $\log_e$RR are -0.7115 to -0.0894; taking exponentials the 95% confidence limits for the RR are 0.49 to 0.91

5.5.5.3 Analysis of mean values

The sampling error of a mean (x) based on n observations, is .... /n where ....is the standard deviation of the observations. In consequence, the 95% confidence interval of the mean x $\pm$ 1.96 (..//n). If .. is not known and a sample estimate (s) is used, the approximate value of t should be

substituted for 1.96; i.e. the 95% confidence interval will be x-t (s/n) to x + t (s^2//n); if n is large, 1.96 itself can be employed instead of t. For example, if the parasite density of 25 observations has a standard deviation of 10 and a mean of 40, the standard error of the mean is 10//25 = 2; the appropriate value of t with 24 degrees of freedom is 2.064 and the 95% confidence interval is 40 ± 2.06 (2), i.e. approximately 36 to 44.

To compare two mean values based on different groups of subjects of size n_1 and n_2 (is the difference between the two population means likely to be zero?), a t-test, appropriate to independent observations, must be employed.

The formula for t is given by:

$$[\bar{x}_1 - \bar{x}_2]/[sp/1)1/n_1 + 1/n_2]$$

where $sp^2 = \frac{[(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{(n_1 + n_2 - 2)}$ and s_1^2 and s_2^2 are the sample variances in the two groups. If the t value so obtained exceeds the tabulated t value with $(n_1 + n_2 - 2)$ degrees of freedom, the null hypothesis of equality of the two population means must be rejected. If not, the null hypothesis may be accepted.

Erythrocyte sedimentation rates were measured in an infected group of patients as well as in a control group; the details are set out below:

	Infected group	Control group
No. of subjects (n)	10	12
Mean ESR (x)	9.7	6.5
Standard deviation (s)	2.41	2.54
Variance s^2	5.808	6.452
SE difference =	1.063	

$$t = (9.7 - 6.5)/1.058 = 3.025$$

As this value is appreciably higher than the tabulated critical value ((20 degrees of freedom) at 5%, namely 2.09, we conclude that the ESR in the infected group is significantly higher than that in the control group.

The sampling error of the difference between two means is given by SE (the denominator in the above formula for t). In the above example, this is equal to 1.058.

The 95% confidence interval of the difference between the two means is given by [difference ± 2.09 SE]. In the above example, this is 3.2 ± (2.09 x 1.06), i.e. 1.0 to 5.4.

5.5.5.4 Analysis of rates

When calculating rates, the concept of person-time-at-risk is frequently used. The denominator in these computations takes into account the number of persons at risk as well as the duration of observation for each person, and is expressed as person-years (or person-months) at risk. For instance 20 persons observed for 3 years each would be 60 person-years, as would be 12 persons observed for 5 years each.

The reason for using this concept of person-time-at-risk is that in field studies involving large numbers of subjects (as opposed to small-scale clinical trials of inpatients), a cohort will not always retain the same strength throughout the study period, which could often be several years. In other words, different persons may have different periods of follow-up. This could be due to the following reasons:

(a) Different persons may have been inducted into the study at different times, e.g. from 1980 to 1983, but the cut-off for analysis is the same for all persons, e.g. 31 December 1985.

(b) Some persons may have gone out of the study on account of death, migration or non-cooperation, and these could be after varying periods of observation.

If varying durations of follow-up are not taken into account and the longest duration is taken as the common denominator, estimated rates will be biased downwards (i.e. underestimates). For example, in a 5-year follow-up of 1000 subjects, if all 1000 are observed for 1 year, 950 for 2 years, 900 for 3 years, 800 for 4 years and 700 for 5 years, and the disease incidence stays constant at 5% per year, the number of cases observed will be 50, 47.5, 45, 40 and 35 from the 1st to the 5th year respectively, totalling 217.5, which works out to an annual incidence of 4.35%; this is 0.65% less than the true figure of 5%, i.e. there is a downward bias of 13%.

The validity of the concept of person-time-at-risk rests on the assumption that the risk of the event occurring (e.g. development of disease) is constant throughout the observation period.

An example is set out below to illustrate the method of computing person-years-at-risk.

Date (a)	No. of persons under observation (b)	Average of successive numbers (c)	Years of observation (d)	person-years (c x d)
1.11.1951	10140			
1.11.1952	9145	9643	1	9643
1.11.1953	8232	8688	1	8688
1.11.1954	7389	7811	1	7811
1.11.1955	6281	6835	1	6835
1.04.1956	5779	6030	5/12	2512
			Total:	35489

If 10140 men were alive on 1.11.51 and 9145 on 1.11.52 and if death occurred evenly throughout the year, there must have been on average (10140 + 9145), i.e. 9643 persons alive during the first year, and each contributed 1 year of observation, hence 9643 person-years.

If e is the number of events that have occurred over a period of y person-years, the rate (per year), r is given by e/y. For example, consider a cohort of 5000 subjects who have received a new antileprosy vaccine and are to be followed up for 5 years; due to losses in follow-up, the person-years-at-risk turn out to be 20 000 (instead of 25 000 if every subject had been followed up throughout); the number of leprosy cases that developed is reported as 80, and so the rate is 80/20 000, i.e. 4 per thousand per year.

The sampling error of the rate (r) is $\sqrt{r/y}$, where y is the person-years-at-risk; in the above example, this works out to be 0.4472 per thousand.

The 95% confidence interval for the rate (r) is given by $r \pm 1.96 \sqrt{(r/y)}$; in the above example, this becomes 3.12 to 4.88 per thousand.

5.5.5.5 Comparison of two rates

Comparison of two rates e_1/y_1 and $e_2/y2$ (see below)

	No. of leprosy cases that developed	Person-years	Rate (per 1000)
Vaccinated	e_1 (80)	Y_1 (20,000)	r_1(4)
Not vaccinated	e_2 (160)	y_2 (20,000)	r_2(8)
Total	e (240)	Y (40,000)	r

The expression can be transformed into a chi square with one degree of freedom (see 5.5.5.2 above); if it exceeds 3.84, we conclude that the difference between the rates is significant at the 5% level.

If $e_2 = 80$, $y_1 = 20{,}000$ in the vacinnated and

$e_2 = 160$, $y_2 = 20{,}000$ in unvaccinated group

$x^2 = 26.83$

which is highly significant (P less than 0.001). We conclude that the rate in the vaccinated group is significantly different (lower) from that in the unvaccinated group.

The sampling error of the difference between the two rates is given by SE = $\sqrt{(r_1/Y_1+r_2/Y_2)}$; in the above example, this is 0.0007745, i.e. 0.7745 per thousand.

The 95% confidence interval is given by $(r_1-r_2) \pm 1.96$ s.e.; in the above example, it works out to be 2.5 to 5.5 per thousand.

The ratio of two rates is e_1Y_2/e_2Y_1 and the sampling error of the logarithm of this ratio is $[1/r_1Y_1)+(1/r_2Y_2)]$. In the above example, the ratio is 1/2 and the sampling error of the log of the ratio is 0.1369. The 95% confidence limits of the log ratio are given by -0.693 ± 1.96 (0.1369), i.e. -0.962 to -0.425 therefore, the 95% confidence interval for the ratio of the two rates is 0.38 to 0.65.

REFERENCES TO CHAPTER 5

Altman, D.G. (1982) Statiistics and ethnics in medical research. In: Statistics in practice. Published by British medical journal, p.7.

Ambroz, A., Chalmers, T.C., Smith, H., Schroeder, B., Freiman, J.A. & Shareck, E.P. (1978) Deficiencies of randomised control trials. Clinical research, 26: 280A.

Anon. (1948) Streptomycin treatment of pulmonary tuberculosis. A Medical Research Council Investigation. British medical journal, ii: 769-782.

Anon. (1950) Treatment of pulmonary tuberculosis with streptomycin and para-amino-salicylic acid. A Medical Research Council Investigation. British medical journal, ii: 1073-1085.

Anon. (1977) Aide-memoire for preparing clinical trial protocols. British medical journal, i: 1323-1324.

Anon. (1983) Code of practice for the clinical assessment of licensed medicinal products in general practice. British medical journal, 286: 1295-1297.

Armitage, P. (1975) Sequential medical trials, Oxford, BlacKwell Scientific Publications.

Bross, I. (1952) Sequential medical plans. Biometrics, 8: 188.

Bross, I. (1958) Sequential medical trials. Journal of chronic diseases, 8: 349.

Bull, J.P. (1959) The historical development of clinical therapeutic trials. Journal of chronic diseases, 10: 218-248

Burkhardt, R. & Kienle, G. (1978) Controlled clinical trials and medical ethics. Lancet, iii: 1356-1359.

Burley, D.M. (1970) Preliminary design - the ideal clinical trial. In: Principles and practice of clinical trials. Edinburgh and London, E. & S. Livingstone.

Burley, D.M. (1976) Designing the correct protocol. In: Principles and practice of clinical trials. Good, C.S., ed. Edinburgh, Churchill Livingstone.

Chaput de Saintonge, D.M. & Vere, D.W. (1982). Measurement in clinical tirals. British journal of clinical pharmacology, 13: 775.

CIOMS (1982) Proposal international guidelines for biomedical research involving human subjects. Geneva, Council for International Organizations of Medical Sciences.

CIOMS (1983) Safety requirements for the first use of new drugs and diagnostic agents in man. Geneva, Council for International Organizations of Medical Sciences.

Chongsuphajaisiddhi, T., Sabchareon, A., Chantavanich, P., Singhasivanon, V., Attanath, P., Wernsdorfer, W.H. & Sheth, U.K. (1987) A phase III clinical trial of mefloquine in children with chloroquine-resistant falciparum malaria in Thailand. Bulletin of the World Health Organization, 65: 223-226.

Clarke, C.J. & Downie, C.C. (1966) A method for the rapid determination of the number of patients to include in a controlled clinical trial. Lancet, ii: 1357.

Clayton, D.G. (1982) Ethically optimised designs. British journal of clinical pharmacology, 13: 469-480.

Cox, K.R. (1968) Planning clinical experiments. Springfield, Illinois, USA, Charles C. Thomas.

Cranberg, L. (1979) Do retrospective controls make clinical trials inherently fallacious? British medical journal, ii: 1265-1266.

Curson, D.A. & Curson, V.H. (1983) Rating scales in clinical research: use and misuse. Second World Conference on Clinical Pharmacology and Therapeutics, Washington D.C.

Day, N.E. (1969) A comparison of some sequential designs. Biometrika, 56: 301.

Doberstyn, E.B., Phintuyothin, P., Noeypatimanondh, S. & Teerakiartkamjorn, C. (1979) Single-dose therapy of falciparum malaria with mefloquine or pyrimethamineOsulfadoxine. Bulletin of the World Health Organization, 57: 275-279.

Ellard, G.A., Jenner, P.J. & Downs, P.A. (1980) Evaluation of the potential of isoniazid, acetyl isoniazid, and isonicotonic acid for monitoring the self administration of drugs. British journal of clinical pharmacology, 10: 369.

Evans, S.J.W. (1982) What can we do with the data we throw away? British journal of clinical pharmacology, 14: 653.

Fisher, R.A. & Yates, F. (1974) Statistical tables for biological, agricultural and medical research. Edinburgh, Olvier and Boyd.

Freiman, J.A., Chalmers, T.C., Smith, H. & Kuebler, R.R. (1978) The importance of beta, the type II error and sample size in the design and interpretation of the randomized controlled trial. New England journal of medicine, 299: 690.

Greenberg, G. (1982) Logistics and management of clinical trials. British journal of clinical pharmacology, 14: 25.

Hampton, J.R. (1981a) Organization of multicentre clinical trials. In: Evered, D. O'Connor, M. Collaboration in medical research in Europe. London, Ciba Foundation/Pitman.

Hampton, J.R. (1981b) Presentation and analysis of the results of clinical trials in cardiovascular disease. British medical journal, 282: 1371.

Hampton, J.R. (1981) The use of beta-blockers for the reduction of mortality aftermyocardial infacction. European heart journal, ii: 259.

Hall, A.P., Doberstyn, E.B., Karnchanachetanee, C., Samransamruajkit, S., Laixuthai, B., Pearlman, E.J., Lampe, R.M., Miller, C.F. & Phintuyothin, P. (1977) Sequential treatment with quinine and mefloquine or quinine and pyrimethamine-sulfadoxine for falciparum malaria. British medical journal, 1: 1626-1628

Hill, A.B., ed. (1960) Controlled clinical trials. Oxford, BlacKwell Scientific Publications.

Harinasuta, T., Bunnag, D. & Wernsdorfer, W.H. (1983) A Phase II clinical trial of mefloquine in patients with chloroquine-resistant falciparum malaria in Thailand. Bulletin of the World Health Organization, 61: 299-305.

Hill, A.B. (1971) Principles of medical statistics, 9th ed. London, The Lancet Ltd

Hill, L.E., Nunn, A.J. & Fox, W. (1976) Matching quality of agents employed in double-blind controlled clinical trials. Lancet, i: 352.

Hills, M. & Armitage, P. (1979) The two-period cross-over clinical trial. British journal of clinical pharmacology, 8: 7.

Joyce, C.R.B. (1968) Psychopharmacology: dimensions and perspectives. London, Tavistock Publications.

Joyce, C.R.B. (1982) Placebos and other comparative treatments. British journal of clinical pharmacology, 13: 313.

Klimt, C.R. (1979) Principles of multicentre clinical studies. In: Boissel, J.P. & Klimt, C.R., ed. Multicentre Controlled Trials. Principles and problems. Paris, INSERM.

Kofi Ekue, J.M., Ulrich, A.M., Rwabwogo-Atenyi, J. & Sheth, U.K. (1983) A double-blind comparative clinical trial of mefloquine and chloroquine in symtomatic falciparum malaria. Bulletin of the World Health Organizzation, 61: 713-718.

Langley, R. (1970) Practical statistics for non-mathematical people. London, Pan Books Ltd.

Lasagna, I. (1974) A plea for the "naturalistic" study of medicines. European journal of clinical pharmacology, 7: 153.

Leck, I. (1983) Spinal bifida and anencephaly: fewer patients, more problems. British medical journal, 286: 1670.

Lellouch, J. & Schwartz, D. (1971) L'essai therapeutique: ethique individuelle ou ethique collective? Revue de l Institut international de Statisique, 39: 127.

Lovell, R.R.H. (1977) Problems of interpretation in secondary prevention trials in coronary heart disease. Medical journal of Australia, i: 224.

Mainland, D. (1956) Measurement of bone density. Annals of rheumatic diseases, 15: 115.

McPherson, K. (1974). Statistics: the problems of examining accumulating data more than once. New England journal of medicine, 290: 501.

Medical Research Council Working Party on Mild to Moderate Hypertension (1981). Adverse reactions to bendrofluazide and propranolol for the treatment of mild hypertension. Lancet, ii: 539-542.

Mitchell, J.R.A. (1981) Timolol after myocardial infarction: an answer or a new set of questions? British medical journal, 282: 1565.

Peto, R., Pike, M.C., Armitage, P., Breslow, N.E., Cox, D.R., Howard, S.V., Mantel, N., McPherson, K., Peto, J. and Smith, P.J. (1976) Design and analysis of randomised clinical trials requiring prolonged observation of each patient. British journal of cancer, 34: 585.

Pocock, S.J. (1978) The size of cancer clinical trials and stopping rules. British journal of cancer, 39: 757.

Rose, G. (1982). Bias. British journal of clinical pharmacology, 13: 157.

Schwartz, D.E., EcKort, G., Hartmann, D., Weber, B., Richard-Lenoble, D.,EKue, J.M. & Gentilini, M. (1982) Single dose kinetics of mefloquine in man. Plasma levels of the unchanged drug and of one of its metabolites. Chemotherapy, 27: 70-84.

Schwartz, D.E., EcKert, G. & EKue, J.M. (1987) Urinary excretion of mefloquine and some of its metabolites in African volunteers at steady state. Chemotheraphy, 33: 305-308.

Scott, P.J. & Huskisson, E.C. (1977) Measurement of functional capacity with visual analogue scales. Rheumatology and rehabilitation, 16: 257-259.

Seldrup, J. & Beaumont, G. (1975) A methodological study of the visual analogue scale applied to normals. Quoted in: Curson, D.A. & Curson, V.H. (1983).

Siegel, S. (1965) Non-parametric statistics in the behavioural sciences. New York, McGraw Hill.

Souza, J. M. de (1983a) A Phase I clinical trial of mefloquine in Brazilian male subjects. Bulletin of the World Health Organization, 61: 809-814.

Souza, J. M. de (1983b) A Phase II clinical trial of mefloquine in Brazilian male subjects. Bulletin of the World Health Organization, 61: 815-820.

Souza, J.M. de, Heizmann, P. & Schwartz, D.E. (1987) Single dose kinetics of mefloquine in Brazilian male subjects. Bulletin of the World Health Organization, 65: 353-356.

Taves, D.R. (1974) Minimization: a new method of assigning patients to treatment and control groups. Clinical pharmacology and therapeutics, 15: 443.

Temple, R. (1982) Government viewpoint of clinical trials. Drug information journal, January/June, page 10.

Trenholme, C.M., Williams, R.L., Desjardins, R.E., Frischer, H., Carson, P.E., RiecKmann, K.H. & Canfield, C.J. (1975) Mefloquine (WR 142,490) in the treatment of human malaria. Science, 190: 792-794.

Weinstein, M.C. (1974) Allocation of subjects in medical experiments. New England journal of medicine, 291: 1278-1285.

WHO/CTD (1990) Severe and complicated malaria. Second edition. Transactions of the Royal Society of Tropical Medicine and Hygiene, 84: (Suppl. 2) : 1-65.

Wynn, J. (1982) Spinal bifida: trials ahead. Nature, 299: 198

Zelen, M. (1969) Play the winner rule and the controlled clinical trial. Journal of the American Statistical Association, 64: 131.

Zelen, M. (1979) New design for randomized clinial trials. New England journal of medicine, 300: 1242-1245.

CHAPTER 6 : POST REGISTRATION SURVEILLANCE*

CONTENTS

* Based on working papers prepared by Professor U.K. Sheth and Professor A. Breckenridge and discussions.

6.1 POST MARKETING SURVEILLANCE

6.1.1 Introduction

Marketing permission is granted for a new drug if the preclinical data and the data for safety, effectiveness and tolerance from Phase I, II and III clinical trials are satisfactory.

Sometimes, a drug may be released for restricted use and after further closely monitored evaluation in few thousand patients, it is released for general clinical use. The first three phases of clinical drug evaluation can only establish toxicity with frequencies around 1:100 - 300 because of the small sample size. Thus, licencing of a drug in most countries is only granted after data on 2000 or more patients are presented from pooled clinical trials. However, rare events with frequencies of 1:1000 or under still cannot be detected with any precision although mild reactions observed during clinical trials may alert the specialist that more serious reactions may occur when drugs are marketed on a larger scale.

