Field application of in vitro assays *for the* sensitivity of human malaria parasites *to* antimalarial drugs

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World Health Organization

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Abbreviations used

ADP	adenosine diphosphate
cpm	counts per minute
DELI	double-site enzyme-linked lactate dehydrogenase immunodetection
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FW	formula weight
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)
HPLC	high-performance liquid chromatography
HRP II	histidine-rich protein II
IC_{50}	50% inhibitory concentration
LDH	lactate dehydrogenase
MIC	minimal inhibitory concentration
NAD	nicotinamide adenine dinucleotide
OD	optical density
PABA	*para*-aminobenzoic acid
PCR	polymerase chain reaction
pfcrt	*P. falciparum* chloroquine resistance transporter gene
*pfmdr*1	*P. falciparum* multidrug resistance gene 1
pvcrt	*P. vivax* chloroquine resistance transporter
RPMI	Roswell Park Memorial Institute
TDR	Special Programme for Research and Training in Tropical Diseases
Tween 80	polyoxyethylenesorbitan monooleate
vs	versus
WHO	World Health Organization

Executive summary

In vitro assays for the sensitivity of human malaria parasites to antimalarial drugs provide information complementary to that derived from the epidemiology of drug-resistant malaria. Its principles were initially based on a suboptimal short-term culture method reported in 1912, applied for the World Health Organization (WHO) macrotest system in the 1960s and used until the 1980s. The principles of in vitro culture underwent a major modification in 1976, when a new method for continuous culture of *Plasmodium falciparum* was reported by Trager & Jensen (1976). The essential components of the complete blood-medium mixture are Roswell Park Memorial Institute (RPMI) 1640 medium buffered with *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and sodium bicarbonate, human serum (or serum substitutes, including animal sera and lipid-enriched bovine albumin) and *P. falciparum*-infected human erythrocytes. This technical improvement led to the elaboration of several in vitro assay systems in the late 1970s and in the 1980s, including the WHO microtest system (morphological assay), the 48-h test (morphological assay) and the radioisotope microtest. Over the past few years, two novel in vitro drug sensitivity assays have been evaluated: an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies directed against either plasmodial lactate dehydrogenase (LDH) or histidine-rich protein II (HRP II) and a fluorometric assay with DNA-binding fluorescent dyes. These two assays are non-morphological and non-radioactive and are also based on Trager & Jensen's (1976) culture method.

Continuous culture of *P. vivax* requires a modified or alternative culture medium and regular addition of reticulocytes. The minimal requirement for performing in vitro drug sensitivity assays is schizont maturation during the first intraerythrocytic cycle, without necessarily attaining the ring stage of the second intraerythrocytic cycle after merozoite invasion. Addition of reticulocytes is not necessary for in vitro assays with only a 48-h incubation period. As all intraerythrocytic stages, including gametocytes, may be present in *P. vivax*-infected patients, it is recommended that blood samples containing predominantly ring stages be selected for in vitro assays. At least three culture media have been proposed for culturing *P. vivax*. Further comparative studies with these media and better understanding of the specific nutritional needs of this *Plasmodium* species will be required in order to design an optimal protocol for in vitro assays for *P. vivax*.

The specific nutrient requirements and the optimal in vitro conditions for short- and long-term continuous culture of *P. ovale* and *P. malariae* have

not been studied. As with *P. vivax*, all intraerythrocytic stages may be present at the time of blood collection. Schizont maturation can be attained in vitro for these two *Plasmodium* spp. by adapting the same protocol (mixture of RPMI and Waymouth media) as used for *P. vivax*; a 72-h incubation is required for *P. malariae* owing to its quartan periodicity. Further understanding of the biochemistry of *P. ovale* and *P. malariae* is needed before the optimal method for in vitro culture can be determined.

The in vitro drug sensitivity assay is a research tool:

- for drug development: screening, in vitro interaction of drug combinations, cross-resistance studies, phenotypic comparisons of pre-treatment and post-treatment isolates, baseline sensitivity to new drugs before introduction into a country;
- as a complementary test to support arguments that drug resistance is the probable cause of therapeutic failure;
- to validate candidate molecular markers of drug resistance;
- for an indirect measure of plasma drug concentrations in bioassays; and
- for monitoring drug resistance (emergence of drug resistance, changing trends of drug sensitivity or resistance over time and space, in vitro responses of individual drugs that are currently administered in combination therapies), in particular for drugs for which there is so far no validated molecular marker, including amodiaquine, artemisinin derivatives and amino alcohols (quinine, halofantrine, lumefantrine).

This report examines the past, present and possible future roles of in vitro drug sensitivity assays in malaria control and their possible contribution to monitoring drug resistance. The report also includes a critical examination of past errors and present weaknesses in order to improve the technical aspects of existing assays, with the ultimate goal of establishing a standardized protocol in the near future.

Four major types of assay system have either been evaluated in the field or are under evaluation in field conditions: morphological assays, radioisotope assays, ELISA-based assays and fluorometric assays. Other novel assay systems (e.g. flow cytometry) are not suitable for field application. Because the assay systems, in particular morphological assays, have different end-points and indirect indicators of parasite growth ([^{3}H]hypoxanthine incorporation, plasmodial LDH production, HRP II secretion and DNA proliferation) reflect different aspects of parasite metabolism and maturation, they do not necessarily yield directly comparable results. The morphology-based assay is the most tedious and least objective method for studying drug response, especially if the minimal inhibitory concentration (MIC) is determined by different readers. Interpretation of the results of the other three major assay systems is based on objective instrumental measures

(i.e. liquid scintillation counter, spectrophotometer or fluorometer). A number of correlation studies with these assay systems are under way in sophisticated laboratories and in the field. The results of these studies are expected to orient investigators' choices and result in harmonized technical procedures. A limited number of laboratories in endemic countries have the capacity to perform radioisotope assays. This method is therefore not appropriate for most such countries, and ELISA-based assays and fluorometric assays are recommended for long-term monitoring for drug-resistant malaria, especilly for researchers who plan to start in vitro testing in a laboratory without radioisotope facilities.

There is no single, universally accepted, standardized protocol for in vitro assays. Different research laboratories take into account different factors, each of which can profoundly influence the level of drug response. These factors include modifications to RPMI 1640 medium (e.g. concentrations of folic acid and *para*-aminobenzoic acid [PABA]), the type of serum supplement or serum substitutes, the volume of blood-medium mixture, the erythrocyte volume fraction, the range of starting parasitaemias, the incubation period, the gas mixture, previous cultivation of the parasites and preparation of drug solutions and test plates. Other factors that can influence the outcome and which vary with the epidemiological setting include the multiplicity of isolates (i.e. the presence of several distinct parasite populations with different drug sensitivity profiles within an isolate), inclusion of blood samples from patients who have recently taken antimalarial drugs, and the level of acquired immunity, which may influence drug response if autologous serum is used for the assay. The in vitro drug response of a given clinical isolate of *P. falciparum* is the result of various interactions among these factors. Even a slight change in any one of them influences parasite growth in vitro and can be detected by the sensitive, specific indicators of schizont maturation used in current non-morphological assays, leading to changes in the level of drug response. These factors explain in part the technical difficulties in standardizing in vitro drug sensitivity assays, especially when they are used to monitor the response of field isolates over long periods in various sentinel sites.

A standardized protocol for conducting in vitro drug sensitivity assays for field monitoring of drug-resistant malaria (*P. falciparum* and *P. vivax*) would allow direct comparisons of in vitro results from different laboratories and help validate the correlations among the phenotype (in vitro response), the genotype (molecular markers of drug resistance) and the therapeutic outcome of the patient from whom the parasites were isolated provided that quality control of data is ensured. The current gold standard for determining and updating drug policy, and the most relevant information for public health, is therapeutic efficacy tests (also called in vivo tests). In vitro drug sensitivity assays (and molecular analysis of resistance genes) are laboratory tools that provide complementary data to the epidemiology of drug-resistant malaria but cannot replace clinical studies. The in vitro response and the

presence of distinct point mutations in established molecular markers (*P. falciparum* chloroquine resistance transporter [*pfcrt*], dihydrofolate reductase [*dhfr*], dihydropteroate synthase [*dhps*], and cytochrome *b*) are highly correlated, whereas in vitro response is generally not highly concordant with clinical and parasitological outcomes. Neither in vitro assays nor molecular markers can accurately predict the treatment outcome of patients.

Monitoring the rapidly changing patterns of drug resistance in many parts of the world where malaria is endemic has become a key component of efficient malaria control and case management. The in vitro drug sensitivity assay and molecular markers for monitoring drug resistance are particularly useful at community, national and regional levels. These laboratory investigations would, however, be more meaningful if they were standardized and conducted in parallel with clinical studies and within a regional antimalarial drug resistance surveillance system. The existing in vitro assays, in particular those that are non-radioactive, clearly need to be updated and standardized. Their goal is to provide complementary data and warning signals of the possible emergence of resistance or trends of declining drug efficacy. In vitro monitoring of the activity of individual antimalarial drugs might be particularly valuable in countries or regions where combination therapies have been adopted or are being considered, especially if there is no established molecular marker for resistance to a drug. Important resources, training and expertise will be required to initiate and perform such laboratory investigations and to manage a regional database in a reference laboratory situated in an endemic country.

1. In vitro assays for the sensitivity of human malaria parasites to drugs

1.1 Assays for the sensitivity of *P. falciparum*

1.1.1 History of assay development

The first assay of the activity of an antimalarial drug on human malaria parasites cultured in vitro was performed by Bass (1922). With technical procedures developed earlier (Bass, 1911; Bass & Johns, 1912), *P. falciparum* isolated from a patient was cultured in vitro and exposed to a single concentration of quinine during a 29-h incubation to demonstrate the schizontocidal effect. Use of this prototype assay was limited for several decades.

About 40 years later, when the problem of drug-resistant *P. falciparum* emerged, the "macrotest", also called the "macrotechnique", was developed by K. Rieckmann on the basis of Bass' method (Rieckmann et al., 1968; Rieckmann, 1971; Rieckmann & Lopez Antuñano, 1971). The main objective of the macrotest was to detect and follow the evolution of chloroquine-resistant *P. falciparum* in various parts of the world. In this test, venous blood (10–12 ml) is collected from malaria-infected persons and immediately defibrinated. Aliquots (1 ml) are then placed in flat-bottomed glass tubes containing 5 mg of glucose and various concentrations of test compounds and placed in an incubator or water-bath at 38–40 °C for 24 h. After incubation, the erythrocytes are resuspended in plasma, and Giemsa-stained thick films are prepared for microscopic examination. Growth inhibition is determined by comparing the schizont count in test vials with that of a drug-free control vial.

A standard test kit and a standard procedure for the macrotest, known as the "WHO standard macrotest", were established under the sponsorship of WHO, and the test was widely used in field studies in several endemic countries between 1976 and 1987 (WHO, 1979; Wernsdorfer & Kouznetsov, 1980; WHO, 1984; Wernsdorfer & Payne, 1988). The macrotest is relatively simple to perform in the field and does not require sophisticated equipment. The percentage of asexual parasites that develop into schizonts with at least two nuclei serves as the end-point for interpreting and quantifying the effect. This assay system was, however, abandoned in the late 1980s because of several disadvantages: the requirements for 10 ml of venous blood, a parasite count of between 1000 and 80 000 asexual parasites per µl with a predominance of relatively older or larger rings that will mature into schizonts

within 24 h, and the low success rate (< 70%) of short-term culture (without buffered culture medium) (Nguyen-Dinh et al., 1985a). The method is described here merely for historical interest.

Before the advent of the culture technique of Trager & Jensen (1976), other attempts had been made to design an in vitro assay system. In one of these early systems, *P. falciparum* strains maintained in *Aotus* monkeys were cultured in vitro for a single complete cycle in the presence of several drug concentrations in Harvard medium with the rocker-dilution technique (Siddiqui, Schnell & Geiman, 1972). The effects of the drug on maturation of rings to trophozoites and schizonts were assessed by microscopic examination. A finding that is of some interest to investigators involved in antimalarial drug research today was the discordance of the results for a *P. falciparum* strain obtained with the macrotest and with the rocker-dilution assay system. These contradictory results, observed several decades ago, were due to differences in the culture technique, emphasizing the importance of establishing a standardized protocol for determining drug response.

All the current assays are based on an in vitro culture technique designed by Trager & Jensen (1976) (and not on the alternative method of Haynes et al., 1976). Researchers working on malaria rapidly realized that this culture technique for *P. falciparum* can be used to determine the in vitro sensitivity of the parasites to both existing drugs and new drug candidates. One of the major reasons for finding new assay methods was for primary screening of new antimalarial drugs that would be effective against *P. falciparum* resistant to chloroquine and antifolates, and also because of the declining efficacy of quinine in South-East Asia. Within seven years of the publication of Trager and Jensen's method, several other assays were reported. More recently, other assay methods, in particular ELISA-based systems, have been designed, on the basis of new biochemical and immunological findings.

The currently available assay systems can be grouped according to the methods used to quantify parasite growth in relation to drug concentrations:

- direct, visual counting of parasites under a microscope;
- incorporation of radioisotope precursors into the parasite; and
- non-radioactive methods.

The last technique includes fluorescence-activated cell sorter assays (FACS), fluorometric assays, ELISA-based methods and non-ELISA-based colorimetric methods (Table 1). All these in vitro methods involve direct exposure of human malaria parasites to drugs in culture plates. In addition to these purely in vitro methods, several in vivo-in vitro bioassays based on continuous culture of reference strains of *P. falciparum* are available. In these bioassays, a test compound is administered to a malaria-free vertebrate host, blood is extracted at various times and in vitro assays are performed with serum containing the test compound or its metabolite(s).

In sections 1.1.2–1.1.7, the essential characteristics of each technique and its variants are described (Table 2). In vitro drug sensitivity assays, including those based on microscopic examination of blood smears, have been developed in "advanced" countries (Europe, India, Japan and the United States), usually with the modern technology and instruments commonly available in universities, hospitals and specialized research laboratories in those areas. The advantages and disadvantages of these methods and their potential interest for field workers are discussed.

1.1.2 Microscopic or visual examination

48-h test and its variants

One of the earliest attempts to apply modern in vitro culture methods to determine a drug sensitivity pattern was made by W. Trager himself. In the method referred to as the "48-h test" (also called the "48-h in vitro re-invasion test"), a laboratory-adapted strain of *P. falciparum* was cultured in 35-mm Petri dishes or 16-mm flat-bottomed multiwell plates (final volume, 0.5 ml; erythrocyte volume fraction, 8%) and exposed to drug-free control medium or medium containing various concentrations of the test compound for 48 h in a candle jar (Nguyen-Dinh & Trager, 1978; see Ponnudurai, Leeuwenberg & Meuwissen, 1981, for a similar assay). The medium, with or without drug concentrations, is changed after 24 h. Parasitaemia is determined by microscopic examination of thin blood films before and after exposure to the drug. This assay requires relatively large quantities of culture reagents and parasites to test five concentrations and a drug-free control in duplicate or quadruplicate. In the modified "single-step 48-h test", a lower erythrocyte volume fraction of 2% (final volume, 0.5 ml; starting parasitaemia, 0.5–0.8%) allows continuous 48-h incubation in a candle jar with no change of medium (Nguyen-Dinh & Trager, 1980).

A modified version of the 48-h test was evaluated in the field (Nguyen-Dinh, Hobbs & Campbell, 1981; Nguyen-Dinh et al., 1982; Spencer et al., 1983; Nguyen-Dinh et al., 1985a,b, 1987). Venous or capillary blood (≥0.3 ml) is collected from malaria-infected patients in a heparinized tube, and 0.2 ml of whole blood are diluted to 1/25 in 4.8 ml of a mixture of RPMI, HEPES and 10% non-immune human serum. If an erythrocyte volume fraction of 50% is assumed for the collected blood sample, the final erythrocyte volume fraction in the mixture will be about 2% and the final serum concentration will be 12%. The erythrocyte suspension is distributed (volume, 0.5 ml per well) in 24-well (16-mm) plates and incubated for 48 h. In the microtitre version, parasitized whole blood is diluted to 1/20 in the mixture of RPMI, HEPES and 10% human serum (final erythrocyte volume fraction, about 2.5%), and 100-µl aliquots are distributed in 96-well plates. After incubation for 48 h at 37 °C in a candle jar, thin blood smears are prepared from each well, and the inhibitory end-point (minimal inhibitory concentration, MIC, i.e. the drug concentration at which there is no

parasite multiplication) is determined. Performance of the assay can be simplified by using a field kit, which includes a lyophilized mixture of RPMI 1640 medium, HEPES and 10% human serum, to which 200 µl of finger-prick capillary blood are added (Nguyen-Dinh, Magloire & Chin, 1983).

Richards & Maples (1979) used the culture technique to study the inhibitory effects of chloroquine and pyrimethamine. A laboratory-adapted strain was cultured in 3.5-cm Petri dishes (1% initial parasitaemia, approximately 7% erythrocyte suspension) and exposed to various drug concentrations for the first 48 h. The erythrocytes were washed at the end of the first intraerythrocytic cycle, and the parasite was cultured in drug-free medium during the next 48 h (i.e. the second intraerythrocytic cycle). The medium was changed daily, and parasite growth was assessed by microscopic examination of blood films made every 24 h until 96 h. The parasitaemia in the drug-free control culture and in the drug-treated cultures was plotted against time in order to compare the effects of the drug. Although the parasite strain used (Liverpool strain of *P. falciparum*) is known to be sensitive to drugs in *Aotus* monkeys, visual inspection of the blood films indicated inhibition only with ≥ 500 nmol/l of chloroquine; no inhibitory effect was seen at 100 nmol/l. Moreover, this method is long (96-h incubation vs 24–48-h incubation in other assays), imprecise (the results are not expressed as 50% inhibitory concentration [IC_{50}] or MIC) and labour-intensive for large-scale screening. It also requires more material than microtechniques.

Thaithong, Beale & Chutmongkonkul (1983) proposed a variation of the 48-h test for evaluating the response of clinical isolates that were collected in the field, transported to a central laboratory in Bangkok and adapted to in vitro culture before assay. The assays were performed in 96-well microtitre plates (5% erythrocyte volume fraction, 0.3–0.8% starting parasitaemia, 100 µl per well). During the 72-h incubation, the medium (with or without drugs) was changed daily. The MIC was determined by microscopic examination of thin blood films.

J. Le Bras and P. Deloron designed a similar method, the "semi-micro test", which was used in studies on laboratory-adapted *P. falciparum* strains, field isolates and clinical isolates from imported cases of malaria (Deloron et al., 1982; Le Bras & Deloron, 1983; Le Bras et al., 1984a; Deloron et al., 1985; Le Bras & Savel, 1987). The method requires collection of venous blood, which is washed with RPMI 1640 before assay. Parasites are exposed to various drug concentrations in triplicate in 24-well culture plates and incubated for 24–48 h (2.5–5% erythrocyte volume fraction; 0.7 ml per well) in either a candle jar or an incubator set at 6% CO_2–5% O_2. The end-point is determined when more than 10% of the parasites have developed into schizonts, and the number of schizonts is counted on Giemsa-stained thin blood films. The results are expressed as the percentage of schizont maturation in comparison with drug-free control wells, plotted on a graph, and the IC_{50} is determined from the graph. The success rate of this assay with field isolates and isolates from imported malaria cases was reported to

be about 50%. Because determinations are made in triplicate and the final volume is 0.7 ml per well, the semi-microtest requires more RPMI, HEPES and 10% human serum-blood mixture than microtechniques. This method has not been used since the mid-1980s, and a radioisotope version has replaced the original method.

The 48-h test and its variations might be useful for an initial evaluation of the potential activity of a limited number of drugs in some moderately equipped laboratories. It does not fulfill the objective criteria for data interpretation and is less accurate than isotope and non-radioactive methods. Nevertheless, valid conclusions can be drawn from the 48-h test. Some investigators still resort to this method, with some modifications, as seen in recent publications (Ang, Chan & Mak, 1995; Biswas, 2002).

Microtechnique and WHO test

The in vitro microtest was developed by Rieckmann et al. (1978) at about the same time as the 48-h test. Owl monkeys (*Aotus trivirgatus*) were infected with *P. falciparum* strains pre-adapted to this monkey species, and capillary blood (50 µl total volume per experiment, 17–58% erythrocyte volume fraction, 940–106 000 asexual parasites per µl) was collected several times from the same monkeys once infection was established. Microtitre plates pre-coated with six to seven concentrations of chloroquine (0.1–2.5 ng chloroquine diphosphate per well for chloroquine-sensitive strain and 2.5–25 ng chloroquine diphosphate per well for chloroquine-resistant strain), and two drug-free control wells per assay were prepared. Whole capillary blood (5 µl per well) was added to buffered RPMI 1640 medium (50 µl per well) in microtitre plates and incubated in a candle jar at 38–39 °C for 24–30 h. Immediately after incubation, thick blood films were prepared from each well, and the number of schizonts was counted against 500 leukocytes. The in vitro response was expressed as the percentage of the number of schizonts with each drug concentration, compared with the number of schizonts in drug-free controls (an alternative criterion of more than two nuclei is recommended in the WHO protocol). There was a 10-fold difference in the chloroquine concentrations at which maturation of the chloroquine-sensitive strain and the chloroquine-resistant strain was completely inhibited. This method was also used to evaluate the in vitro response of a culture-adapted strain to pyrimethamine and sulfadoxine-pyrimethamine in combination, with standard RPMI 1640 containing 1 mg/l folic acid and 1 mg/l PABA (Yisunsri & Rieckmann, 1980; Eastham & Rieckmann, 1983).

Rieckmann's microtechnique has the important advantages of requiring only a small quantity of capillary blood and involving simplified in vitro procedures. Fingerprick capillary blood samples from young children can be tested, while venepuncture is necessary for the macrotest. Other major advantages over the previous macrotechnique include the suitability for ring

forms of all ages (small, medium and large rings, while the macrotest requires large rings) and the greater success rate due to direct application of Trager and Jensen's culture methods. Because of the better parasite growth in the microtechnique, the incubation period should be carefully controlled so that mature schizonts do not rupture and release merozoites (re-invasion usually does not occur in the macrotest because of suboptimal culture conditions), leading to an invalid end-point.

Rieckmann's microtechnique was adopted by W. Wernsdorfer to design a field-applicable microtest, under WHO sponsorship (Wernsdorfer, 1980; Wernsdorfer, Kouznetsov, 1980; WHO, 1982; Wernsdorfer & Payne, 1988). The WHO assay systems (macrotest and microtest) are laboratory tools designed to assist description of the epidemiology of drug-resistant malaria as part of the global monitoring programme. The assay system can be used to determine baseline levels of drug sensitivity and to follow the evolution of the drug resistance of malaria parasites by regular monitoring. The WHO system is not intended for drug screening or for individual diagnosis.

The earliest version of the WHO microtest underwent field trials in the late 1970s (Lopez Antuñano & Wernsdorfer, 1979; Kouznetsov et al., 1980). Malaria-infected persons (asymptomatic carriers or symptomatic patients) are enrolled after informed consent if the following conditions are fulfilled: no recent intake of antimalarial drugs, confirmed by a negative result in a urine test for antimalarial drugs, pure *P. falciparum* infection and parasitaemia > 500 asexual parasites per µl (no specified upper limit in the early version of the WHO microtest). It should be noted that the WHO macrotest and microtest ("Mark I") co-existed between 1981 and 1987.

The 96-well assay plates are prepared by distributing 50 µl of RPMI 1640, HEPES, $NaHCO_3$ and gentamicin solution into wells 2–12 (wells 2 and 3, drug-free; wells 4–12, pre-dosed with increasing quantities of chloroquine [1–32 pmol] or mefloquine [0.5–16 pmol per 50-µl well]). Well 1 contains the anticoagulant ethylenediaminetetraacetic acid (EDTA), into which 100 µl of fingerprick capillary blood are placed immediately after blood collection (the microtest requires one sample of 100 µl capillary blood per drug). Whole blood (5 µl per well) is transferred to the wells in the same row (wells 2–12). The culture plates are incubated in a candle jar at 37–38 °C for 24–30 h. After removal of the supernatant, two Romanowsky- or Giemsa-stained thick blood films are prepared from each well (see WHO, 1991 for details of preparation and staining). The number of schizonts (more than two nuclei) is counted against 200 asexual parasites by microscopy. The result for individual isolates is expressed as the percentage of schizonts in comparison with drug-free control wells (i.e. the number of schizonts with a given drug concentration divided by the mean number of schizonts in two drug-free control wells x 100).

On the basis of this protocol, the WHO standard in vitro microtest kit was produced between 1981 and 2000, the first series being called Mark I,

the second Mark II and the third Mark III. The main features of the assay procedures were maintained in all three versions; some of the minor modifications in the Mark II and Mark III versions include (WHO, 1987, 1997):

- lower (1000 asexual parasites per µl blood) and upper (80 000 asexual parasites per µl blood) limits of initial parasitaemia;
- ready-to-use, sterile, low-PABA, low-folic acid RPMI 1640 liquid medium, which is compatible for testing antifolate drugs;
- additional plates (the first version included plates for chloroquine, amodiaquine, quinine and mefloquine; in later versions, plates for sulfadoxine-pyrimethamine and artemisinin were added and plates for halofantrine, pyrimethamine and pyronaridine were available on request);
- lay-out of culture plates;
- range of drug concentrations;
- collection of blood in heparinized capillary tubes (an EDTA-coated well does not always ensure anticoagulation and is no longer needed in the culture plate);
- direct mixture of capillary blood (0.1 ml) in low-PABA, low-folic acid RPMI 1640 (0.9 ml) before distribution into pre-coated wells;
- final volume of 50 µl of blood-medium mixture (not 55 µl as in the early version);
- drug concentrations expressed as pmol/well and the corresponding concentrations in either µmol/l blood (i.e. µmol/l in the 10% of blood in the blood-medium mixture) for drugs that are concentrated in malaria-infected erythrocytes (chloroquine, amodiaquine, mefloquine) or µmol/l (or nmol/l) blood-medium mixture (1/10 the micromolar concentration reported for the blood-medium mixture for chloroquine, amodiaquine and mefloquine above) for drugs that "are not concentrated in infected erythrocytes" (*sic*) (quinine, halofantrine, artemisinin, sulfadoxine-pyrimethamine and pyronaridine); and
- arbitrary thresholds for drug sensitivity or resistance (e.g. chloroquine-sensitive if ≤ 4 pmol/well; mefloquine-resistant if ≥ 32 pmol/well).