Health policy makers are thus principally dependent on Phase IV studies as well as post marketing surveillance through prescription event monitoring and spontaneous reporting to establish the risk of less common but serious toxic reactions associated with a drug. Estimates of risk of serious reactions may be conducted by monitoring the specific drug reaction of all users of the drug. Both systematic and ongoing monitoring of drug reactions most frequently conducted through national registers for adverse reactions, prescription event monitoring and medical record linkages.

6.1.2 Phase IV studies

Phase IV studies are prospective trials in clinical patients, comparing the effects of the new drug against an appropriate control group. This is generally carried out by enrolling either a large number of general practitioners who agree to keep records and to report back to the sponsors or a number of hospitals depending upon the disease for which the drug is indicated. The number of patients covered is much larger, compared to the total number of patients studied in Phases I-III. The patients are also not selected with the rigidity of admissions used in Phase III studies. Protocols are standardized. Adverse reactions are studied closely to differentiate between unwanted pharmacological effects, toxic effects and idiosyncratic reactions. Patient questionnaires should be designed so that bias and suggestibility are avoided and symptoms are recorded in the patients' own words.

Phase IV studies will further confirm efficacy, tolerance and side effects commonly observed in earlier studies. The evidence on risk/benefit ratio will be better confirmed. Less common adverse reactions are however less likely to be discovered in these studies and if suspected or observed will need a large number of cases to confirm them.

Serious reactions are principally rare disorders and therefore, measurement of risk through any type of post-marketing surveillance takes time. In order to identify a reaction with 95% confidence, a population three times the size of the expected rate must be studied. Thus, with a true reaction rate of 1 : 5 000 at least 15 000 patients would need to be exposed to the drug before it becomes possible to detect one reaction with 95% confidence.

6.1.3 Post marketing surveillance

This has been defined as "close observation of drug effects following marketing". The information obtained includes: the beneficial effects, adverse effects, social aspects, economic aspects, quality of life after drug usage, and the more delayed effects of drug exposure. Data is collected on drug benefit and risk under conditions of actual use of the drug so that the risk/benefit ratio can be defined. In the case of anti-infective drugs, e.g. antimalarials or antibiotics, there is a continuous review of information on their effectivenness or on development and spread of drug resistance. Rare adverse drug reactions are likely to be observed but these require special investigation and validation to confirm that the observed adverse drug reaction is drug related and not due to a variety of other factors not related to the drug use.

Post marketing surveillance is concerned with detecting adverse reactions and changes in efficacies, validating suspected adverse reactions and providing an estimate of the balance between benefit and risk involved in continued and widespread usage of the new drug. The following are various aspects of special relevance to the conduct of post-marketing surveillance.

6.1.3.1 Spontaneous recording

Spontaneous reports of adverse reactions may come from a variety of sources; the patients, the medical practitioner, the correspondence columns of medical journals, presentations at scientific meetings and even articles in the lay press. National centres for recovering such reports exist in Australia, Canada, the Netherlands, Scandinavia, the United Kingdom, and United States of America. Spontaneous reporting to the manufacturers or to WHO is also a major source of information.

6.1.3.2 National registers

Post marketing surveillance through national registers is dependent on voluntary and spontaneous reporting of adverse reactions to drugs. Reports are received from five main sources: case report forms (as with the yellow card systems in the United Kingdom), pharmaceutical companies, correspondence, death certificates and medical journals. To establish rates and facilitate risk-benefit judgements, data on adverse reactions need to be linked with denominators. Estimates also need to

take into account the background incidence. Primary denominators consist of all users of the drug and are seldom available as a direct measure. Secondary denominators are achieved through collating drug sales or prescriptions. Pharmaceutical data, however, often overestimate users because they include export sales data and unsold drugs. Prescription data may achieve high precision if estimates are based on random samples of all prescriptions. The use of travel statistics and traveller surveys to estimate the proportion of users requires caution because of the biases associated with self-reported use and selective responses.

Comprehensive national registries provide a unique opportunity to monitor adverse drug reactions. The strengths and weaknesses of the systems, however, must be identified before data are used for policy decisions. Under-reporting and misclassification of causality are the two principle biases associated with all post marketing surveillance systems. Reporting bias also includes the reporting of inadequate information. Many confounding risk factors need to be collected, including the age of patients, genetic factors, concomitant drug use, presence of other disease, and drug dose.

6.1.3.3 Monitored release

Drugs are released in some countries in a restricted fashion and doctors are required to send feedback information designed to clarify specific questions within a specified time. The objective is to seek additional information on predicted adverse reactions already observed in toxicity studies and in earlier clinical trials. The number of patients involved may be 20 000 or more.

6.1.3.4 Prescription event monitoring and medical record linkage

This involves identification of patients treated with the drug and a follow-up system to record unexpected events including adverse drug reactions. These patients would be followed up over a long time period. Identification of the patient who received the drug can be achieved by using the prescription form, this information is obtained from the prescribing doctor or the pharmacist in hospital practice. A monitoring centre sends confidential record forms to the prescribing doctor for the recording of events. These should be returned to the centre. Each trial drug under investigation is compared with an "active control" which should be a drug of a similar class used for the same indications. This system is expensive and depends on the response of the prescribing physician and the pharmacist. Responses are usually around 50-60%.

6.1.3.5 Cohort studies

A cohort study is concerned with identifying and following patients and recording the relevant aspects of their history. It is a prospective study and requires follow-up over

a given time. No reference is made to drug administration in the identification of patient groups. Such groups may include all patients in an area of District Health Authorities or in designated teaching hospitals, or again all patients in a list of general practitioners of a certain age group. The group(s) is then followed over a period of time to determine the frequency of the event, adverse drug reaction or the disease under investigation. The information can be obtained for one or two drugs, or many drugs at one time. Examples of cohort studies are :

(a) a prospective randomized clinical trial recording all events (multicentre);

(b) a non-randomized prospective study e.g. oral contraceptive investigations; or

(c) part of an intensive hospital monitoring system, including comparison with a "control" group.

Cohort studies are expensive since they involve large numbers of patients to detect rare adverse reactions. Monitoring and observation of cohorts is further handicapped by the fact that only a non-randomized proportion of the population sampled will have taken the suspect drug. Moreover, self-reported information cannot be verified on all occasions.

6.1.3.6 Case control studies

These are studies in which patients who are developing for the first time a specified illness are identified and the frequency of exposure to a postulated cause is determined. The frequency of exposure is then compared to a series of patients without the illness under investigation.

6.1.3.7 Under-reporting

Under-reporting is the most important bias associated with the ascertainment of risk. It occurs when either the patient or the doctor fails to recognize or report. Non-reporting by the doctor may result from ignorance of the severity of the case or of the necessity to report, from fear and guilt of the reaction, or from secrecy (retaining data for publication). As a result of under-reporting, calculations of rates provide the lowest limit of risk using a drug. While up to 90% of reactions to non-steroidal anti-inflammatory drugs were estimated to be missed because of reporting bias, reporting of reactions to antimalarials is thought to be considerable higher. Reporting rates of cutaneous reactions to pyrimethamine/sulfadoxine based on national registries in Sweden the United Kingdom and were similar to those achieved during an intense investigation in the USA. Reporting may be high because

there is increased recognition of adverse reactions in travellers taking monotherapy, compared with elderly and infirm people on multiple therapy. It may also be attributed to the substantial publicity describing cutaneous reactions. Reporting rates for conditions receiving little publicity may thus be lower; in the United Kingdom six of the nine cutaneous reactions to pyrimethamine/ sulfadoxine were published but none of the three fetal abnormalities and only one of the nine hepatic reactions. Reporting is also temporal. It is likely that ascertainment is low during the early post marketing phase, when reactions are not attributed to a new drug, and again later when associations are well established. This has been shown in the United Kingdom where reported rates for adverse drug reactions associated with pyrimethamine/dapsone have decreased tenfold in recent years.

6.1.3.8 Misclassification

Reports need to be scrutinized for causality. Questions include: were other drugs administered?; was the drug really taken?; if taken on a previous occasion, was a similar reaction experienced?; what occurred after the report?; were full details submitted?; and has the doctor recently seen similar reactions? Based on this assessment, causality may be described as definite, probable, possible, conditional or doubtful. Causality is strengthened if the event or reaction:

(a) was temporally associated with the administration of the suspect drug;

(b) followed a known response pattern of the suspect drug;

(c) improved when the suspect drug was removed;

(d) recurred when the patient was rechallenged with the suspect drug;

(e) could not be explained by another reason

Misclassification may also occur with regard to the degree of reactions. The clinical decision of serious or mild reaction, although made by experts, is a value judgement and difficult to quantitate.

6.1.3.9 Post marketing surveillance in developing countries

Post marketing surveillance in many developing countries is rarely carried out. Malaria is endemic and affects a large population in tropical countries. Surveillance on the use of antimalarial drugs in these vast areas either does not exist or is very inadequate. It is difficult to organize, very expensive and needs a concentrated effort of the government to set up such schemes. This may not be possible due to financial constraints or not considered within the priorities of the countries concerned. The need is however no less than that in the developed world.

6.2 ADVERSE EFFECTS OF ANTIMALARIAL DRUGS AND THEIR MONITORING

Antimalarial drugs have been used for many decades in vast numbers of people. Generally these drugs have been well tolerated, without exhibiting serious side effects, except in specific circumstances.

The most important aspects of a new drug evaluation are efficacy, safety, tolerance and incidence of adverse drug reactions. Signs and symptoms of overdosage, either accidental or intentional, are included in the term "toxic actions".

All drugs are molecules which have a potential for reacting with various living cells and for producing some actions. The desirable actions which are beneficial in a disease condition are termed "therapeutic actions" - all other actions observed may be called "unwanted actions". Some unwanted actions may be useful in the treatment of other diseases and may be welcome. Others which are harmful to the patient are termed "adverse reactions"

Some of these adverse reactions may be mild, transient, self-limited, e.g. nausea, vomiting, abdominal pains, mild dizziness, mild diarrhoea and may be acceptable, considering the benefit derived from treating the disease. Some may however be serious and life-threatening, e.g. bone marrow depression or hepatic damage. Such a drug would not be acceptable, even though it may effectively treat the disease. A necessary objective in studying the new drug and its adverse effects is related to determining the type of undesirable actions produced at the effective dose, their intensity, duration and the extent to which these reactions are dangerous to the host. There is a need to determine the risk/benefit ratio. The limits of acceptability of drug associated risk will depend upon the risk associated with the disease and the efficacy of other available drugs.

Even some of the reactions which are not life-threatening like dizziness or vomiting may not, if severe, be acceptable by patients when a drug is used extensively in real life situations, although they may be accepted by selected hospitalized patients. Thus "patient acceptance" also becomes an important aspect contributing to the continued widespread use of drugs, like antimalarials.

6.2.1 Occurrence of adverse reactions

Adverse reactions to antimalarial drugs may occur in various circumstances:

(a) Adverse reactions may be similar to the clinical symptoms of the disease itself, e.g. nausea, vomiting, dizziness. They assume importance only if these symptoms are significantly increased after drug administration, and may lead to a loss of the drug from the body, e.g. in vomiting when inadequate absorption occurs. For example, there are reports of vomiting following mefloquine administration in doses higher than 750 mg.

(b) Addverse reactions may be associated with overdosage either accidental or intentional. These would be really "toxic actions" and not "adverse reactions" of clinical significance.

(c) Adverse reactions may be related to a wrong method of drug administration, e.g. intravenous quinine or chloroquine given at a fast rate. These could be reduced by giving the drugs slowly.

(d) Adverse reactions may occur unpredictably in some individuals, e.g. urticaria and angioneurotic oedema after quinine, which are classified as "idiosyncratic" in the absence of a better understanding of their causation.

(e) Adverse reactions can be "predicted" in certain populations, e.g. haemolytic anaemia following primaquine, or dapsone administration in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. These reactions occur only in certain variants of enzyme deficiency found in populations in specific geographical areas. These reactions may also be dependent on the dose and the dose regimen, e.g. primaquine 45 mg weekly may be better tolerated than a daily dose of 15 mg in Thai subjects.

(f) Some adverse reactions occur only after prolonged use of a drug for prophylaxis in endemic areas, e.g. ophthalmic lesions consisting of punctate keratitis, lens opacities and atrophy of the optical nerve following a total life time dosage of chloroquine exceeding 100g base.

(g) Use of some antimalarials in high doses for prolonged periods in the treatment of other diseases, e.g. chloroquine in the treatment of arthritis, or pyrimethamine for treating toxoplasmosis is associated with serious side effects. These are, however, not associated with their use for treating malaria.

(h) In certain populations, some adverse reactions are more frequently seen, e.g. skin itching following chloroquine use in Africans.

All the above reactions are to a certain extent

predictable, depending upon the length of use, on whether the use of the drug is for prophylaxis or treatment, on geographical and ethnic factors, on the dose given and the method of administration. Some of these factors are amenable to changes which would reduce incidence and severity of the adverse drug reactions.

Of great concern in the use of antimalarial drugs, however, is the sudden appearance of serious, or even fatal adverse reactions associated with the administration of a drug which has been effective and considered safe in earlier use, or of serious adverse reactions manifested in newly introduced drugs. These need serious consideration.

Occasional occurrence of Stevens Johnson syndrome, toxic epidermal necrolysis or Lyell's syndrome have been well documented with the use of sulfonamides and particulary with long-acting sulfonamides. A combination of sulfadoxine and pyrimethamine Fansidar^{R}, has been in clinical use since 1971 for the treatment and prophylaxis of multidrug-resistant Plasomodium falciparum in South-East Asia and South America. Serious side-effects with an appreciable mortality rate have been only recently been reported in tourists using sulfadoxine/pyrimethamine for prophylaxis (Miller, et al., 1986; Phillips-Howard & West, 1990). The absence of such reports from endemic areas may be explained partially by poor follow-up and a failure to recognize the syndrome and connect it with the use of Sulfadoxine/pyrimethamine. This does not seem to be the only explanation. A serious event with a frequency of 1:8000 should have been noticed by some clinicians. The occurrence of these serious adverse effects has created great concern. Use of mefloquine/sulfadoxine/pyrimethamine (MSP) has also been associated with serious cutaneous reactions following its use for treatment in outpatients in Thailand (Anon., 1976).

In 1986 the use of amodiaquine for prophylaxis was implicated in agranulocytosis and hepatic necrosis occurring in British and Swiss travellers who visited malarious areas. The frequency of these reactions was calculated to be 1:2000 with death occurring in 1:15 000 users (Hatton et al., 1986, Steffen & Heusser, 1986). Most of the cases occurred after very few doses which might be simulated by repeated amodiaquine therapy of malaria in endemic areas.

Behavioural disorders following the use of mefloquine for treatment have been reported from Thailand and Zambia.

Clindamycin and lincomycin are both known to cause pseudo-membraneous colitis. Clindamycin is being used as an antimalarial in South American countries and certain other parts of the world.

An important aspect of adverse drug reactions is to review and select the approaches to the problems raised by adverse drug reactions and to make the necessary decisions using the available data. Unfortunately reaction in these circumstances

can be highly emotional, leading to an unjustifiable scare and wrong decisions. The use of drugs is inherently associated with some adverse reactions. A decision must therefore be made on a studied risk/benefit analysis.

Each adverse reaction report needs to be investigated, documenting the conditions under which the drug was used, e.g. for prophylaxis or for treatment, the dosage used and whether other drugs were used simultaneously. There is also a need to study the mechanisms of adverse drug reactions and for investigating the blood levels of the drug or its metabolites associated with adverse reactions so that properly collected, reliable data can be used to redefine the use of a particular drug. For example a drug may be too risky for repeated use in prophylaxis, but it may be extremely useful for treating acute attacks and therefore reducing the mortality from malaria. Another drug may be used safely in some populations, but contraindicated in another. A serious adverse effect, of course, would not be acceptable under any circumstances.

Another important area which is not well studied is the question of drug interactions which may lead to serious adverse reactions. Patients on long-term treatment for diabetes, tuberculosis, leprosy, chronic renal failure or hypertension and women on oral contraceptives might use antimalarial drugs for prophylaxis and treatment. For example, the use of mefloquine is contraindicated in persons using beta-blockers.

6.2.2 Monitoring adverse drug reactions

The objective of the clinical evaluation of a new drug, as stated earlier is to determine its efficacy, safety and tolerance. Clinical safety and patient acceptance are determined by monitoring subjective adverse drug reactions, various haematological and biochemical parameters and repeated physical examination of the patients.

During Phase I, II and III clinical trials, adverse drug reactions have to be distinguished from those which are disease-related in order to correctly evaluate "drug related" side effects.

Disease-related symptoms and abnormalities in haematological and biochemical parameters used for evaluation of safety are recorded as "baseline data" before drug administration. In Phase I studies involving healthy subjects these should be recorded for two days prior to drug administration and then after drug administration.

The "normal" range of laboratory parameters is usually determined in a non-infected target population of about 100 subjects, the mean values and range obtained are then used as the standard for comparing the values obtained during the study. For subjective symptoms, the patient's spontaneous complaints are recorded.

Adverse reaction monitoring involves a judgement on the part of the investigator of the causal relationship between the administration of a drug and the observed adverse drug reactions. The relationship could be 1) causative (definitely drug related); 2) probable; 3) possible; or 4) coincidental. In some cases the relationship could be established either by "re-challenging" the patient with the same drug at a later date and reproducing the reaction, or by "de-challenging", stopping the drug and noting if the reaction subsides. If the reaction has been severe, rechallenging cannot be considered, and if the treatment involves one or a few doses, dosing is likely to have already been stopped by the time the adverse reactions occur.

When evaluating a new drug for which no previous experience is available, investigators have to depend on the evaluation of data from several trials. Even then, an association between use of a drug and the observed adverse reaction may be very difficult to prove. Judgement must therefore be based on circumstantial evidence.

The frequency and pattern of adverse drug reactions to the same drug may differ in different ethnic populations, according to various factors such as genetic differences in drug disposition, increased susceptibility to the drug due to genetic enzyme deficiences (e.g. G6PD deficiency), or dietary habits.

6.3 DRUG INTERACTIONS

This section reviews the various classifications of drug interactions, the theoretical basis of drug interactions with antimalarial drugs and the known interactions involving antimalarial therapy.

6.3.1 Classification of drug interactions

Drug interactions must first be differentiated from additive drug effects. As shown in Fig. 6.1, if the effect of drug A when given together with drug B is merely the sum of the effects of A + B, this is not an interaction although the additive effect may be therapeutically useful. An interaction has occurred only if the combined effect is either greater or less than the sum of effects of A plus B. If greater this is a positive interaction and, if less, a negative interaction.

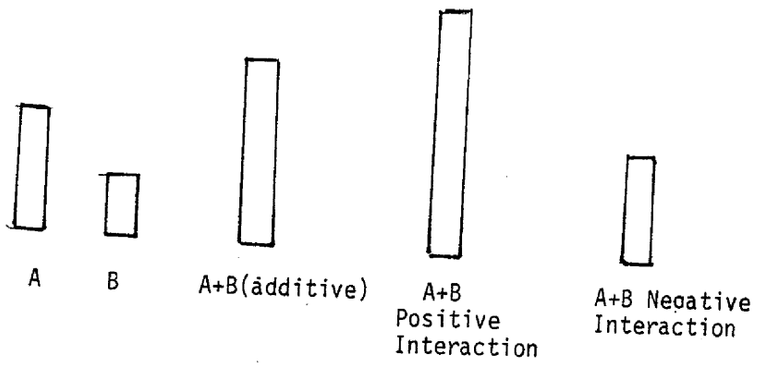


Fig. 6.1 Difference between additive effects and positive and negative interactions

Further, drug interactions may be either beneficial or harmful, depending on whether the result of the drug combination enhances efficacy or toxicity. Examples of beneficial interactions are the combination of a dopadecarboxylase inhibitor with levodopa used in the treatment of parkinsonism to enhance the accumulation of dopamine within the brain, or the combination of two folic acid antagonists, a sulfonamide and trimethoprim, in co-trimoxazole. In this second example, however, current evidence might suggest that the efficacy of trimethoprim is equal to that of the combination and moreover, is less toxic than the combination. An example of an adverse interaction is the combination of cimetidine with an oral anticoagulant whereby plasma levels of the latter drug are increased, resulting in the possibility of haemorrhage.

Clearly, the likelihood of adverse reactions occurring because of drug interactions increases with the number of coadministered drugs. It has been calculated that if five drugs are coadministered, there is a 75% chance of perpetrating an interaction which may be dangerous. In many respects, drug interactions have attracted attention disproportionate to their clinical importance; but, on the other hand, they may illustrate important pharmacological principles. Important clinical interactions occur with a relatively restricted number of drugs with a narrow therapeutic ratio, i.e. whose dose must be adjusted within a small range and whose effects (beneficial or adverse) are pronounced when this range is either exceeded or not attained.

Various classifications of drug interactions have been proposed and three of these are given in Tables 6.1, 6.2 and 6.3. According to the classification used in Table 6.1, the underlying basis of interactions may be either pharmacokinetic or pharmacodynamic. Pharmacokinetic interactions depend on a change in free drug concentration either in the plasma or at the site of drug action in the tissue. These changes are due to alteration of drug absorption, distribution including binding, metabolism or excretion by a coadministered agent. Pharmacodynamic interactions result from a modification of drug occupancy of a receptor site or a modification of an underlying physiological control mechanism.

TABLE 6.1 CLASSIFICATION OF DRUG INTERACTION BY SITE

1. Prior to administration
2. During drug absorption
   - within the gut lumen
   - By altering gut motility
   - By altering gut flora
   - Within the gut wall
3. Protein binding
4. Drug metabolism
   - Stimulation
   - Inhibition
5. During excretion
6. At receptor sites

TABLE 6.2 CLASSIFICATION OF DRUG INTERACTIONS BY CLINICAL IMPORTANCE

1. Drug has major effect on a vital process
   e.g., respiration
   blood presssure
   blood coagulation
2. Drug has steep dose response curve

TABLE 6.3 CLASSIFICATION OF DRUG INTERACTION BY PREDICTABILITY

1. Predictable from existing knowledge of pharmacological effects.
2. Predictable from existing knowledge of pharmacokinetic properties.
3. Unpredictable from present knowledge.
4. Predictable but not predicted.
5. Overlooked or forgotten.

The classification in Table 6.2 depends on the predictability of the interaction. If current knowledge allows the physician to predict an interaction, then he/she should be able to take steps to avoid it should it result in an adverse effect. Too frequently this knowledge has been gathered but is either not available to the physician or has been forgotten by him/her. If a novel drug is being coadministered with another agent, the possibility of causing a drug interaction must always be kept in mind and great care taken.

The classification used in Table 6.3 stresses that it is mainly where a drug has an effect on vital processes, e.g. respiration, blood coagulation, blood pressure, and has a steep dose response curve, that interactions with it are clinically important. It is of course, important to remember that interactions can occur by more than one mechanism.

6.3.1.1 Interactions prior to drug administration

The low pH of fluid in which a drug such as heparin or penicillin is administered may cause loss of drug activity by chemical inactivation. Further, since drugs may interact in the infusion bottle, e.g. carbenicillin is inactivated by gentamicin, administration of more than one drug in the same infusion should be avoided wherever possible.

6.3.1.2 Interactions during drug absorption

This subject has been discussed in Section 1.5.4.

6.3.1.3 Interactions involving protein binding

Many drugs are transported in blood bound either to albumin (acidic drugs) or to alpha acid glycoprotein (basic drugs). The significance of displacement of drugs from binding sites as a cause of drug interaction has probably been exaggerated. Any effect will be transient since the displaced free drug will be eliminated in the normal way and a new and lower steady state plasma concentration results. Further, the drug displaced must be tightly bound with a low volume of distribution for displacement to have any therapeutic significance. However, when the displacing drug is injected intravenously, the concentration of unbound drug may be increased instantaneously and highly perfused organs, such as heart, brain and liver, exposed to high free drug concentration. Not only may drugs be displaced from binding sites by other drugs with a high affinity for the site but endogenous substances which may accumulate in disease may also compete for these sites. Bilirubin in liver failure and various endogenous metabolites in renal failure may displace such drugs as warfarin and phenytoin from albumin binding.

6.3.1.4 Interactions involving metabolism

(a) Stimulation

Many lipid soluble drugs, following long-term administration cause non specific stimulation of drug metabolism in both humans and experimental animals. The number of such drugs used in therapeutics is small, e.g. anticonvulsants such as phenobarbitone, phenytoin and carbamazepine and the antibiotic rifampicin. This process by which drug metabolism is stimulated within the smooth endoplasmic reticulum occurs is known as enzyme induction and in many tissues, mainly liver but also gut wall, kidney and skin.

Increasing the rate of drug metabolism of a substrate such as warfarin results in its inactivation with a consequent decrease in therapeutic effect, but, if the drug has active metabolites, effects can be enhanced by induction. For example, hepatic necrosis following paracetamol overdose is more severe in patients previously given inducing agents due to the increased production of toxic metabolites of paracetamol.

An interesting aspect of enzyme induction is the increase in portal venous blood flow caused by the administration of inducing agents such as pheno- barbitone. For drugs which are largely cleared by the liver, e.g. propranolol, the increase in liver blood flow is a more important determinant of their increased elimination than an increase in liver microsomal enzyme activity when an inducing agent is given.

(b) Inhibition

Inhibition of drug metabolism may result in prolonged and exaggerated drug responses and an increased risk of toxicity. The time course of changes is quite different from that seen with induction since it depends only on the rate of elimination of the drug whose metabolism has been inhibited. Inhibition of drug metabolism may be competitive or non-competitive. An important example of the former type is the effect of cimetidine, a potent inhibitor of warfarin, diazepam and other drugs. This effect is due to its imidazole structure; similar antagonists with different chemical structures do not show this effect. Non-competitive inhibitors can be exemplified by the destruction of cytochrome P450 by quinalbarbitone.

6.3.1.5 Interactions at the level of the kidney

(a) Changes in urine pH

The renal clearance of weak acids i.e. those with pKa 4.0-7.5, is increased in alkaline urine, and the renal clearance of weak bases i.e. those with pKa 7.5-10.0 in increased in acid urine due to the impermeability of renal tubules to ionized molecules and their permeability to non-ionized molecules. The half-life of the antifilarial drug, diethylcarbamazine, can be increased up to threefold in alkaline urine and this has produced useful therapeutic results.

If drugs are totally eliminated by hepatic metabolism rather than by renal excretion, changes in urine pH in the normal clinical situation are irrelevant, irrespective of pKa.

(b) Competition for active tubular secretion

The proximal part of the renal tubule has an active transport mechanism which is used by many drugs. There is probably one transport system for acidic drugs and another for

basic drugs. Examples of acidic drugs using this transport mechanism include penicillin, probenecid and thiazide diuretics. Probenecid will block the renal tubular secretion of penicillin, thus augmenting its activity.

6.3.1.6 Interactions at receptor sites

There are many examples of pharmacodynamic interactions where one drug alters the effect of another by virtue of its greater affinity for a receptor site. The antagonism of warfarin by vitamin K, of morphine by naloxone, of acetylcholine by atropine or tubocurarine are such examples. The receptors concerned are better defined in some instances than in others and in some instances the underlying mechanism is complex, involving perturbation of other physiological control mechanisms.

6.3.2 Interactions involving antimalarial drugs

The clinical pharmacology of most antimalarial drugs has only been elucidated over the past five years or so, and in many instances, knowledge remains very incomplete. In theory, however, several types of interaction can be envisaged:

- those in which the administration of one antimalarial drug causes an interaction with another antimalarial drug;
- those in which administration of an antimalarial drug causes an interaction with another non antimalarial drug;
- those in which administration of a non-antimalarial drug causes an interaction with an antimalarial drug;

Several of the examples given below are more theoretical than factual since appropriate studies have either not been performed or are in the process of being carried out. However, the examples cited do illustrate important principles which will be appropriate for new antimalarial drugs.

It is important to distinguish, wherever possible, between additive effects and true interactions, which may be positive or negative.

The classification of drug interactions involving antimalarial drugs is that given in Table 6.1.

6.3.2.1 Interactions prior to administration

No studies have been made on the effect of altering the pH of infusion fluids containing antimalarial drugs. Quinine has two pKa values, i.e. 4.3 and 8.4, and while the degree or ionization will change with the pH, it is not clear whether

pharmacological efficacy changes too. This occurs with other drugs such as heparin and penicillin cited earlier in section 6.3.1.1 (White, 1985).

6.3.2.2 Interaction during absorption

Most antimalarial drugs which are given by mouth are well absorbed in the small intestine. The major exception is halofantrine which is poorly and erratically absorbed; preliminary data suggesting that a meal with a high fat content may promote its absorption. No drug-drug interactions involving changes in the absorption of halofantrine have so far been reported but it is likely that some will emerge.

With respect to alteration of gut motility, patients with severe malaria may well have decreased gastric emptying and it would not be surprising if the rate of antimalarial absorption was decreased; in this situation, of course, most antimalarials are given parenterally to avoid delayed or diminished absorption.

No studies have been made on the enterohepatic circulation of antimalarial drugs or on the effect of changing gut flora by antibiotic administration.

6.3.2.3 Interactions involving protein binding

It has been demonstrated by many workers that plasma levels of digoxin are increased by coadministration of quinidine or quinine and this is of clinical importance. The basis of the interaction is twofold. Firstly, there is a decrease of about 30-40% in the apparent volume of distribution of digoxin and, secondly, a change occurs in its renal excretion. The change in the volume of distribution presumably implies altered binding of digoxin to the tissues, though this has not been proven. Other antimalarials such as mefloquine are highly bound but no interactions involving this mechanism have been reported (White 1985). In acute severe malaria, the concentration of acids and glycoprotein increases, and this may increase the extent of binding of drugs such as quinine.

6.3.2.4 Interactions involving metabolism

(a) Stimulation

No antimalarial drug is an enzyme inducing agent but inducing agents such as phenobarbitone may affect the disposition of antimalarials such as quinine (Murray, 1987). This is of more than theroretical interest since phenobarbitone is used for the treatment of convulsions in children who are likely to be given quinine for severe malaria. Studies of the mangnitude and frequency of the interactions are required.

(b) Inhibition

Most work on interactions involving antimalarial drugs has concentrated on the effect many of these agents have in inhibiting drug oxidation (Murray, 1987). Many of the quinoline antimalarials will inhibit drug oxidation in vitro or in vivo. In rats, primaquine has been shown to be a more potent inhibitor of drug oxidation than chloroquine (Back et al., 1983). In experiments to evaluate a series of quinoline drugs for their ability to inhibit rat liver microsomal aminopyrine N-demethylase in vitro, quinine and primaquine were found to be potent inhibitors, amodiaquine relatively potent, while quinidine (the isomer of quinine) and chloroquine were essentially non inhibitory. Primaquine, mefloquine and quinine, to a lower extent quinidine and amodiaquine but not chloroquine have been shown to inhibit ethinyloestradiol and tolbutamide metabolism in vitro.

In humans primaquine and quinidine have been shown to be potent inhibitors of antipyrine and debrisoquine respectively. While mefloquine has little effect on antipyrine metabolism, chloroquine may inhibit the metabolism of metoprolol.

Clearly, the structure activity relationship of quinoline antimalarials with respect to microsomal enzyme inhibitors requires further investigation, and the specificity of these inhibitors with respect to various forms of cytochrome P450 is an especially interesting problem. The biguanides, proguanil and chlorproguanil, are known to act through the oxidative metabolites, cycloguanil and chlorcycloguanil. Further, it has been shown that the conversion of proguanil to cycloguanil is genetically controlled and a polymorphism has been demonstrated. Sulfonamides are known to inhibit drug oxidation and it is a matter of considerable practical importance to establish whether the conversion of the biguanides to their active metabolites may be inhibited in certain individuals by concomitant sulfonamide administration.

6.3.2.5 Interactions at the level of the kidney

Quinine, as noted above, has pKa values of 4.3 and 8.4. Approximately 5% of quinine is excreted unchanged at a urine pH of 7.4, but, in acid urine, both N^+ groups will be ionized and renal excretion of quinine is twice as rapid as in alkaline urine.

As mentioned in section 6.3.2.3, the other component of the digoxin and quinidine/quinine interaction is at the level of the kidney where quinine or quinidine will decrease the tubular secretion of digoxin by some 30%.

6.3.2.6 Interactions at receptor sites

Since little is known about the receptors on which most antimalarials act, pharmacodynamic interactions remain largely

conjecture rather than fact. At present, there is great interest in the way in which drugs such as verapamil and desimpramine reverse the resistance to chloroquine in Plasmodium falciparum. The basis of these interactions is obscure but they do not involve alteration in chloroquine pharmacokinetics.

6.3.3. Conclusion

By understanding the mechanisms by which antimalarial drugs may interact, studies can be performed at a preclinical and clinical level, with new antimalarial agents and other drugs with which they are likely to be co-administered. Clinical investigation of established antimalarials used in combination with other agents is also of great importance to increase their efficacy and decrease toxicity.

REFERENCES TO CHAPTER 6

Anon.(1986) Antimalarial drug risk. Bulletin of the World Health Organization, 64: 504-506.

Back,D.J., Purba, H.S., Park, B.K., Ward, S.A. & Orme M.L'E (1983) Effect of chloroquine and primaquine on antipyrine metabolism. British journal of clinical pharmacology, 16: 497-502.

Hatton. C.S.R., Peto. T., Bunch, C., Pasvol, G., Russel, S.L., Singer, C.R.J., Edwards, G. & Winstanley, P. (1986) Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria, Lancet, 1: 411-414.

Miller, K.D., Lobel, H.O., Satriale, R.F., Kinitsky, J.N., Stern, R. & Campbell, C.C. (1980) Severe cutaneous reactions among American travellers using pyrimethamine-sulfadoxine (Fansidar) for malaria prophylaxis. American journal of tropical medicine and hygiene, 35: 451-458.

Murray, M. (1987) Mechanism of inhibition of cytochrome P450 mediated drug oxidation by therapeutic agents. Drug metabolism reviews, 18: 55-81.

Phillips -Howard, P.A. & West, R.J.(1990) Severe adverse reactions to pyrimethamine-sulfadoxine, pyrimethamine-dapsone and to amodiaquine in Britain. Journal of the Royal Society of Medicine, 83: 82-85.

Steffen, R. & Heusser, R. (1986) Fiabilite' et effets secondaires de la prophylaxie du palodisme. Medecine et hygiene, 44: 2125-2127.

White, N. (1985) Clinical pharmacology of antimalarial drugs. Clinical pharmacokinetics, 10: 187-215.

CHAPTER 7 : CONCLUSIONS

CONTENTS

Malaria is still one of the major health problems in large parts of the tropics, where 110 million clinical cases are estimated to occur every year. The complexity of malaria has enabled it to resist successfully the many and varied attempts to eradicate or control it. With this in mind, WHO has promoted an epidemiological approach to the design of control operations, priorities for action and the orientation of control being determined by the local epidemiological situation rather than by general control axioms. The necessary minimum is, however, to provide easily accessible and appropriate diagnostic and treatment facilties to meet the most basic health requirements of the population.

The increasing incidence of Plasmodium falciparum resistant to chloroquine has made the appropriate choice of drugs both for treatment and prophylaxis ,difficult since alternative drugs or drug combinations may be more expensive, more toxic and difficult to administer. Resistance to these alternative drugs also occurs.

Chemotherapeutic research, therefore, deserves high priority since it has to provide the tools for an effective treatment of malaria. Besides the review of the current state of the clinical pharmacology of antimalarial drugs, the workshop has also addressed the orientation of future work. In doing so it identified lacunae in the knowledge about currently available drugs and obstacles to the acquisition of such knowledge. It also emphasized the need for stimulating the practice of clinical pharmacology in malarious countries and for orienting developmental and research efforts in accordance with the practical problems of the countries where malaria is endemic. Specific suggestions pertain to drug assays, pharmacokinetics, trials of antimalarials, drug interactions, adverse drug reactions and post marketing surveillance.

7.1 DRUG ASSAYS

The development of sufficiently sensitive assay methods for new candidate compounds is a prerequisite for pharmacokinetic studies. In normal circumstances this is adequately taken care of by the pharmaceutical industry and cooperating research laboratories. The methods are usually sophisticated. Constraints exist, however, with regard to relatively simple, yet reliable methods for use in the field, the standardization of analytic procedures between laboratories, the preservation of biological material prior to analysis, assays for older compounds introduced before the advent of modern clinical pharmacology, and assays for new compounds being developed outside industry.

7.1.1 Simple Assays

Simple, rapid, sensitive and reliable tests for antimalarial drugs in blood and urine are required in the context of patient management in order to verify or exclude previous drug intake. Such screening can assist in the

identification of potential drug resistance and direct the choice of an alternative medicament when a previous regimen has been ineffective. It can also indicate a potential for toxicity upon retreatment if a high concentration of a previously administered drug is found. For such simple assays in urine, colorimetric and dipstick methods should be explored, while thin layer chromatography (TLC), especially high performance TLC, and colorimetric tests should be useful for semi-quantitative drugs assays in blood.