The basic procedures, such as the conditions and approximate duration of incubation, preparation and examination of post-test thick films and graphical representation of the probit analysis, have not undergone major modification. An individual assay is considered to be acceptable if at least 10% of asexual parasites develop into schizonts with three or more (i.e. more than two) nuclei (eight or more [i.e. more than seven] nuclei for pyrimethamine and sulfadoxine-pyrimethamine) in the drug-free control well (i.e. 20 or more schizonts among 200 asexual parasites).

Alternative microtechniques

The assay protocol designed by Desjardins et al. (1979) for drug screening (see [^{3}H]hypoxanthine-based assays, below) can be adapted, with slight modifications, to monitor the in vitro response of field isolates by microscopic examination of blood smears (Childs et al., 1987; Childs, Wimonwattrawatee & Pooyindee 1988; Childs et al., 1989). Twofold dilutions of drugs are prepared as described by Desjardins et al. (1979), with each drug concentration in duplicate. Blood samples from patients are washed three times in RPMI, HEPES and $NaHCO_3$ medium by centrifugation and resuspended in medium supplemented with 10% human plasma to a 1.4% erythrocyte volume fraction. The erythrocyte suspension is distributed in 96-well microtitre plates (200 µl per well plus 25 µl of drug solution per well) and incubated in a candle jar. The incubation period required to attain schizont maturation can vary from 24 to 48 h. A total of 100 asexual parasites per well are counted in Giemsa-stained thick smears (i.e. 200 asexual parasites per drug concentration, as duplicate wells are used), and the proportion of schizonts with at least three nuclei is noted. The dose-effect is expressed as the IC_{50} and the MIC.

Congpuong et al. (1998) evaluated a hybrid microtechnique, which is a combination of Desjardin's method, the 48-h test and the WHO microtest. Blood samples from patients were washed three times in RPMI medium and resuspended at an erythrocyte volume fraction of 1.5% with the starting parasitaemia adjusted to 0.2–0.5% (as in Desjardin's technique). Blood-medium mixture (50 µl per well) was distributed in microtitre plates that had been pre-dosed by WHO. The plates were incubated for 48 h, and thin blood films were prepared from each well for microscopic examination (as in the 48-h test). The results were analysed by log probit linear regression. The success rate was moderate (188 of 245 Thai isolates, 77%), possibly because of the high proportion (34%) of patients with pretreatment mefloquine or quinine in their plasma.

Visual agglutination test

A simple visual agglutination test applicable for detection of haemozoin production during maturation of malaria parasites was designed for initial drug screening (Rieckmann, 1982; Kotecka & Rieckmann, 1992). A synchronized laboratory-adapted *P. falciparum* strain predominantly in the ring stage is suspended in complete RPMI 1640 medium (2% erythrocyte volume fraction, 0.02–1.0% initial parasitaemia) and distributed (55–100 µl per well) in 96-well microtitre plates pre-coated with test compounds. After incubation in a candle jar or incubator for 24–48 h, 25 µl of 1 N NaOH–1 N NaCl solution (v/v 1:1) are added to each well. The solution denatures proteins and disintegrates cells within a few minutes. The plate is agitated for several minutes and allowed to stand for 10–60 min. Upon agitation, dark-pigmented precipitates formed by haemozoin aggre-

gation become visible to the naked eye in drug-free control wells, which are an indicator of schizont maturation. In wells containing drug concentrations that inhibit schizont formation, dark precipitates do not form. Kotecka & Rieckmann (1992) showed that comparable results are obtained with the visual agglutination test, a microtechnique followed by microscopic examination of thick blood films, and a radioisotope assay. The visual test is not, however, accurate, and its authors have suggested its use only for initial drug screening, as compounds with low or no activity can be eliminated immediately, and compounds with high activity can be tested further by more accurate assay methods. The visual agglutination test has a major drawback: leukocytes agglutinate in an alkaline solution and interfere with precipitate formation. Therefore, the visual test is not applicable for routine evaluation of the in vitro response of fresh clinical isolates in field laboratories.

Current use of microscope-based tests

Of the methods based on visual or microscopic examination, the 48-h test, and in a lesser extent, the microtechnique are still practised today. The microtechnique was used in the field until a few years ago, partly because WHO provided a standard kit containing all the necessary materials and reagents, including pre-dosed plates. The WHO kit is no longer being produced, and alternative methods are proposed below.

With the exception of the visual agglutination test, the assay methods described above require visual counting of parasites under a microscope, which is tedious and time-consuming, unless the results are expressed as the MIC. If schizont (i.e. more than two nuclei) maturation is not used as a criterion, the decision about whether parasites are viable tends to be subjective in microscope-based tests. Specific indicators of parasite growth are required for an assay system that provides an objective measure and can be used for large numbers of samples. On the basis of the biology and biochemistry of malaria parasites, several radiolabelled precursors, enzymatic reactions and monoclonal antibodies have been proposed for use in alternative assays.

1.1.3 Radioisotope methods

Choice of radioisotopes

Several radiolabelled precursors have been used to assess parasite maturation, including precursors of nucleic acids (i.e. purines and pyrimidines), proteins (i.e. amino acids) and phospholipids (i.e. polar head groups). In cultures containing malaria parasites, uninfected mature human erythrocytes and platelets do not synthesize DNA, RNA, proteins or membranes; human leukocytes do not multiply and tend to disintegrate over a few days. The only actively dividing cells are the parasites themselves. Despite a low

background level in uninfected erythrocytes, incorporation of radioactive precursors is an indirect measure of the metabolic activity of the parasites.

Plasmodium spp. use exogenous purines and do not synthesize purines de novo (Sherman, 1977). Of the DNA precursors, [^{3}H]adenosine, [^{3}H]xanthine and [^{3}H]hypoxanthine were shown to be incorporated into malarial nucleic acids in early studies with avian, rodent, and simian malaria models. [^{3}H]Hypoxanthine is the main purine base used by *P. falciparum* and is the preferred radioisotope for in vitro drug sensitivity assays. Other purines are also specifically incorporated into the parasite's nucleic acid. [2,8–^{3}H]Adenosine was used for in vitro drug screening by some investigators working on animal malaria models during the 1960s (Van Dyke et al., 1970a,b; Lantz & Van Dyke, 1971). According to Ye, Van Dyke & Wimmer (1987), [2,8–^{3}H]adenosine and [G–^{3}H]hypoxanthine can be used interchangeably for determining drug activity in *P. falciparum* cultured in vitro; however, use of this labelled purine can result in a slightly lower level of radiolabelled nucleic acids and does not offer any particular advantage over [^{3}H]hypoxanthine, with the possible exception of lower cost because of wider use of [2,8–^{3}H]adenosine in cell biology.

Pyrimidines (uridine, thymidine and cytidine) and pyrimidine analogues are not suitable DNA or RNA precursors for radiolabelling malaria parasites. Pyrimidine bases enter the infected erythrocytes, but no further incorporation occurs in parasites. In one study, *P. falciparum* was found to be capable of using uracil (Pérignon, Hamet & Druilhe, 1994), but these results could not be replicated with various laboratory-adapted clones and fresh clinical isolates cultured with [5,6–^{3}H]uracil for 42 h (Basco, 1996). In fact, the artefactual results obtained by Pérignon, Hamet & Druilhe (1994) were due to use of a mycoplasma-contaminated *P. falciparum* strain. Mycoplasmas, which are invisible under the light microscope, use the salvage pathway of pyrimidines, and mycoplasma-contaminated cultures are a common source of artefacts in biochemical and DNA-based studies (Rowe et al., 1998).

For certain experiments, it may be more appropriate to use radiolabelled amino acids. For example, in biochemical studies, [^{35}S]methionine can be added to methionine-free RPMI 1640 medium for analysis of the proteins produced by parasites by autoradiography of gels after sodium dodecyl sulfate-polyacrylamide gel electrophoresis or in the quantification of radioactivity incorporated into proteins in the trichloracetic acid precipitation method (Shyamala, 1985). In some early studies on *P. falciparum* culture and drug assays, [^{3}H]leucine or [^{14}C]isoleucine was used as an indicator of parasite growth (Iber et al., 1975; Richards & Williams, 1975). Carbon-14 has a long half-life and is more expensive than tritium, and therefore its use is discouraged, especially for routine experiments. [^{3}H]Isoleucine has been used as an indicator of protein synthesis in some studies of the mode of action of drugs (Gu, Warhurst & Peters, 1983; Elabbadi, Ancelin & Vial, 1992; ter Kuile et al., 1993), but there is no particular advantage in using this radiolabelled amino acid instead of [^{3}H]hypoxanthine in routine drug

screening. RPMI 1640 contains 50 mg/l of cold L-isoleucine, so that relatively little [^{3}H]isoleucine may be incorporated into malaria parasites unless isoleucine-free RPMI 1640 is used.

Radiolabelled precursors of phospholipids, such as the sources of phospholipid polar head groups, [^{3}H]ethanolamine, [^{3}H]choline, [^{14}C]serine and [^{3}H]inositol and a fatty acid, [^{3}H]palmitate, have been used to measure parasite growth (Elabbadi, Ancelin & Vial, 1992). After the initial 24-h incubation, a laboratory-adapted *P. falciparum* strain was incubated with these precursors (1 µCi), as well as [^{3}H]isoleucine and [^{3}H]hypoxanthine, for an additional 4 h. [^{3}H]Palmitate appeared to be unsuitable as a growth indicator because of its extensive incorporation into uninfected erythrocytes. [^{14}C]Serine also appeared not to be a suitable alternative owing to the long half-life of carbon-14. More incorporation into parasitized erythrocytes was observed with [^{3}H]ethanolamine than with [^{3}H]hypoxanthine. The levels of incorporation of the other phospholipid precursors, [^{3}H]choline and [^{3}H]inositol, and of [^{3}H]isoleucine were more than 20-fold lower than that of [^{3}H]ethanolamine, probably because of the presence of cold choline, inositol and isoleucine in RPMI 1640. Incorporation of both [^{3}H]ethanolamine and [^{3}H]hypoxanthine increased linearly in relation to the starting parasitaemia (0.1–1%) and erythrocyte volume fraction (0.1–3%). Although these precursors reflect different metabolic activities of the parasite, the findings suggest that [^{3}H]ethanolamine might be a suitable substitute for [^{3}H]hypoxanthine for assessing parasite viability. Moreover, the [^{3}H]ethanolamine radioisotope assay and SYBR® Green I-based fluoroassay (see later) yield similar in vitro responses with a reference *P. falciparum* clone (Smilkstein et al., 2004).

Use of [^{3}H]ethanolamine, instead of [^{3}H]hypoxanthine, offers the advantage that RPMI 1640 can be supplemented with cold hypoxanthine to optimize the in vitro growth of parasites. Before these two radiolabelled precursors are used interchangeably, however, additional studies are required to compare various factors, including the sensitivity pattern of different parasite strains. The final concentration of ethanolamine must be adjusted carefully, as human plasma can contain up to 20 µmol/l of ethanolamine, and Asahi et al. (1996) found that addition of 10 µmol/l of ethanolamine to RPMI 1640 inhibits parasite growth.

As mentioned above, [^{3}H]hypoxanthine is the preferred purine base for radiolabelling parasite DNA and RNA for isotope microtests. [^{3}H]Hypoxanthine incorporation is directly proportional to the number of *P. falciparum*-infected erythrocytes under the usual conditions of in vitro drug sensitivity assays (i.e. initial parasitaemia between 0.1 and 1.0% at 1.5% erythrocyte volume fraction) during the 42-h incubation period (Chulay, Haynes & Diggs, 1983; Geary, Divo & Jensen, 1983). At an initial parasitaemia > 1.0% (1.5% erythrocyte volume fraction), incorporation becomes non-linear and tends to be lower than expected.

In a synchronized culture, [^{3}H]hypoxanthine incorporation is stage-dependent and closely follows DNA replication and nuclear division: low incorporation during the ring stage, followed by increased incorporation as mature trophozoites develop, reaching a maximum with schizont formation (Campbell et al., 1979; Chulay, Haynes & Diggs, 1983; Geary, Divo & Jensen, 1983; Nivet, Guillotte & Pereira da Silva, 1983). At the beginnning of the second schizogonic cycle, i.e. after re-invasion, [^{3}H]hypoxanthine incorporation decreases, even though the parasitaemia might increase as compared with the first intraerythrocytic cycle (Campbell et al., 1979; Chulay, Haynes & Diggs, 1983). These observations imply that the final quantity of [^{3}H]hypoxanthine incorporation varies little in relation to the moment when synchronous cultures, including fresh clinical isolates, are pulsed during the first 24 h of the 48-h incubation period. Therefore, in drug assays in the field, it is convenient to add [^{3}H]hypoxanthine to the complete culture medium at the beginning of the incubation period, avoiding the extra step of taking the culture plates out after the initial 18–24 h of incubation and distributing [^{3}H]hypoxanthine into each well (Basco, 2004b).

[^{3}H]Hypoxanthine enters uninfected erythrocytes at a low level. Incubation of uninfected human erythrocytes (1.5–2% erythrocyte volume fraction) with 0.5 μCi of [G–^{3}H]hypoxanthine per well in a 96-well plate for 18 h results in background incorporation of 50–400 counts per minute (cpm) or < 2% of the quantity of [^{3}H]hypoxanthine incorporated into drug-free control wells (Desjardins et al., 1979; Chulay, Haynes & Diggs, 1983; Petersen, 1986; Ye, Van Dyke & Wimmer, 1987). Before calculating IC_{50} values, the usual practice is to subtract the background level of [^{3}H]hypoxanthine incorporation from all values of cpm in the raw data, in order to fulfill one of the mathematical conditions (minimum/maximum of 0–100% on the *Y* axis) of the logistic function in a non-linear regression analysis. Calculation of the IC_{50} is discussed in more detail in section 3.3.

[^{3}H]Hypoxanthine-based assays

Use of [^{3}H]hypoxanthine in drug assays allows rapid, sensitive, accurate determination of the effects of a drug on parasite growth. The first radioisotope, semi-automated assay was that of Desjardins et al. (1979). This method has been adopted for primary drug screening in the United States Army Antimalarial Drug Development Program at the Walter Reed Army Institute of Research (Washington DC, United States) and is now considered the "gold standard" for in vitro drug sensitivity assays. The original technique was applied to the chloroquine- and pyrimethamine-sensitive African Uganda I strain and the chloroquine- and pyrimethamine-resistant Vietnam Smith strain. In later in vitro screening studies, the chloroquine-sensitive D6/Sierra Leone clone and the chloroquine-resistant W2/Indochina clone were used regularly. The assay method has been adapted for epidemiological studies on fresh clinical isolates and is now one of the most commonly used assay methods in well-equipped malaria research laboratories.

Before assay, cultures are maintained under optimal conditions by frequent (i.e. every 2–3 days) dilution with uninfected erythrocytes and by limiting the maximum parasitaemia to 2% at any given time. For drug assays, cultures are diluted in complete RPMI 1640 medium with uninfected erythrocytes to a final erythrocyte volume fraction of 1.5% and a starting parasitaemia of 0.25–0.5%. Twofold serial dilutions of drug solutions in complete RPMI 1640 medium (25 µl per well) are prepared in duplicate in 96-well microtitre plates with an automatic diluter (rows B–H; row A consists of drug-free control wells). This format allows evaluation of seven drug concentrations over a 64-fold range. An alternative format with drug-free control wells in row 1 and twofold dilutions in duplicate in rows 2–12 allows evaluation of 11 drug concentrations over a 1024-fold range. The parasite suspension, 200 µl per well plus 25 µl drug solution, i.e. a final volume of 225 µl, is distributed in each well. The plates are incubated at 37 °C for 24 h in a 5% O_2–5% CO_2–90% N_2 gas mixture. [^{3}H]Hypoxanthine (25 µl per well of 20 µCi/ml solution in RPMI 1640, i.e. 0.5 µCi per well) is added to each well after the first 24-h incubation period. When the parasites are asynchronous, this delay in adding the radioactive indicator of parasite growth allows mature trophozoites and schizonts present at the beginning of the assay to develop and initiate a new, second blood cycle without incorporating [^{3}H]hypoxanthine during the first 24 h. After an additional 18 h incubation, during which [^{3}H]hypoxanthine incorporation occurs essentially in developing mature trophozoites and schizonts of the first blood cycle (parasites in ring stage at the beginning of the assay), the assay is terminated. Incorporation of [^{3}H]hypoxanthine is quantified with a liquid scintillation counter.

Several similar [^{3}H]hypoxanthine-based isotope assays have been reported, as described below. The modifications include the volume of erythrocyte suspension (500–700 µl per well in 24-well culture plates, instead of 200 µl per well in 96-well microtitre plates) and erythrocyte volume fraction (2–2.5% instead of 1.5%).

In the in vitro system developed by Geary, Divo & Jensen (1983), the culture medium is supplemented with 10% pooled human serum, 10% pooled bovine serum with cold hypoxanthine or 20% pooled rabbit serum. In 24-well plates, the erythrocyte volume fraction is adjusted to 2% in a final volume of 520 µl (initial parasitaemia, 0.2–0.3%). In 96-well culture plates, the erythrocyte volume fraction is adjusted to 0.9% in a final volume of 220 µl (initial parasitaemia, 1.0%), and [^{3}H]hypoxanthine (10 µCi/ml) is added. The parasites are incubated for 48 h at 37 °C.

The semi-microtest, initially interpreted by microscopic examination of thin blood films (Le Bras & Deloron, 1983), was later modified to a radioisotope method (Le Bras et al., 1984b). The parasite suspension (2.5% erythrocyte volume fraction [1.5% erythrocyte volume fraction from 1992 onwards]) is distributed in 24-well culture plates and, as in Desjardins' procedure, [G–^{3}H]hypoxanthine (25 µl of 40 µCi/ml solution per well) is added after the initial incubation period of 18 h. The assay is terminated

after an additional 24 h incubation (total of 42 h). The IC_{50} values derived in the isotope semi-microtest (700 µl of erythrocyte suspension per well in 24-well plates) and Desjardins' microtest (200 µl per well in 96-well plates) are almost identical under similar culture conditions (Bickii, Basco & Ringwald, 1998).

The assay procedure of Desjardins et al. (1979) was initially designed for drug screening in well-characterized, laboratory-adapted *P. falciparum* clones. The versatility of the [^{3}H]hypoxanthine-based assay is evident from its multiple applications, including drug screening, experimental studies (e.g. of drug combinations), evaluation of potential cross-resistance and monitoring of the responses to drugs of field isolates. The disadvantages of radioisotope assays are use of a radioactive product, requiring a special disposal system, and the requirement for a relatively high (i.e. ≥ 0.1%) starting parasitaemia (Desjardins et al., 1979; Chaparro & Wasserman, 1999). At a high starting parasitaemia, > 1%, the relation between incorporation of [^{3}H]hypoxanthine and parasite density is not linear (Chulay, Haynes & Diggs, 1983); in addition, an inoculum effect is observed. In [^{3}H]hypoxanthine assays, the starting parasitaemia must be adjusted to 0.1–1% for optimal measurement of parasite growth and drug response. [^{3}H]Hypoxanthine can be added at any time during the first 24 h of incubation of synchronous parasites (i.e. during the ring and trophozoite stages) (Chaparro & Wasserman, 1999; Basco, 2004b).

The radioactivity of tritium results in relatively weak emission at high counts, which is completely blocked by plastic tubes and culture plates and hence does not represent a direct, serious hazard to the person handling [^{3}H]hypoxanthine under the usual laboratory procedures for biosafety. Nevertheless, special care is necessary in waste disposal owing to the half-life for radioactive decay of tritium of approximately 11 years.

Although it is not an appropriate technique for most field laboratories, the radioisotope method can be used on field isolates by:

- performing the assay in a relatively well-equipped field laboratory, collecting radiolabelled parasites on glass-fibre filters and transporting the dried filters for counting at a central laboratory (Brasseur et al., 1986; Brandicourt et al., 1986; Brasseur et al., 1988, 1992a,b) or
- transporting fresh samples or cryopreserved clinical isolates to a well-equipped central laboratory (Webster et al., 1985; Pradines et al., 1998a,b,c, 1999a,b, 2000). It should be recalled that particular clones might be selected during subsequent culture of cryopreserved isolates.

Although the radioactive assay has become the reference method for assaying sensitivity to drugs in advanced countries, it cannot be the reference method for most countries in which malaria is endemic. It is therefore essential that non-radioactive assays become standard methods in the near future.

[^{3}H]Chloroquine-based assays

A rapid in vitro test that is specific for chloroquine resistance in *P. falciparum* was reported by Gluzman et al. (1990). Its principle is based on two observations. First, chloroquine-resistant parasites actively expel chloroquine, leading to lower drug uptake, while chloroquine-sensitive parasites accumulate the drug (Verdier et al., 1985; Krogstad et al., 1987). Second, addition of verapamil (and other calcium channel blockers, tricyclic antidepressants, tricyclic antihistaminics and phenothiazines) inhibits chloroquine efflux in chloroquine-resistant *P. falciparum* strains but not in chloroquine-sensitive parasites (Martin, Oduola & Milhous, 1987; Bitonti et al., 1988; Basco & Le Bras, 1991a). The latter effect is indirectly assessed by comparing the IC_{50} of chloroquine alone with that of chloroquine in combination with a modulator. A similar phenomenon is observed in fresh clinical isolates of *P. falciparum* cultured without prior adaptation to in vitro conditions (Basco & Le Bras, 1990, 1991b, 1994a).

Modulation and circumvention of drug resistance were initially observed in lines of multidrug-resistant malignant mammalian cells, which became "sensitive" to a cytotoxic agent to which they were originally resistant after the cytotoxic agent was combined with diverse pharmacological agents without cytotoxic effects (Deeley & Cole, 1997; Tew & Speicher, 1997). Verapamil and other drugs, collectively known as "reversal agents", "modulating agents" or "resistance modulators", are thought to inhibit drug efflux in cancer cells (and in resistant *P. falciparum* and some other organisms, including yeast) by binding to the transporter protein (P-glycoprotein in mammalian cells), thereby interfering with the influx-efflux process. This results in accumulation of drugs within the drug-resistant cell, leading to its death. In chloroquine-resistant *P. falciparum*, the involvement of another site on transporter protein PfCRT is now recognized (Sidhu, Verdier-Pinard & Fidock, 2002).

In the rapid in vitro test of Gluzman et al. (1990), the intracellular and extracellular distribution of [^{3}H]chloroquine in *P. falciparum* cultures is analysed after incubation for 1 h to deduce sensitivity or resistance to the drug. If a given *P. falciparum* isolate incorporates a similar quantity of [^{3}H]chloroquine, with or without 25 µmol/l of verapamil, the isolate is chloroquine-sensitive. In contrast, if the isolate incorporates a greater quantity of [^{3}H]chloroquine in the presence of verapamil, the isolate is chloroquine-resistant.

Although the rapid in vitro test is simple in its concept and is designed on a rational basis, it can fail to distinguish between chloroquine-sensitive and chloroquine-resistant clinical isolates, as compared with the response in the [^{3}H]hypoxanthine-based assay (Bickii et al., 1998). There are several possible reasons for this failure. Fresh clinical isolates are at the ring stage at the time of blood collection, and incubation of metabolically less active ring forms for a few hours is probably insufficient to detect parasite metabolism,

especially for chloroquine, which has stage-dependent activity that attains a maximum during the trophozoite and early schizont stages (Yayon et al., 1983). Furthermore, the rapid in vitro test is probably not sensitive enough to detect the difference in [^{3}H]chloroquine incorporation in chloroquine-resistant isolates when the parasitaemia is below 1%. The initial experiments were done with laboratory-adapted parasites at 1% parasitaemia, including rings and trophozoites (Gluzman et al., 1990). The poor performance of the rapid in vitro test precludes any practical application to field isolates without further improvements or modifications of the protocol. A longer incubation period of 24–36 h would probably improve the capacity of the assay to distinguish resistant from sensitive field isolates, but at the expense of rapidity.

1.1.4 Non-radioactive methods

Flow cytometry

Flow cytometry can be used to measure various properties of cells suspended in a suitable medium as they flow through a column one by one. The modern flow cytometer is a highly sophisticated instrument that combines modern techniques in fluidics, laser optics, electronics and computerized data processing, and modifications (see below) can often be made for active sorting after the cell characteristics have been determined. The sample is usually fixed and tagged with monoclonal antibodies conjugated with fluorochromes for cell surface marker analysis, DNA- or RNA-binding dyes for cell counts (e.g. reticulocyte count), measurements of DNA content and cell cycle analysis, and other special dyes to study particular cellular components (e.g. intracellular free Ca^{++}) or metabolic activity (e.g. oxidative burst). A flow cytometer generates a fluid column with a laminar flow into which cells are injected, aligned in single file and presented to a beam of laser light. Two physical events occur when a particle tagged with a fluorescent dye (i.e. fluorochromes) interrupts the light beam: light scattering and fluorescence. Scattering of the light occurs when any particle (labelled or unlabelled) interrupts the light beam. The properties of scattering at different angles indicate cell size and granularity (e.g. lymphocytes, monocytes, granulocytes). The beam also excites fluorochromes, which absorb the laser light and emit light at longer wavelengths (i.e. fluorescence). The photons with different wavelengths from scatter signals and fluorescence are separated by optical filters, collected by sensors, amplified by photomultipliers and converted to electrical impulses and digital signals for data processing. Some flow cytometers (cell sorters) can physically separate cells on the basis of pre-programmed parameters. Instead of a continuous flow, droplets are formed, and each cell is isolated in a separate droplet. When a droplet containing the cell to be sorted is detected, the droplet is electrically charged and deflected for separate collection.

Laser-based FACS can be used to detect and count malaria-infected erythrocytes and, in the presence of a DNA-targeted fluorescent dye, differ-

entiate the asexual developmental stage on the basis of the amount of DNA in individual host erythrocytes: uninfected erythrocytes have no DNA content and are therefore not fluorescent, while fluorescence intensity increases in direct proportion as trophozoites develop into schizonts. Raw data from flow cytometric analysis are presented in histograms showing the number of cell counts against increasing intensity of fluorescence. A sample containing asynchronous asexual erythrocytic stages would typically show three peaks: a large, non-fluorescent peak consisting of uninfected erythrocytes; the first, less intense fluorescent peak, representing trophozoite-infected erythrocytes (it is sometimes possible to differentiate two peaks, one corresponding to rings and early trophozoites and the other to late trophozoites); and the second, intense fluorescent peak, representing schizont-infected erythrocytes (another intense fluorescent peak may be present if the culture sample contains leukocytes). This technique has been used by several investigators to detect the presence of *P. falciparum*-infected erythrocytes, separate infected from uninfected erythrocytes, separate the infected erythrocytes according to developmental stage and quantify the absolute number of parasites in a given blood sample (Brown, Battye & Howard, 1980; Saul et al., 1982; Whaun, Rittershaus & Ip, 1983; Franklin, Brun & Grieder, 1986; Hare, 1986; Janse et al., 1987; Makler, Lee & Recktenwald, 1987; Pattanapanyasat et al., 1992; Piper, Roberts & Day, 1999; Saito-Ito et al., 2001). Flow cytometry has also been used to determine the sensitivity of *P. falciparum* to drugs (Franklin, Brun & Grieder, 1986; van Vianen et al., 1990; Pattanapanyasat et al., 1997; Saito-Ito et al., 2001; Contreras et al., 2004).

The assay for drug sensitivity is performed in the same way as the isotope microtest of Desjardins et al. (1979), except for omission of [^{3}H]hypoxanthine, in standard 96-well microtitre plates pre-coated with test compounds. At the end of the incubation period, the infected erythrocytes are fixed with glutaraldehyde and stained with a fluorescent DNA dye. Several fluorochromes can be used, including thiazole orange, acridine orange, ethidium bromide, fluorescein isothiocyanate (conjugated to specific antibodies), propidium iodide, the bisbenzimidazole dyes Hoechst 33258 and Hoechst 33342, and 4´,6-diamidino-2-phenylindole. The stained cells are excited with laser or ultra-violet light, depending on the dye used. The fluorescence intensity of individual wells is measured, and the data are analysed by a computer linked to the FACS. Once the cells are fixed and stained, the entire procedure, including data processing and analysis, can be automated and completed within less than 3 h (van Vianen et al., 1990). The effect-concentration curves generated from data obtained by microscopic examination of thin or thick smears or the radioisotope method and by flow cytometry were shown to be similar (van Vianen et al., 1990; Reinders et al., 1995; Pattanapanyasat et al., 1997; Saito-Ito et al., 2001; Contreras et al., 2004). Further drug assays performed with flow cytometry, isotope precursors and other methods should be conducted in order to validate this alternative method.

Flow cytometry is rapid, accurate, highly sensitive, highly DNA-specific, objective, automated and non-radioactive. This technique can be used to distinguish different asexual stages on the basis of their DNA content. FACS is, however, costly, requires a highly specialized technician for maintenance and operation, and is too sophisticated for routine application in the field. Moreover, the standard FACS is not adapted for installation in a tropical climate unless the laboratory is well-equipped with air-conditioners and a constant, regulated electrical source. At present, it is not an appropriate technique for most laboratories in malaria-endemic countries.

Fluorometric assay

A FACS can be used to detect and measure the DNA content of intact (but fixed) malaria-infected erythrocytes with DNA-specific fluorochromes. Nevertheless, it is a costly instrument, and alternative methods based on the principle of DNA labelling with fluorochromes but which do not require a FACS have been generated (Waki et al., 1986; Smeijsters et al., 1996; Bennett et al., 2004; Corbett et al., 2004; Smilkstein et al., 2004). In these fluorescence-based assays, or fluoroassays, drugs are tested in 24- or 96-well culture plates with no additional reagents. At the end of the incubation period, the erythrocytes are lysed by addition of distilled water or saponin. After centrifugation, the packed pellets are dissolved in guanidinium or sodium dodecyl sulfate solution and stained with DNA-binding fluorochromes (ethidium bromide or Hoechst 33358). The procedure may require an extra step of DNA extraction in chloroform to eliminate haemozoin, which can cause quenching. If PicoGreen® is used, the erythrocyte suspension can be mixed with the fluorochrome solution in tris(hydroxymethyl)aminomethane (Tris)–EDTA buffer containing Triton X-100 for lysis and direct DNA labelling. If SYBR® Green I is used to stain the DNA, lysing of erythrocytes and washing are not necessary (Bennett et al., 2004). The fluorescence intensity, which reflects the amount of DNA in individual samples, is measured with a minifluorometer, fluorescence spectrophotometer or fluorescence-activated microplate reader. Smilkstein et al. (2004) reported similar IC_{50} values for chloroquine, quinine, mefloquine and artemisinin in a reference *P. falciparum* clone with SYBR® Green I-based fluoroassay and [^{3}H]ethanolamine incorporation.

Fluoroassays are less costly than FACS-based assays; however, the DNA extraction protocol must be optimized to ensure complete recovery of DNA from each sample. The protocol must be executed by a skilled technician experienced in the manipulation of small volumes of reagents. To minimize artefacts, contaminating human leukocytes must be removed completely. In the studies of Waki et al. (1986) and Smeijsters et al. (1996), the volumes of cultured laboratory-adapted strains grown were larger than the volumes in the wells of a 96-well microtitre plate, in order to ensure an adequate amount of DNA after extraction. Bennett et al. (2004), Corbett et al. (2004) and Smilkstein et al. (2004) have shown that fluoroassays can be adapted to the

96-well microtitre plate format and the DNA extraction steps can be eliminated, considerably simplifying the assay procedure.

This non-radioactive assay is accurate, rapid and simple, and the instrument is less expensive than a liquid scintillation counter or FACS. It might therefore be promising for use in antimalarial drug sensitivity studies in field laboratories as well as for drug screening. The available fluoroassays are not, however, as sensitive as other methods. Waki et al. (1986) used an initial parasitaemia of 2% ring-infected erythrocytes. Smeijsters et al. (1996) reported that a minimum of 1% ring-infected erythrocytes or 0.4% schizont-infected erythrocytes was required for accurate measurement. Corbett et al. (2004) conducted their studies at 2% erythrocyte volume fraction and a starting parasitaemia of 1% in 96-well plates. Even with PicoGreen®, the fluorescence intensity was too low for accurate data interpretation when the parasitaemia was below 1%. This major disadvantage limits application of fluoroassays to laboratory-adapted *P. falciparum* strains. SYBR® Green I is an alternative fluorophore dye that might be sensitive enough for detection of parasitaemia < 1.0 % at 1–4% erythrocyte volume fraction (Bennett et al., 2004; Smilkstein et al., 2004). Preliminary data suggest that SYBR® Green-based fluoroassays are more sensitive than ELISA-based assays for detecting parasites, but the presence of leukocytes might result in high background fluorescence. Further studies on field isolates are needed with SYBR® Green I (and other new fluorophore dyes) to assess the sensitivity of fluoroassays for detecting malaria-infected parasites in vitro.

The toxicity of some of these dyes should also be taken into account. For example, ethidium bromide is mutagenic and should be handled with care; furthermore, wastes containing ethidium bromide require a special disposal system. It is likely that particular precautions might be needed for other DNA-binding dyes. Fluoroassays must be improved further before they can be used as alternative assay methods suitable for field use.

Non-ELISA-based colorimetric assay

LDH (L-lactate NAD^+-oxidoreductase, EC 1.1.1.27) plays an important role in the carbohydrate metabolism of human malaria parasites. In the final step of the anaerobic Embden-Meyerhoff pathway, LDH metabolizes pyruvate to lactic acid, regenerating NAD, which is necessary for production of ATP. The LDH enzymes of various prokaryotes and eukaryotes, including *P. falciparum* and humans, have a similar relative molecular mass of 35 kDa (Vander Jagt, Hunsaker & Heidrich, 1981). The translated sequence of the *P. falciparum* LDH gene, however, displays distinctive differences in amino acid composition from that of the LDH of other organisms (Simmons et al., 1985; Bzik, Fox & Gonyer, 1993), and it can be separated electrophoretically from five isoenzymes of human LDH present in the erythrocytes (Vander Jagt, Hunsaker & Heidrich, 1981; Vander Jagt et al., 1990).

One of the biochemical characteristics that distinguishes malarial LDH from human LDH is the ability of malarial LDH to use rapidly 3-acetylpyridine adenine dinucleotide, an analogue of NAD, as the coenzyme in a reaction leading to the formation of pyruvate from lactate (Makler & Hinrichs, 1993). Human erythrocyte LDH can also use 3-acetylpyridine adenine dinucleotide instead of NAD, but at a much slower rate. On the basis of measurements of malarial LDH activity in the presence of 3-acetylpyridine adenine dinucleotide, Makler and co-workers demonstrated the specificity and sensitivity of the method for detecting the presence of *P. falciparum* and designed the first-generation LDH-based diagnostic method and drug sensitivity assay (Makler & Hinrichs, 1993; Makler et al., 1993; Basco et al., 1995a). In another study, a diagnostic procedure based on malarial LDH performed less well (sensitivity, 76%; specificity, 97%) than microscopic examination of blood films in non-immune patients returning to Germany with imported malaria (Knobloch & Henk, 1995).

The in vitro drug sensitivity assay with LDH is performed with laboratory-adapted strains and fresh clinical isolates of *P. falciparum* and *P. vivax* in 24- or 96-well culture plates, like the radioisotope method, except that [^{3}H]hypoxanthine is omitted. At the end of the incubation period, the plates are frozen and thawed. The haemolysed suspension from each well (10 µl) is transferred to another 96-well microtitre plate and mixed with 100 µl of Malstat® solution, consisting of 3-acetylpyridine adenine dinucleotide coenzyme and L-lactate substrate. Nitro blue tetrazolium (240 µmol/l), which turns blue in reduced form, and phenazine ethosulfate (33 µmol/l) are added in order to measure formation of the reduced form of 3-acetylpyridine adenine dinucleotide at 650 nm with a spectrophotometer. The raw data are expressed as optical density (OD). With a sophisticated spectrophotometer, the kinetics of the enzymatic reaction can also be measured and the raw data expressed as the maximal velocity (mOD/min). The OD or maximal velocity values are plotted against the logarithm of drug concentrations to determine the IC_{50}.

The results of radioisotope and enzymatic drug assays are strongly correlated (Makler et al., 1993; Basco et al., 1995a). The main limitation of the enzymatic assay is its relatively low sensitivity for detecting malarial LDH. At an erythrocyte volume fraction of 1.5%, no significant LDH activity was observed at a starting parasitaemia of < 0.4% in fresh clinical isolates (Basco et al., 1995a). Increasing the erythrocyte volume fraction to 3.0% did not resolve the problem of sensitivity, as the increase in malarial LDH activity was accompanied by an increase in background human LDH activity. The time- and parasitaemia-dependent increase in malarial LDH activity indicates that interpretable tests based on LDH activity can be performed with fresh isolates of *P. falciparum* and *P. vivax* at an initial parasitaemia of 1–2%, as shown in studies on reference clones of *P. falciparum* (Makler et al., 1993). This range of starting parasitaemia is too high for most field isolates. The assay method is rapid and reliable but is not sensitive

enough for routine field application. The use of related dyes, such as sodium 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, instead of nitro blue tetrazolium, does not improve the sensitivity of the LDH-based colorimetric assay, which still requires a minimal starting parasitaemia of 1–2% (Lazaro & Gay, 1998; Delhaes et al., 1999).

Owing to the relatively low sensitivity of this colorimetric assay and the difficulty in improving its sensitivity, the first-generation LDH-based drug sensitivity assay has been largely abandoned. In the second-generation LDH-based drug sensitivity assay, monoclonal antibodies against malarial LDH are used to enhance sensitivity (see "ELISA-based assays").

ELISA-based assays

Several ELISA-based assays have been reported. As the reagents and basic equipment required to perform these assays are relatively cheap, non-radioactive and widely available in moderately equipped laboratories, they hold promise for field use. ELISA-based in vitro drug sensitivity assays can conveniently be performed with 96-well microtitre culture plates (or 24-well plates) in exactly the same way as the radioisotope method, except that a radioactive precursor is unnecessary. At the end of the incubation period, aliquots of the contents of each well are transferred to another 96-well microtitre plate for washing and ELISA reading. One disadvantage of ELISA-based assays is the numerous washing steps, which can be tedious to perform unless an automated washer is used.

One of the first ELISA-based systems was that of Doi, Ishii & Shimono (1988). Ring-stage parasites are cultured in RPMI 1640 and 10% human serum medium (4.5% erythrocyte volume fraction, 1.0–1.5% starting parasitaemia) containing 2 mmol/l of 5-bromo-2´-deoxyuridine (bromodeoxyuridine), a thymidine analogue, in 24-well plates pre-dosed with test compounds. The unique feature of this protocol is the short incubation period of only 3 h. After incubation, the erythrocytes are lysed and washed, and the standard ELISA procedures are performed with mouse anti-bromodeoxyuridine antibody, horseradish peroxidase anti-rabbit immunoglobulin G conjugate and 2,2´-azino-di(3-ethylbenzylthiazoline sulfonic acid)-6-diammonium salt substrate. The raw data are expressed as OD, determined with a spectrophotometer. Human leukocytes do not incorporate bromodeoxyuridine. A correlation was found between bromodeoxyuridine incorporation and a starting parasitaemia of between 5000 (approximately 0.1% infected erythrocytes) and 50 000 (approximately 1%) asexual parasites per well. The assays were, however, performed with a relatively high starting parasitaemia (1.0–1.5%), and the sensitivity of the assay system was not determined with a lower parasitaemia.

Further studies on the effects of concentration, starting parasitaemia and incubation time on bromodeoxyuridine incorporation were conducted by Biswas & Valecha (1996). Bromodeoxyuridine incorporation was optimal for a synchronized culture of laboratory-adapted *P. falciparum* strains with a starting parasitaemia of 1% ring stage, as determined by an immunofluorescent assay and ELISA, at a concentration of 5 mmol/l after a 24-h incubation, when most parasites develop into schizonts. The lowest detectable parasitaemia was 0.02 %. In contrast to the study of Doi, Ishii & Shimono (1988), a minimal incubation period of 8 h was required for bromodeoxyuridine incorporation to be detectable. On the basis of their findings, Biswas & Valecha (1996) performed ELISA-based in vitro drug sensitivity assays but concluded that the assay system is not highly sensitive, as it requires a relatively high starting parasitaemia of 1 %.

A word of caution is needed here. One research team was unable to reproduce the results of ELISA-based experiments with *P. berghei* and *P. falciparum* incubated in vitro with non-radioactive bromodeoxyuridine (20 µmol/l) for 24 h (Janse et al., 1991). They also demonstrated that, in contrast to the positive control labelled with [^{3}H]hypoxanthine, [^{3}H]bromodeoxyuridine (10 mmol/l) is not incorporated into *P. berghei* DNA. On the basis of these experimental findings, bromodeoxyuridine does not appear to be a suitable indicator of DNA synthesis in malaria parasites.

M. Makler developed and improved the first-generation LDH-based malaria detection system (see "Non-ELISA-based colorimetric assay"above) by using monoclonal antibodies specific for *Plasmodium* LDH in an antigen-capture immunochromatographic assay (Quintana et al., 1998; Piper et al., 1999). Antibodies immobilized on a solid surface (dipstick, culture plate) bind to the malaria antigen with high specificity. This technique allows rapid (< 20 min), simple, reliable diagnosis for a parasitaemia > 0.01% of *P. falciparum* and other human *Plasmodium* species, without requiring microscopic examination of blood smears (Palmer et al., 1998; Hunt-Cooke et al., 1999; Palmer et al., 1999; Moody et al., 2000).

The enzymatic activity of malarial LDH reflects the general metabolic activity of viable parasites. When parasites are exposed in vitro to increasingly effective concentrations of blood schizontocidal drugs, increasing numbers of parasites are killed. The activity and quantity of plasmodial LDH correspond to the number of surviving parasites with intact glycolytic activity in relation to the drug concentration. The relative amount of plasmodial LDH as a function of drug concentration, i.e. the dose-effect relation, is an alternative measure of drug activity.

The same principles as those for the LDH-based immunoassay with dipsticks were used to develop an ELISA-based in vitro drug sensitivity assay system called the "double-site enzyme-linked lactate dehydrogenase immunodetection" (DELI) assay (Brasseur et al., 2001; Druilhe et al., 2001). The assay is performed as described by Desjardins et al. (1979) in 96-well

microtitre plates, except that [^{3}H]hypoxanthine is omitted. Albumax® (lipid-enriched bovine albumin) (w/v 0.5%) can be added to RPMI 1640 medium as a replacement for 10% human serum. At the end of the 48-h incubation in a candle jar, the test plates are frozen and thawed three times to ensure complete haemolysis and liberation of LDH. The lysate in each well is diluted 1/20–1/200 in phosphate-buffered salt solution for a starting parasitaemia > 0.01%. This is a key step, which requires adjustment to obtain the desired OD reading from drug-free control wells. The diluted lysate is transferred into another 96-well microtitre plate pre-coated with the first monoclonal antibody that specifically binds to *P. falciparum* LDH. The subsequent steps are those of a typical ELISA assay, including a series of incubations with the second biotinylated monoclonal antibody that reacts with *Plasmodium* LDH, streptavidin-peroxidase conjugate, and peroxidase substrate (tetramethylbenzidine), with a series of washes between each incubation. The resulting colour reaction is quantified with a spectrophotometer at 450 nm.

Preliminary studies have shown that the DELI test and the radioisotope assay yield closely similar IC_{50} values. Under optimal laboratory conditions, the minimum starting parasitaemia for laboratory-adapted *P. falciparum* strains was reported to be 0.005% (Druilhe et al., 2001; Kaddouri et al., 2006), and this finding has been confirmed in several field studies (Moreno et al., 2001a,b; Thomas et al., 2002). In a study conducted by another group (Brockman et al., 2004), the DELI assay gave more variable results for a *P. falciparum* clone (K1), with wider ranges of IC_{50} values, than the radioisotope method. These investigators also showed that, in fresh clinical isolates from Thailand, the two assay methods do not yield the same IC_{50} values for most commonly used drugs, except for quinine and mefloquine. The mean values with the DELI test were significantly higher than those with the radioisotope method for chloroquine, lumefantrine and atovaquone and lower for artesunate and dihydroartemisinin. The culture conditions (erythrocyte volume fraction, culture medium, incubation period) were identical in the two assays, except for the starting parasitaemia, which was adjusted to 0.5–1.0% for the radioisotope microtest and to 0.2% for the DELI test. If a different starting parasitaemia was the only cause of the different in vitro responses, higher IC_{50} values would have been expected for all drugs with the radioisotope method. As this was not the case, further studies are required to seek other explanations. In another recent study (Kaddouri et al., 2006), the IC_{50}s determined with the DELI test and the radioisotope assay were highly correlated; however, as in the study of Brockman et al. (2004), chloroquine, monodesethylamodiaquine, and lumefantrine IC_{50}s (but not dihydroartemisinin IC_{50}) determined with the DELI test were higher than those determined with the radioisotope method.

The greater sensitivity of the DELI assay than the radioisotope assay to detect low parasitaemia, the non-radioactive procedure and the relatively cheaper instrument used for data interpretation (i.e. spectrophotometer or

ELISA plate reader, as compared with a liquid scintillation counter for radioisotope assays) are definite advantages of this new in vitro drug sensitivity assay. It has been claimed that up to 15 microtitre plates can be treated and interpreted in a single day (Moreno et al., 2001a). In an average field laboratory with few technical personnel, such a high output of work would seem to be unrealistic. The original procedures described by Druilhe et al. (2001) are labour-intensive and time-consuming (three cycles of freeze-thaw to haemolyse assay plates, numerous washes and incubation periods), especially if an automated ELISA plate washer is not available, as in most field laboratories. Incubations at 37 °C are also required to obtain optimal results. Moreover, before the OD of the entire microtitre plate can be measured, an extra step of trial-and-error dilution is required to adjust the absorbance readings of drug-free control wells. Despite these disadvantages, the DELI assay shows promise for regular in vitro surveillance of drug-resistant *P. falciparum* (and *P. vivax*, see later) in the field, particularly if the test kit becomes commercially available at a reasonable price.

Another ELISA assay system, which is based on the detection and quantification of *P. falciparum* HRP II, has become available. Histidine-rich proteins (HRPs) appear to be produced specifically by *P. falciparum,* and not by other *Plasmodium* species. HRP I has been shown to be concentrated in erythrocyte membrane protuberances, called "knobs", and may have functions related to cytoadherence. The functions of HRP II and HRP III are unknown, but it has been suggested that they mediate haemozoin formation (Sullivan, Gluzman & Goldberg, 1996). HRP II is secreted into the host erythrocyte and the extracellular compartment (plasma, culture medium) (Rock et al., 1987), and it has been exploited as a specific target protein for detecting the presence of *P. falciparum* for diagnostic purposes by immunochromatography. A number of commercial manufacturers have produced dipstick methods that allow simple, rapid (usually less than 10–15 min) diagnosis, with low rates of false-positives and false-negatives (WHO, 2000a; Wongsrichanalai, 2001). Unlike LDH, HRP II is a stable antigen which persists in patients, despite parasite and fever clearance, up to two weeks after effective therapy.

HRP II is secreted most actively during the late ring and trophozoite stages (Noedl et al., 2002a). In synchronized laboratory-adapted strains (starting parasitaemia, 0.05–0.1%; 1.5% erythrocyte volume fraction), the HRP II concentration remains relatively low during the first 48 h but reaches high levels after 60–72 h. Inhibition of parasite growth in vitro in the presence of a drug arrests parasite metabolism and HRP II production. HRP II is therefore an indicator of parasite multiplication in vitro. Its concentration reflects the cumulative effect of parasite metabolism and does not appear to increase in parallel with the developmental stages during the first schizogonic cycle.

In vitro drug sensitivity assays are performed in 96-well microtitre plates, as described by Desjardins et al. (1979), with some modifications,

including a lower starting parasitaemia of 0.05% and a longer incubation period of 72 h, without [^{3}H]hypoxanthine (Noedl et al., 2002b). At the end of the incubation, the erythrocytes are lysed by two freezing–thawing cycles. The lysate (100 µl per well) is transferred to another 96-well microtitre plate pre-coated with HRP II-specific monoclonal antibodies and incubated for 1 h. After four successive washes, a conjugate is added, and the plate is incubated for 1 h. The plate is washed four times, and chromogen is added and incubated for 15 min. The reaction is stopped, and the absorbance is measured with an ELISA plate reader.

As in the DELI system, samples are diluted either before assay by adjusting the starting parasitaemia to 0.02–0.1% or after incubation by mixing the lysate and culture medium. Noedl et al. (2002a, 2004a) found that the IC_{50} values determined by HRP II, radioisotope and morphological assays were similar and correlated. The HRP II ELISA assay has fewer steps than the DELI system. A simplified protocol for field use, with minimal handling of blood samples before culture, has also been reported (Noedl et al., 2004a). Further studies on field isolates and comparison with other ELISA-based, [^{3}H]hypoxanthine and morphological assays are required. The price of the commercial kit (in 2004), containing 10 microtitre plates pre-dosed with HRP II monoclonal antibodies, is prohibitive (US$ 800) for most laboratories in endemic countries; however, the cost can be reduced by more than 80% (i.e. about US$ 100 for 10 plates) if the assay plates are prepared in-house from commercial monoclonal antibodies (Noedl et al., 2005).

1.1.5 Bioassays

In bioassays, a drug is administered to a malaria-free vertebrate at various doses by the oral or parenteral route. After the drug has been absorbed, possibly metabolized and distributed in the blood circulation, venous blood samples are obtained at various times. The serum is diluted in a mixture of RPMI 1640, HEPES and $NaHCO_3$, in which a laboratory-adapted reference strain of *P. falciparum* is cultured to assess the inhibitory effect of the serum. In contrast to the in vitro assays described in the previous sections, bioassays are used to test the absorption efficiency of a compound in an animal host and the effects of metabolites, although these cannot be examined separately. The vertebrate host to which a test compound is administered is usually a rabbit or monkey, as their serum is compatible with human erythrocytes and supports the in vitro growth of *P. falciparum* (Sax & Rieckmann, 1980; Kotecka & Rieckmann, 1995). Sera from several other animals are also compatible with continuous culture system of *P. falciparum*, but administration of experimental drugs to relatively large animals (goat, horse, calf) is not common. If the drug being assessed in a bioassay is approved for clinical use, sera can be obtained from human volunteers (Yeo & Rieckmann, 1992; Kotecka & Rieckmann, 1993; Teja-Isavadharm et al.,

1996; Na-Bangchang et al., 1997; Tan-ariya et al., 1997; Traore, Lazaro & Gay, 1997; Ubalee et al., 1999; Kotecka et al., 2003).