7.1.2 Standards

Drug assays in the context of clinical pharmacology require the highest possible precision and reproducibility. This cannot be ensured without a sound calibration and the use of standards for the antimalarial drugs and their major metabolites. It will therefore be essential to create a source of quality-controlled internal standards of all antimalarial drugs and their major metabolites, including blood samples spiked with known amounts of drug (freeze-dried). This material should be available without prejudice to all research laboratories. When internal standards and metabolites are not yet available or are unsatisfactory for technical reasons, suitable compounds should be synthesized.

7.1.3 Partition

Clinical pharmacological studies of antimalarial drugs usually employ assays requiring plasma or serum. There is an increasing need for methods which use whole blood and are suitable for field investigations. This includes the use of dried specimens on filter paper. Since several antimalarial drugs, e.g. chloroquine and mefloquine, show marked concentration differences in serum, plasma and corpuscular blood elements, there is a need for conducting partition studies, and eventually elution studies, to assess the parameters of variability under standardized conditions. This will render interpretation and comparison of assay results feasible.

7.1.4 Preservation of biological material

The preservation of biological material prior to drug assays poses problems especially in the context of field investigations. This calls for the development and standardization of suitable methods. New approaches may be required for the handling of specimens containing compounds which may rapidly decompose in the biological material, e.g. artemisinin and its derivatives.

7.2. PHARMACOKINETICS

Despite the remarkable progress in the clinical pharmacology of antimalarial drugs over the past 20 years, major lacunae exist not only with regard to new compounds, but also in respect of the antimalarials which has been used for many

years. In part, this stems from the fact that most investigations were carried out in healthy adult volunteers and relatively few in malaria patients. Drug regimens derived from observations in healthy volunteers may not be optimum for malaria patients. This highlights the important role of investigations in malarious countries.

7.2.1 Pharmacokinetics in malaria patients

Future pharmacokinetic studies should be geared to elucidate the fate of antimalarial drugs and their metabolites not only in healthy volunteers, but especially in patients with malaria, including specific groups such as children, elderly persons and patients suffering concurrently from other conditions, e.g. nutritional, hepatic, renal or gastrointestinal disorders.

7.2.2 Data base

A data base of pharmacokinetic parameters of the standard antimalarial drugs should be developed. This should reflect also specific group characteristics, e.g. age, sex, pregnancy, fever. Based on this information an attempt should be made to produce computer simulations requiring only a small number of concentration time point data.

7.2.3 Drug concentration and efficacy

With the exception of chloroquine, little is known about the correlation between blood levels of drugs and the *in vitro* and *in vivo* response of *Plasmodium falciparum*. Such information should not only be obtained for the new antimalarial compounds but also for standard drugs such as quinine and sulfadoxine/pyrimethamine.

7.2.4 Drug formulations

It is not possible to measure absolute bioavailability of some drugs, e.g. halofantrine and mefloquine, due to the lack of formulations which can be administered by the intravenous route. Stable suspensions of microparticles may assist in the development of such formulations which may also be of therapeutic value. Similarly, suppository formations of various antimalarial compounds, e.g. of artemisinin and its derivatives, would be particularly useful in the management of severe and complicated malaria at the peripheral levels of the health care system.

7.3 CLINICAL AND FIELD TRIALS

7.3.1 Standardization

Clinical and field trials of antimalarial drugs are being conducted in various parts of the world and under widely varying conditions. In the interest of the comparability of results there is need for the standardization not only of drug regimens,

but also of the range of laboratory parameters required and the methods used for their assessment. This technical standardization relates to haematological, clinical chemical and parasitological methodology.

7.3.2 Interactions between antimalarials and other drugs

It is often unavoidable that antimalarial drugs be administered in addition to medication for other conditions, e.g. diabetes, cardiovascular disorders or bacterial infections. It is therefore necessary to study potential interactions between antimalarials and those drugs commonly prescribed for such conditions.

7.3.3 Interactions between antimalarials

The potential interaction between different antimalarial compounds should be investigated for toxicological and antiparasitic effects. All associations which are conceivable from an operational point of view should be examined.

7.4 POST MARKETING SURVEILLANCE AND ADVERSE DRUG REACTIONS

Both post marketing surveillance and monitoring for adverse drug reactions are grossly deficient, especially in developing countries, where appropriate mechanisms either do not exist or are in a rudimentary state of development.

7.4.1 Post marketing surveillance

The post marketing surveillance of antimalarial drugs in developing countries is rarely carried out by industry; the majority of drugs originate from generic manufacture devoid of post marketing surveillance systems. Ministries of health, the appropriate national pharmaceutical associations and international associations and organizations such as International Federation of Pharmaceutical Manufacturers Association (IFPMA) and WHO should cooperate in implementing and maintaining viable and reliable post marketing surveillance for antimalarial drugs.

7.4.2 Adverse drug reactions

The monitoring for adverse drug reactions related to antimalarials needs to be implemented in those areas where these drugs are routinely used. This should be done with the objective of devising ways in which the occurrence of adverse drug reactions can be minimized without withholding eventually life-saving malaria treatment. Monitoring of adverse drug reactions should also be used to determine the risk/benefit ratio of those compounds to which there exists a feasible, albeit more expensive, alternative.

7.5 TECHNICAL COORDINATION AND TRAINING

There is a need for promoting research capability in clinical pharmacology, especially in malarious countries where much of the target-oriented research should take place. This requires training, interinstitutional cooperation as well as the identification of studies which are of priority and relevant to the operation of antimalaria programmes.

APPENDIX 2. A CURVE FITTING PROGRAMME*

For a set of data points (X_i, Y_i), = 1, 2, ...n, this programme can be used to fit the data to any of the following curves:

1. Straight line (linear regression); $y = a + bx$.
2. Exponential curve; $y = ae^{bx}$ $(a > 0)$.
3. Logaithmic curve; $y = a + b \ln x$.
4. Power curve; $y = ax^b$ $(a > 0)$.

The regression coefficients a and b are found from solving the following system of linear equations:

$$\begin{bmatrix} N & \Sigma X_i \\ \Sigma X_i & \Sigma X_i \end{bmatrix} \quad \begin{bmatrix} A \\ b \end{bmatrix} \quad \begin{bmatrix} \Sigma Y_i \\ \Sigma Y_i \quad X_i \end{bmatrix}$$

where the variables are defined as follows:

Regression	A	X_i	Y_i
Linear	a	x_i	Y_i
Exponential	ln a	x_i	ln Y_i
Logarithmic	a	ln x_i	Y_i
Power	ln a	ln x_i	ln Y_i

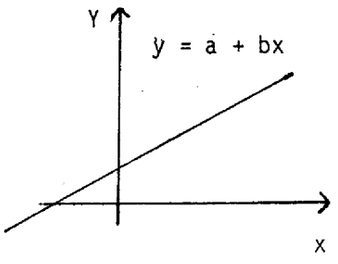
The coefficient of determination is :

$$R^2 = \frac{A \Sigma Y_i + B \Sigma Y_i Y_i - \frac{1}{n} (\Sigma Y_i)^2}{\Sigma(Y_i^2) - \frac{1}{2} (\Sigma Y_i)^2}$$

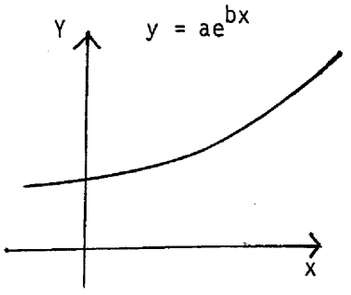
FROM
THE HEWLETT PACKARD HP-41C STAT PAC HANDBOOK
PORTABLE COMPUTER DIVISION, 1000 N.E. CIRCLE BLVD,
CORVALLIS, OR 97330, U.S.A.

to take extract from appendix 2

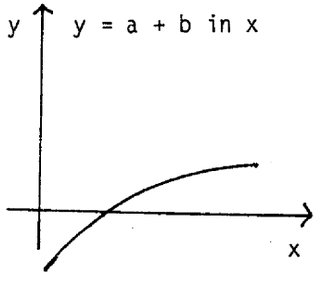
Linear Regression



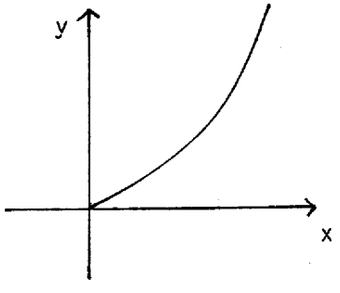
Exponential Curve Fit



Logarithmic Curve Fit



Power Curve Fit



Remarks:

- The programme applies the least square method, either to the original equations (straight line and logarithmic curve) or to the transformed equations (exponential curve and power curve).
- Negative and zero values of x_i will cause a machine error for logarithmic curve fits. Negative and zero values of y_i will cause a machine error for exponential curve fits. For power curve fits, both x_i and y_i must be positive, non-zero values.
- As the differences between x and/or y values become small, the accuracy of the regression coefficient will decrease.

SIZE: 016

STEP	INSTRUCTIONS	INPUT	FUNCTION	DISPLAY
1.	Initialize the programme. . for STRAIGHT LINE -->| or . for EXPONENTIAL CURVE -->| or . for LOGARITHMIC CURVE -->| or . for POWER CURVE -->|	XEQ ΣLIN XEQ ΣEXP XEQ ΣLOG XEQ ΣPOW	ΣLIN ΣEXP ΣLOG ΣPOW
2.	Repeat step 2-3 for 1, 2 ... n. Input x_i y_i	x_i y_i	ENTER 1 A	(i)
3.	If you made a mistake in inputting Xk and Yk_i then correct by	Xk Yk	ENTER 1 C	(k - 1)
4.	Calculate R^2 and regression coefficients a and b.		E R/S R/S	R2 = (R^2) a = (a) b = (b)
5.	Calculate estimated y from regression. Input x	x	R/S	Y = (y)
6.	Repeat step 5 for different x's			
7.	Repeat step 4 if you want the results again.			
8.	To use the same programme for another set of data, initialize the programme by -->| then go to step 2.		■ A	LIN or EXP or LOG or POW
9.	To use another programme, go to step 1.			

Example 1:

Fit the following set of data into a straight line

x_i	40.5	38.6	37.9	36.2	35.1	34.6
y_i	104.5	102	100	97.5	95.5	94

Solution:

$a = 33.53$, $b = 1.76$

$R^2 = 0.99$

i.e., $y = 33.53 + 1.76\,x$

For $x = 37$, $y = 98.65$

For $x = 35$, $y = 95.13$

Keystrokes:

				Display:
XEQ	ALPHA	SIZE	ALPHA	016
XEQ	ALPHA	ΣLIN	ALPHA	ΣLIN
40.5	ENTER ↑	104.5	A	
38.6	ENTER ↑	102	A	
37.9	ENTER ↑	100	A	
36.2	ENTER ↑	97.5	A	
35.2	ENTER ↑	95.5	A	
35.2	ENTER ↑	95.5	C	
35.1	ENTER ↑	95.5	A	
34.6	ENTER ↑	94	A	6.00
E				
R/S				R2 = 0.99
R/S				a = 33.53
37 R/S				b = 1.76
35 R/S				Y. = 98.65
				Y. = 95.13

Example 2:

Fit the following set of data into an exponential curve.

x_i	72	1.31	1.95	2.58	3.14
y_i	2.16	1.61	1.16	.85	0.5

Solution:

$a = 3.45$, $b = -0.58$

$y = 3.45\,e^{-0.58x}$

$R^2 = 0.98$

For $x = 1.5$, $\hat{y} = 1.44$

For $x = 2$, $\hat{y} = 1.08$

APPENDIX 3. ANALYSIS OF NONLINEAR DATA*

Example 1: Fitting a model defined in the pharmacokinetic library (single dose).

In this example a set of data was fitted to Model 13 in the pharmacokinetic library. Note that three constants, number of doses, dose and time of dosing had to be entered. (Note that the units for dose must be consistent with the concentration data.)

The pharmacokinetic library was stored on disk and referred to as file "ONE". Note that a listing of all statements read from the library are printed on the sample output. This is to enable the user to verify easily that the data were fitted to the desired model.

```
TITLE
EXAMPLE ONE - FITTING MODEL 13
MODEL NUM = 13, FILE = 'ONE'
CONSTANT:  dose = 2
INITIAL = -1, 8, 1.5, 13, .25
NOBS 15
DATA
.1  1.59
.25 2.81
.5  3.96
.75 4.6
1   4.93
1.5 5.03
2   4.78
2.5 4.39
3   3.98
4   3.2
5   2.55
6   2.03
8   1.28
12   .509
14   .321
BEGIN
FINISH
```

(output begins on next page)

*C.M. Metzler & D.L. Winer,
Statistical Consultant Inc.,
462, East High Streer,
Lexington, Kentucky 40508,
United States Of America

From the PCNONLIN Handbook

```
LISTING OF INPUT COMMANDS

TITLE
EXAMPLE ONE - FITTING MODEL 13
MODEL NUM = 13, FILE = 'ONE'
MODEL 13
REMARK TWO COMPARTMENT MODEL - FIRST ORDER INPUT AND OUTPUT
REMARK DEFINED IN TERMS OF A, B, ALPHA, BETA, K01
REMA
REMA      NO.      PARAMETER  CONSTANT         SECONDARY PARM
REMA      --       ---------  --------         --------------
REMA      1         A           DOSE           K10
REMA      2         B                          K12
REMA      3         K01                        K21
REMA      4         ALPHA                      AUC
REMA      5         BETA                       K10 HALF LIFE
REMA      6                                    K01 HALF LIFE
REMA      7                                    ALPHA HALF LIFE
REMA      8                                    BETA HALF LIFE
REMA      9                                    VOLUME
REMA*************************************************************
REMA              I---------------I
REMA              I               I
REMA      K01 --> I COMPARTMENT   I -------> K10
REMA              I               I
REMA              I---------------I
REMA                  I         I
REMA              K12 I         I K21
REMA                  I         I
REMA                  I         I
REMA              I---------------I
REMA              I               I
REMA              I COMPARTMENT 2 I
REMA              I               I
REMA              I---------------I
REMA*************************************************************
COMM
NPARM 5
NCON I
NSEC 9
PNAMES 'A', 'B', 'K01', 'ALPHA', 'BETA'
SNAMES 'K10', 'K12', 'K21', 'AUC', 'K10-HL', 'K01-HL', &
       'ALPHA-HL', 'BETA-HL', 'VOLUME'
END
TEMP
D=CON(1)
A=P(1)
B=P(2)
C=-1*(A+B)
```



```
K01=P(3)
ALPHA=P(4)
BETA=P(5)
T=X
D1=(A*(K01-ALPHA) + B*(K01-BETA))
V=K01*D/D1
D2=(A*(K01-ALPHA) + B*(K01-BETA))
K21=(A*BETA*K01 + B*ALPHA*K01 + C*ALPHA*BETA) / D2
K10=ALPHA*BETA/K21
K12=ALPHA+BETA-K21-K10
END
FUNC1
F=A*DEXP(-ALPHA*T) = B*DEXP(-BETA*T) + C*DEXP(-K01*T)
END
SECO
S(1)=K10
S(2)=K12
S(3)=K21
S(4)=D/V/K10
S(5)=-DLOF(.5)/K10
S(6)=-DLOG(.5)/K01
S(7)=-DLOG(.5)/ALPHA
S(8)=-DLOG(.5)/BETA
S(9)=V
END
EOM
CONSTANT: dose = 2
INITIAL = -1, 8, 1.5, 13, .25
NOBS 15
DATA
BEGIN


EXAMPLE ONE - FITTING MODEL 13
PCNONLIN NONLINEAR ESTIMATION PROGRAM

ITERATION WEIGHTED SS   A          B          K01        ALPHA
                        BETA

    0     .760294       -1.000     8.000      1.500      13.00
                         .2500
    1     .113536E-02   -1.011     7.999      1.572      13.60
                         .2274
    2     .442823E-04   -1.020     8.057      1.562      13.48
                         .2299
    3     .438646E-04   -1.020     8.058      1.562      13.48
                         .2299

CONVERGENCE ACHIEVED
RELATIVE CHANGE IN WEIGHTED SUM OF SQUARES LESS THAN  .000100
    3     .438646E-04   -1.020     8.058      1.562      13.48
                         .2299
```

EXAMPLE ONE - FITTING MODEL 13
PCNONLIN NONLINEAR ESTIMATION PROGRAM

PARAMETER	ESTIMATE	STANDARD ERROR	95% CONFIDENCE LIMITS		
A	-1.020010	.008612	-1.039198	-1.000821	UNIVARIATE
			-1.055433	-.984586	PLANAR
B	8.057619	.007397	8.041138	8.074101	UNIVARIATE
			8.027193	8.088046	PLANAR
K01	1.562430	.003494	1.554645	1.570216	UNIVARIATE
			1.548057	1.576803	PLANAR
ALPHA	13.477182	.179763	13.076644	13.877721	UNIVARIATE
			12.737742	14.216623	PLANAR
BETA	.229884	.000210	.229416	.230352	UNIVARIATE
			.229020	.230748	PLANAR

EXAMPLE ONE - FITTING MODEL 13
PCNONLIN NONLINEAR ESTIMATION PROGRAM

*** CORRELATION MATRIX OF THE ESTIMATES ***

1.00000				
-.57507	1.00000			
.86709	-.86712	1.00000		
.89197	-.42729	.69709	1.00000	
-.45297	.93995	-.73658	-.33149	1.00000

*** EIGENVALUES OF (A TRANSPOSE A) MATRIX ***

NUMBER	EIGENVALUE
1	1178.
2	10.04
3	.3995
4	.8019E-01
5	.1354E-03

EXAMPLE ONE - FITTING MODEL 13
PCNONLIN NONLINEAR ESTIMATION PROGRAM

*** SUMMARY OF NONLINEAR ESTIMATION ***

FUNCTION 1

X	OBSERVED Y	CALCULATED Y	RESIDUAL	WEIGHT	SD-YHAT	STANDARIZED RESIDUAL
.1000	1.590	1.590	.1596E-03	1.000	.2068E-02	.4816
.2500	2.810	2.811	-.5253E-03	1.000	.1781E.02	-.4770
.5000	3.960	3.959	.6666E-03	1.000	.1301E-02	.4062
.75000	4.6000	4.601	-.1242E-02	1.000	.1243E-02	-.7366
1.000	4.930	4.928	.2474E-02	1.000	.1263E-02	1.481
1.500	5.032	5.032	-.2115E-02	1.000	.1052E-02	-1.168
2.000	4.780	4.779	.1429E-02	1.000	.1033E-02	.7841
2.500	4.390	4.394	-.3787E-02	1.000	.1078E-02	-2.109
3.000	3.980	3.978	.1910E-02	1.000	.1048E-02	1.053
4.000	3.200	3.199	.9821E-03	1.000	.9176E-03	.5217
5.000	2.550	2.550	.2756E-04	1.000	.9303E-03	.1469E-01
6.000	2.030	2.028	.2059E-02	1.000	.1029E-02	1.129
8.00	1.280	1.281	-.8542E-03	1.000	.1120E-02	-.4826
12.00	.5090	.5107	-.1692E-02	1.000	.8597E-03	-.8862
14.00	.3210	.3225	-.1467E-02	1.000	.6760E-03	-.7399

CORRECTED SUM OF SQUARED OBSERVATIONS = 36.8750
WEIGHTED CORRECTED SUM OF SQUARED OBSERVATIONS = 36.8750
SUM OF SQUARED RESIDUALS = .438646E-04
SUM OF WEIGHTED SQUARED RESIDUALS = .438646E-04
S = .209439E-02 WITH 10 DEGREES OF FREEDOM
CORRELATION (Y,YHAT) = 1.00

EXAMPLE ONE - FITTING MODEL 13
PCNONLIN NONLINEAR ESTIMATION PROGRAM

SUMMARY OF ESTIMATED SECONDARY PARAMETERS

PARAMETER	ESTIMATE	STANDARD ERROR
K10	.480802	.002062
K12	6.782475	.114426
K21	6.443790	.067057
AUC	30.470879	.013348
K10-HL	1.441648	.006179
K01-HL	.443634	.000991
ALPHA-HL	.051431	.000685
BETA-HL	3.015207	.002752
VOLUME	.136515	.000576

LISTING OF INPUT COMMANDS

FINISH

NORMAL ENDING

APPENDIX 4. ASSAY METHODS FOR SOME ANTIMALARIAL COMPOUNDS

Developed or practised at the
National Drug Research Centre,
Universiti Sains Malaysia,
Penang, Malaysia.