Pioneering studies with serum from drug-treated monkeys and humans were conducted in the 1940s (Black, 1946), but the in vitro culture methods used at that time were not optimal and allowed only short-term culture. Since that time, several bioassay methods have been reported. Rabbits are relatively easy to procure and are commonly used for medical purposes. Mrema & Rieckmann (1983) administered several dosages of chloroquine or mefloquine and a single dosage of antifolates to rabbits, collected blood samples at various intervals between 4 and 24 days and measured the growth of laboratory-adapted *P. falciparum* strains in the presence of serum samples diluted to 5% in RPMI 1640 by either microscopic examination of thin smears or the [^{3}H]hypoxanthine assay. The authors showed that combined rabbit-in vitro model might be of some value for studying the kinetics of drug activity.

Interpretation of the results of some experiments, such as fluctuation of mefloquine activity over time, is not always straightforward. Geary, Divo & Jensen (1983) conducted similar experiments with antibiotics and experimental drugs in rabbits and tested the inhibitory effect of rabbit serum, collected at times corresponding to the peak plasma drug concentration, on a *P. falciparum* strain maintained in vitro. More recently, researchers in the Australian Army performed bioassays with chloroquine, amodiaquine, pyronaridine and experimental Mannich-base drugs administered to *Aotus* or *Saimiri* monkeys (Kotecka & Rieckmann, 1995; Kotecka et al., 1997). The scientific value of the results obtained by bioassays is less clear than that for the results of other assays. In the author's opinion, the benefits of bioassays do not outweigh the unnecessary harm done to animals, especially monkeys.

Use of bioassays for studying antimalarial drugs has become less common for several obvious reasons. Maintenance of animals solely for studying antimalarial drugs requires a large budget and technical support and is illegal in most countries if a protected animal species, such as *Aotus* monkeys, is involved. Ethically, it might be questionable to administer an experimental drug to monkeys without prior complete toxicological studies on small laboratory animals. Only a few, selected drugs can be studied in bioassays. The pharmacokinetics of the test compound might differ considerably in humans and monkeys, rendering interpretation and extrapolation of the results hazardous. It is difficult to standardize bioassay protocols because of the inherent biological variation. Drug and metabolite concentrations in test samples must be measured by high-performance liquid chromatography (HPLC). Furthermore, information about the antimalarial activity of drugs after biotransformation can be obtained by other experimental means, and chemists might be able to find a way to synthesize drug metabolites to determine their in vitro activity directly.

In contrast, use of bioassays to estimate the plasma concentrations of clinically approved drugs and to evaluate the antimalarial activity of both the parent drug and its biologically active metabolites in humans has provided important insights into pharmacodynamic interactions. For optimal interpretation, measurement of both the drug and its metabolites remains vital. The use of bioassays for indirect measurement of plasma drug concentrations in malaria-free persons receiving chemoprophylaxis or in healthy volunteers to whom a drug is administered for pharmacological studies might be a simple, affordable alternative to HPLC, which is the gold standard for qualitative and quantitative determination of drug concentrations.

Bioassays can be performed with the WHO microtest protocol in the field, i.e. with a small volume of fingerprick capillary blood (Yeo & Rieckmann, 1992; Kotecka & Rieckmann, 1993). Kotecka, Edstein & Rieckmann (1996) and Edstein et al. (1997) obtained plasma samples from soldiers receiving prophylaxis with chloroquine-doxycycline, proguanil or proguanil-dapsone and determined the inhibitory effect of serially diluted plasma against laboratory-adapted *P. falciparum* strains. The plasma chloroquine concentrations estimated from bioassays were strongly correlated with the drug concentration determined by HPLC. Moreover, the bioassay results were in agreement with those of in vitro and in vivo tests. Na-Bangchang et al. (1997) administered a single oral dose of dihydroartemisinin to healthy volunteers and collected serial samples of venous blood through an indwelling catheter. Serum samples were serially diluted with normal human serum and evaluated for their inhibitory effect on a reference *P. falciparum* strain. In parallel, pharmacokinetic profiles were characterized by HPLC. The undiluted (1:1) sera (v/v 20% in RPMI medium) completely inhibited schizont maturation 15 min to 12 h after the dose in most cases. During and a few hours after the mean peak plasma concentration had been attained, serum dilutions of 1:4 to 1:64 resulted in complete inhibition of schizont maturation. The antimalarial activity of serum closely followed the plasma concentrations of dihydroartemisinin. Newton et al. (2000) conducted a similar study in malaria-infected patients treated with artesunate and determined the pharmacokinetic profiles by both HPLC and bioassays. The results were similar to those of other studies. For field application, the HRP II-based bioassay might be more convenient to perform (Noedl, Teja-Isavadharm & Miller, 2004; Noedl et al., 2005).

Unlike HPLC determination of most commonly used antimalarial drugs, reliable, reproducible measurement of artemisinin derivatives requires great skill with a method such as HPLC and reductive mode electrochemical detection. Kotecka et al. (2003) and Teja-Isavadharm et al. (2004) showed that bioassay of artemisinin derivatives in plasma is strongly correlated with HPLC measurements. Furthermore, Teja-Isavadharm et al. (1996) and Bethell et al. (1997) established the pharmacokinetic parameters of artemether and dihydroartemisinin from HPLC and bioassays. Plasma samples from healthy volunteers who received artemether were serially

diluted with control plasma, and a reference *P. falciparum* clone was cultured in 96-well plates. Parasite growth was assessed by incorporation of [^{3}H]hypoxanthine. The IC_{50} values were calculated from the standard curve of parasite growth vs known drug concentration, and the plasma concentration of artemisinin derivatives was calculated from a second standard curve of IC_{50} value vs known plasma concentration. These investigators concluded that the two methods yielded similar results and that bioassay was more sensitive than the HPLC methods existing at the time of the study.

These studies suggest that bioassays might be a useful adjunct in the field to distinguish prophylactic failures due to drug resistance (with an adequate plasma drug concentration) or other causes (poor compliance, poor absorption, inadequate hepatic biotransformation) associated with low plasma concentration. Furthermore, field laboratories with in vitro culture facilities might find bioassays a useful alternative to HPLC.

1.1.6 Ex vivo assays

An early form of ex vivo assay was performed in experimentally infected *Aotus trivirgatus* monkeys treated with experimental drugs (Richards & Williams, 1975). A single venous blood sample was collected 18 h after the first dose, diluted in culture medium and incubated for 24 h. Parasite growth was estimated by [^{3}H]leucine incorporation. The only information obtained was that control parasites from untreated monkeys that died from *P. falciparum* infection within 10 days developed into mature schizonts, whereas parasites from treated monkeys did not develop beyond schizonts with two to four nuclei.

More recent ex vivo assays bear a superficial resemblance to in vivo-in vitro bioassays. Instead of a test compound being administered to uninfected animals, however, a drug that has been registered for clinical use is administered to malaria-infected patients. Instead of serial blood samples being collected to test the inhibitory effect of serum, they are collected to obtain malaria-infected erythrocytes that have been exposed to a test compound for various times. The serial samples of clinical isolates of *P. falciparum* are then cultured. In bioassays, the inhibitory effect of serum is determined in order to estimate the level and duration of drug activity and possibly to identify biologically active metabolites or pro-drugs, i.e. a weak antimalarial drug that undergoes hepatic biotransformation to a highly active antimalarial drug (e.g. proguanil). In ex vivo assays, serially collected isolates are cultured in vitro to assess their capacity to develop into mature trophozoites and schizonts after exposure to a test compound in vivo.

The standard method for assessing a parasitological response in a patient under treatment is to quantify and follow the evolution of asexual parasite density by microscopic examination of blood smears until parasite clearance is attained. Viable and non-viable young trophozoites from a patient under

therapy cannot be distinguished by morphological criteria on microscopic examination of blood films. In mammalian cells, a simple, rapid trypan blue dye exclusion test can be used to differentiate functionally viable cells from dead cells; an equivalent test for malaria parasites is uptake of rhodamine 123.

The "modern" version of the ex vivo assay, to assess the viability of *P. falciparum* isolates in patients under treatment and to determine the rapidity of drug action, was described by Watkins, Woodrow & Marsh (1993). Patients were treated with quinine or with a combination of quinine, sulfadoxine and pyrimethamine for severe malaria and with sulfadoxine and pyrimethamine or halofantrine for uncomplicated malaria. Venous blood samples (0.2 ml) were collected through an indwelling catheter every 6 h until 30 h after the first dose. The parasites were cultured for 72 h (RPMI 1640, HEPES and $NaHCO_3$ with 10% human serum; 8% erythrocyte volume fraction; 5% O_2–3% CO_2; medium changed daily), and thin blood smears were examined every 24 h. Functional viability was determined on the basis of the percentage of ring forms that developed to trophozoites or schizonts after 24–48 h of culture in vitro. During the first 24 h of treatment, quinine, quinine-sulfadoxine-pyrimethamine and sulfadoxine-pyrimethamine had no effect on viability; a large proportion of ex vivo parasites matured to trophozoites and schizonts. In contrast, parasites exposed to halofantrine in vivo for 6–12 h failed to mature. The results of the experiments with quinine are in agreement with the findings of Mapaba et al. (1995, 1996), who showed that some parasites remain viable even after continuous exposure to high quinine concentrations for up to 96 h.

Using a modified ex vivo assay incorporating aphidicolin to arrest parasite development at the early schizont stage, Murphy et al. (1995) confirmed the finding that there is no reduction in the ex vivo viability of ring-stage parasites during the first 24 h of quinine therapy. In contrast, in children with cerebral malaria treated with intramuscular artemether, a significant reduction in parasite viability was observed 6 h after the start of treatment, and most of the isolates were not viable 24 h after the start of treatment. Gachot et al. (1995) reported that parasite viability is inversely correlated with plasma quinine level and that there might be a marked decrease in viability 12 h after the start of intravenous quinine therapy in non-immune patients with severe malaria, provided that a loading dose is administered. They also observed that parasite viability increases if a loading dose of quinine is not administered. In a comparison of the rapidity of action of artemether and sulfadoxine-pyrimethamine, the results of the ex vivo test reflected the clinical response (Sowunmi & Oduola, 1998). After 8–12 h of exposure to artemether, viability ex vivo was considerably reduced in most isolates, and no viable parasites were found 30 h after treatment. In contrast, some parasites were viable even 36 h after sulfadoxine-pyrimethamine treatment. These results are in general agreement with the finding of stage-specific inhibition by quinine, mefloquine and artemisinin derivatives (Jiang et al., 1982; Alin & Björkman, 1994; ter Kuile et al., 1993; Skinner et al., 1996).

The ex vivo assay allows evaluation of the stage-specificity of drugs, the rapidity of drug action and pharmacodynamics in patients under therapy. These results contribute to understanding of antimalarial drug actions and are highly relevant to clinical pharmacology. As the assay requires serial collection of venous blood samples, it should be performed in a hospital setting or in a malaria research laboratory adjacent to a hospital. Only drugs approved for clinical use should be evaluated in ex vivo assays.

1.1.7 Target-specific assays

In the assays described above, whole living parasites are used. If the molecular target of candidate drugs is known, the target can be purified and exposed directly to drugs. This approach is particularly useful for screening drugs belonging to chemical classes known to inhibit the target. At present, several potentially promising substances serve as molecular targets: haemozoin for quinoline-type drugs, dihydrofolate reductase (DHFR) for antifolate drugs and 1-deoxy-D-xylulose 5-phosphate reductoisomerase for fosmidomycin derivatives.

A biochemical process that is unique to malaria parasites is formation of haemozoin crystals in the acidic food vacuole (*Haemoproteus columbae*, an apicomplexan protozoan, and *Schistosoma mansoni* also produce ß-haematin: Chen, Shi & Sullivan, 2001). The hypothesis has been proposed that haemozoin formation is involved in the detoxification of haem molecules, more precisely known as ferriprotoporphyrin IX, derived from haemoglobin digestion. The main mechanism of action of quinoline-containing drugs (e.g. chloroquine, amodiaquine, quinine, mefloquine) has long been thought to involve inhibition of the formation of haemozoin from haematin, a toxic derivative of ferriprotoporphyrin IX. The precise target ("haem polymerase" or other, unidentified enzymes, haematin or haemozoin itself) and mode of action resulting in parasite death remain unknown (Sullivan, 2002; Egan, 2003). In vitro assays for inhibition of formation of β-haematin, the synthetic equivalent of naturally occurring haemozoin, are important alternative tools for accelerated, high-output drug screening (Egan, Ross & Adams, 1994; Basilico et al., 1998; Kurosawa et al., 2000; Deharo et al., 2002; Kalkanidis et al., 2002; Sahal, Kannan & Chauhan, 2003).

Malarial enzymes with characteristics that differ considerably from those of their human counterparts are particularly suitable for target-specific assays. This is the case for DHFR. The corresponding gene (wild-type and mutants with various combinations of mutations that confer drug resistance) can be amplified by a polymerase chain reaction (PCR), inserted into an expression vector and transformed into bacteria or yeast. Point mutations can be introduced by mutagenesis. The protein produced by the heterologous system is purified, and the enzyme activity is determined with natural substrates and drug candidates. The complete DNA sequences of *P. falciparum* and *P. vivax dhfr* genes are now known (Bzik et al., 1987; Snewin et

al., 1989; Eldin de Pécoulas et al., 1998a). Human DHFR can be used as a control to anticipate cytotoxicity. This approach has been used to screen novel antifolates that inhibit mutant *P. falciparum* and *P. vivax* DHFR associated with pyrimethamine resistance (Brobey et al., 1996; Wooden et al., 1997; Brobey et al., 1998; Ferlan et al., 2001; Hastings & Sibley, 2002). Antifolate drugs that are effective against other pathogens can also be screened by inserting the corresponding *dhfr* gene into the heterologous system (Sibley et al., 1997; Brophy et al., 2000; Lau et al., 2001; Gerum et al., 2002; Ma, Jia & Kovacs, 2002).

Similar approaches can be devised to screen drugs that inhibit other malarial enzymes, e.g. 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Haemers et al., 2006). The main disadvantage is that the current assay systems are designed to detect only those drugs that have mechanisms of action similar to those of the quinoline-type antimalarial drugs, antifolates and fosmidomycin derivatives. These in vitro assays are complementary methods for primary drug screening. They have little relevance for field workers, whose main concern is to track drug-resistant parasites.

1.2 Assays for the sensitivity of other human *Plasmodium* spp.

1.2.1 *P. vivax*

Historical background of drug-resistant P. vivax

Clinical observations suggest that South-East Asia and the Amazonian basin are the original foci of chloroquine-resistant *P. falciparum*, identified in the late 1950s, from where drug-resistant parasites spread to other endemic regions (Maberti, 1960; Moore & Lanier, 1961; Rodrigues, 1961; Young & Moore, 1961; Harinasuta, Suntharasamai & Viravan, 1965; Peters, 1987). The spread of chloroquine-resistant and antifolate-resistant *P. falciparum* from South-East Asia to Africa was suggested by recent genetic studies (Mehlotra et al., 2001; Wootton et al., 2002; Nair et al., 2003). Antifolate drugs (pyrimethamine, proguanil, cycloguanil, sulfonamides) have been widely used to treat chloroquine-resistant malaria, exposing both *P. falciparum* and *P. vivax*, which are highly prevalent and often co-exist in endemic regions, to 4-aminoquinolines and antifolates. Early work showed that *P. falciparum* and *P. vivax* can acquire resistance to proguanil and pyrimethamine (Seaton & Adams, 1949; Seaton & Lourie, 1949; Edeson & Field, 1950; Canet, 1953; Clyde & Shute, 1954; Burgess & Young, 1959; Young & Burgess, 1959), and these reports were confirmed by clinical observation of slow parasite clearance and early recrudescence (i.e. before day 28) after antifolate therapy, in particular with *P. vivax* infection, a few years after massive introduction of antifolates for malaria control (McGregor & Smith,

1952; Hernandez et al., 1953; Maberti, 1960; Doberstyn et al., 1979; Darlow et al., 1982; Ponnampalan & Frank, 1983).

Suspected clinical cases of chloroquine-resistant *P. vivax* were reported from South Asia in the late 1970s and early 1980s (Gupta et al., 1978; Talib et al., 1979; Ali & Uppal, 1981). About a decade later, a series of reports on *P. vivax* infections suspected to be resistant to chloroquine or mefloquine followed and continue to be reported from various parts of the world (Arias & Corredor, 1989; Rieckmann, Davis & Hutton, 1989; Schuurkamp et al., 1989; Baird et al. 1991; Collingnon, 1991; Schwartz, Lackritz & Patchen, 1991; Amor & Richards, 1992; Garavelli & Corti, 1992; Schuurkamp et al., 1992; Kyaw et al., 1993; Murphy et al. 1993; Garg et al., 1995; Than et al., 1995; Baird et al., 1996; Fryauff et al., 1998; Alecrim, Alecrim & Macêdo, 1999; Singh, 2000; Soto et al., 2001). At least some of the reported cases are probably due to relapse rather than true drug resistance. Chloroquine-resistant *P. vivax* is currently limited to a few foci. For example, in Thailand, chloroquine-resistant *P. vivax* has not been reported, although a clinical study confirmed the poor therapeutic response of *P. vivax* to sulfadoxine-pyrimethamine (Tan-ariya et al., 1995; Pukrittayakamee et al., 2000; Congpuong et al., 2002).

Antifolate-resistant *P. vivax* is found frequently in South-East Asia and South America and less frequently or rarely in South Asia, East Africa and elsewhere in the world, while chloroquine-resistant *P. vivax* occurs sporadically in restricted foci in the Pacific region and possibly in some parts in Asia and South America. These observations confirm the concern of malariologists that human plasmodial species besides *P. falciparum* can well develop drug resistance.

Most of the reports cited above are based on clinical observations. Some were also supported by measurements of plasma levels of the drug. More recent studies based on analysis of the *P. vivax dhfr* gene and characterization of its protein product have revealed the genetic and biochemical mechanism of antifolate resistance (Eldin de Pécoulas et al., 1998a,b; Tahar et al., 2001; Leartsakulpanich et al., 2002). The results of these studies are in general agreement with clinical observations. The possible role of amino acid substitutions in *P. vivax* DHPS in conferring acquired or innate resistance to sulfonamides is not clear at present (Korsinczky et al., 2004; Imwong et al., 2005; Anliff et al., 2006). As for chloroquine-resistant *P. vivax*, the molecular target of the drug has still not been identified with certainty, so that an approach like that used for antifolate drugs cannot be used to elucidate the molecular mechanism of resistance.

In vitro assay systems for P. vivax

In order to document drug-resistant *P. vivax* fully, measurements of plasma drug concentrations after supervised drug administration, analysis of

the genetic profile of pre-treatment and post-treatment isolates by PCR and in vitro drug sensitivity patterns are required. These laboratory tests are particularly important for *P. vivax* and, to a lesser extent, *P. ovale* infections, as one of the fundamental biological and clinical characteristics of these malaria species is to produce repeated erythrocytic phase infections from "reactivation" of hypnozoites, a dormant hepatic stage (i.e. relapse).

Long-term culture of *P. vivax* is not feasible without frequent addition of reticulocytes and modified culture media. For in vitro assays, however, short-term culture for a single erythrocytic cycle, without re-invasion and initiation of the second schizogonic cycle, is the minimum requirement. In practice, re-invasion can occur, and rings of the second schizogonic cycle may be present, in spite of an incubation period < 48 h, if trophozoites and schizonts are present at the time of blood collection. It should be remembered that, unlike *P. falciparum*, *P. vivax* isolates often present several developmental stages of both asexual and sexual erythrocytic phases in the peripheral circulation of the human host, including schizonts.

To avoid confusion when counting parasites after incubation, it is advisable to select *P. vivax* isolates with a predominance of rings at the time of blood collection, to shorten the incubation period to < 36 h or to use non-microscopic methods (e.g. [^{3}H]hypoxanthine or LDH; HRP II is specific for *P. falciparum* and hence inapplicable to assess the growth of *P. vivax*). For practical purposes, the low levels of [^{3}H]hypoxanthine incorporation and LDH production during the ring stage of the second schizogonic cycle do not affect the results. Synchronization of *P. vivax* by sorbitol treatment is deleterious for subsequent in vitro growth (Hamedi et al., 2002). Another technical problem that *P. vivax* infections may pose is the generally low parasitaemia in the peripheral circulation, which is due to the limited proportion of reticulocytes (mean, 2.0–2.2% of circulating erythrocytes; maximum, 5% in normal healthy children and young adults) (Geaghan, 2000).

Several in vitro drug sensitivity assays for *P. vivax*, derived from the culture techniques of Trager & Jensen (1976) and from the microtechnique of Rieckmann et al. (1978), have been reported. Attempts to culture *P. vivax* indicate that the standard growth medium for *P. falciparum*, RPMI 1640 medium and 10% human serum, without further supplements, generally does not support growth of the parasites (Chotivanich et al., 2001). Fair-to-satisfactory growth of the parasites for a single erythrocytic cycle can be obtained by using a mixture of RPMI 1640 and Waymouth MB 752/1 media; McCoy 5A medium; or RPMI 1640 supplemented with glucose, inorganic salts, vitamins and hypoxanthine (Chotivanich et al., 2001). Supplementation of these modified media with high concentrations of human serum (v/v, 20–50%) further enhances parasite growth. For in vitro drug sensitivity assays terminated within the first schizogonic cycle, additional reticulocytes are not required. For short-term culture, the growth of *P. vivax* isolates is similar in Waymouth and RPMI 1640 media (v/v 1:2) and in McCoy 5A medium (Hamedi et al., 2002).

Gajanana & Raichowdhuri (1984) cultured monolayers of feeder cells in microtitre plates to supplement RPMI and 10% human serum mixture less than 3 days before in vitro assays. Fingerprick capillary blood (10 µl per well; starting parasitaemia, 4300–34 300 asexual parasites per µl) from 19 patients was mixed with RPMI and 10% human serum mixture (200 µl per well) and incubated at 37 °C for 16–24 h. The end-point was the number of schizonts per 100 asexual parasites. The authors reported that most of the trophozoites matured into schizonts in drug-free control wells. A number of technical details were not reported in this short communication. The requirement for feeder cells precludes field application of this method.

Brockelman, Tan-Ariya & Bunnag (1989) introduced several improvements to Rieckmann's microtechnique, including use of Waymouth MB 752/1 and RPMI 1640 medium (v/v 1:3 mixture) buffered with HEPES and $NaHCO_3$, supplementation with 15% type AB^+ human serum and incubation for 24, 30 or 44 h in a candle jar at 39 °C. Relatively synchronous isolates with a predominance of rings and a parasitaemia of ≥ 0.5% were selected for this study. Blood-medium mixture (2.5% erythrocyte volume fraction, 50 µl per well) was distributed in pre-dosed WHO microtitre plates. After incubation, parasites were counted under a microscope. Interpretation of the results was more consistent when the results of the 44-h incubation, rather than the 24-h incubation, were expressed as a percentage of pre-schizonts and schizonts (per 200 asexual parasites) instead of as the number of parasites per 100 leukocytes. The relatively low rate (23 of 34 isolates, 68%) of schizont maturation in drug-free control wells was ascribed, at least partly, to repeated washes by centrifugation at 4 °C, which might have ruptured the relatively fragile plasma membrane of *P. vivax*-infected erythrocytes. Other possible causes include a delay between blood collection and culture and transport on wet ice. Under similar assay conditions, Basco & Le Bras (1994b) obtained interpretable results with the radioisotope method in three of four *P. vivax* isolates.

Tan-ariya et al. (1995) and Hamedi et al. (2002, 2003) used the modified assay of Brockelman, Tan-ariya & Bunnag (1989) to evaluate the in vitro response to chloroquine of *P. vivax* isolates from Thailand and the Islamic Republic of Iran, in parallel with the clinical response to therapy with chloroquine and primaquine (Hamedi et al., 2003, used only in vitro assays). Venous blood was washed once, instead of thrice in the original protocol. The erythrocytes were suspended in Waymouth and RPMI 1640 medium (v/v 1:2, instead of 1:3) supplemented with 10% human serum (instead of 15%; 25% in the study of Hamedi et al., 2003). The blood-medium mixture (5% erythrocyte volume fraction and 50 or 100 µl per well, instead of 2.5% erythrocyte volume fraction and 50 µl per well) was distributed in WHO microtitre plates and incubated at 37.5–38.0 °C (not 39 °C) in a candle jar for 30–40 h (instead of 24 or 44 h). The numbers of pre-schizonts and schizonts were counted for 200 asexual parasites in each well. Most patients cleared parasitaemia within 48–72 h, and none showed reappearance of *P. vivax*

parasitaemia before day 30 after chloroquine treatment. The proportion of successful in vitro assays was low (33 of 57, 58% [34 of 42, or 81% in the study of Hamedi et al., 2003]) in Thailand and satisfactory (31 of 39, 79%) in the Islamic Republic of Iran. As in the earlier study of Brockelman, Tan-Ariya & Bunnag (1989), *P. vivax* isolates were highly sensitive in vitro to chloroquine (as extrapolated from the arbitrary fixed cut-off values for *P. falciparum*). Tan-ariya et al. (1995) noted that the chloroquine IC_{50} values were significantly higher in 1992–1993 than in the study of Brockelman, Tan-Ariya & Bunnag (1989), conducted in another Thai province in 1982–1985, suggesting a trend to declining chloroquine sensitivity. The two in vitro studies are not, however, directly comparable because of various technical differences.

Tasanor et al. (2002) and Congpuong et al. (2002) further modified the technique of Brockelman, Tan-Ariya & Bunnag (1989). Whole blood (100 µl capillary blood) containing > 133 per 200 asexual parasites in ring or trophozoite stages was immediately diluted in 1.9 ml of Waymouth and RPMI 1640 medium (v/v 1:1), without serum supplementation. There was no washing step. The blood-medium mixture (approximately 2.0–2.5% erythrocyte volume fraction; 50 µl per well) was distributed in the WHO microtitre plates and incubated at 37.5 °C in a candle jar for 30 h or 42 h. Rings, trophozoites, pre-schizonts with fewer than eight nuclei and schizonts with more than eight nuclei were counted in 200 asexual parasites in thick blood films. For each drug concentration, the number of parasites in these four stages was multiplied by stage-dependent factors (0.75, 2.75, 4.50 and 5.50, respectively), and the sum ("population indicator") was used to evaluate the validity of the assay (e.g. < 400 before assay, difference > 100 between post-incubation drug-free control and pre-incubation control) and to calculate the IC_{50}, IC_{90} and IC_{99} values by log-probit analysis (see Tasanor et al., 2002, for details). These modifications did not increase the rate of success (61–66%).