1. QUININE/QUINIDINE (high-performance liquid chromatography-HPLC method)

Introduction

Quinine is the oldest known pure antimalarial drug and still remains the only consistently effective drug for the emergency treatment of severe and complicated falciparum malaria.

Quinidine was rediscovered only a few years ago as a potential antimalarial compound and its efficacy *in vivo* and *in vitro* against *Plasmodium falciparum* has been shown to be significantly higher than that of quinine. Quinidine has a spectrum of side-effects similar to that of quinine, but it is more expensive and more likely to cause cardiac effects such as arrhythmias and hyper sensitivity reactions. It is therefore not recommended as an alternative to quinine unless quinine is not available.

Objectives

(1) Development of an analytical method for quinine and quinidine in plasma.

(2) To use the analytical method developed to analyse data obtained from a kinetic study with different formulations of quinine and quinidine.

Structure

The structures of quinine and qunidine are shown in Fig. 1.

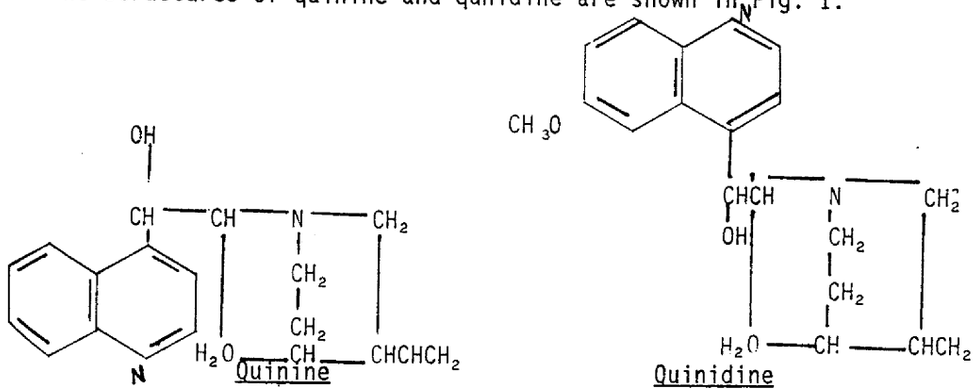


Fig 1. Structure of quinine and quinidine

Extraction procedure

The extraction procedure used is given in Fig. 2.

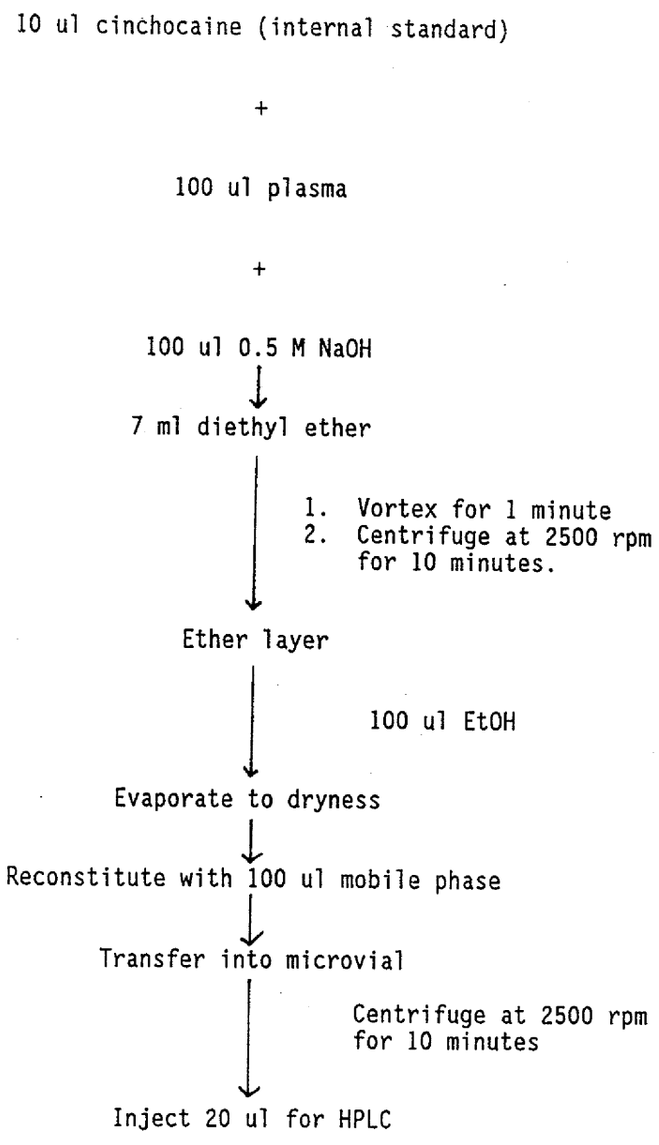


Fig. 2 Extraction procedure

HPLC conditions

Detector	:	Fluorometer with excitation at 309 - 395nM and emission at 430 - 470nM
Column	:	Novapak Alkyl Phenyl 150 mm x 3.9 mm, 5 um particle size
Mobile phase	:	Phosphate buffer (pH 3.00): methanol (45 : 55)
Flow rate	:	1.0 ml/minute
Injection volume	:	20 ul
Chart-speed	:	0.2 cm/minute

The quinine and quinidine analyses were not done simultaneously as the HPLC conditions used did not resolve the quinine and quinidine peaks.

Detector linearity

The detector response of quinine/quinidine over the range 2 ng to 800 ng was linear.

Standard calibration curve

It was found that a linear correlation existed within the range 0.25 to 8 ug/ml with a coefficient of determination of 0.994 and 0.999 for quinine and quinidine, respectively.

Sensitivity

Starting from a 100 ul ssample the minimum detection limit is 40 ng/ml for both quinine and quinidine.

Recovery of quinine in spiked plasma samples

Concentration (ug/ml)	Replicates (N)	% recovery	CV% reproducibility
0.50	3	104.7	5.29
1.00	3	97.9	2.15
2.00	3	101.7	5.85
4.00	3	93.1	5.34
8.00	3	99.4	5.27

N = number
CV = coefficient of variance

Within day precision of assay of quinine in spiked plasma samples

Concentration (ug/ml)	Replicates (N)	Mean concentration determined (ug/ml)	CV% reproducibility
0.50	3	0.49	5.17
1.00	3	0.98	5.10
2.00	3	1.98	4.13
4.00	3	3.99	1.38
8.00	3	7.96	1.20

N = number
CV = coefficient of variance

Day to day precision of assay of quinine in spiked plasma samples.

Concentration (ug/ml)	Replicates (N)	Mean concentration determined (ug/ml)	CV% reproducibility
0.25	6	0.24	4.46
2.00	6	2.02	4.74
8.00	6	7.99	1.31

N = number
CV = coefficient of variance

Recovery of quinidine in spiked plasma samples

Concentration (ug/ml)	Replicates (N)	% recovery	CV% reproducibility
0.50	3	98.6	1.39
1.00	3	91.6	5.73
2.00	3	93.7	2.96
4.00	3	95.9	6.28
8.00	3	103.5	3.39

N = number
CV = coefficient of variance

Within day precision of assay of quinidine in spiked plasma samples.

Concentration (ug/ml)	Replicates (N)	Mean concentration determined (ug/ml)	CV% reproducibility
0.50	3	0.50	4.99
1.00	3	1.03	5.91
2.00	3	1.98	4.63
4.00	3	3.86	3.00
8.00	3	8.02	3.40

N = number
CV = coefficient of variance

Day to day precision of assay of quinidine in spiked plasma samples.

Concentration (ug/ml)	Replicates (N)	Mean concentration determined (ug/ml)	CV% reproducibility
0.25	6	0.24	5.54
2.00	6	1.99	2.87
8.00	6	7.99	1.46

N = number
CV = coefficient of variance

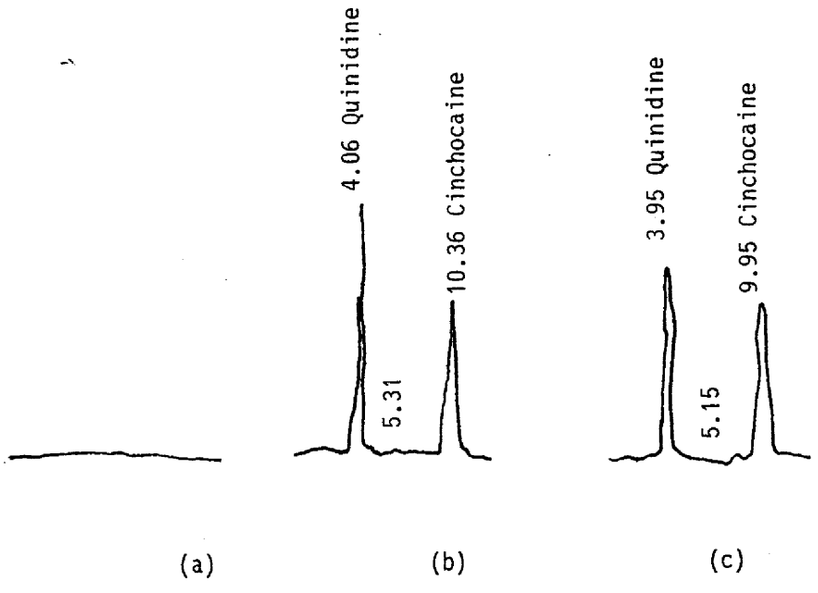


Fig. 3 Chromatograms of :

(a) Blank diethyl ether extract of plasma

(b) Aqueous standards of quinine and cinchocaine

(c) Diethyl ether extract of quinine and cinchocaine

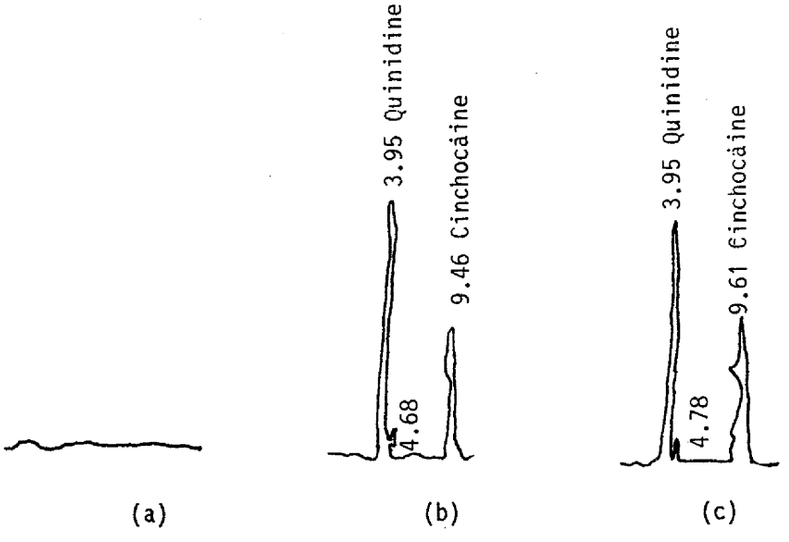


Fig. 4. Chromatograms of:

(a) Blank diethyl ether extract of plasma

(b) Aqueous standards of quinidine and cinchocaine

(c) Diethyl ether extract of quinidine and cinchocaine

Conclusion of a comparative study of quinine and two quinidine formulations

The rate of absorption of quinine or quinidine from both capsule formulations were rapid (see Fig. 5).

The quinidine "retard" tablet appears to produce a steady level of quinidine from 2 to 9 hours and then follow a steady decline after 9 hours. The rate of absorption of quinidine was also slower than that from quinidine capsules.

The study showed that quinidine "retard" was successful in sustaining the levels of quinidine for 9 hours. This suggests that dosing every 12 hours will be adequate for therapy.

The capsules with quinine or quinidine require dosing every 8 hours when quinine hydrochloride is administered and every 6 hours when quinidine sulfate is given since the release of quinine or quinidine is rapid.

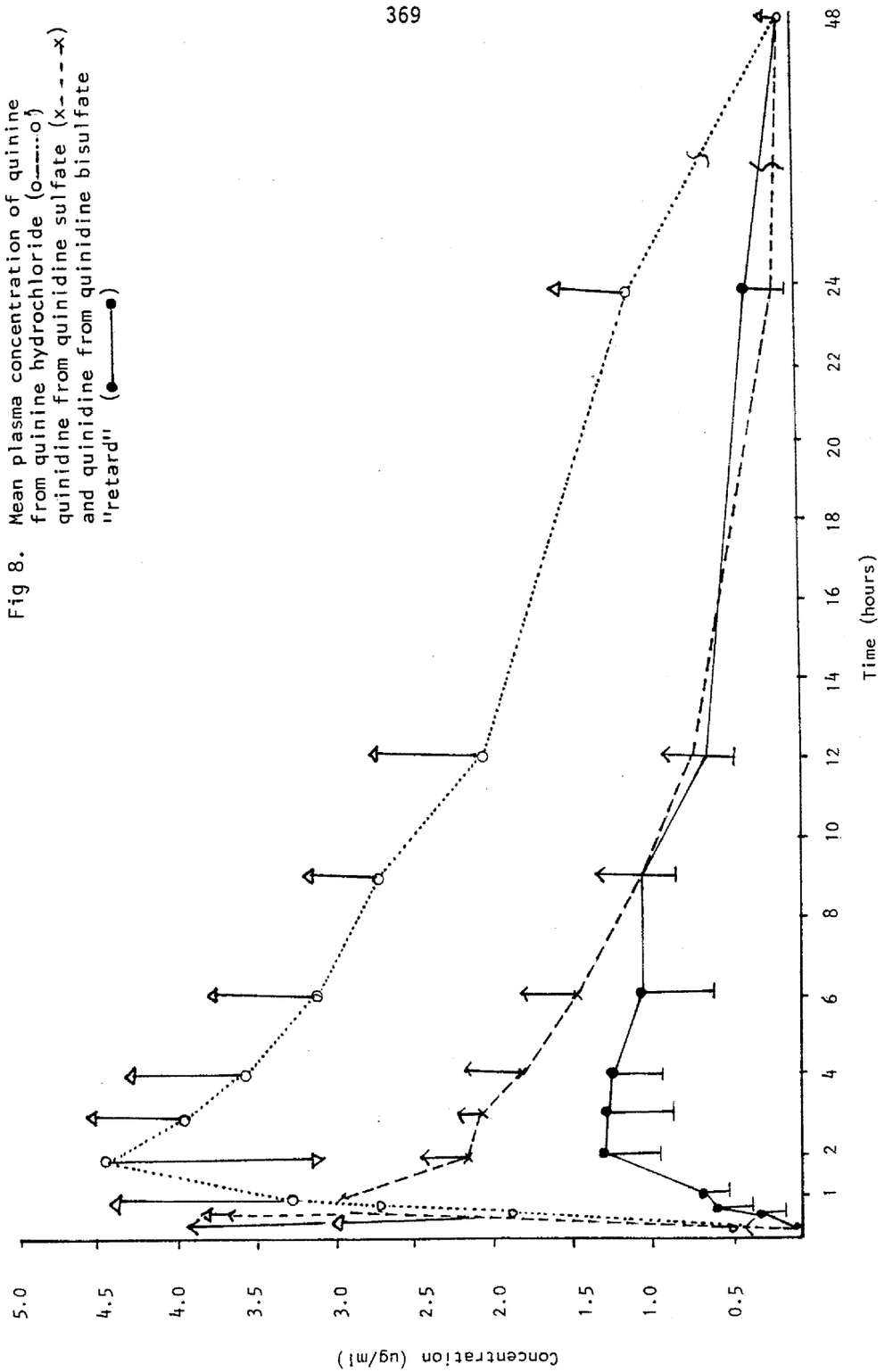


Fig 8. Mean plasma concentration of quinine from quinine hydrochloride (o—o) quinidine from quinidine sulfate (x- - -x) and quinidine from quinidine bisulfate "retard" (●——●)

2. MEFLOQUINE (gas chromatography-electron capture detection-GC-ECD- method)

Introduction

Mefloquine is a 4-quinoline methanol derivative and its hydrochloride salt is a white, odourless and slightly bitter tasting powder.

In its un-ionized form, mefloquine is a weak base and is slightly soluble in water, sparingly soluble in dichloromethane but more soluble in ethyl acetate and ethanol.

Objective

To operationalize the GC-ECD analytical method of mefloquine in plasma.

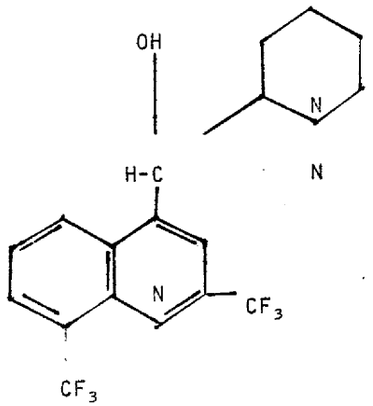


Fig. 6. Structure of mefloquine

Experimental

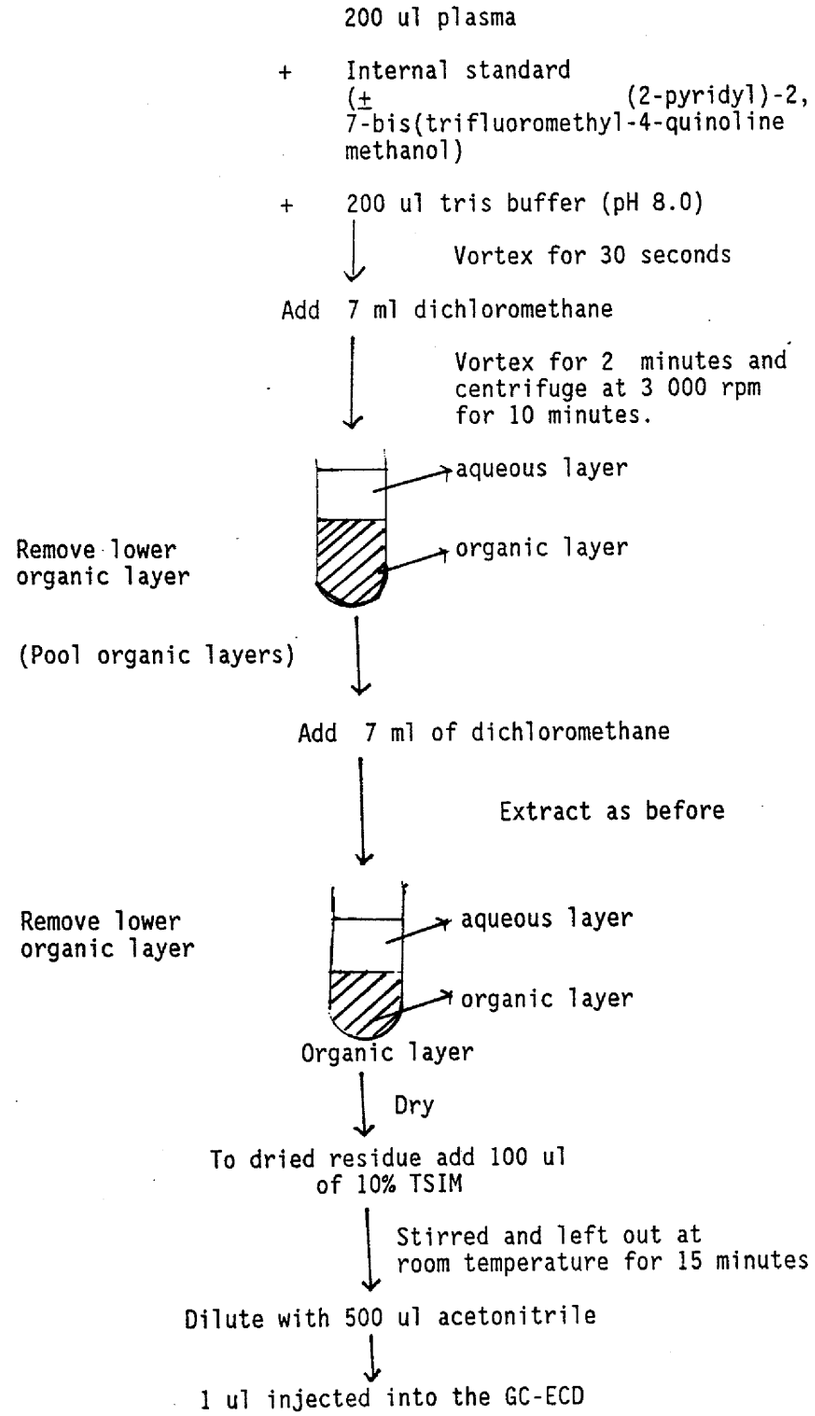


Fig. 7. Extraction procedure

GC-ECD conditions

Detector	:	Electon-capture detector (ECD) Ni^{63}
Column	:	3% Ov-17 on 100/120 Chromosorb 6' x 1/4" x 2 mm
Detector temperature	:	320°C
Injector Temperature	:	280°C
Column oven temperature	:	185°C
Programme rate	:	8°C/minute
Carrier gas flow rate	:	Nitrogen at 25 ml/minute
Initial time	:	4 minutes
Final time	:	4 minutes
Equilibrium time	:	3 minutes
Run time	:	10.50 minutes

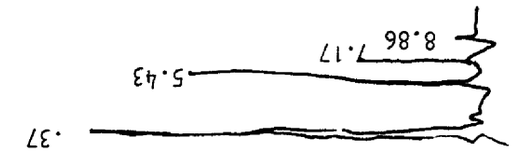


Fig. 10. Dichloromethaneextract of plasma containing 500 ng internal standard and 500 ng mefloquine

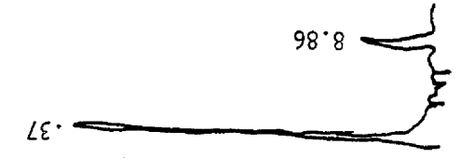


Fig. 9. Dichloromethane extract of plasma only

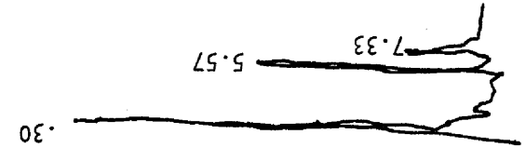


Fig. 8 Solution 500 ng of internal standard and 500 ng of standard

5.57 - Internal Standard
7.33 - Mefloquine

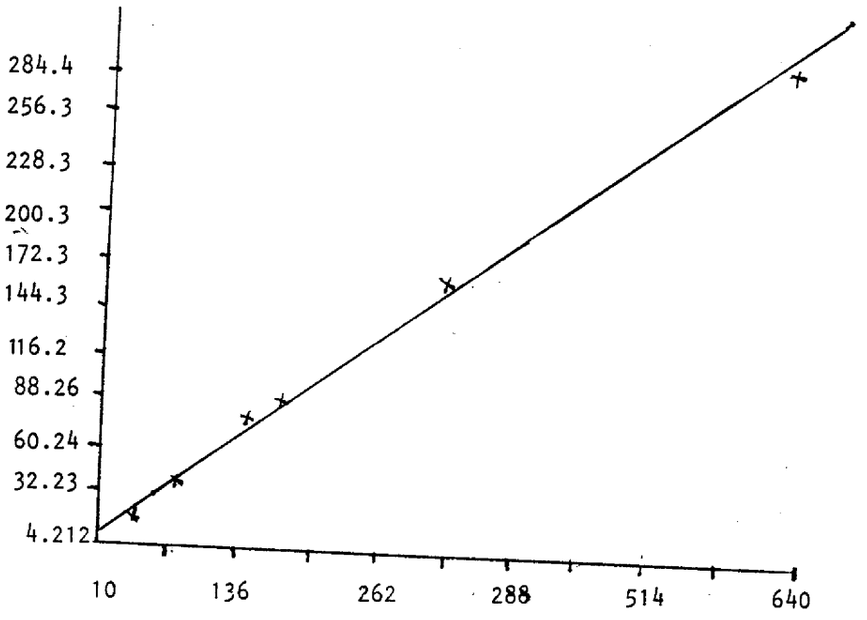


Fig. 11. Detector linearity of mefloquine.

3. Proguanil/cycloguanil (HPLC)

Introduction

Proguanil hydrochloride (Paludrin^{R}) has been used in several parts of the world as a prophylactic drug against malaria.

It is usually administered in one daily dose of 100 mg or in certain places, 200 mg, most commonly in combination with chloroquine.

Proguanil is metabolized principally to cycloguanil and it is this metabolite rather than proguanil that possesses the prophylactic activity.

Since proguanil is administered on a daily basis over a long period of time and since metabolism plays an important role in producing the active compound, a multiple dose pharmacokinetic study was conducted.

Objective

Use of a sensitive and specific analytical method for measuring proguanil and cycloguanil in plasma samples obtained from the multiple dose study.

Structure

The structures of proguanil and cycloguanil are shown in Fig. 12.

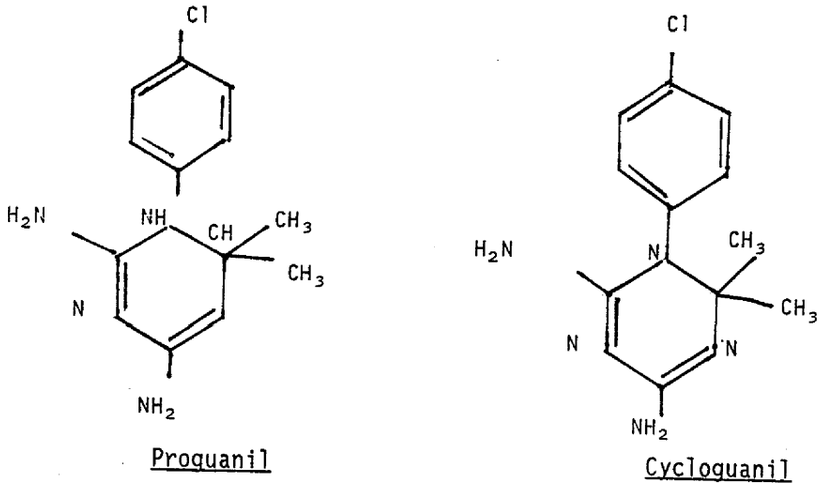
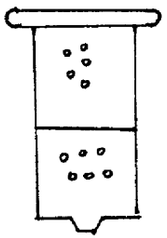


Fig. 12. Structure of proguanil and cycloguanil

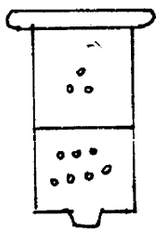
Solid phase extraction procedure

This procedure is shown, step by step, in Fig. 13.

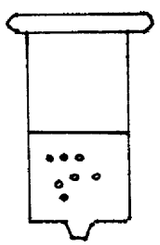
Step 1 Column conditioning (1) 1 ml methanol
(2) 1 ml water
(3) 1 ml buffer pH 8.00



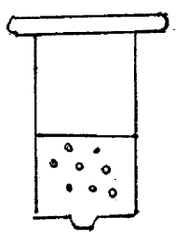
Step 2 Pass sample through



Step 3 Wash with 3 ml water

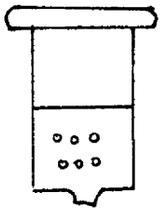


Step 4 Wash with 125 ul acetonitrile

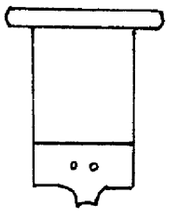


Step 5 Wash with 1 ml DEA: acetic acid in water

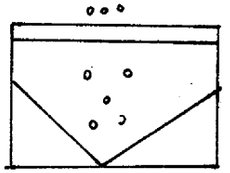
Fig. 13. Solid phase extraction procedure



Step 6 Centrifuge for a few minutes



Step 7 Collect with 1 ml methanol
Add 3 drops of DEA: acetic acid in methanol



Step 8 Dry under gentle stream of nitrogen gas at 60°C.

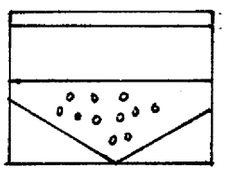


Fig. 13. Solid phase extraction procedure

HPLC conditions

Detector	:	UV detector at 248 nm
Column	:	Lichrospher 250 - 4 mm, 5 um particle size
Mobile phase	:	1% TEA : acetonitrile (76 : 24)
Flow rate	:	1.5 ml/minutes
Injection volume	:	50 ul

Recoveries of Proguanil in spiked plasma samples

Concentration (ng/ml)	% recovery	CV % reproducibility
50	72.5	6.83
100	82.0	3.95
200	76.0	1.86
400	73.5	6.73

CV = coefficient of variance

Recoveries of cycloguanil in spiked plasma samples

Concentration (ng/ml)	% recovery	CV % reproducibility
50	87	11.4
100	95	0.75
200	89	6.35
400	90.5	7.03

CV = coefficient of variance

Within day precision of the assay of proguanil in plasma

Concentration ng/ml	Replicates (N)	Mean concentration determined ng/ml	CV% reproducibility
50	3	54.4	5.4
100	3	110.1	2.8
200	3	220.7	5.4

N = number
CV = coefficient of variance

Day to day precision of the assay of proguanil in plasma

Concentration (ng/ml)	Replicates (N)	Mean concentration determined (ng/ml)	CV% reproducibility
50	5	42.8	6.23
100	5	98.3	6.69
200	5	191.1	3.34

N = number
CV = coefficient of variance

Within day precision of the assay of cycloguanil in plasma

Concentration (ng/ml)	Replicates (N)	Mean concentration determined (ng/ml)	CV% reproducibillity
50	3	48.9	7.42
100	3	96.5	2.60
200	3	193.5	5.51

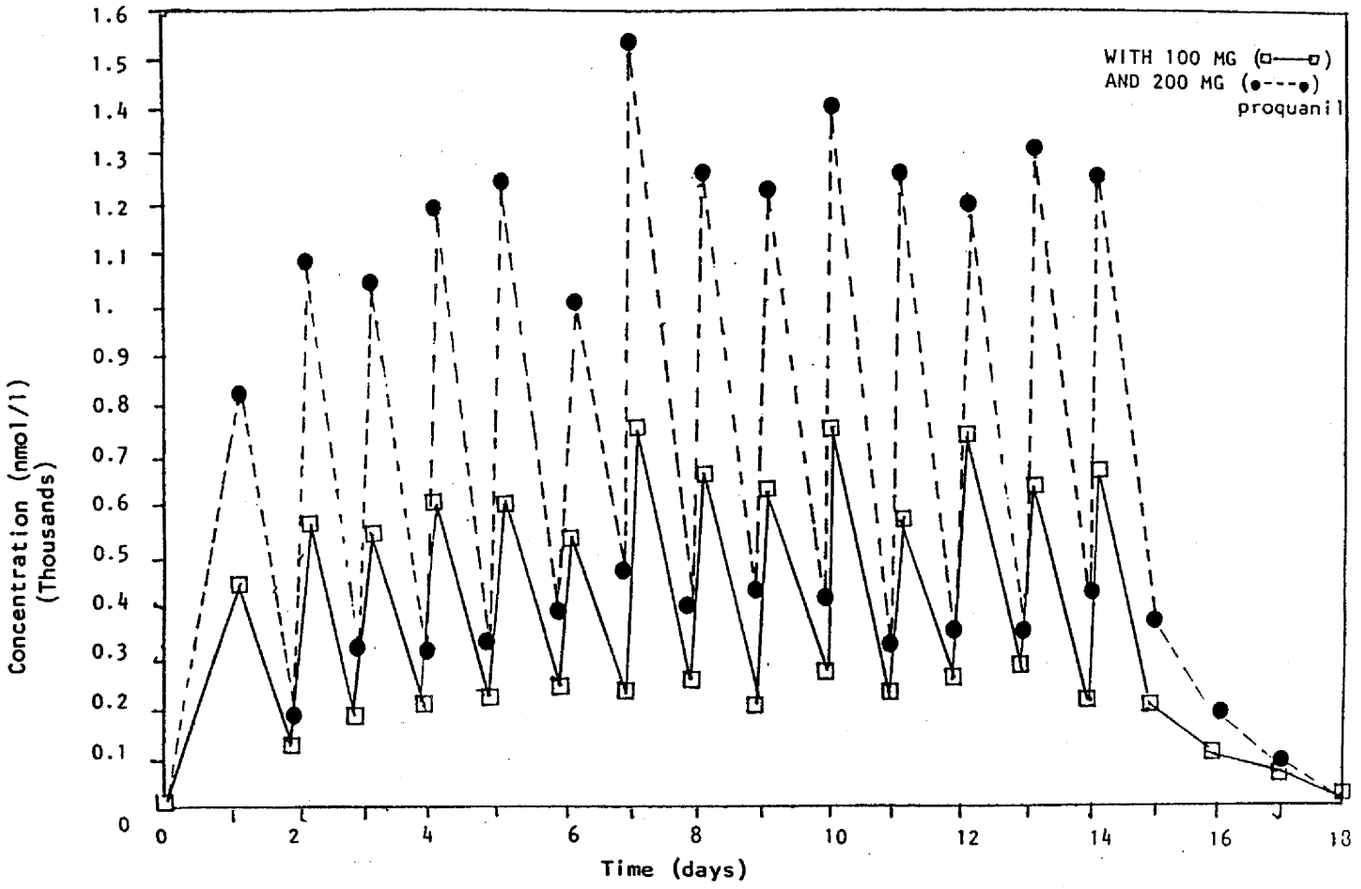
N = number
CV = coefficient of variance

Day to day precision of the assay of cycloguanil in plasma

Concentration (ng/ml)	Replicates (N)	Mean Concentration Determined (ng/ml)	CV% reproducibility
50	5	51.7	4.68
100	5	101.7	8.38
200	5	198.5	3.50

N = number
CV = coefficient of variance

Fig. 14. Proguanil plasma concentrations (nmol l^{-1}) obtained with daily oral dose of 100 mg and 200 mg proguanil hydrochloride in adult male volunteers



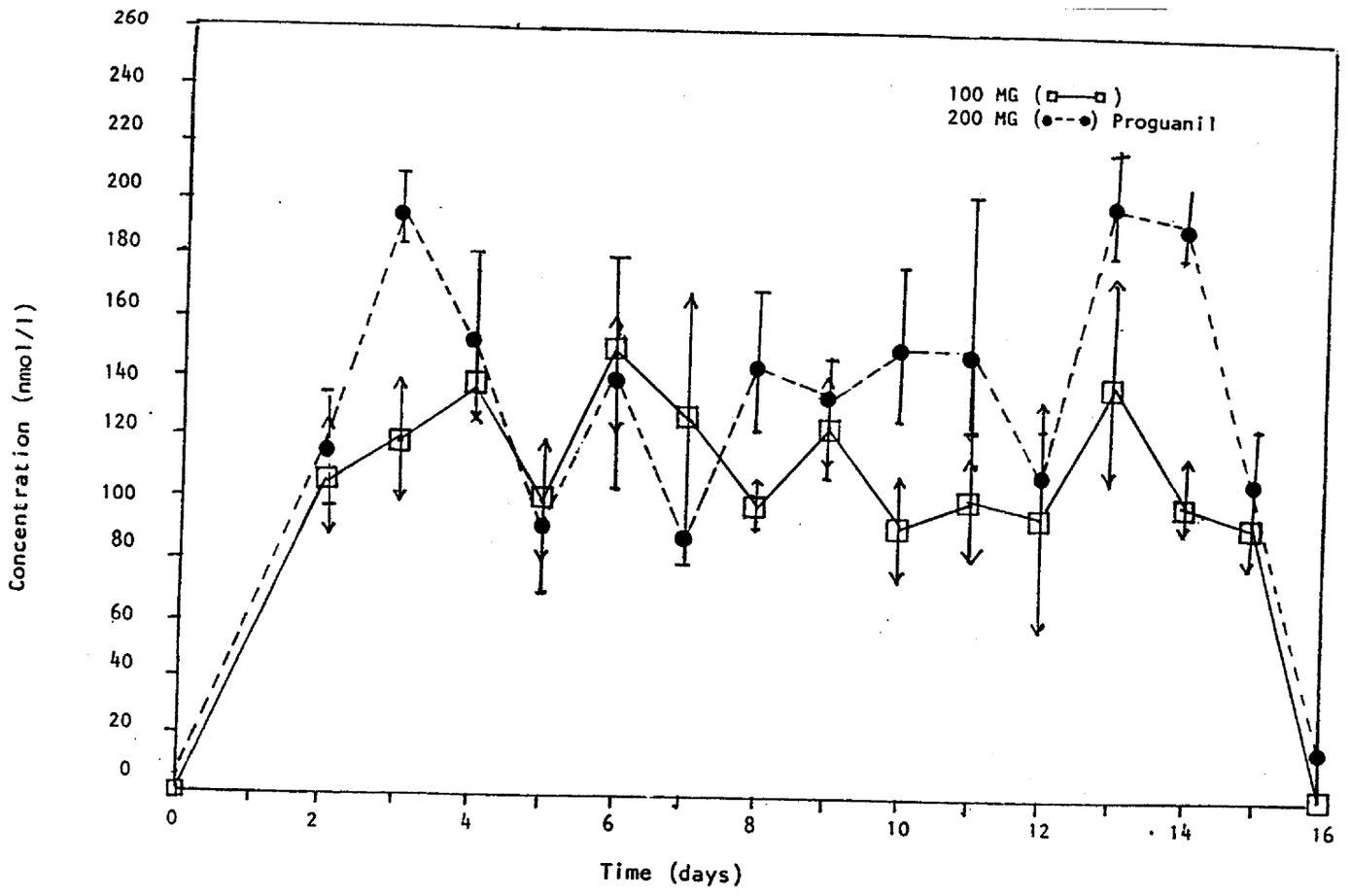


Fig. 15. Cycloquanil plasma concentrations (nmol l^{-1}) obtained with daily oral doses of 100 mg and 200 mg proguanil hydrochloride in adult male volunteers.