Russell et al. (2003) developed a modified WHO microtest method based on the findings of Golenda, Li & Rosenberg (1997). Venous blood was collected (2 ml) from patients with 0.02–1% *P. vivax* rings, and leukocytes were removed by passage through a CF11 cellulose powder column. The leukocyte-free blood was washed twice, and blood-serum mixture was reconstituted in type AB^+ non-immune human serum (40% erythrocyte volume fraction). The blood-serum mixture was added to McCoy 5A medium supplemented with 20% human serum to obtain the blood-medium mixture (v/v 1:9; 4% erythrocyte volume fraction; final serum concentration, 24%), which was added (50 µl per well) to each well of the pre-dosed microtitre plates. After an incubation period of 24–36 h, when ≥ 50% of the rings had developed into schizonts, thick blood films were made from each well to determine the MIC. The preliminary field study showed a high rate of parasite growth (34 of 39, 87%) and good in vitro activity of dihydroartemisinin against *P. vivax* isolates. Direct comparison of the MICs of chloroquine for *P. vivax* and *P. falciparum* showed inconsistent results, possibly due to differences in the genetic and biochemical mechanisms of resistance in

these two *Plasmodium* species. In a further study (Kosaisavee et al., 2006), it was shown that a slightly modified method yields similar chloroquine IC_{50}s using microscopic, isotope, and PicoGreen assays conducted in parallel with the same *P. vivax* isolates. However, the morphological method was more successful in producing valid assays.

Chotivanich et al. (2001, 2004) reported a short-term culture system for *P. vivax* in a modified RPMI 1640 medium. A high percentage of rings underwent complete schizogony with this technique. Use of this culture medium for determining in vitro drug sensitivity profiles might prove to be at least as satisfactory as, or better than, RPMI and Waymouth mixture or McCoy 5A medium. These publications indicate that researchers have not yet found the best protocol for determining the drug response of *P. vivax* isolates.

Ex vivo assay for P. vivax

Ridley et al. (1997) induced an experimental *P. vivax* infection in splenectomized owl monkeys, *Aotus boliviensis*, to obtain trophozoites for short-term in vitro culture. Leukocytes were removed by filtration, and no supplementary reticulocytes were added. The culture conditions were similar to those for *P. falciparum* (RPMI 1640, HEPES and $NaHCO_3$ medium; 11-h incubation in an atmosphere of 3% O_2, 4% CO_2 and 93% N_2), except that a higher concentration (20%) of type AB^+ human serum and 2 g/l of glucose were added. The assays were carried out in 96-well microtitre plates, and parasite growth was measured by the incorporation of [^{3}H]hypoxanthine. The incubation period of 11 h was sufficient for schizont maturation. A bisquinoline candidate drug was highly active against *P. vivax* but was about threefold less active against a chloroquine-resistant *P. falciparum* strain, due at least partly to cross-resistance between chloroquine and bisquinolines. The ex vivo assay for *P. vivax* is still in an early experimental stage and any future application of this type of assay will require further improvement of the culture conditions for this *Plasmodium* species.

1.2.2 *P. malariae*

P. malariae can be cultured for a short period with techniques similar to those used for *P. vivax* to determine the in vitro response. On the basis of the modified method of Brockelman, Tan-ariya & Bunnag (1989), Tan-ariya & Pasuralertsakul (1994) determined the in vitro response of three fresh clinical isolates of *P. malariae* to chloroquine. Venous blood samples were washed once in Waymouth medium, and the erythrocytes were suspended in Waymouth and RPMI 1640 medium (v/v 1:2 mixture; 5% erythrocyte volume fraction; starting parasitaemia, 0.02–0.05%, mostly rings) supplemented with 10% type AB^+ human serum and distributed (100 µl per well) in chloroquine-pre-dosed WHO microtitre plates. After an incubation period of 24 h in a candle jar at 37.5–38.0 °C, the assay was

terminated. Thick blood films showed pre-schizonts and schizonts (48–59% of asexual parasites in drug-free control wells) in all three isolates. In vitro schizont maturation was inhibited at low chloroquine concentrations, corresponding to the rapid clinical response (parasite clearance within 48 h) to chloroquine in the corresponding patients. Similar results were obtained in 4 of 10 radioisotope assays (Ringwald et al., 1997).

It has been reported that, in cases of mixed *P. falciparum*–*P. malariae* infections, rings of *P. malariae* can develop into schizonts in a RPMI–blood mixture, even in wells containing high concentrations of chloroquine in which schizonts usually signify in vitro chloroquine resistance for *P. falciparum* (WHO, 1984). For this reason, the WHO protocol for the microtest requires a careful search for malaria parasites, an accurate microscopic diagnosis and exclusion of blood samples with mixed infections. Genetic tests are needed to prove that *P. malariae* was indeed present in these cases and to exclude a misdiagnosis of *P. falciparum*, which is commonly seen in so-called mixed infections. Morphology alone might not be sufficiently reliable for a specific diagnosis. More studies are needed to define the nutritional requirements and culture conditions of *P. malariae* to optimize its in vitro proliferation.

1.2.3 *P. ovale*

Owing to its limited geographical distribution, its relative rarity and difficulties in distinguishing this malaria species from *P. vivax* on the basis of morphological features, the in vitro drug response of *P. ovale* has not been studied extensively. Basco & Le Bras (1994b) and Ringwald et al. (1997) reported limited success in determining the in vitro response of four of nine and five of eight *P. ovale* isolates, respectively, to chloroquine with a radioisotope assay derived from the method originally developed for *P. vivax* by Brockelman, Tan-Ariya & Bunnag (1989). [^{3}H]Hypoxanthine was an appropriate growth indicator. The level of [^{3}H]adenosine incorporation was low, i.e. < 10-fold that of [^{3}H]hypoxanthine in drug-free control wells. Microscopic examination of the control wells showed a predominance of rings and trophozoites before the assays and schizont formation after 42 h. Further improvements are necessary to achieve a higher rate of schizont formation.

1.3 Factors that influence sensitivity to drugs

1.3.1 Effect of culture medium and supplements

Testing quinoline-type drugs

In the majority of the in vitro studies conducted since 1976, RPMI 1640 medium was used. Reconstituted RPMI 1640 medium lacking several

amino acids and supplemented with dialysed human serum (i.e. without amino acids), as described by Divo et al. (1985a), does not change drug effects (Geary, Divo & Jensen, 1985). Few published data are available on the IC_{50} values obtained when parasites are cultured in media other than RPMI 1640. Microscopic examination of Giemsa-stained thin smears after a 72-h incubation period showed similar IC_{50} values for various antimalarial drugs determined in RPMI 1640 supplemented with 10% human serum and in GIT medium (equal volumes of Iscove and F-12 medium, 10% GF21 and other supplements) (Kim et al., 1998). In another study based on the radioisotope assay, it was shown that chloroquine IC_{50} does not differ when fresh clinical isolates are cultivated in RPMI 1640, Dulbecco's modified Eagle's medium, or Iscove's modified Dulbecco's medium (Basco, 2006).

Unlabelled (or cold) hypoxanthine is routinely added to RPMI 1640 medium in some laboratories to optimize the growth of reference strains maintained in continuous culture. When radioisotope assays are performed in the presence of unlabelled hypoxanthine at concentrations ≥ 2 µmol/l, incorporation of [^{3}H]hypoxanthine decreases, without affecting growth rates (Chulay, Haynes & Diggs, 1983). Addition of other unlabelled purines, such as adenine, adenosine, guanosine, inosine, ADP and NAD, at concentrations ≥ 1 µmol/l to the culture medium also diminishes [^{3}H]hypoxanthine incorporation. To obtain maximal incorporation of [^{3}H]hypoxanthine in drug assays on laboratory-adapted strains, the usual practice is to wash the infected erythrocytes thoroughly with hypoxanthine-free RPMI 1640 just before the assay or to culture the parasites for a complete schizogonic cycle with hypoxanthine-free medium before assay. With fresh clinical isolates cultured for a single schizogonic cycle, there is no need to add unlabelled hypoxanthine to determine a drug response. Human erythrocytes store hypoxanthine, and an additional source of hypoxanthine is provided if human serum or plasma is added to the culture medium.

Testing antifolate drugs

Standard RPMI 1640 contains folic acid (1 mg/l) and PABA (1 mg/l). These substrates antagonize the blood schizontocidal activity of antifolate drugs, which include sulfonamides, sulfones and DHFR inhibitors (pyrimethamine, trimethoprim and cycloguanil, the biologically active metabolite of proguanil). To measure the in vitro activity of these drugs, a modified RPMI 1640 medium with little or no folic acid and PABA is used (Desjardins et al., 1979; Brockelman & Tan-Ariya, 1982a; Chulay, Watkins & Sixsmith, 1984; Sixsmith et al., 1984; Spencer et al., 1984; Milhous et al., 1985; Watkins et al., 1985; Schapira et al., 1986; Petersen, 1987; Tan-ariya, Brockelman & Menabandhu, 1987; Watkins et al., 1987; Payne & Wernsdorfer, 1989). In some studies, Waymouth medium was used in the place of RPMI 1640 because the former contains a low concentration of folic acid and lacks PABA (Tan-ariya & Brockelman, 1983; Sabchareon et al., 1985)(see also annex 3).

Even if the exogenous supply of folic acid and PABA in culture medium is reduced or eliminated, human plasma contains 6–20 ng/ml of folic acid and highly variable concentrations (< 20–820 ng/ml) of PABA (Milhous et al., 1985; Schapira et al., 1986). Furthermore, human erythrocytes store PABA and folic acid (160–640 ng/ml of packed cells), which can be used by the parasites. These alternative sources apparently supply a sufficient amount of folate cofactors to most laboratory-adapted *P. falciparum* strains and isolates cultured in PABA- and folic acid-free RPMI 1640 medium. Some laboratory-adapted *P. falciparum* strains (and possibly fresh clinical isolates) do not survive in a PABA- and folic acid-free environment (i.e. both medium and plasma depleted of these folate cofactors) (Milhous et al., 1985). High concentrations of PABA (≥ 1 mg/l) inhibit parasite growth, whereas high concentrations of folic acid (up to 8 mg/l) have no effect (Chulay, Watkins & Sixsmith, 1984).

The in vitro activities of sulfonamides and sulfones appear to be particularly sensitive to the presence of PABA and folic acid. The IC_{50} values (or MICs) of sulfa drugs determined in culture medium containing even a trace amount of folate cofactors may not accurately reflect their "real" inhibitory effect because of antagonism. Pyrimethamine activity is similar in assays performed with folic acid-free RPMI 1640 medium and with medium containing 10 µg/l folic acid, which corresponds to the normal physiological concentration of folate in human plasma (Chulay, Watkins & Sixsmith, 1984). The same concentration of folic acid reduces sulfadoxine activity by 1000-fold. It follows that the results of some early studies on in vitro antifolate drug activity in which the standard RPMI 1640 was used are not comparable to those of later studies with modified RPMI 1640 with low PABA and low folic acid concentrations (Nguyen-Dinh & Payne, 1980; Yisunsri & Rieckmann, 1980; Thaithong & Beale, 1981; Brockelman & Tan-Ariya, 1982a,b; Lamont & Darlow, 1982; Eastham & Rieckmann, 1983; Schapira, 1984).

In vitro assays for sulfadoxine pose a technical challenge. Even with PABA- and folic acid-free RPMI medium, a wide range of IC_{50} values has been reported for sulfadoxine-sensitive isolates and clones (Schapira et al., 1986; Winstanley et al., 1995). The problem is due, at least partly, to apparent inhibition of drug activity by the presence of trace amounts of folic acid or PABA in human serum and erythrocytes and the poor solubility of sulfadoxine in aqueous solutions. Wang, Sims & Hyde (1997) proposed a protocol which involves the following modifications:

- period of adaptation (several days to a few weeks) from the standard medium supplemented with human plasma to Albumax®-supplemented medium devoid of PABA and folic acid before assays;
- use of laboratory-adapted strains during the phase of rapid multiplication;
- use of PABA- and folic acid-free RPMI supplemented with Albumax®;

- prior testing of several batches of erythrocytes from non-immune donors;
- washing and suspension of uninfected erythrocytes in phosphate-buffered saline overnight;
- use of pure dimethyl sulfoxide as the drug solvent (up to 1 µl per well);
- an incubation period of 64 h;
- use of [^{3}H]hypoxanthine solution with no trace of ethanol; and
- [^{3}H]hypoxanthine pulsing after the initial 48-h incubation.

To evaluate the in vitro activity of sulfadoxine in field isolates, this protocol requires an initial adaptation to in vitro culture (Mberu et al., 2002). In our experience, adaptation of the protocol to test the sensitivity of fresh clinical isolates to sulfadoxine without prior adaptation to in vitro culture was partially successful (Ndounga, Basco & Ringwald, 2001).

The WHO basic kit for the Mark II and Mark III in vitro microtests includes sulfadoxine-pyrimethamine plates, RPMI 1640 with low PABA (0.5 µg/l) and low folic acid (10 µg/l) concentrations (designated low-PABA, low-folic acid medium) for testing all drugs (chloroquine, amodiaquine, quinine, mefloquine, artemisinin). The concentrations of PABA and folic acid in this medium correspond to the average levels in human plasma. The in vitro activity of drugs belonging to other chemical classes is not affected by the lower concentrations of PABA and folic acid in the culture medium, and the growth of field isolates in low-PABA, low folic-acid RPMI 1640 is comparable to that in standard RPMI 1640 (Tan-Ariya, Brockelman & Menabandhu, 1987; Payne & Wernsdorfer, 1989). Several modifications to the WHO assay protocol yield optimal results for sulfadoxine-pyrimethamine: a longer incubation period of 48 h, 2% erythrocyte volume fraction (5% for other drugs), 12% serum supplement (10% for other drugs), 100 µl final volume per well (50 µl per well for other drugs) (Nguyen-Dinh et al., 1985b).

Researchers who wish to evaluate the in vitro activity of antifolate drugs must place a special order with the suppliers for low-PABA, low-folic acid RPMI 1640 or RPMI devoid of folate cofactors, incurring higher costs because of the minimum amount required for a bulk order (up to 100 litres). An alternative solution is to forego in vitro testing of classical antifolate drugs and resort to molecular analysis of *P. falciparum dhps* and *dhfr* genes. At least for DHFR inhibitors, a strong correlation has been established between *dhfr* mutations and the in vitro response to these drugs (Foote, Galatas & Cowman, 1990; Peterson, Milhous & Wellems, 1990; Basco et al., 1995b; Reeder et al., 1996; Basco & Ringwald, 2000a; Basco, 2003b).

1.3.2 Effect of uninfected erythrocytes: erythrocyte volume fraction

In radioisotope assays, [^{3}H]hypoxanthine incorporation decreases in a non-linear manner as the number of uninfected erythrocytes increases for

identical numbers of infected erythrocytes; i.e. the erythrocyte volume fraction increases from 0.1% to 10%, but the parasitaemia decreases because of dilution in uninfected erythrocytes (Chulay, Haynes & Diggs, 1983). In contrast, an increase in the fixed proportion of infected to uninfected erythrocytes (i.e. the same parasitaemia for increasing erythrocyte volume fraction) results in increasing incorporation of [^{3}H]hypoxanthine from 1.0% to 2.0% erythrocyte volume fraction but tends to attain a plateau at ≥ 2.5% (Basco, 2004b).

1.3.3 Effect of infected erythrocytes

Initial parasitaemia and erythrocyte volume fraction

For a constant erythrocyte volume fraction (i.e. the same absolute number of both uninfected and infected erythrocytes), the in vitro activity of drugs tends to decrease, resulting in a higher IC_{50} value or MIC when the proportion of infected to uninfected erythrocytes increases. The need for an increased drug concentration to inhibit the growth of larger numbers of parasites for a constant volume is called the "inoculum effect", and this has been observed with most antimalarial drugs.

Thaithong, Beale & Chutmongkonkul (1983) studied the effects of initial parasitaemia ranging from 0.1% to 2% on the in vitro response of a laboratory-adapted strain, measured by microscopic examination of thin blood films. Parasitaemia > 1% was associated with decreased inhibitory activity of chloroquine, amodiaquine, quinine and mefloquine but not that of pyrimethamine. These investigators recommended that the initial parasitaemia be adjusted to 0.3– 0.8%. Gluzman, Schlesinger & Krogstad (1987) observed that the IC_{50} values of chloroquine in sensitive and resistant parasites increased by up to five- to sevenfold as the parasitaemia increased from 0.1% to 10%, with a constant erythrocyte volume fraction of 2%. Similar findings were made by Chaparro & Wasserman (1999) and Basco (2004b) with laboratory-adapted strains and field isolates, respectively. The inoculum effect has also been observed with quinine, mefloquine, halofantrine, artemisinin and dihydroartemisinin (Ritchie et al., 1996; Duraisingh et al., 1999).

For the same starting parasitaemia, increasing the erythrocyte volume fraction from 1.0% to ≥ 2.5% resulted in higher IC_{50} values for chloroquine and amodiaquine (Chaparro & Wasserman, 1999; Basco, 2004b). A confounding factor that can be observed at erythrocyte volume fractions > 2.5% in 96-well microtitre plates is suboptimal parasite growth during a 48-h incubation period without medium change, owing to depletion of nutrients, accumulation of lactic acid, poor gas exchange in the thicker layer of erythrocytes or combinations of these factors.

The fact that the inoculum effect leads to higher IC_{50} values or MICs implies that some isolates might be wrongly classified as resistant in vitro unless the erythrocyte volume fraction and starting parasitaemia are adjusted

for samples with a high parasitaemia. In addition, a low erythrocyte volume fraction (< 2.5%) and a low starting parasitaemia (< 1.0%) ensure optimal growth conditions and provide adequate nutrients to the parasites for up to 48 h without an intermediate change of culture medium.

Biological fitness, selection and chloroquine resistance

In short-term culture in in vitro drug sensitivity assays, rings of both chloroquine-sensitive and chloroquine-resistant isolates mature into schizonts over a 48-h incubation period. It has been suggested, however, that chloroquine-resistant *P. falciparum* isolates adapt more readily to in vitro culture conditions than chloroquine-sensitive parasites, as reflected in the larger number of rings that develop into schizonts during the first schizogonic cycle (Wernsdorfer et al., 1995). In the same study, asymptomatic carriers infected with chloroquine-resistant parasites tended to have significantly higher parasitaemia than those infected with chloroquine-sensitive parasites.

These observations support the unproven hypothesis that drug-resistant parasites might have some biological advantages, propagating more rapidly and in larger numbers, over their drug-sensitive counterparts. It is not clear what would happen if there were no more drug pressure to maintain their selection. Biological fitness might influence in vitro results for mixed parasite populations with varying proportions of sensitive and resistant parasites.

Several studies have shown that the phenotype and genotype of *P. falciparum* parasites determined during the early phase of in vitro adaptation can undergo profound modification during long-term culture, including loss of knobs and changes in drug sensitivity patterns (Langreth et al., 1979; Jensen, Capps & Carlin, 1981; Le Bras et al., 1983). Part of the explanation lies in the initial presence of multiple parasite populations, followed by the loss of populations by selection during adaptation to in vitro culture conditions (Thaithong, 1983; Thaithong et al., 1984; Thaithong, Beale & Chutmongkonkul, 1988; Viriyakosol et al., 1994; Ang, Chan & Mak, 1996, 1997; Druilhe et al., 1998). The biochemical and genetic mechanisms involved in adaptation to in vitro culture and the dynamics of the winnowing process during long-term culture have not been studied.

Intrinsic characteristics other than drug resistance

The concept of "virulence" with respect to malaria parasites is not yet well understood. In clinical practice, a virulent *P. falciparum* strain is associated with greater severity of disease. In biological terms, virulence may be associated with a high capacity for cytoadherence and rosetting, a high multiplication rate or a large repertoire and strong expression of genes responsible for antigenic variation to evade the host's immune response to the parasites.

The multiplication rates in vitro of *P. falciparum* isolates from Thai patients with severe and complicated malaria were threefold higher than those of isolates from patients with uncomplicated malaria (Chotivanich et al., 2000). There is some evidence that, in severe and complicated malaria, parasites are unselective for erythrocyte invasion (i.e. the number of erythrocytes infected with multiple rings is relatively lower), while the larger number of erythrocytes with multiple rings in uncomplicated malaria suggests that an average of 40 % of erythrocytes are susceptible to invasion at the beginning of the second schizogonic cycle in vitro (Simpson et al., 1999; Chotivanich et al., 2000). The direct relevance of these data for in vitro drug sensitivity assays has not been determined.

1.3.4 Effect of leukocytes

Depending on the experiment, in particular in biochemistry and immunology, the presence of leukocytes can produce artefactual results. Most PCR protocols for molecular biology are specific, despite the overwhelming presence of human DNA in blood samples, but some experiments (e.g. preparation of DNA library) are best performed without contaminating human DNA.

Although many early in vitro studies were performed with leukocyte-free blood, the growth of *P. falciparum* is not inhibited when it is maintained in the laboratory with non-immune human serum and leukocytes in blood from non-immune healthy donors (Capps & Jensen, 1983). In contrast, Binh, Luty & Kremsner (1997) showed that the presence of peripheral blood mononuclear cells (2×10^5 cells per well in 96-well plates) from healthy semi-immune African volunteers and African patients with acute malaria enhanced the in vitro growth of a laboratory-adapted *P. falciparum* strain over 6 days. Mononuclear cells from non-African, non-immune, healthy donors were associated with slightly enhanced parasite growth. The mechanisms involved (specific antibodies, cytokine production) are not yet known.

Leukocytes in freshly collected blood undergo rapid degradation within a few days, and the same phenomenon can be observed in cultured malaria-infected blood. In isotope assays, the presence of leukocytes in freshly collected venous blood samples does not influence the level of [^{3}H]hypoxanthine incorporation (Petersen, 1986; Chaparro & Wasserman, 1999). Thus, for most field-based research, elimination of leukocytes is unnecessary, and untreated erythrocytes (i.e. with leukocytes) can be used for routine culture and in vitro drug sensitivity assays.

Investigators wishing to work with leukocyte-free blood can resort to a CF11 cellulose powder column, cell affinity chromatography with protein A sepharose or commercial filters (Homewood & Neame, 1976; Waki & Suzuki, 1986; Ihalamulla & Mendis, 1987; Mons et al., 1988a,b; Janse et al., 1994; Mishra & Tekwani, 1996; Carlton et al., 1999). Although these studies

on leukocyte removal pertained to certain *Plasmodium* species, the techniques are generally applicable to all types of host blood and can eliminate close to 100% of contaminating leukocytes. It should be noted that leukocyte-free blood prepared by passage over a powdered cellulose column can result in suboptimal parasite growth (Capps & Jensen, 1983). Another technique, biomagnetic separation, is host-specific and might be useful for eliminating the trace amounts of leukocytes left after use of one of the above-mentioned techniques. In a study conducted by Carlton et al. (2001), mouse leukocytes were tagged with a magnetic particle-antibody complex that recognizes the specific antigen on the surface of mouse leukocytes. Passage of mouse blood through a magnetic field resulted in elimination of the tagged leukocytes. Of the various available methods for leukocyte removal, commercially available filtration units are the most convenient.

The possible influence of leukocytes on drug concentration has not been well studied. Bergqvist & Domeij-Nyberg (1983) showed that 70–85% of chloroquine administered to healthy volunteers or incubated with whole blood in vitro can be recovered from blood cells, mostly from granulocytes and platelets. Further studies might be needed to determine whether IC_{50} values and MICs are modified by the white blood cell and platelet count of patients. It has been assumed until now that the blood count has no effect on antimalarial drug activity in vitro.

1.3.5 Effect of serum factors

When drugs are administered to humans or animals, some of the drug that is absorbed and enters the blood circulation is bound to blood proteins, in particular albumin, while the rest remains free in the blood. The effects of the drug are mediated by the unbound fraction. The proportions of bound and unbound fractions depend on each drug; however, as the drug is removed from the unbound fraction into the parasite, more dissociates from the plasma proteins, as the affinity of this binding may not be strong.

For antimalarial drugs, it is assumed that the free, unbound fraction passes through the various biological membranes to reach the target site within the parasite and exert its inhibitory actions. The bound fraction is sequestered by blood proteins and must dissociate before it can enter the cells to act on the target. Any factor that influences the proportion of the bound:unbound fraction of the drug modifies the degree of inhibition of in vitro growth of the parasite.

Compounds can be characterized as lipophilic or hydrophilic; e.g. halofantrine is highly lipophilic, while chloroquine and amodiaquine are highly hydrophilic. Halofantrine is strongly bound to serum proteins (83%), mostly to low- and high-density lipoproteins (Cenni et al., 1995). When the medium used to culture malaria parasites contains human serum with high concentrations of triglyceride-rich lipoproteins (as during the postprandial

state), the unbound fraction of halofantrine hydrochloride decreases because more drug binds to serum proteins. The diminution of the free, unbound fraction implies that there will be less drug to inhibit parasite growth, leading to an increased IC_{50} value for halofantrine (Humberstone et al. 1998). The opposite phenomenon, an increased unbound fraction, can occur when Albumax® is used to supplement the culture medium, resulting in a lower halofantrine IC_{50} value (Ringwald et al., 1999a). The in vitro activity of a highly hydrophilic drug such as chloroquine is not influenced by the amount of triglycerides present in the serum supplement but is modified by use of Albumax® or autologous serum.

Another serum factor that influences the in vitro response to quinine is α_1-acid glycoprotein, also called orosomucoid. Many pharmacological studies have confirmed that the plasma concentration of α_1-acid glycoprotein is elevated during the acute phase of malarial infection and that the unbound, free fraction of quinine is reduced because of greater binding to this protein (Silamut et al., 1985; Mansor et al., 1991; Silamut et al., 1991; Supanaranond et al., 1991; Graninger et al., 1992; Pussard et al., 1999; Beesley et al., 2000). Quinine binds preferentially to α_1-acid glycoprotein rather than albumin. When acute-phase autologous serum is used to supplement RPMI 1640 medium, instead of non-immune human serum from healthy donors, for in vitro assays like the WHO macrotest and microtest systems, drug-protein binding properties are altered, resulting in high quinine IC_{50} values (Birley et al., 1992; Ringwald et al., 1999a). In earlier studies, it has been reported that chloroquine and halofantrine bind weakly to α_1-acid glycoprotein (Walker et al., 1983; Cenni et al., 1995). However, a recent study has suggested that the presence of α_1-acid glycoprotein (1.25 g/l) leads to an increased chloroquine IC_{50} (Gbotosho et al., 2006). Further studies are required to understand the interactions between various antimalarial drugs and α_1-acid glycoprotein. The available data suggest that acute-phase autologous serum is not suitable for in vitro drug sensitivity assays, particularly when quinine is being evaluated.

1.3.6 Effect of serum substitutes

In radioisotope assays, use of human plasma instead of human serum results in a three- to fourfold increase in [^{3}H]hypoxanthine incorporation for similar growth rates (Chulay, Haynes & Diggs, 1983). The possible cause is release of ADP from platelets during clot formation. Like unlabelled hypoxanthine, ADP at concentrations ≥ 1 µmol/l reduces [^{3}H]hypoxanthine incorporation. Another possible explanation is a difference between serum and plasma in cold hypoxanthine.

In a study conducted by Divo et al. (1985b), the sensitivity of 10 Sudanese isolates to chloroquine was identical with pooled human serum and with pooled adult bovine serum. No data were provided in the paper, however, and there was a high (40%) failure rate in parasite growth, probably

due to the long delay associated with the transport of blood samples from Sudan to the United States. Smalley & Brown (1982) determined the in vitro activity of pyrimethamine against fresh isolates of *P. falciparum* using RPMI 1640 supplemented with 15% fetal calf serum in a 48-h test. The activity in RPMI supplemented with human serum was not determined. In our experience, use of fetal calf serum as a replacement for human serum can enhance parasite growth during the first schizogonic cycle, depending on the serum batch, but is associated with consistent decreases or increases in the IC_{50} values for different antimalarial drugs (Basco, 2003a,c).

With regard to animal sources of serum substitutes, Geary, Divo & Jensen (1983) reported that use of 10% pooled human serum, 20% pooled rabbit serum or 10% hypoxanthine-supplemented (10 µmol/l) pooled bovine serum yielded comparable results (IC_{50} values not calculated). Smrkovski et al. (1983) observed that, with the WHO assay system, addition of 10% rabbit serum to the whole blood-RPMI mixture (v/v 1:9) generally enhanced parasite growth but could result in discordant results from those obtained with the whole blood-RPMI mixture without rabbit serum. Moreover, the low proportion (65%) of isolates that produced schizonts in their study and the surprisingly long incubation period required before schizonts were observed (≥ 60 h and up to 91 h, in 19 of 35 successful assays) render interpretation of these results difficult. Geary, Divo & Jensen (1983) compared in vitro responses to chloroquine and to antibiotics in culture media supplemented with 10% pooled human serum, 10% pooled bovine serum with cold hypoxanthine or 20% pooled rabbit serum. Although the IC_{50} values were not presented in the paper, the concentration-effect curves determined with human and animal sera were superposable for chloroquine and antibiotics. Oduola et al. (1985) found comparable IC_{50} values for chloroquine and mefloquine in two reference clones cultured in RPMI 1640 supplemented with either 10% human or 10% goat plasma.

Ofulla et al. (1994) determined the in vitro drug sensitivity pattern of laboratory-adapted and fresh field isolates in serum-free RPMI, HEPES and $NaHCO_3$ medium containing 5 g/l Cohn fraction V of bovine albumin and 10 ml/l of a lipid- and cholesterol-rich mixture and in the standard RPMI, HEPES and $NaHCO_3$ medium with 10% human serum by the isotope microtest of Desjardins et al. (1979). Although the IC_{50} values for chloroquine, amodiaquine, quinine and sulfadoxine-pyrimethamine were strongly correlated, the IC_{50} values were generally higher in serum-free medium, except that for sulfadoxine-pyrimethamine, which showed the opposite trend. Laboratory-adapted strains also grew in serum-free medium with only 5 g/l Cohn fraction V of bovine albumin (no lipid- and cholesterol-rich mixture). The IC_{50} values for chloroquine and quinine were much higher in serum-free medium, possibly because of the greater drug-protein affinity with bovine albumin than with human albumin, leading to smaller unbound fractions of drugs that exert schizontocidal action. In our experience, use of Albumax® in the place of human serum results in unpredictable

growth of an isolate (sometimes better than in human serum-supplemented RPMI medium, sometimes worse), and the IC_{50} values of different drugs are profoundly affected (Ringwald et al., 1999a; Basco, 2003a,c, 2004a).

1.3.7 Effect of incubation period

In vivo, *P. falciparum* completes the erythrocytic cycle in 48 h, and the duration of the complete schizogonic cycle is approximately the same in vitro. This observation is based on use of tightly synchronous parasites fully adapted to in vitro culture. Clinical isolates freshly obtained from patients might not be perfectly synchronous, as there may be two or more broods, each following its own synchronicity.

Some investigators have subdivided the ring stage into "tiny rings" (approximate age, 0–6 h), "small rings" (6–16 h) and "large rings" (16–26 h) on the basis of the morphological appearance of the visible portion of asexual parasites by microscopic examination of smears of peripheral blood (Jiang et al., 1982; Rieckmann, Suebsaeng & Rooney, 1987; Silamut & White, 1993). The "invisible" portion consists of mature trophozoites (26–36 h) and schizonts (36–48 h). These three subsets of rings are roughly equivalent to the "small, medium and large" classification scheme that was used much earlier, in particular in evaluating the feasibility of the macrotest, which requires large rings at the outset. Subdivision of the ring stage into small, medium and large phases is, however, arbitrary because, in reality, the ring stage is a continuum. Nevertheless, it helps understanding of the asynchronicity of malaria parasites in the human host.

The incubation period for in vitro drug sensitivity assays varies from 24 to 96 h, depending on the method, the drugs being tested and the researchers. In most studies, the incubation time is ≤ 48 h, i.e. within the first intraerythrocytic cycle of freshly isolated parasites. Some investigators using the reinvasion rate as the end-point prefer an incubation time of 48 h (Nguyen-Dinh & Trager, 1980). Still others have used a prolonged incubation time of 72–96 h for drugs that appear to have a delayed inhibitory effect on parasites after the first complete cycle (e.g. antibiotics and antimetabolites) (Geary & Jensen, 1983; Divo, Geary & Jensen, 1985; Geary, Divo & Jensen, 1985).

Many researchers working with laboratory-adapted *P. falciparum* strains prefer to analyse the drug response of synchronous parasites, for several good reasons. The in vitro drug sensitivity assays is designed to assess the inhibitory effect of drugs on schizont maturation. To apply this principle in practice and to obtain results that are comparable with those from other laboratories, assays should be performed with parasites most of which are at the ring stage at the beginning of the incubation period. Furthermore, drug effects are generally stage-dependent. The commonly used metabolic markers that indirectly reflect a parasite's metabolic activity are also stage-dependent.

Often, a particular erythrocytic stage tends to predominate in laboratory-adapted parasites, especially when medium changes and incubation conditions are regular. Several synchronization techniques exist for obtaining parasites in a narrow window of stage development (Jensen, 1978; Pasvol et al., 1978; Lambros & Vanderberg, 1979; Mrema et al., 1979; Reese, Langreth & Trager, 1979; Jensen, 1988). Sequential treatment of infected erythrocytes with two or more synchronization techniques is effective for obtaining parasites in the range of a 2–4-h difference in developmental stage.

After a single D-sorbitol treatment of asynchronous laboratory-adapted parasites, ring stages ≤ 18 h old are obtained. Depending on the proportion of early- and late-ring stage parasites after sorbitol treatment, preschizonts (defined by the presence of two to four nuclei) can appear as early as 16 h after synchronization, and the maximum number of mature schizonts is attained at 22–30 h (Srinivas & Puri, 2002).

Fresh clinical isolates of *P. falciparum* are usually predominantly rings, and synchronization procedures are thus unnecessary. The time required to attain a mature schizont stage is variable as there are various levels of development of the ring stage (tiny, small or large rings) at the time of blood collection. The "tiny ring" form can require as much as 24 h of additional incubation as compared with the "large ring" form. Other possible reasons for the variable period required to attain mature forms include biological fitness, recent intake of antimalarial drugs and the quality of serum.

In assays based on schizont counts by microscopy, the incubation time must be adjusted for individual isolates in order to fulfill the criterion of an interpretable test; i.e. ≥ 10% schizonts with three or more nuclei (eight or more nuclei when testing antifolates) of 200 asexual parasites. In practice, a control smear is examined after an initial incubation of 24 h. If the criterion is not met at this time, further smears are prepared several hours later. Most isolates develop into schizonts within 24–30 h (Lopez Antuñano & Wernsdorfer, 1979). Some isolates require ≥ 48 h (up to 96 h) to undergo schizogony and fulfill the WHO criterion (Smrkovski et al., 1983, 1985).

In a study conducted in the Solomon Islands (Inaba et al., 2001), about 50% of fresh isolates cultured within 30 min after collection developed into schizonts within 27 h, while the other 50% required between 28 and 63 h to reach the schizont stage. In this study, 43 (35 chloroquine-sensitive and 8 chloroquine-resistant) of 69 isolates (62%) were successfully adapted to a single cycle in vitro culture. The time lag to attain schizonts was not related to the pre-culture delay between collection and processing.

Some investigators prefer an extended incubation period (i.e. 48–96 h) for in vitro assay of certain drugs, in particular slowly acting drugs (antibiotics, fosmidomycin, inhibitors of phospholipid metabolism). Laboratory-adapted strains may show a slight decrease in [^{3}H]hypoxanthine uptake during the second schizogonic cycle, but the results are not profoundly affected because of the high multiplication rates. With fresh field isolates,

an incubation period extending into the second intraerythrocytic cycle may be associated with a higher parasitaemia than the starting value, depending on the capacity of individual isolates to adapt to in vitro conditions (Chin & Collins, 1980). As, in general, the adaptation process takes several schizogonic cycles, evaluation of drug activity over two schigozonic cycles is usually not optimal for fresh isolates.

As a general rule, it is best to evaluate in vitro activity in fresh isolates within the first schizogonic cycle. The in vitro activity of antifolate drugs has been assessed over a relatively long incubation period of 66 h in PABA-free RPMI 1640 with a reduced concentration of folic acid, in PABA- and folic acid-free RPMI 1640 and in dialysed human plasma depleted of these folate cofactors (Chulay, Watkins & Sixsmith, 1984; Sixsmith et al., 1984; Milhous et al., 1985; Watkins et al., 1987). Petersen (1987) reported no difference in schizont count or pyrimethamine IC_{50} value determined by both microscopy and [^{3}H]hypoxanthine incorporation, after 48-h and 72-h incubation periods; he used an asynchronous laboratory-adapted strain cultured in low-PABA (0.5 µg/l), low-folic acid (10 µg/l) RPMI medium. Using the radioisotope assay, Ndounga et al. (1999) also reported that there was no difference in chloroquine and cycloguanil IC_{50}s determined after 48-h and 66-h incubation periods, but there was a slight but significant decrease in pyrimethamine IC_{50} after a 66-h incubation. In the ELISA-based HRP II detection assay, an incubation period of 72 h is recommended by H. Noedl for all antimalarial drugs in order to attain maximum secretion of HRP II in field isolates, regardless of the initial stage (i.e. small, medium or large rings), including "slowly growing" parasites requiring > 48 h to adapt to in vitro culture conditions and attain the schizont stage (Noedl et al., 2002a). For consistency as well as for a standardized protocol, it is nonetheless preferable to set the total incubation time for all established drugs and test compounds to either 42–48 h or 72 h.

1.3.8 Effect of gas mixtures

The standard medium, RPMI 1640, 25 mmol/l HEPES and 25 mmol/l $NaHCO_3$, was optimized to maintain the pH within the physiological range in an atmosphere containing 5% CO_2. Any modification of the CO_2 concentration alters the pH of the medium, which in turn can influence the IC_{50} values of pH-dependent drugs, such as chloroquine, but not of those that are not dependent on pH, such as pyrimethamine. He et al. (2000) determined the IC_{50} values of chloroquine in laboratory-adapted strains (1.5% erythrocyte volume fraction, 0.2–0.5% starting parasitaemia) at three CO_2 concentrations (2.7%, 5% and 7%) with a constant O_2 concentration (5%) by the [^{3}H]hypoxanthine incorporation method of Desjardins et al. (1979). Despite similar growth and [^{3}H]hypoxanthine incorporation rates in drug-free control wells, increasing the CO_2 concentration from 2.7% (pH 7.65; measured in standard buffered RPMI 1640 medium after 24 h) to 5% (pH

7.50) and 7% (pH 7.39) resulted in significantly higher IC_{50} values. A chloroquine-resistant strain showed a 2.5-fold difference in IC_{50} values when the CO_2 concentration was increased from 2.7% to 5% and a ninefold difference when the concentration was increased from 2.7% to 7%. In a chloroquine-sensitive strain, the difference with 2.7% and 7% CO_2 was less than twofold.

The oxygen concentration can considerably modify the in vitro activities of some compounds. Divo, Geary & Jensen (1985) observed that the O_2 concentration (1% O_2–3% CO_2 vs candle jar) did not affect the IC_{50} values of quinoline-containing antimalarial drugs. In contrast, the in vitro antimalarial activity of some antibiotics is dependent on the O_2 concentration. In a preliminary study by Haruki et al. (1997), the chloroquine IC_{50} values were similar when assayed in an incubator set at 4% CO_2–3% O_2 or in a 10% CO_2–10% O_2 mixture generated by an AnaeroPack system. The IC_{50} values obtained with these two incubation systems were, however, about twofold higher than those obtained with a candle jar. Although the CO_2–O_2 concentrations were different in later studies, the results of Haruki et al. (1997) were not confirmed by other investigators. He et al. (2000) reported that the chloroquine IC_{50} values determined in a candle jar (2.7% CO_2–17.5% O_2) were similar to those determined in an incubator set at 2.7% CO_2–5% O_2. Lin et al. (1999) also observed similar chloroquine IC_{50} values with parasites incubated in 5% CO_2 and 5% or 15% O_2 (5% CO_2–15% O_2 generated by an AnaeroPack system). Therefore, most studies have not shown any changes in chloroquine IC_{50} when the parasites were incubated at O_2 concentrations between 1% and 17.5%.

To maintain a pH of 7.4 with various CO_2 concentrations, He et al. (2000) suggested that the $NaHCO_3$ concentration in RPMI 1640 medium should be adjusted according to the CO_2 concentration used. Another approach is to use the standard buffered RPMI 1640 medium for all in vitro assays and specify the CO_2 concentration. In practice, most researchers incubate malaria parasites in an incubator set at 5% CO_2, with an option for variable O_2 concentrations, or in a candle jar. The latter is more affordable in most endemic countries. More information is needed on the influence of CO_2 concentration on the schizontocidal effects of drugs.

1.3.9 Effect of previous drug intake

Inhibition of parasite growth

The success rate in many reports on in vitro drug sensitivity in field isolates and clinical isolates from imported malaria rarely surpasses 70%, regardless of the geographical area in which the studies were conducted (Deloron et al., 1985; Nguyen-Dinh et al., 1985a; Smrkovski et al., 1985; Wery, Ngimbi & Hendrix, 1986; Slutsker et al., 1990; Afari et al., 1993; Trenholme et al., 1993; Adagu et al., 1995; Brasseur et al., 1995; Sowunmi

et al., 1995; Wernsdorfer et al., 1995; Noedl et al., 2001; Borrmann et al., 2002; Ralaimazava et al., 2002). Most of these studies were conducted with the WHO microtest system, but in some the proportion of interpretable assays was < 40%. Despite this low success rates, extrapolations and generalizations have been made on the basis of the minority of isolates that developed into schizonts. In the WHO microtest system, inadequate schizont maturation might be due, at least partly, to cell harvesting after 24 h. Blood samples containing early ring-stage must be cultured for > 24 h to attain the schizont stage.

One of the principal reasons for poor parasite growth is previous intake of an antimalarial drug and, less commonly, antibiotics with some antimalarial activity. A patient might have taken a non-prescribed antimalarial drug before consultation, as often occurs in many malaria-endemic countries, or might have taken a prescribed antimalarial drug but failed to respond to it. In non-endemic countries, malaria-infected patients attending clinics and hospitals might have received chemoprophylaxis. In either case, the presence of antimalarial drugs in the blood and exposure of the parasites to drugs in vivo before blood collection profoundly affect parasite growth, usually leading to failure of schizont maturation. Recent intake of drugs is not the only reason for the generally low success rate of in vitro assays, as in some of the studies cited above urine was screened for antimalarial drugs before the assays were performed.

Smrkovski et al. (1985) reported that 12 of 21 (57%) fresh clinical isolates from patients with a positive Dill-Glazko urine test for 4-aminoquinolines developed into schizonts in vitro, while the remaining 9 isolates (43%) failed to undergo schizogony. Conversely, 28 of 34 (82%) isolates from patients with a negative urine test developed into schizonts.

Thorough washing of malaria-infected blood samples does not eliminate drugs taken previously by patients, especially those that are concentrated in malaria-infected erythrocytes (Basco et al., 2002). Le Bras & Savel (1987) estimated that, if a low erythrocyte volume fraction (≤ 2.5%) is used in the assay, the IC_{50} values of chloroquine and quinine determined in blood samples from patients with recent intake of antimalarial drugs can be underestimated by ≤ 50 nmol/l. Likewise, a brief exposure in vitro (up to 30 minutes) of fresh isolates to chloroquine or amodiaquine, followed by several washings of the infected erythrocytes just before performing the in vitro drug sensitivity assay, diminishes the IC_{50} values, as compared with the control IC_{50} values obtained with unexposed isolates (P. Ringwald, unpublished data, 2002).

Bustos et al. (1993) conducted in vitro drug sensitivity assays on a chloroquine-sensitive *P. falciparum* strain diluted with erythrocytes obtained serially from a healthy volunteer under chloroquine chemoprophylaxis for 4 weeks (100 mg/day, a prophylactic regimen recommended by the French health authorities at the time), who received the standard therapeutic dose

of chloroquine (25 mg/kg over 3 days) after a washout period. The IC_{50} values of chloroquine, quinine, mefloquine and halofantrine did not differ significantly when the infected erythrocytes were diluted with normal erythrocytes or with erythrocytes from the healthy volunteer (washed twice in culture medium) during the prophylactic period (total chloroquine concentration in plasma and erythrocytes, 28–300 ng/ml, as determined by HPLC). During the therapeutic period, the chloroquine concentration rose to 470–1124 ng/ml, and most of the IC_{50} values were significantly higher with chloroquine-containing erythrocytes from the healthy volunteer (up to 30-fold) than with normal erythrocytes.

In the experiments conducted by Bustos et al. (1993), the parasites were exposed to the drug-containing erythrocytes only during the in vitro assays and not before. Close analysis of the dose-response curve reveals that growth of the parasites was poor in chloroquine-containing erythrocytes (< 2000 disintegrations per minute, corresponding to < 1000 cpm in control wells) from the healthy volunteer and that the sigmoidal curve is almost flat. These observations suggest that the results are artefactual and invalid and should have been interpreted as such, rather than used to calculate IC_{50} values. The experiment mimics, to some extent, the in vitro response of parasites exposed to a drug in vivo after self-medication or prophylaxis, assuming that, during the 48-h incubation period, chloroquine in the uninfected erythrocytes of the healthy volunteer freely diffuses into the culture medium and into the infected erythrocytes.

In another study, Bustos et al. (1994) found that the main reason why only 40 of 73 (55%) in vitro radioisotope tests with Filipino clinical isolates gave interpretable results with complete or partial parasite growth was the presence of antimalarial drugs (in 43 of 73 patients, 59%), mostly chloroquine, in the plasma of pre-treatment samples, as measured by HPLC. Some of the isolates from patients who had presumably self-medicated before consultation developed into schizonts. As in the previous study (Bustos et al., 1993), the chloroquine IC_{50} values were positively correlated with the plasma concentrations of chloroquine and desethylchloroquine (main metabolite of chloroquine with schizontocidal activity). No data on parasite growth in samples with detectable antimalarial drugs were presented.

Congpuong et al. (1998) studied the in vitro response of isolates obtained from 382 Thai patients, of whom 34% had detectable levels of quinine or mefloquine before treatment. None of the patients had detectable levels of artemisinin derivatives by HPLC analysis, but previous intake of these drugs cannot be ruled out owing to their rapid elimination. In vitro tests for mefloquine, quinine and artemisinin were interpretable in 47/62 (76%), 43/57 (75%) and 98/126 (78%) isolates, respectively. The authors reported that the IC_{50} values of quinine and mefloquine in isolates from patients with or without pre-treatment quinine or mefloquine in their plasma did not differ significantly; however, the in vitro response of 24–25%

of the isolates is unknown because of inadequate parasite growth associated with previous treatment.

These studies support the hypothesis that antimalarial drug intake before blood collection suppresses parasite growth in vitro and that the in vitro response cannot be determined with accuracy in such blood samples. Drugs that concentrate in malaria-infected erythrocytes and have a relatively long elimination half-life (e.g. chloroquine and mefloquine) are particularly damaging to the parasites and practically impossible to wash out from the blood sample.

This idea may not hold true during the early hours of treatment (possibly up to 24 h after the first dose) with quinine or sulfadoxine-pyrimethamine (see section 1.1.6). Quinine is not heavily concentrated in parasite-infected erythrocytes. White, Looareesuwan and Silamut (1983) reported that the ratio of quinine concentration in red cells (uninfected and infected) and plasma is between 0.2 and 1.3. In another study, the mean ratio of quinine concentrations in erythrocytes (uninfected and infected) and medium was 3.6 (range 3.0–4.3) (Mapaba et al., 1996). This ratio does not change significantly with duration of exposure (0.5–24 h), with or without 3% of infected erythrocytes. Quinine can be removed almost completely from infected erythrocytes exposed in vitro to the drug by thorough washing (at least three times by centrifugation with a large volume of culture medium or isotonic buffer), except at high drug concentrations (> 300 µmol/l) (Mapaba et al., 1995). For consistency and the highest possible rate of in vitro growth, the in vitro response to antimalarial drugs is best studied in parasites obtained from patients without recent intake of antimalarial drugs.

Urine screening test

Methods for measuring antimalarial drug levels in blood or urine are important laboratory tools in field research. In tests of therapeutic efficacy, these methods provide data on recent intake of drugs, indicate compliance with prescribed medication and, with sensitive and specific techniques (e.g. HPLC), can be used to determine pharmacokinetics. For in vitro drug sensitivity assays, patients with malaria should be screened for the presence of antimalarial drugs that would inhibit growth of the parasite. Measurement of drug levels in malaria-infected patients also contributes to correct interpretation of in vivo and in vitro drug responses. Clinical histories of recent drug intake are unreliable when the information is provided by the patient or by accompanying relatives (Silamut et al., 1995).

The biological samples commonly obtained for drug dosage are blood and urine. As urine is easily and rapidly obtained from many patients, it is the preferred sample for screening in the field. There are several methods for determining drug levels in urine that are applicable in the field, such as colorimetric tests, ELISA and thin-layer chromatography (Churchill, 1989).

The last two methods can be used to detect chloroquine with high specificity but require expensive instruments (ultra-violet light source, spot luminance meter or ELISA plate reader).

The most appropriate technique for most field studies is simple, qualitative colorimetry, including: the Wilson and Edeson test (Wilson & Edeson, 1954), the Haskins test (Haskins, 1958; Steketee et al., 1988), the Dill-Galzko test (Lelijveld & Kortmann, 1970), the bromthymol blue test (Bergqvist et al., 1985) and the Saker-Solomons test (Saker & Solomons, 1979; Mount et al., 1989). Each test has its advantages and disadvantages (Rombo et al., 1986; Shenton et al., 1988; Wernsdorfer & Payne, 1988; Mount et al., 1989). None is highly sensitive and highly specific for chloroquine, but their performance is considered to be satisfactory for field studies. In addition to these tests for detecting 4-aminoquinolines, the lignin test and the modified Bratton-Marshall technique have been reported for detecting sulfonamides in urine (de Almeida-Filho & de Souza, 1983). The Dill–Galzko test was commonly used in field studies in the past but is the least sensitive colorimetric test. Readers interested in the Wilson and Edeson test, the Haskins test, the Dill-Galzko test and the lignin test can find concise protocols in the annex to a WHO document (WHO, 1979). Another method of potential interest for detecting chloroquine, pyrimethamine and quinine in blood and urine in the field is drug-specific dipsticks (Silamut et al., 1995; Schwick et al., 1998). These are not commercially available and have to be prepared by specialized research laboratories.

Three colorimetric tests that are more sensitive than the Dill-Glazko test are the bromthymol blue test, Haskins methods and the Saker-Solomons test. A negative result with these three tests indicates < 1 μg/ml of chloroquine and its metabolites in a urine sample, i.e. < 100 ng/ml of chloroquine in blood (Churchill, 1989). These three methods can be adapted for quantitative determination of chloroquine in urine.