Results

With both cycloguanil and proguanil, there is greater fluctuation between peak and trough levels at the higher dose of 200 mg compared to that of 100 mg (see Fig. 14 and 15)

Since cycloguanil is the active species, it is important to compare the trough levels of cycloguanil at both doses. The mean trough levels of cycloguanil at 100 mg and 200 mg proguanil do not differ significantly.

Conclusion

To reduce fluctuations between peak and trough levels of both proguanil and cycloguanil, it would probably be better to use 12 hourly dosing with 100 mg proguanil. This would also raise the trough levels and ascertain higher efficacy.

4. HALOFANTRINE (HPLC)

Introduction

Halofantrine, a 9-phenanthrenemethanol with a dialkylaminopropanol side chain, is a new antimalarial drug. It chemical structure is shown in Fig. 16.

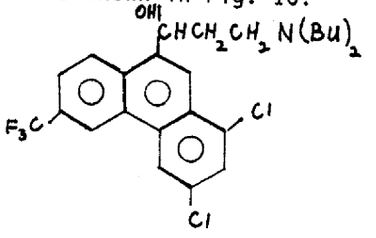


Fig. 16 Structure of halofantrine

Objective

To develop a sensitive and specific method for the detection of halofantrine in plasma.

Extraction procedure

The extraction procedure is shown in Fig. 17.

1 ml plasma + 1000 ng halofantrine + 1 ml buffer

1. Vortex for 1 minute.
2. On addition of 250 ul Na_2SO_4 solution (saturated), vortex for 30 seconds
3. Add 5 ml hexane :CH_2Cl_2 (5:4)
4. Vortex for 3 minutes (moderate speed).

Centrifuge
(at 2,500 rpm, 10 minutes)

Aqueous phase

Organic layer

Dry under a stream of nitrogen gas at room temperature

Residue

1. Reconstitute in 1000 ul of methyl alcohol.
2. A volume of 20 ul is injected

HPLC

Fig. 17. Extraction procedure (experimental)

Instrument and chromatographic conditions [experimental]

Instrument	-	Liquid chromatograph with binary pumps (Model 510, Waters Associates).
Detector	-	Fixed wavelength UV absorbance detector (Model 440, Waters), set at 254 nm.
Column	-	Rp-18 (250 x 4.6 mm, partisil 10 ODS-3 Whatman).
Mobile phase	-	MeOH-O, 1M CH_3COONH_4 at pH4 (75:25).
Flow	-	2ml/minute.
Column pressure	-	2,600psi.

Results

(1) Quantitation

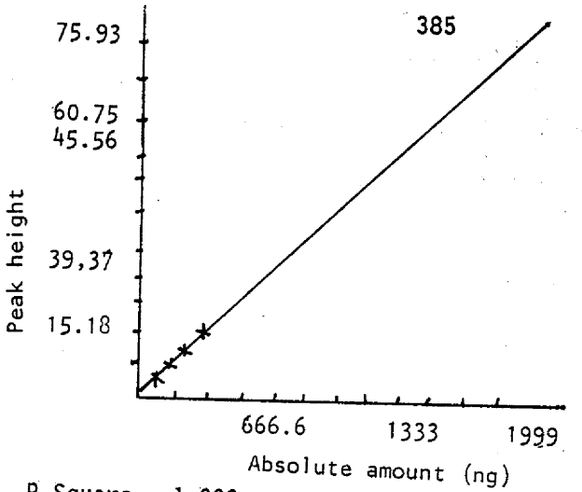
Due to unavailability of an internal standard, a bracketing method was used, whereby a fixed amount of standard (i.e. halofantrine) was injected after every injection of a spiked sample. The ratio of the peak height of halofantrine in the spiked sample to the peak height of the standard was then calculated.

Calibration curves were prepared by analysing replicate samples of plasma spiked with halofantrine at 5 concentration levels over the range of 25-1000 ng/ml. Quadruplicate samples were analysed at the two lowest concentrations (25 and 50 ng/ml) and triplicate samples were analysed at the higher concentrations (100, 500 and 1000 ng/ml).

(2) Detector linearity

On column concentration	Average peak height	Standard Deviation	CV %
2000 ng	75.9376×10^4	$\pm$ 4005.80	0.53
1000 ng	38.6225×10^4	$\pm$ 4557.30	1.18
200 ng	6.4535×10^4	$\pm$ 197.28	0.31
100 ng	3.6045×10^4	$\pm$ 303.35	0.84
50 ng	1.8081×10^4	$\pm$ 289.36	4.43
7.5 ng	0.2989×10^4	$\pm$ 22.60	0.76
5 ng	0.1774×10^4	$\pm$ 43.33	2.44

CV = coefficient of variance



R Square = 1,000

Fig. 18. Detector linearity of peak height with different amounts of halofantrine.

(3) Standard calibration curve

The peak height ratios at various concentrations are tabulated below:

N Replicates	Spiked amount (ng/ml)	On column concentration (ng)	Average peak height ratio	Standard deviation	CV (%)
3	1000	200	2.8032	0.1106	3.95
3	500	100	1.5941	0.0348	2.18
3	100	20	0.3175	3.41×10^{-3}	1.075
4	50	10	0.1258	3.17×10^{-3}	2.52
4	25	5	0.0685	3.35×10^{-3}	4.89

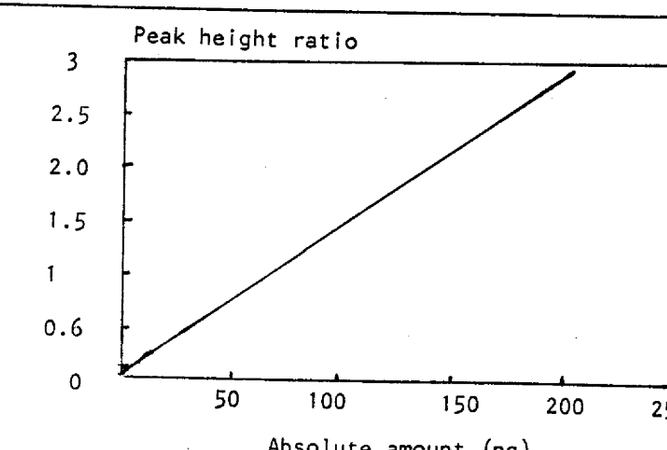


Fig. 19. A Plot of peak height ratios with different amounts of halofantrine

Within day precision of the analysis

Column Concentration (ng)	Mean column concentration determined (ng)	Replicates (N)	Standard deviation	CV (%)
200	194.37	3	7.72	3.97
20	20.89	3	0.24	1.13
5	3.51	4	0.24	6.69

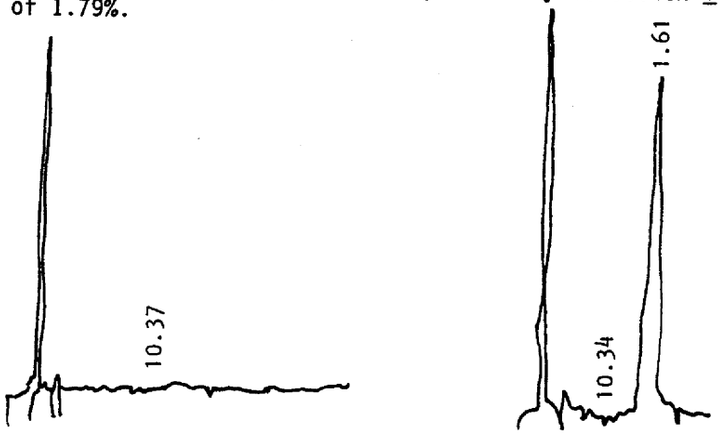
N = number
CV = coefficient of variance

Recovery

Replicates (N)	Spiked amount (ng/ml)	Column concentration (ng)	Average recovery (%)	Standard deviation	CV %
3	1000	200	92.59	±3.31	3.58
3	500	100	92.50	±2.06	2.23
3	100	20	92.08	±0.06	0.06
4	50	10	88.52	±1.06	1.19
4	25	5	86.56	±1.63	1.89

N = number
CV = coefficient of variance

The overall average within day recovery is 90.45% ± 2.75 with a CV of 1.79%.



[A] Blank plasma [B] Sppiked plasma (1000 ng/ml)

Fig. 20. Chromatogram of blank plasma (A) and plasma spiked with halofantrine (B)

THE CHROMATOGRAM OF A SPIKED PLASMA SAMPLE

Chromatographic conditions

Mobile phase	-	MeOH-O, $IMCH_3COONH_4$ (pH 4)
Composition	-	75 : 25
Flow	-	2 ml/minutes
Attenuation	-	2 $\hat{1}$ = 3
Threshold	-	2

Retention time of halofantrine = 11.61 minutes

On column concentration = 200 ng

Conclusion

The extraction procedure developed is simple and rapid.

The HPLC method allows quantitation of halofantrine in plasma at therapeutic levels and will be very useful in future pharmacokinetic studies. The lowest limit of sensitivity in plasma is 25 ng/ml. The on column concentration detected in plasma is 5 ng.

5. PYRONARIDINE (HPLC)

Introduction

Pyronaridine is an antimalarial candidate drug originally developed in the People's Republic of China.. In vitro tests have shown that it possesses high activity against both chloroquine-resistant and -sensitive strains of Plasmodium falciparum. There is also clinical evidence to this fact. The chemical structure of pyronaridine is shown in Fig. 21.

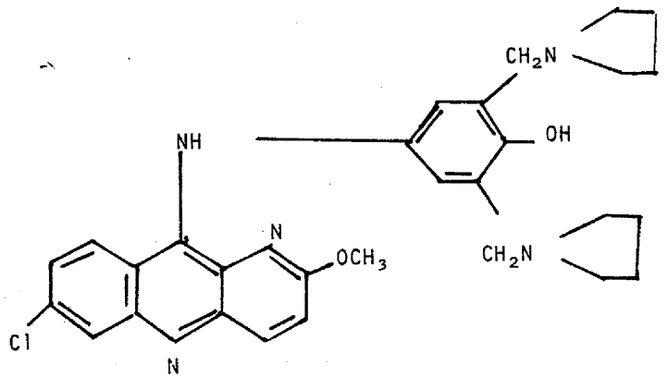


Fig. 21 Structure of pyronaridine

Objective

To develop a sensitive and specific analytical method for the analysis of pyronaridine in plasma.

Sample preparation

200 ul plasma sample containing pyronaridine

\+ 250 ng chloroquine diphosphate as internal standard

\+ 500 ul of buffer, pH 9 + 3 ml diethyl ether

↓

The mixture is vortexed for 2 minutes

↓

Centrifuge for 10 minutes at 2500 rpm

↓

Organic layer is aspirated and dried under nitrogen gas

↓

Residue is reconstituted in 100 ul mobile phase

↓

50 ul aliquot is injected into HPLC

Analytical method

A HPLC-UV detector method was developed for measuring pyronaridine in plasma.

Chromatographic conditions

Mobile phase	85% of 0.08M KH_2PO4 and 1% TEA 15% acetonitrile
pH of mobile phase	3
Flow Rate	1.5 ml/minute
Column	Whatman Partisil 10 ODS-3 250 x 4.8 mm
Maximum	278nm

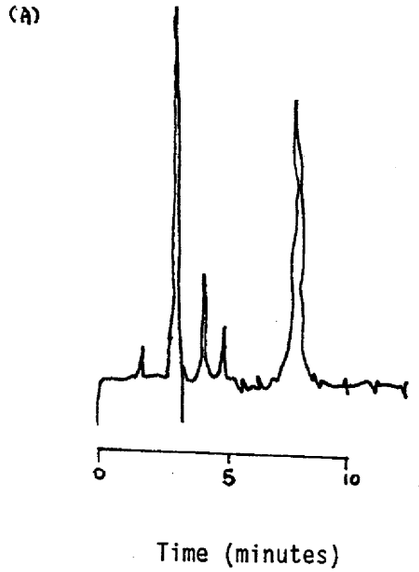


Fig. 22. Representative chromatograms of: (A) an extracted blank plasma; (B) an extracted plasma containing (i) pyronaridine and (ii) chloroquine.

Detector linearity

Concentration of Pyronaridine (ng)	Peak height (units)
5	1 569.33
10	3 781.00
25	8 840.00
50	18 609.00
100	40 516.00
200	79 379.00
400	173 902.00
800	369 606.00
1 000	467 098.00

R square = 0.999

Y = -547.403 + 468.177 x

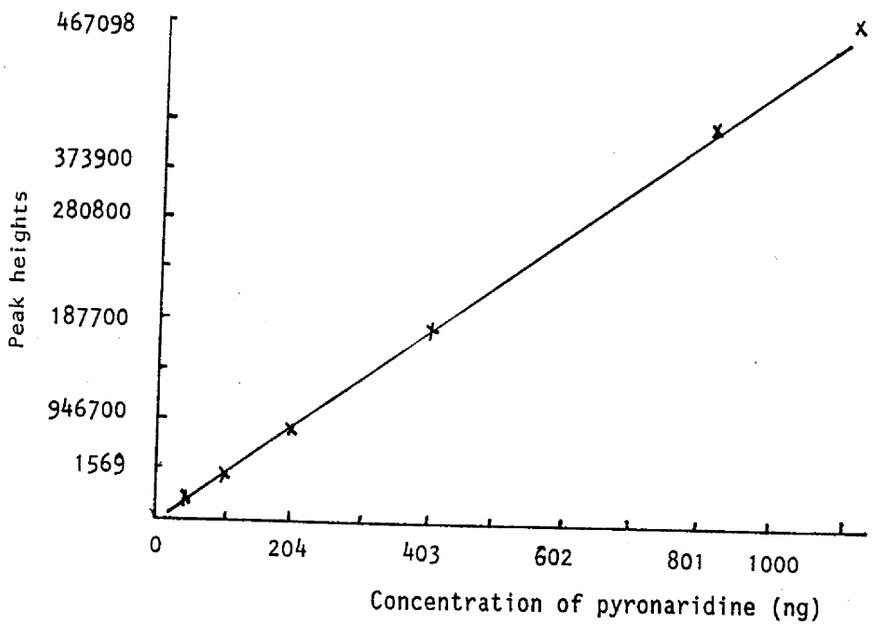


Fig. 23 Detector linearity for pyronaridine tetraphosphate.

Standard Calibration Curve (High Concentration Range)

Concentration of Pyronaridine, ng/200 ul plasma	Ratio of Spike to internal standard	Recovery, % [Average]	Coefficient of Variance (%)
100	0.954 ± 0.037	86.30	4.3
200	1.948 ± 0.074	85.24	3.8
400	4.059 ± 0.190	84.5	3.4
800	9.134 ± 0.142	88.16	3.4
1000	10.771 ± 0.283	85.90	3.0

R Square = 0.997

Y = -0.259 + 0.011 x

N = 4 at each concentration

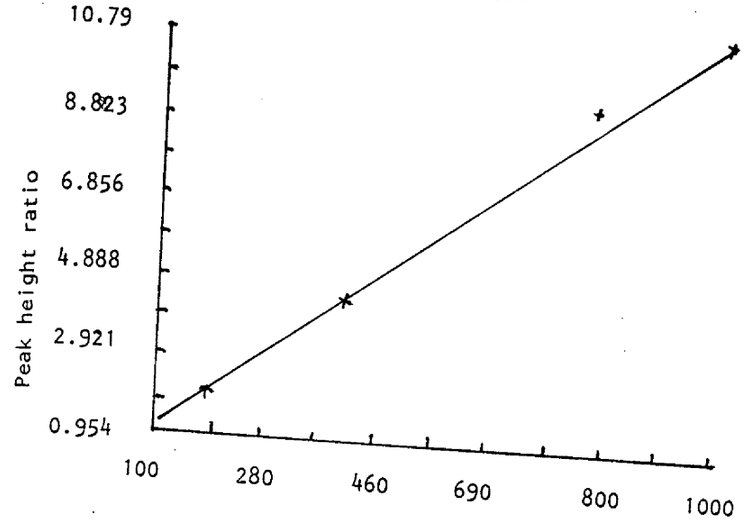


Fig. 24. Calibration curve for pyronaridine tetraphosphate (high concentration range)

Standard calibration curve (low concentration range)

Concentration of pyronaridine, ng/200 ul plasma	Ratio of spike to internal standard	(%) recovery [average]	Coefficient of variance (%)
14	0.1087 ± 0.0090	87.1	9.3
25	0.2147 ± 0.0209	84.7	10.4
50	0.4860 ± 0.0047	83.0	4.0
100	0.9390 ± 0.0260	85.8	1.1

R square = 0.998

Y = -0.021 + 0.010 x

N = 4 at each concentration

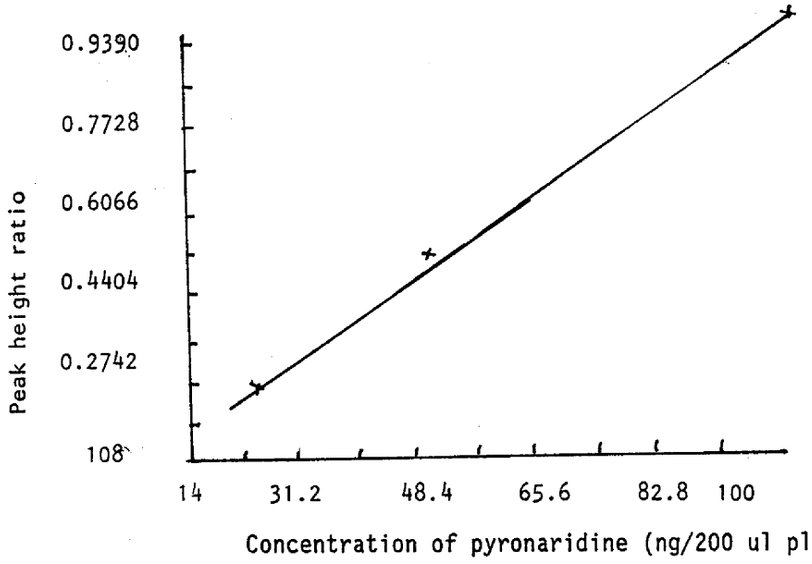


Fig. 25. Calibration curve for pyronaridine tetraphosphate [low concentration range]

Detection limit

The detection limit of the method is 14 ng/200 ul plasma or 70 ng/1 ml plasma.

Conclusion

An accurate, reproducible and precise reversed-phase HPLC-UV detection assay has been developed for the determination of pyronaridine in plasma. The detection limit of this method is 14ng/200ul plasma. A possible way to improve the sensitivity of the method is to use a fluorescence detector because pyronaridine possesses fluorescent properties.

APPENDIX 5. RANDOM NUMBERS*

03 47 43 73 86	36 96 47 36 61	46 98 63 71 52	33 26 16 80 45	60 11 14 10 95					
97 74 24 67 62	42 81 14 57 20	42 53 32 37 32	27 07 36 07 51	24 51 79 89 73					
16 76 62 27 66	56 50 26 71 07	32 90 79 78 53	13 55 38 58 59	88 97 54 14 10					
12 56 85 99 26	96 96 68 27 31	05 03 72 93 15	57 12 10 14 21	88 26 49 81 76					
55 59 56 35 64	38 54 82 46 22	31 62 43 09 90	06 18 44 32 53	23 83 01 30 30					
16 22 77 94 39	49 54 43 54 82	17 37 93 23 78	87 35 20 96 43	84 26 34 91 64					
84 42 17 53 31	57 24 55 06 88	77 04 74 47 67	21 76 33 50 25	83 92 12 06 76					
63 01 63 78 59	16 95 55 67 19	98 10 50 71 75	12 86 73 58 07	44 39 52 38 79					
33 21 12 34 29	78 64 56 07 82	52 42 07 44 38	15 51 00 13 42	99 66 02 79 54					
57 60 86 32 44	09 47 27 96 54	49 17 46 09 62	90 52 84 77 27	08 02 73 43 28					
18 18 07 92 46	44 17 16 58 09	79 83 86 19 62	06 76 50 03 10	55 23 64 05 05					
26 62 38 97 75	84 16 07 44 99	83 11 46 32 24	20 14 85 88 45	10 93 72 88 71					
23 42 40 64 74	82 97 77 77 81	07 45 32 14 08	32 98 94 07 72	93 85 79 10 75					
52 36 28 19 95	50 92 26 11 97	00 56 76 31 38	80 22 02 53 53	86 60 42 04 53					
37 85 94 35 12	83 39 50 08 30	42 34 07 96 88	54 42 06 87 98	35 85 29 48 39					
70 29 17 12 13	40 33 20 38 26	13 89 51 03 74	17 76 37 13 04	07 74 21 19 30					
56 62 18 37 35	96 83 50 87 75	97 12 25 93 47	70 33 24 03 54	97 77 46 44 80					
99 49 57 22 77	88 42 95 45 72	16 64 36 16 00	04 43 18 66 79	94 77 24 21 90					
16 08 15 04 72	33 27 14 34 09	45 59 34 68 49	12 72 07 34 45	99 27 72 95 14					
31 16 93 32 43	50 27 89 87 19	20 15 37 00 49	52 85 66 60 44	38 68 88 11 80					
68 34 30 13 70	55 74 30 77 40	44 22 78 84 26	04 33 46 09 52	68 07 97 06 57					
74 57 25 65 76	59 29 97 68 60	71 91 38 67 54	13 58 18 24 76	15 54 55 95 52					
27 42 37 86 53	48 55 90 65 72	96 57 69 36 10	96 46 92 42 45	97 60 49 04 91					
00 39 68 29 61	66 37 32 20 30	77 84 57 03 29	10 45 65 04 26	11 04 96 67 24					
29 94 98 94 24	68 49 69 10 82	53 75 91 93 30	34 25 20 57 27	40 48 73 51 92					
16 90 82 66 59	83 52 64 11 12	67 19 00 71 74	60 47 21 29 68	02 02 37 03 31					
11 27 94 75 06	06 09 19 74 66	02 94 37 34 02	76 70 90 30 86	38 45 94 30 38					
35 24 10 16 20	33 32 51 26 38	79 78 45 04 91	16 92 53 56 16	02 75 50 95 98					
38 23 16 86 38	42 38 97 01 50	87 75 66 81 41	40 01 74 91 62	48 51 84 08 32					
31 96 25 91 47	96 44 33 49 13	34 86 82 53 91	00 52 43 48 85	27 55 26 89 62					

66 67 40 67 14	64 05 71 95 86	11 05 65 09 68	76 83 20 37 90	57 16 00 11 66
14 90 84 45 11	75 73 88 05 90	52 27 41 14 86	22 98 12 22 08	07 52 74 95 80
68 05 51 18 00	33 96 02 75 19	07 60 62 93 55	59 33 82 43 90	49 37 38 44 59
20 46 78 73 90	97 51 40 14 02	04 02 33 31 08	39 54 16 49 36	47 95 93 13 30
64 19 58 97 79	15 06 15 93 10	01 90 10 75 06	40 78 78 89 62	02 67 74 17 33
05 26 93 70 60	22 35 85 15 13	92 03 51 59 77	59 56 78 06 83	52 91 05 70 74
07 97 10 88 23	09 98 42 99 64	61 71 62 99 15	06 51 29 16 93	58 05 77 09 51
68 71 86 85 85	54 87 66 47 54	73 32 08 11 12	44 95 92 63 16	29 56 24 29 48
26 99 61 65 53	58 37 78 80 70	42 10 50 67 40	32 17 55 85 74	94 44 67 16 94
14 65 52 68 75	87 59 36 22 41	26 78 63 06 55	13 08 27 01 50	15 29 39 39 43
17 53 77 58 71	71 41 61 50 72	12 41 94 96 26	44 95 27 36 99	02 96 74 30 83
90 26 59 21 19	23 52 23 33 12	96 93 02 18 39	07 02 18 36 07	25 99 32 70 23
41 23 52 55 99	31 04 49 69 96	10 47 48 45 88	13 41 43 89 20	97 17 14 49 17
60 20 50 81 69	31 99 73 68 68	35 81 33 03 76	24 30 12 48 60	18 99 10 72 34
91 25 38 05 90	94 58 28 41 36	45 37 59 03 09	90 35 57 29 12	82 62 54 65 60
34 50 57 74 37	98 80 33 00 91	09 77 93 19 82	74 94 80 04 04	45 07 31 66 49
85 22 04 39 43	73 81 53 94 79	33 62 46 86 28	08 31 54 46 34	53 94 13 38 47
09 79 13 77 48	73 82 97 22 21	05 03 27 24 83	72 89 44 05 60	35 80 39 94 88
88 75 80 18 14	22 95 75 42 49	39 32 82 22 49	02 48 07 70 37	16 04 61 67 87
90 96 23 70 00	39 00 03 06 90	55 85 78 38 36	94 27 30 69 32	90 89 00 76 93
53 74 23 99 67	61 32 28 69 84	94 62 67 86 24	98 33 41 19 95	47 53 53 38 09
63 38 06 86 54	99 00 65 26 94	02 82 90 23 07	79 62 67 80 60	75 91 12 81 19
35 30 58 21 46	06 72 17 10 94	25 21 31 75 96	49 28 24 00 49	55 65 79 78 07
63 43 36 82 69	65 51 18 37 88	61 38 44 12 45	32 92 85 88 65	54 34 81 85 35
98 25 37 55 26	01 91 82 81 46	74 71 12 94 97	24 02 71 37 07	03 92 18 66 75
02 63 21 17 69	71 50 80 89 56	38 15 70 11 48	43 40 45 86 98	00 83 26 91 03
64 55 22 21 82	48 22 28 06 00	61 54 13 43 91	82 78 12 23 29	06 66 24 12 27
85 07 26 13 89	01 10 07 82 04	59 63 69 36 03	69 11 15 83 80	13 29 54 19 28
58 54 16 24 15	51 54 44 82 00	62 61 65 04 69	38 18 65 18 97	85 72 13 49 21
34 85 27 84 87	61 48 64 56 26	90 18 48 13 26	37 70 15 42 57	65 65 80 39 07

03 92 18 27 46	57 99 16 96 56	30 33 72 85 22	84 64 38 56 98	99 01 30 98 64
62 95 30 27 59	37 75 41 66 48	86 97 80 61 45	23 53 04 01 63	45 76 08 64 27
08 45 93 15 22	60 21 75 46 91	98 77 27 85 42	28 88 61 08 84	69 62 03 42 73
07 08 55 18 40	45 44 75 13 90	24 94 96 61 02	57 55 66 83 15	73 42 37 11 61
01 85 89 95 66	51 10 19 34 88	15 84 97 19 75	12 76 39 43 78	64 63 91 08 25
72 84 71 14 35	19 11 58 49 26	50 11 17 17 76	86 31 57 20 18	95 60 78 46 75
88 78 28 16 84	13 52 53 94 53	75 45 69 30 96	73 89 65 70 31	99 17 43 48 76
45 17 75 65 57	28 40 19 72 12	25 12 74 75 67	60 40 60 81 19	24 62 01 61 16
96 76 28 12 54	22 01 11 94 25	71 96 16 16 88	68 64 36 74 45	19 59 50 88 92
43 31 67 72 30	24 02 94 08 63	38 32 36 66 02	69 36 38 25 39	48 03 45 15 22
50 44 66 44 21	66 06 58 05 62	68 15 54 35 02	42 35 48 96 32	14 52 41 52 48
22 66 22 15 86	26 63 75 41 99	58 42 36 72 24	58 37 52 18 51	03 37 18 39 11
96 24 40 14 51	23 22 30 88 57	95 67 47 29 83	94 69 40 06 07	18 16 36 78 86
31 73 91 61 19	60 20 72 93 48	98 57 07 23 69	65 95 39 69 58	56 80 30 19 44
78 60 73 99 84	43 89 94 36 45	56 69 47 07 41	90 22 91 07 12	78 35 34 08 72
84 37 90 61 56	70 10 23 98 05	85 11 34 76 60	76 48 45 34 60	01 64 18 39 96
36 67 10 08 23	98 93 35 08 86	99 29 76 29 84	33 34 91 58 93	63 14 52 32 52
07 28 59 07 48	89 64 58 89 75	83 85 62 27 89	30 14 78 56 27	86 63 59 80 02
10 15 83 87 60	79 24 31 66 56	21 48 24 06 93	91 98 94 05 49	01 47 59 38 00
55 19 68 97 65	03 73 52 16 56	00 53 55 90 27	33 42 29 38 87	22 13 88 83 34
53 81 29 13 39	35 01 20 71 34	62 33 74 82 14	53 73 19 09 03	56 54 29 56 93
51 86 32 68 92	33 98 74 66 99	40 14 71 94 58	45 94 19 38 81	14 44 99 81 07
35 91 70 29 13	80 03 54 07 27	96 94 78 32 66	50 95 52 74 33	13 80 55 62 54
37 71 67 95 13	20 02 44 95 94	64 85 04 05 72	01 32 90 76 14	53 89 74 60 41
93 66 13 83 17	92 79 64 64 72	28 54 96 53 84	48 14 52 98 94	56 07 93 89 30
02 96 08 45 65	13 05 00 41 84	93 07 54 72 59	21 45 57 09 77	19 48 56 27 44
49 83 43 48 35	82 88 33 69 96	72 36 04 19 76	47 45 15 18 60	82 11 08 95 97
84 60 71 62 46	40 80 81 30 37	34 39 23 05 38	25 15 35 71 30	88 12 57 21 77
18 17 30 88 71	44 91 14 88 47	89 23 30 63 15	56 34 20 47 89	99 82 93 24 98
79 69 10 61 78	71 32 76 95 62	87 00 22 58 40	92 54 01 75 25	43 11 71 99 31

75 93 36 57 83	56 20 14 82 11	74 21 97 90 65	96 42 68 63 86	74 54 13 26 94
38 30 92 29 03	06 28 81 39 38	62 25 06 84 63	61 29 08 93 67	04 32 92 08 09
51 29 50 10 34	31 57 75 95 80	51 97 02 74 77	76 15 48 49 44	18 55 63 77 09
21 31 38 86 24	37 79 81 53 74	73 24 16 10 33	52 83 90 94 76	70 47 14 54 36
29 01 23 87 88	58 02 39 37 67	42 10 14 20 92	16 55 23 42 45	54 96 09 11 06
95 33 95 22 00	18 74 75 00 18	38 79 58 69 32	81 76 80 26 92	82 80 84 25 39
90 84 60 79 80	24 36 59 87 38	82 07 53 89 35	96 35 23 79 18	05 98 90 07 35
46 40 62 98 82	54 97 20 56 95	15 74 80 08 32	16 46 70 50 80	67 72 16 42 79
20 31 89 03 43	38 46 82 68 72	32 14 82 99 70	80 60 47 18 97	63 49 30 21 30
71 59 73 05 50	08 22 23 71 77	91 01 93 20 49	82 96 59 26 94	66 39 67 98 60

*Reproduced from Table XXXIII of Fisher and Yates (1963) following Armitage (1971) by permission of the authors and publishers.

LIST OF PARTICIPANTS

1. Professor A.M. Breckenridge
Department of Pharmacology and Therapeutics
University of Liverpool
New Medical Building
Ashton Street
Liverpool L69, 3BX
United Kingdom

2. Dr. D.M. Burley
The Centre of Pharmaceutical Medicine
Dorna House
West End
Woking
Surrey
United Kingdom

3. Dr. Brian G. Schuster
Director
Division of Experimental Therapeutics
Walter Reed Army Institute of Research
Washington, D.C. 20307-5100
United States of America

4. Dr. Frederick C. Churchill
Formerly: Control Technology Branch
Division of Parasitic Diseases
Centre for Infectious Diseases
Centres for Disease Control
Atlanta GA. 30333
United States of America

5. Dr. E.B. Doberstyn
Formerly: Chief
Malaria Control
Division of Control of Tropical Diseases
World Health Organization
Geneva
Switzerland

6. Dr. J.M. Kofi Ekue
Tropical Diseases Research Centre (IDRC)
P.O. Box 71769
Ndola
Zambia

7. Dr. Lawrence Fleckenstein
Department of Pharmacology
Division of Experimental Therapeutics
Walter Reed Army Institute of Research
Washington, DC, 20307-5100
United States of America

8. Dr. Ailene Jamaludin
Centre for Drug Research
Universiti Sains Malaysia
Penang, Malaysia

9. Dr. (Mrs) Juntra Karbwang
Department of Clinical Tropical Medicine
Faculty of Tropical Medicine
Mahidol University
Bangkok 100400
Thailand

10. Dr. (Mrs) Nilima A. Kshirsagar
Department of Pharmacology
Seth G.S. Medical College
Parel
Bombay 400012
India

11. Dr. Normaznah Yahya
Division of Malaria and Filarial Research
Institute of Medical Research
Kuala Lumpur
Malaysia

12. Ms. Kesara Nabanchong
Department of Clinical Tropical Medicine
Faculty of Tropical Medicine
Mahidol University
Bangkok 100400
Thailand

13. Professor (Dr.) V. Navaratnam
Centre for Drug Research
Universiti Sains Malaysia
Penang, Malaysia

14. Dr. (Mrs) J.E. Ogbuokiri
Department of Pharmacology/Therapeutics
College of Medicine
University of Nigeria Teaching Hospital
Enugu
Nigeria

15. Professor L.A. Salako
Department of Pharmacology and Therapeutics
College of Medicine
Universiti of Ibadan
Ibadan
Nigeria

16. Professor U.K. Sheth
14 Modern Villa, 7th Road
Santa Cruz (East)
Bombay 400055
India

17. Dr. Vanvarothai Singhasivanon
Department of Tropical Paediatrics
Faculty of Tropical Medicine
Mahidol University
Bangkok 100400
Thailand

18. Dr. Akintunde Sowunmi
Department of Clinical Pharmacology
University College Hospital
Ibadan
Nigeria

19. Dr. Franco Tin
Department of Health (Malaria Control Division)
Ministry of Health
Yangon
Myanmar

20. Dr. P.I. Trigg
Malaria Unit
Division of Tropical Diseases Control
World Health Organization
Geneva
Switzerland

21. Dr. Ines Vasquez
c/o Dr. William Rojas
Corporacion para Investigaciones Biologicas (CIB)
Calle 51 No. 50-30
Medellin
Colombia

22. Dr. W.M. Watkins
Wellcome Trust Research Laboratories
P.O. Box 43640
Nairobi
Kenya

23. Dr. K. Weerasuriya
Department of Pharmacology
Faculty of Medicine
University of Colombo
P.O. Box 271
Colombo 8
Sri Lanka

24. Professor W.H. Wernsdorfer
Institute of Specific Prophylaxis
and Tropical Medicine
Kinderspitalgasse 15
A-1095 Vienna
Austria