In well-equipped field laboratories and central laboratories in endemic countries, assays based on ELISA and thin-layer chromatography can be used to detect chloroquine in urine or blood if highly sensitive, specific methods are required for clinical protocols rather than for simple screening of patients (Mount et al., 1987a,b; Mount, Patchen & Churchill 1988; Rowell et al., 1988; Shenton et al., 1988; Witte et al., 1990). These assays cannot be used to detect other antimalarial drugs. In endemic areas where antifolate drugs are commonly used for self-medication, the lignin test might be a useful adjunct for detecting sulfonamides in urine.

In our laboratory, we use the Saker-Solomons test as part of routine procedures. In Yaoundé, Cameroon, where self-medication is widespread, the only practical, reliable, cost-effective method for performing in vitro drug sensitivity assays with a high success rate is to screen malaria-infected patients for the presence of antimalarial drugs with a simple urine test (Mount et al., 1989). While this type of screening test cannot match the

high sensitivity and specificity of HPLC and other sophisticated pharmacological techniques, it is simple and rapid to perform, the results are easy to interpret without special training, and it is relatively cheap. In endemic areas where chloroquine and quinine are the antimalarial drugs usually used for self-medication, this urine test is adequate. Systematic screening of patients with this urine test has allowed us to maintain a success rate > 95% with in vitro drug sensitivity assays for the past 10 years. Some of its disadvantages include non-detection of sulfadoxine-pyrimethamine, use of chloroform, the requirement for a reagent that must be ordered from a major supplier in developed countries, the relatively rapid decomposition of tetrabromophenolphthalein ethyl ester solution and the requirement for storage of the solution in the dark at 4 °C.

The Saker-Solomons urine test is neither highly sensitive nor highly specific for chloroquine. The lowest level of detection of chloroquine plus desethylchloroquine was reported to be 1 μg/ml (Mount et al., 1989). In practical terms, a patient who has taken chloroquine during the past few days would have a positive urine test. The relatively low specificity of the Saker-Solomons test might be an advantage in the field, however, as it can also be used to detect the presence of other antimalarial drugs, including quinine and, to a lesser extent, mefloquine. Owing to its rapid elimination, quinine might not be detectable in the urine of a patient who has taken the drug more than 12 h before consultation. The urine test can also be used to detect pyrimethamine, but not sulfonamides or sulfones. In practice, a patient who has taken sulfadoxine-pyrimethamine more than 24 h before consultation is likely to have a negative Saker-Solomons test. Proguanil and cycloguanil (the major metabolite of proguanil with high schizontocidal activity against antifolate-sensitive parasites) also give positive results; however, in practice, one is unlikely to encounter a malaria-infected patient with proguanil or cycloguanil in the urine in the field unless he or she has been given chemoprophylaxis (non-immune travellers and perhaps local pregnant women).

The cross-reactivity of antimalarial drugs in the Saker-Solomons colorimetric test is summarized in Table 3. Experiments were carried out with aqueous solutions of active principles, with the protocol of Mount et al. (1989). Stock solutions (1 mg/ml) were prepared in sterile demineralized water or absolute ethanol, whereas subsequent 10-fold dilutions were prepared in water. A drug-free aqueous solution with 10% ethanol did not affect the colour reaction. As the modified Saker-Solomons test was originally designed to detect chloroquine plus desethylchloroquine in urine samples, experiments with aqueous solutions prepared in the laboratory do not exactly reproduce the results found with urine samples (e.g. the minimum detection level of 1 μg/ml is for chloroquine plus desethylchloroquine). Drugs that are poorly soluble in water or alcohol were tested directly by mixing 10 mg of active principle in the test reagent (0.2 ml tetrabromophenolphthalein ethyl ester solution in chloroform plus 1 ml phosphate buffer).

This qualitative test gave positive results with mefloquine, halofantrine and desbutylhalofantrine (positive controls) and negative results with sulfadoxine, sulfamethoxazole and dapsone.

As mentioned above, patients' histories are not a very reliable source of information about recent intake of antimalarial drugs. Any discrepancy between what patients claim to have taken and what they have really taken might be due to either a deliberate effort to provide false information to satisfy the inquirer or inexact information, as low-quality or counterfeit drugs are largely available in informal drug outlets in most developing countries (Basco, 2004a). To obtain more accurate information, routine screening of urine with the Saker-Solomons test is strongly recommended. Exclusion of patients for in vitro drug sensitivity assays with this simple test saves much time and effort, which could be focused on blood samples that would yield an interpretable result.

1.3.10 Delay after blood collection, anticoagulants and parasite viability

Investigators have used various anticoagulants in collecting capillary or venous blood from patients: heparin, acid citrate dextrose and EDTA. Parasites collected on these anticoagulants stain normally with Giemsa (or May-Grunwald-Giemsa), and their growth is not affected if isolates are cultured immediately after blood collection. As heparin inhibits the activity of *Taq* DNA polymerase, its use should be avoided if the blood samples are to be examined by molecular analysis.

EDTA was included in the WHO microtest kit (Lopez Antuñano & Wernsdorfer, 1979). One advantage of EDTA over acid citrate dextrose is that it does not modify the total volume of collected whole blood. Blood collection tubes containing acid citrate dextrose are designed to be filled completely, as, otherwise, the blood is diluted with the anticoagulant. EDTA and acid citrate dextrose do not interfere with *Taq* DNA polymerase activity.

The viability of freshly collected clinical isolates diminishes considerably after several hours, and most parasites do not survive after 24 h at room temperature in endemic countries (25–30 °C). In our experience, EDTA (and heparin) has the disadvantage of decreasing parasite viability when malaria-infected blood is stored at 4 °C for more than 24 h (Basco, 2004b). After storage for 24–96 h at 4 °C before in vitro assays, parasite viability was better when whole blood was stored in acid citrate dextrose-containing tubes (LK Basco, unpublished data, 2004). Feng & Liu (2003) also observed that parasite viability decreases after a ≥ 2 h delay at room temperature (25–35 °C) and after a 24–48 h delay when parasites are stored at 4 °C.

1.3.11 Sources of antimalarial drugs

In some early studies, commercial formulations were used to prepare solutions (Siddiqui, Schnell & Geiman, 1972). A tablet with a known quantity of free base was ground and dissolved in an appropriate solvent, the suspension was filtered to remove insoluble excipients, and the transparent filtrate was further diluted. Such procedures are not recommended because the quantity of active principle(s) in each tablet varies slightly, an unknown proportion of active principle can be lost during filtration, unknown quantities of excipients are present in the working solutions, and pure active principles are available from commercial suppliers, pharmaceutical firms or non-profit organizations, such as WHO.

Some of the active principles are available in salt forms. For example, chloroquine is available as the sulfate or phosphate salt and rarely as the base. Others are available as a single type of salt (e.g. halofantrine hydrochloride, mefloquine hydrochloride) or base (e.g. pyrimethamine base). Several salts of quinine are available from commercial suppliers. The solubility of different salts might differ in aqueous solutions. The choice of drug (i.e. base vs salt, salt form, drug purity and possible supplier) should be standardized.

1.3.12 Artefactual sources

Drug binding

Some drugs adsorb on glass or a plastic surface, and the amount of adsorbed drug depends on the concentration. Generally, higher drug concentrations are associated with less loss due to surface adsorption. For in vitro drug sensitivity assays of established antimalarial drugs, the final concentrations are usually in the picomolar or nanomolar range, so, if the possibility of drug adsorption is not taken into account, inconsistent results might be obtained, such as abnormally high IC_{50} values (or MICs).

Chloroquine binds extensively to non-silicone-treated glassware and cellulose acetate filters (Yayon & Ginsburg, 1980; Geary, Akood & Jensen, 1983). Chloroquine appears to bind to glass test tubes and glass pipettes immediately; regardless of the aqueous solvent used (distilled water, RPMI 1640, phosphate-buffered saline), 30–40% of chloroquine (1 µmol/l) binds to glass (Geary, Akood & Jensen, 1983). Filtration of a very dilute chloroquine solution through cellulose acetate membrane removed 60–70% of the drug. As chloroquine (1 µmol/l) does not bind to plastics (polystyrene, polypropylene, polycarbonate) at temperatures of –20 °C to 37 °C, these materials are recommended for preparing and storing chloroquine solutions. The property of binding to glass and plastic differs for each compound. Like chloroquine, amodiaquine adsorbs onto glass surface (Thaithong, Beale & Chutmongkonkul, 1983). Mefloquine has been shown to bind to polystyrene plastic plates (Kouznetsov et al., 1980). The extent of drug binding or

loss due to adsorption depends on the concentration of drug, individual drug properties and the type of material (glass, plastic). The loss is minimal for millimolar stock solutions but is considerable for very dilute solutions.

Another source of drug binding is the filtration units fitted to syringes to sterilize solutions and culture media. Solutions of amodiaquine, mefloquine, antibiotics and experimental drugs have been sterilized by filtration through a 0.45-µm polycarbonate filter (Geary, Divo & Jensen, 1985). Baird & Lambros (1984) filter-sterilized solutions containing chloroquine, mefloquine, amodiaquine and quinine and compared the in vitro activity of these solutions with that of solutions prepared in 70% ethanol and diluted without filtration. Drug losses due to binding to membrane filters were evident with fluorocarbon (67–98% binding), as reflected by high IC_{50} values, in particular when solutions with a low drug concentration (10 mg/l) were filtered. When solutions with a high concentration (2000 mg/l) were filtered through a membrane made of fluorocarbon, the drug losses were minimal. Drug binding to membrane filters made of cellulose acetate and polycarbonate was limited (2–11% binding); however, the IC_{50} values were abnormally high, possibly due to chemical impurities in the membrane filters, such as nonionic detergents (e.g. Triton X-100), which were extracted into the filtrates. These investigators recommended filtration of drug solutions at high concentration, followed by aseptic dilution of the filtrate without further filtration, or preparation of stock solutions in 70% ethanol (filtration is unnecessary as ethanol at this concentration sterilizes the solution) followed by aseptic dilution.

In our laboratory, filtration of drug solutions is avoided. Not all drugs dissolve in 70% ethanol, and some drugs are highly soluble in water (and less soluble in alcohol). It is preferable to use water throughout the preparation of stock and working solutions. Bacteria and fungi do not grow on dry, crystalline active principles stored in airtight vials. Filtration of stock solutions is not necessary, even if the active principle is exposed to air for a short time for weighing on an analytical balance, as long as the materials that come into direct contact with the active principles are sterile. Distilled water used to prepare stock and working solutions is sterilized by membrane filtration before addition to the drug powder, and solutions are prepared under a laminar flow hood. When drug solutions are prepared as described, bacterial or fungal contamination of stock or working solutions is extremely rare.

Solvent

Water, alcohol and dimethyl sulfoxide are the three most commonly used solvents for preparing drug solutions. In some studies, acidic or basic aqueous solutions containing lactic acid or NaOH have been used to prepare stock solutions, depending on the pKa of the drug. Desjardins et al. (1979) recommended preparation of stock solutions in 70% ethanol. After the solution has been allowed to stand at room temperature for 30 min, further

dilutions are prepared in RPMI 1640, HEPES, $NaHCO_3$ and 10% human plasma (or serum) mixture with constant mixing.

When fresh aqueous drug solutions are mixed with medium-blood suspension, care should be taken that the hypotonic drug solution is well diluted in the suspension, to avoid large changes in osmolarity. When fresh drug dilutions containing alcohol or dimethyl sulfoxide are used, the final concentrations of these solvents should generally be ≤ 0.1%, in order to avoid artefactual toxic effects on the parasites (Ndounga, Basco & Ringwald, 2001). Dimethyl sulfoxide cannot be evaporated owing to its viscosity. In case of doubt, parallel tests on parasite growth, with and without the solvent at various concentrations, should be carried out in drug-free wells.

If pure or nearly pure alcohol is used to dilute test compounds, the solvent should be removed thoroughly by air-drying in pre-dosed microtitre plates before the assays are performed. An alternative is to dilute the solutions in water and maintain the final alcohol concentration at ≤ 0.1%. Extra precautions are required when working with solutions with a high alcohol content, as they tend to evaporate during dilution, leading to inaccurate concentrations. Lell, Binh & Kremsner (2000) studied the possible effects of alcohol consumption on malaria parasite growth: addition of ethanol (0.08% or 0.39%) to 96-well culture plates inhibited parasite growth by approximately 20–30% during a 48-h incubation.

Reber-Liske (1983a) reported that a 4-year-old mefloquine hydrochloride solution in water (1 mmol/l) had almost identical in vitro activity against *P. falciparum* strains as a freshly prepared solution. The author concluded that mefloquine in aqueous solution remains stable. In our experience, mefloquine hydrochloride is insoluble in water, as indicated by the IC_{50} value for mefloquine (Reber-Liske, 1983a), ranging from 95 to 165 µg/l (229–397 nmol/l). These values are at least 10–20 times higher than the usual IC_{50} reported for mefloquine prepared in alcohol. Each antimalarial compound requires an appropriate solvent if consistent results are to be obtained.

Drug degradation

Test plates are best prepared with freshly prepared drug solutions immediately before assays. A well-equipped laboratory might have an automatic diluter or computer-controlled robotics to prepare test plates. For field workers, it is much more convenient to pre-dose test plates before going to the field. Pre-dosed test plates have various shelf-lives, depending on the drug and storage conditions.

According to Feng & Liu (2003), the recommended shelf-lives of plates pre-dosed with chloroquine, pyronaridine, piperaquine and artesunate stored at 4 °C are 2 years, 3 months, 6 months, and 3 months, respectively. In our experience, plates pre-coated with chloroquine and monodesethylamodi-

aquine have long shelf-lives, > 1 year, even at room temperature. Plates pre-dosed with quinine, pyrimethamine, halofantrine, atovaquone and lumefantrine are also stable and can be stored for several months, at either 4 °C or ambient temperature. The following shelf-lives (storage at 4 °C until use) have been recommended for test plates of the WHO Mark III systems: 2 years for chloroquine, amodiaquine, and quinine; 3 months for artemisinin; and 2 months for mefloquine. Similar shelf-lives of pre-dosed test plates (storage at 4 °C) have been recommended by the Centre National de Référence pour la Chimiosensibilité du Paludisme (Hôpital Bichat-Claude Bernard, Paris) (J. Le Bras, unpublished data, 2004; Kaddouri et al., 2006): chloroquine, 1 year; monodesethylamodiaquine, 1 year; mefloquine, 2 months; lumefantrine, 4 months; and artemisinin and its derivatives (artesunate and dihydroartemisinin), 6 months.

Based on the experience of several investigators, it may be said that test plates pre-dosed with mefloquine have a relatively short shelf-life. Some investigators have reported that dihydroartemisinin is unstable on the plates and that artemisinin is the most appropriate drug for in vitro drug assays due to its stability in pre-dosed plates (Tanariya et al., 2000).

1.4 Applications of in vitro assays

The in vitro drug sensitivity assay is a useful laboratory tool for drug development, particularly for:

- screening new drugs (Desjardins et al., 1979; Vennerstrom et al., 1992);
- studying the interactions of drug combinations (Berenbaum, 1978; Chulay, Watkins & Sixsmith, 1984; Geary et al., 1986; Canfield, Pudney & Gutteridge, 1995; Alin, Björkman & Wernsdorfer, 1999);
- studying potential cross-resistance to different drugs (Winkler et al., 1994; Pradines et al., 1999a); and
- determining the responses of pre-treatment and post-treatment isolates during clinical trials (Webster et al., 1985; Childs et al., 1991).

In the context of drug development, the results of in vitro assays can indicate future studies, notably in vivo, before the observations of clinical trials are validated. Comparison of the in vitro response pattern of pre-treatment and post-treatment isolates is useful for evaluating whether recrudescent parasites represent essentially the same parasite populations as before treatment (i.e. treatment failure due to pharmacokinetic factors, such as inadequate absorption) or selection of resistant populations (i.e. treatment failure possibly due to drug resistance if reinfection is ruled out). This approach is helpful if there is no molecular marker for resistance to a particular drug (or if its DNA sequence is unknown).

In the opinion of some investigators, the results of an in vitro assay on isolates from individual patients can be used to adjust the therapeutic regimen if alternative drugs have been shown to be more active in vitro, and therefore probably more effective in achieving cure (WHO, 1984). Any such modification necessitates the availability of results within 48 h, which is often not the case, and a high correlation between the in vitro response and the expected clinical outcome, which is also not the case. Furthermore, the idea of a therapeutic adjustment based on in vitro antimalarial drug assays, akin to use of antibiotic sensitivity test results to guide the management of microbial infections, has become obsolete because of the current consensus to resort to combination therapies and is unrealistic in many endemic countries where large numbers of patients are treated with limited health resources.

Another relatively new application of in vitro assays is the bioassay. Several studies have shown that bioassays can be as sensitive as, or even more sensitive than, HPLC for measuring the quantity of artemisinin derivatives in a patient's plasma indirectly.

What is the role of in vitro assays in malaria control at present? Monitoring the changing status of antimalarial drug resistance is one of the essential functions of malaria control programmes. The collected data provide a rational basis for recommending, if necessary, modifications to antimalarial drug policy at national or regional level. Malaria experts generally concur that drug resistance can be monitored by tests of therapeutic efficacy (i.e. in vivo tests), in vitro drug sensitivity assays or, more recently, molecular markers. The two related functions often cited as the operational roles of in vitro assays and applied by some investigators (WHO, 2000b) are detection of emergence of drug resistance (Wongsrichanalai et al., 1992a,b) and surveillance of changing trends in drug sensitivity over time and space (Guiguemde et al., 1994; Philipps et al., 1998; Ringwald et al., 2000).

There is no doubt that in vivo tests provide more relevant data to clinicians and health authorities; however, tests of therapeutic efficacy require a 28-day (or longer) follow-up of patients, whose response is usually confounded by immunity, differences in pharmacokinetics and possible reinfections. Ethical considerations preclude the evaluation of new drugs until pre-clinical data, in particular toxicological studies, and the initial phases of clinical trials are complete and the drug has been approved for clinical use by an official body.

In vitro assays by-pass many of these obstacles. In any therapeutic strategy, establishment of the baseline sensitivity of local *P. falciparum* isolates to commonly prescribed drugs that are still effective in some endemic areas and new drugs that are not yet widely available in a country but might be employed in the near future (e.g. artemisinin derivatives) is important. The in vitro assay is particularly useful for fulfilling this function as it provides an objective, quantitative measure. Likewise, for monitoring the emergence and spread of drug resistance, simultaneous determination of the

response of a given isolate to several drugs is an advantage over in vivo tests. As some "old" antimalarial drugs, such as chloroquine and, to a much lesser extent, amodiaquine and sulfadoxine-pyrimethamine, are being phased out gradually in many parts of the world, in vitro assays or molecular markers might become the only means for investigating changing trends of sensitivity to these drugs. Each national malaria control programme is not expected to organize and perform in vitro assays as part of their routine procedures, as the in vitro assay is a specialized laboratory procedure that is best performed by a team of well-trained researchers and technicians.

An additional role that can be assigned to in vitro assays is validation of candidate molecular markers for drug resistance in field isolates. Studies of the correlation between the phenotype (i.e. drug response) and the presence of a point mutation or combinations of mutations indicate that *dhfr* is a valid marker for resistance to pyrimethamine (and cycloguanil) (Cowman et al., 1988; Peterson, Walliker & Wellems, 1988; Foote, Galatas & Cowman, 1990; Hyde, 1990; Peterson, Milhous & Wellems, 1990; Basco et al., 1995b; Reeder et al., 1996), *P. falciparum* chloroquine resistance transporter (*pfcrt*) is valid for chloroquine (Fidock et al., 2000; Basco & Ringwald, 2001; Babiker et al., 2001; Chen et al., 2001; Durand et al., 2001; Basco, 2002b), and cytochrome *b* is valid for atovaquone (Looareesuwan et al., 1996a; Srivastava et al., 1999; Korsinczky et al., 2000; Basco, 2003c). The results of these studies suggest that resistance to these drugs, in particular antifolates, can be surveyed by analysing the corresponding molecular markers, without resorting to in vitro assays. Validation of other molecular markers, such as *dhps* and *P. falciparum* multidrug resistance gene 1 (*pfmdr*1), by comparison with the in vitro response of field isolates to sulfonamides, amino alcohols and artemisinin derivatives, is not yet complete. The corresponding molecular markers of *P. vivax* – *dhfr*, *dhps*, *P. vivax* chloroquine resistance transporter (*pvcrt*) and *P. vivax* multidrug resistance gene (*pvmdr*) – could also be evaluated for correlation with the in vitro response.

At present, the in vitro assay is still an indispensable investigational tool for studying drugs, and it is expected to continue to play a major role in primary drug screening and, to a lesser extent, in studies on the activities of individual drugs that are administered in combination in vivo and cross-resistance, until a rational, computer-assisted drug design based on potential molecular targets derived from the complete *P. falciparum* genome overtakes traditional screening methods. Its possible role in determining plasma drug concentrations in the field by bioassays is still being evaluated. As for its operational role, the in vitro assay is useful, but not indispensable, for monitoring drug resistance; however, inclusion of in vitro studies as an adjunct to clinical evaluation of therapeutic efficacy in which molecular analysis of parasite DNA is also performed is important for the advancement of knowledge on drug-resistant malaria. We may be at the crossroads, where validated molecular markers might render phenotypic determination by in vitro assays "obsolete" in coming years.

1.5 In vitro-in vivo correlation

1.5.1 Notion of drug resistance

Practically, the term "drug resistance" becomes meaningful only in the context of therapeutic failure in malaria-infected patients. The IC_{50} value and the MIC are the commonly used measures of in vitro response of malaria parasites to drugs. The level of sensitivity expressed by these measures is an inherent property of the parasite, independent of various host factors, such as acquired immunity and the pharmacokinetics of antimalarial drugs. The meaning of "decreased in vitro sensitivity" is relatively clear when a given IC_{50} value or MIC determined for an individual isolate or strain can be compared with previous or concurrent measures for other isolates or strains, determined with the same assay protocol. The meaning of "drug resistance" is less clear when applied to the in vitro response of parasites. In the literature, many investigators have used the terms "sensitive" and "resistant" and defined them arbitrarily, usually on the basis of a threshold value, which, in most cases, has not been validated (Table 4). A high IC_{50} or MIC value in a given isolate for an antimalarial drug administered to the patient from whom the isolate was collected might or might not be associated with therapeutic failure and is therefore not synonymous with drug resistance. In endemic zones, it is likely that patients carrying drug-resistant parasites will nevertheless recover after treatment. As clinical and parasitological outcome is largely determined by the immune status of the patient, an association between drug-resistant parasites and therapeutic failure is more likely in small children than in adults.

If the therapeutic response is satisfactory but circulating parasites are still present or reappear without symptoms, "parasitological failure" is concluded. Therapeutic failure is a clinical and parasitological observation that a patient receiving the correct dosage of an antimalarial drug within the limit of tolerance does not fully respond to treatment clinically. The patient is not cured after treatment, i.e. either there is no clinical improvement at all within 2 or 3 days after the start of therapy, or there is temporary relief of symptoms but similar symptoms and signs recur in the presence of parasitaemia, usually within 4 weeks after the start of therapy. The usual end-point of "cure" is complete clearance of fever and parasitaemia. Therapeutic failure is not always due to parasites tolerating antimalarial drugs and persisting in the patient despite correct treatment; it is known to occur in some patients in whom a sufficient plasma drug level is not attained because of inter-individual differences in pharmacokinetics (e.g. rapid drug elimination, inadequate hepatic biotransformation) or other causes (e.g. repeated vomiting). Therapeutic failure can also occur in patients with new infections, a few days before treatment or during or after treatment, as parasites are not affected by most blood schizontocidal drugs during the pre-erythrocytic stages, which in *P. falciparum* can last ≥ 6 days. Moreover, poor compliance with the prescription and use of low-quality antimalarial drugs can lead to therapeutic failure.

Therapeutic success (i.e. cure) is a clinical and parasitological observation that a patient with an initial diagnosis of malaria infection no longer has the signs and symptoms and parasitaemia observed before drug treatment. The notion implicitly supposes that the patient was examined by a clinician and that the diagnosis was supported by laboratory examinations, in particular microscopic examination of blood smears or a malaria parasite-specific dipstick. It is also assumed that the clinically observable signs and symptoms described by the patient and the presence of malaria parasites in the blood are causally related. The latter assumption does not hold true in all cases, especially in areas of stable malaria transmission. Barring such cases, therapeutic success can be attained in patients who are infected with drug-sensitive parasites or with parasites that are inherently resistant to the drug being administered. Acquired immunity can control parasitaemia, with or without drug therapy (Trape et al., 1998). There is also evidence that adults in an endemic area can recover, even with drug-resistant parasites, while the recovery of small children depends on the parasites being susceptible (Omar, Adagu & Warhurst, 2001). The immune system is thought to inhibit parasite growth independently of the drug resistance status of the parasites. Unreported self-medication with another antimalarial drugs or herbal medicines or concurrent treatment with antibiotics during or shortly after the prescribed antimalarial therapy might also explain therapeutic success despite the presence of drug-resistant parasites.

Because of these confounding factors, "drug resistance" is not synonymous with "therapeutic failure". Some cases of therapeutic failure are due to drug-resistant malaria parasites (i.e. parasites with a high IC_{50} value or MIC), but not all cases of failure can be explained by drug-resistant parasites. "Drug resistance" is a more restrictive term than "therapeutic failure".

The classical definition of drug resistance is "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" (WHO, 1973). This definition is somewhat ambiguous. The term "absorption" usually implies oral administration of a drug, whereas some patients might require initial parenteral treatment followed by oral treatment. The "usually recommended" dose of a given drug varies widely in reality. For example, a physician usually sets a limit to the total dose prescribed to overweight patients, and it is well known in medicine that different patients can tolerate a wide range of drug dosages. Furthermore, the definition ignores the possible interference of acquired immunity, which might help clear drug-resistant parasites.

From a more modern viewpoint, a given isolate or strain is "drug-resistant" when the following conditions are satisfied (Basco & Ringwald, 2000b; Basco et al., 2002):

- **Clinical and parasitological criteria:** the patient is not relieved of signs and symptoms, and microscopic examination of blood smears shows

malaria parasites, despite administration of the standard therapeutic regimen (this might have been arbitrarily defined, on the basis of, or without much consideration for, the pharmacokinetic profile).

- **In vitro drug sensitivity assay:** the clinical isolate obtained from the patient has a higher IC_{50} or MIC value than the mean values determined for other isolates.
- **Plasma drug concentration:** measurement of the drug concentration in the patient's blood by HPLC shows an adequate therapeutic level.
- **Genotype analysis:** polymorphic markers of pre-treatment and post-treatment isolates have been compared to demonstrate that there is a high probability that the isolates are identical (therefore excluding a new infection).
- **Drug resistance markers:** with drugs for which there are established molecular markers (chloroquine, pyrimethamine, cycloguanil, sulfadoxine, atovaquone), a point mutation or set of mutations at defined codons is associated with resistance. In some cases (e.g. *pfmdr*1), gene amplification resulting in increased copy numbers, rather than point mutations, might be correlated with drug resistance.

Each of these criteria has several limitations. When they are taken together for individual patients and isolates collected from them, however, the term "drug-resistant" becomes more meaningful. At present, it seems to be the best available approach for defining drug-resistant malaria.

1.5.2 In vitro and in vivo responses

Many parallel studies based on the WHO microtest or its modifications have yielded unexplained discordance between in vitro and in vivo responses to different drugs (Spencer et al., 1984; Nguyen-Dinh et al., 1985a,b; Schapira et al., 1986; Schapira & Schwalbach, 1988; Petersen et al., 1990; Slutsker et al., 1990; Umotong et al., 1991; Trenholme et al., 1993; Sowunmi & Oduola, 1995). In some of these studies, the two tests for drug resistance did not show a correlation. The generally poor correlation might have many causes, and the explanations might differ for each study. Some of the possible explanations include the inadequate protocol of the WHO microtest, poor study design based on the WHO 7-day in vivo test (Kremsner & Wernsdorfer, 1989), inappropriate drug for the in vitro assay (e.g. amodiaquine instead of monodesethylamodiaquine [see Schapira & Schwalbach, 1988]; arbitrary fixed doses for sulfadoxine-pyrimethamine assay), technical difficulties in obtaining a consistent in vitro response to sulfadoxine, irrelevant choice of study population (e.g. parasitological response in asymptomatic carriers), small sample size, enrollment of patients with recent intake of antimalarial drugs, failure to distinguish between reinfection and recru-

descence associated with reappearance of the same isolate as before initial treatment, and acquired immunity.

For example, Noedl et al. (2001) attempted to correlate the therapeutic response to artemisinin combination therapy and in vitro cross-resistance between artemisinin derivatives and mefloquine. The clinical and parasitological response to artemisinin derivatives and the IC_{50} values for artemisinin were correlated, and the IC_{50} values for mefloquine and artemisinin were also correlated, as shown in several previous studies (Gay et al., 1990; Ringwald et al., 1990; Doury et al., 1992; Basco & Le Bras, 1993). The authors concluded that the artemisinin-mefloquine correlation in vitro is reflected in the response to artemisinin treatment. The regimen was not standardized (unspecified artemisinin derivatives, drug(s) used in combination and duration of treatment; delayed administration of mefloquine), and some patients were afebrile throughout treatment and follow-up. Moreover, the success rate of the WHO microtest was low (35/76, 46%). The in vitro responses of isolates from eight patients responding with resistance I (RI) late recrudescence were not specified.

In Yaoundé, Cameroon, we observed about 25% discordance between clinical and parasitological responses to chloroquine and the in vitro chloroquine response of the corresponding isolate determined by the radioisotope method (Ringwald & Basco, 1999). Even when patients (aged > 5 years) with reinfection within 14 days were excluded from the analysis, discordance remained for about 20% of patients. The study was limited to a 14-day follow-up, as recommended by the WHO protocols existing at that time for areas of intense transmission.

The experience of several field researchers tends to reinforce the following conclusions:

- In vitro assays and clinical studies of therapeutic efficacy do not address the same biological and clinical end-points.
- The thresholds for in vitro resistance are fixed arbitrarily, with no reference to predictable clinical and parasitological response.
- For these and other reasons, cited above, it is difficult to establish a strong correlation between measures determined by two methods for studying drug resistance. It is more often difficult to explain therapeutic success than therapeutic failure on the basis of in vitro assays, possibly because of the major confounding host factor, i.e. acquired immunity.

These observations can be extended to current efforts to establish a direct correlation between molecular markers and clinical and parasitological response in patients. In many studies, no marked correlation was found between these two methods for studying drug resistance, especially in terms of predicting the clinical outcome from the DNA sequence of a molecular

marker in the parasite obtained before treatment. In vitro drug sensitivity patterns and molecular markers are more closely related from the biological viewpoint, as the phenotype of the parasite "strain" or population can be considered to be determined by a specific gene that codes for an expression product, which may be an enzyme or transporter protein, that characterizes the observable biological feature. This close relation between the in vitro drug sensitivity profile and molecular markers is one of the reasons for maintaining in vitro drug sensitivity assays as an important research tool in the field.

2. Practical aspects of in vitro assays

2.1 General organization of laboratory work

2.1.1 The central laboratory

Monitoring in vitro drug responses of malaria parasites in the field requires a central laboratory as a support base. Routine monitoring of responses of *P. falciparum* and, in some regions, *P. vivax* is not required in all malaria control programmes (WHO, 2000a); therefore, every country where malaria is endemic, particularly in regions where human, material and financial resources are limited, does not need a specialized laboratory. The current global trend is to share and exchange expertise and limited resources at regional and international levels. The initial investment for specialized equipment, maintenance of a research laboratory and training of the personnel is high, and several countries can share the funding of a regional central laboratory. The role of the central laboratory is described below.

Coordination of surveillance of resistance to antimalarial drugs

The central laboratory is usually a public institution closely linked to the malaria control unit of the ministry of health, the ministry of research, national or foreign armed forces or universities, depending on the country. Because no one laboratory can perform the enormous task of national and regional monitoring and because antimalarial drug resistance is a global problem, links should be established with neighbouring countries where malaria is endemic, regional malaria networks and regional offices of WHO for a more effective surveillance system, exchange of data and implementation of common measures to combat drug-resistant malaria.

The epidemiology of drug-resistant malaria is based mainly on the clinical and parasitological responses of symptomatic patients. The in vitro drug sensitivity assay is a specialized tool for assessing the response of parasites to relevant antimalarial drugs (i.e. drugs used in the country or region, as recommended in the national or regional antimalarial drug policy) in vitro over time and space. In vitro assays do not replace tests for therapeutic efficacy. They are useful for establishing a baseline level before a new antimalarial drug is introduced into a country.

Preparation of a complete in vitro assay kit for field studies

As WHO has ceased production of the microtest kit, various reagents for in vitro assays have to be ordered by a central laboratory in order to

assemble the necessary materials. Culture media and other reagents must be prepared under aseptic conditions and the required volumes aliquoted and stored in ready-to-use form for field work.

Quality control

Before the prepared kit can be used in the field, each item must be controlled for quality and sterility. Aliquots of reference *P. falciparum* strains should be either cryopreserved at the central laboratory or obtained from other reference laboratories. These parasites are cultivated at the central laboratory to test and compare new and old batches in the assay kit in parallel. If a parasite in continuous culture is not available, fresh clinical isolates can be used to test new and old batches in the assay kit in parallel.

The pH of new batches of media and buffers should be checked with a calibrated pH meter, preferably with an automatic temperature compensation system; pH paper is not accurate enough for quality control. Likewise, phenol red (phenolsulfonphthalein), which is incorporated in most culture media, including RPMI 1640, is a rough pH indicator that is helpful for estimating the approximate pH by visual inspection: at pH 7.2, phenol red gives an orange colour, and it turns yellow in acidic pH < 6.8 and deep red or purple at basic pH > 8.2. Other quality control tests, such as the osmolarity of the culture medium, the partial pressures of O_2 and CO_2 in the incubator and the precise temperature of the thermal cycler, require instruments that are usually not available in laboratories in malaria-endemic countries.

Samples of culture medium, human serum, stock solution of Albumax® and erythrocytes from uninfected donors can be inoculated into test medium to exclude the presence of bacteria and fungi. Any batch of solutions shown to be contaminated should be discarded, rather than an attempt made to save it by, for example, re-filtration.

Mycoplasma contaminations are frequent and can pass unnoticed, as these organisms are not readily visible under a microscope (about 0.4 µm). Mycoplasmas can originate from untested serum, cross-contamination from other cultures or the personnel handling cultures. Their indirect effects can be observed by keen personnel who notice an acidic spent medium, slower growth of the parasite or morphological abnormalities. They can be detected by experienced personnel under a light or fluorescence microscope after special staining methods, culture on agar and ELISA or PCR. Mycoplasma detection is probably best performed in collaboration with a microbiologist. If mycoplasma is detected, all potential sources and materials that have come into contact with the contaminated cells should be autoclaved and discarded. Bench surfaces, incubators and hoods should be disinfected; laboratory coats should be thoroughly washed.

Management of the database

Raw data collected in the field and in the central laboratory should be recorded in both printed and electronic forms. Electronic files stored in a computer are analysed by statistical software to calculate IC_{50} values, the commonest expression of in vitro response, or MICs. The results should be expressed as geometric mean IC_{50} values. Data are classified according to study site and year, so that longitudinal analyses of in vitro responses can be performed rapidly.

Management of the database includes regular reports to the ministry of health, presentation of results at national, regional or international scientific meetings and publication of articles in scientific journals.

Training

If the staff members at the central laboratory and the field staff who conduct the in vitro assays are not the same personnel, training of field staff is an important component of the surveillance system. Close contact between the two teams should be maintained for rapid feedback about any technical problems arising in the field. Rapid communication is especially important in areas where malaria transmission occurs seasonally and the field station is far from the central laboratory, in order to avoid having to postpone the field survey for a year.

The central laboratory should also provide advanced training to the coming generation of researchers, including graduate students, postdoctoral fellows and medical practitioners, who will continue the long-term surveillance over the next few decades and apply innovative techniques for research on antimalarial drug resistance.

2.1.2 Organization of the central laboratory

The central laboratory is specialized in cell culture. It should be capable of fulfilling the following basic functions: aseptic handling and manipulation, incubation, storage of reference strains (optional), medium preparation, laboratory diagnosis and sterilization. To ensure sterile conditions in the handling area, cell culture facilities should occupy a closed space, as much as possible: closed doors and windows during operation, air conditioners turned off if they are installed close to the sterile handling area and limited access to persons who are not working at the laboratory.

2.1.3 Specialized equipment

Some specialized equipment and a reliable source of electricity are needed to fulfill the basic laboratory functions and derive data from in vitro assays in cell culture facilities.

Vertical laminar flow hood

Although in vitro assays can be performed aseptically on an open bench close to a gas burner, handling of malaria parasites under a hood considerably reduces the risk of contamination with bacteria and fungi. The hood draws room air and creates a stream of filtered air that passes vertically downwards over the handling area. A typical hood is partially open in front to allow manipulation and provides partial protection of the operator with a shield. Most aseptic handling of parasites, blood samples, media and drug solutions can be performed under the hood.

Incubator and candle jar

An incubator set at 37 °C is required to culture asexual stages of malaria parasites. An incubator with reliable temperature control that can house a candle jar is the minimum requirement, although more sophisticated and expensive incubators with electronic controls and an automated system for regulating temperature and gas control can be used. The CO_2 incubator is specifically designed for open culture vessels, such as Petri dishes, microtitre plates and culture flasks, with a bicarbonate buffer system. The existence in the country of a reliable commercial source of pure CO_2, supplied in a gas cylinder, should be verified before a CO_2 incubator is ordered. A CO_2 incubator is not required if in vitro assays and continuous cultures are performed in a closed system, such as in a candle jar or in closed vessels into which a high CO_2–low O_2 gas mixture is flushed. The inner atmosphere of the incubator or candle jar should be humidified to minimize evaporation of the contents of Petri dishes, microtitre plates and other open vessels.

An ordinary desiccator made of polypropylene or polycarbonate can well be used as a candle jar. To create an airtight seal, silicon-based grease should be applied to the cover and body of the desiccator.

Cold storage system

Cryogenic equipment and a regular supply of liquid nitrogen are required if reference strains of *P. falciparum* are to be maintained in continuous culture or if a collection of "live" parasite strains is to be used. The existence of a reliable source of liquid nitrogen should be ensured before such plans are made. A capacity for cryopreservation and regular maintenance of reference strains in culture are recommended, for example, for quality control of pre-dosed culture plates. The present author does not recommend immediate cryopreservation of fresh isolates in the field and subsequent culture for determining the in vitro drug response at the central laboratory. Such procedures are not only labour-intensive but also lead to selection of subpopulations of parasites. A freezer at –20 °C and a refrigerator are essential for storing various reagents and solutions. A freezer at

– 80 °C is not necessary for most research with in vitro drug sensitivity assays but might be useful for storing collected plasma for HPLC measurements.

Water purification system

Tap water is not suitable for most solutions used in cell culture, and distilled or deionized water is required for the preparation of culture media, drug solutions, buffers and other solutions. Most laboratories have deionizers. Modern deionizers produce ultra-pure water with a resistance of 18 mΩ•cm; for comparison, the resistance of double-distilled water is 1 mΩ•cm. The cartridge system usually consists of a prefilter, activated charcoal, an ion exchanger and a final 0.22-µm filtration unit. Filtered, deionized water is sterile and free of organic substances and particles. The deionizer must be maintained regularly, including changing the cartridges periodically (usually every 3–4 months) and changing the prefilters, as well as testing the resistance of the deionized water.

Filtration equipment is needed to remove possible contaminating bacteria and fungi during preparation of culture media and reagents. Depending on the volume, a wide range of reusable (autoclavable) or disposable filtration equipment is available. To filter volumes of < 200–400 ml, a disposable filtration unit that fits onto a syringe is convenient. For larger volumes, as in the preparation of culture media, a pre-assembled reusable or disposable sterile filtration unit with a 0.22-µm membrane can be used. Filters with a larger pore size (e.g. 0.45 µm) do not remove bacteria and should not be used. Filters with a pore size of 0.1 µm allow the removal of mycoplasma.

ELISA plate reader (spectrophotometer), automated multiwell plate washer, fluorometer or liquid scintillation counter

As a national or regional reference centre, the central laboratory should have the capacity to perform at least ELISA-based in vitro drug sensitivity assays. In the near future, fluorescence-based assays might be at least as simple and economical as ELISA-based assays. At present, however, most of the equipment necessary for fluorescence-based assays is more costly than an ELISA plate reader. The radioisotope assay is the current "gold standard" but is not a promising technique for most malaria-endemic countries. Central laboratories in endemic countries that do not have a liquid scintillation counter at present should envisage use of alternative non-radioactive methods. LDH- and HRP II-based ELISA assays are available and promising.

In addition to the above-mentioned items that are specific for cell culture, procurement of the following apparatus is highly recommended: a thermal cycler, agarose gel electrophoresis unit, ultra-violet transilluminator and numerical image analysis system. Although these expensive items do not form part of a classical cell culture laboratory, PCR is an integral part of

research on antimalarial drug resistance. The core staff of the central laboratory should have additional expertise in the basic techniques of molecular biology (DNA extraction, PCR, agarose gel electrophoresis, restriction fragment length polymorphism) and a working knowledge of DNA sequencing.

2.1.4 Common equipment

The equipment found in most types of biological laboratories includes:

- a light microscope with a x 100 oil immersion objective,
- a centrifuge,
- an analytical balance,
- a vacuum pump and 0.2-µm filtration units,
- a water-bath or heater block,
- a Bunsen burner,
- a set of adjustable- or fixed-volume digital pipettes and a multichannel pipette,
- a pipette controller,
- a magnetic stir plate,
- a shaker,
- an autoclave,
- various glassware (beakers, bottles, volumetric flasks) and disposable culture-grade plasticware (tubes, flasks, graduated cylinders, graduated pipettes of 1–25 ml volumes, Pasteur pipettes, Petri dishes, microtitre plates), and
- a desk-top or laptop computer. This is indispensable for any modern research laboratory, for data analysis, word processing, communication, searching for scientific information on the web and possibly ordering materials directly from international suppliers.

2.2 Preparation of reagents for in vitro assays

2.2.1 RPMI 1640 medium

Powdered and liquid preparations of RPMI 1640 are available from several commercial sources at reasonable cost. The preparation containing HEPES (5.94 g/l or 25 mmol/l) is convenient for field work. The composition of RPMI 1640 is well defined. Unless the effects of one or several of its components are being studied, it is not worth reconstituting the medium from its individual components. For special studies requiring partial or complete depletion of one or several components, bulk quantities of special RPMI 1640 preparation (e.g. RPMI without folic acid and PABA) can be ordered from most major suppliers.

There is no reason for batch-to-batch or quality differences of the commercial product. Liquid preparations are practical and ready for use, but their shelf-life is short (about 9 months when stored at 4 °C), and shipping to malaria-endemic countries can be costly owing to their weight and volume. For many researchers in endemic countries, use of pre-weighed powdered packages (10.4 g/l without HEPES; 16.4 g/l with HEPES) is practical, not only for importation and transport, but also for preparation and long-term storage, either as a dry powder (shelf-life of about 3 years) or as aliquots of prepared liquid medium stored at –20 °C. For several reasons, however, including lack of facilities for obtaining glass-redistilled or demineralized water or lack of a laminar flow hood for preparing liquid medium, some investigators prefer ready-to-use RPMI 1640 solution.

The medium in powdered form is highly hygroscopic, and a package should be opened only immediately before preparation of RPMI 1640 solution. Once the package containing the powdered RPMI 1640 is opened, the entire contents should be used. Preparation of liquid medium from the powdered form is straightforward. After addition of $NaHCO_3$, the osmolality of the medium at room temperature is 292 milli-osmoles per kg H_2O (± 5%) and the pH is 7.0 ± 0.3.

RPMI 1640 powder medium dissolves rapidly and completely when it is added in small quantities to demineralized water with gentle stirring with a magnetic stirrer. Some researchers recommend addition of 1 N HCl to lower the pH to 4.0 in order to dissolve the powder completely. In this case, after complete dissolution, 1 N NaOH should be added to readjust the pH to 7.2 before $NaHCO_3$ is added. Once the solution has been prepared (with or without pH changes), some researchers prefer to adjust the pH by adding either HCl or NaOH; however, when the medium has been correctly prepared, there should be no need to adjust the final pH. An experienced technician or researcher can judge the "right" pH from the bright orange colour of the medium, but the pH of every new batch of culture medium should be verified with an accurate pH meter.

The solution should always be prepared as a 1 x concentration. It is not advisable to prepare concentrated solutions of RPMI 1640 medium, as they can form precipitates. If storage space in the laboratory is limited, smaller quantities (for example, 1 litre) of liquid medium should be prepared regularly, rather than storing concentrated solution. Liquid medium prepared from powder should be sterilized by membrane filtration (0.2-µm filter) and dispensed in sterile containers. If liquid medium is ordered directly from the supplier, there is no need to sterilize it before use unless leakage occurred during transport. RPMI 1640 solution should not be autoclaved.

Depending on the annual consumption of medium, 1, 5 or 10 litres of RPMI 1640 can be prepared periodically and stored. There are several ways of storing liquid RPMI 1640 medium. Aliquots of medium (without serum) for routine use can be stored at 4 °C for up to 2–3 months. Complete medium

containing serum should be stored at 4 °C and be used within 2 weeks. It is not advisable to freeze (–20 °C) and thaw aliquots several times. The required volume of RPMI 1640 should be taken out of the refrigerator and left at room temperature (or heated in a water-bath set at 37 °C) about 1–2 h before use to avoid a sudden temperature change in parasitized erythrocytes. Bottles containing RPMI 1640 should not be exposed to direct sunlight.

Liquid medium can be stored at –20 °C for months or even years. In some laboratories, serum supplements (10% v/v) are added before storage. Alternatively, liquid medium without serum can be frozen for storage and serum can be added just before use of the thawed medium. When RPMI 1640 is stored with $NaHCO_3$ at –20 °C, dry ice should not be placed in the same freezer, to avoid pH modification of the medium.

Trager and Jensen (Jensen & Trager, 1977; Trager & Jensen, 1980) recommended the following procedure for preparing 1 litre of medium:

- weigh 10.4 g of RPMI 1640 powder with L-glutamine;
- weigh 5.94 g of HEPES (final concentration, 25 mmol/l);
- dissolve the powders in 900 ml of glass-redistilled water;
- add glass-redistilled water to 960 ml;
- sterilize by filtration (0.2-µm pore size);
- dispense in 100-ml aliquots and store at 4 °C (for up to 1 month);
- prepare 5% $NaHCO_3$ solution in water; sterilize by filtration through a 0.2-µm filter. The solution is colourless, and water solubility is complete at this concentration;
- before use, add 4.2 ml of 5% $NaHCO_3$ solution to 100 ml of RPMI–HEPES aliquot (pH 7.4). This medium without serum can be stored for up to 1 week at 4 °C;
- prepare complete RPMI medium by adding 11 ml of human serum.

There are other ways of preparing RPMI 1640 medium from powder:

- weigh 10.4 g of RPMI 1640 powder for 1 litre. If HEPES (formula weight [FW] = 238.3) is contained in the powder, weigh 16.4 g for 1 litre.
- weigh 2.1 g of $NaHCO_3$ powder (FW = 84);
- dissolve the powders (RPMI 1640, HEPES and $NaHCO_3$) in 1 litre of demineralized water with stirring at room temperature;
- add 20–50 µg/ml (final concentration) of gentamicin (optional);
- stir gently until the powder is completely dissolved;
- sterilize by filtration (0.2-µm pore size). A 500-ml sterile, disposable filtration unit is convenient, with a vacuum pump to operate it. The solution can also be sterilized manually through a 0.2-µm filter held on a 50-ml sterile syringe;
- store at 4 °C. The medium can be used for months as long as the colour remains bright orange. Aliquots can be stored at –20 °C indefinitely.

2.2.2 Antibiotics

Antibiotics provide some protection against bacterial contamination and are particularly useful for cultures and in vitro drug assays in the field. Workers should be aware that antibiotics do not fully protect cultures from bacterial contamination, particularly from massive amounts of bacteria introduced inadvertently. RPMI 1640-serum-blood mixture is a good medium for bacterial growth. A change in the colour of complete RPMI 1640 medium with erythrocytes to dark-red or black within 24–48 h of incubation is usually due to bacterial contamination or an excessively high CO_2 concentration (> 5%) in the incubator. In such cases, the cultures should be controlled immediately by microscopic examination of Giemsa-stained thin blood films. Contaminated cultures should be discarded immediately, after addition of a disinfectant to kill the bacteria.

The source of bacterial contamination should be identified so that the risk of further contamination is minimized. The usual source of contamination is the culture medium, serum or its substitutes; contamination is sometimes due to the uninfected erythrocytes used to dilute cultures, in particular when all wells of the culture plate are contaminated, indicating a common source of bacterial contamination. A contaminated medium (without erythrocytes) usually turns turbid, and, after shaking, white particles can be seen against the light. An aseptically prepared RPMI 1640 is orange and transparent, with no particles or precipitates. Human serum that is contaminated with bacteria is more difficult to detect by visual inspection, as serum is naturally turbid. If the medium and blood are ruled out as the source of bacterial contamination and the serum is suspected, a control culture with malaria-infected or uninfected erythrocytes is incubated for 24 h and a Giemsa-stained thin blood film is examined. The source of contamination should be disinfected and discarded immediately. It is not advisable to re-filter massively contaminated medium for further use.

Sometimes, only certain wells of a test plate are contaminated with bacteria, and the pipettes used to distribute medium-blood mixture into these wells should be considered potential sources of bacteria. If none is identified, it can be concluded that contamination of individual wells occurred during handling and manipulation. The sterility of pipettes, pipette tips and test plates should be checked.

For prevention of fungal contamination, it is not common practice to add anti-fungal agents to the medium, as some anti-fungal agents, such as amphotericin B and nitroimidazoles, inhibit parasite growth (Pfaller & Krogstad, 1981, 1983; McColm & McHardy, 1984). Fungi grow on almost any humid surface, including tubes and bottles in which liquid RPMI 1640 is stored. Any reagent that is infected with fungi should be disinfected and discarded immediately. The basic rule for avoiding bacterial and fungal contamination is to use an aseptic technique with sterile materials and reagents and have a clean working place (open bench with a gas burner or, preferably, with a laminar flow hood) in a closed room, as for any cell culture method.

Addition of antibiotics, such as penicillin (100 µg/ml) and gentamicin (133 µg/ml), to minimize bacterial contamination is optional (Trager & Jensen, 1977). The antibiotics used most commonly for cell culture in general are penicillin and streptomycin in combination. For malaria culture, gentamicin, a wide-spectrum antibiotic, is commonly used, as it inhibits Gram-positive and Gram-negative bacteria and mycoplasma and has no effect on parasite growth at concentrations up to 200 µg/ml. The actual concentration of gentamicin used by malaria investigators varies widely but is usually within the range of 20–50 µg/ml. For culture of cells other than malaria parasites, the usual working concentration is 50 µg/ml. Neomycin (50–100 µg/ml) has been used by a few investigators to prevent bacterial contamination (Reber-Liske, 1983b; Ridley et al., 1997). The spectrum of activity of neomycin is similar to that of gentamicin, and there is no particular advantage in its use. The same remark holds for streptomycin.

Penicillin is not commonly used in culturing malaria parasites because it has a narrow spectrum of activity (Gram-positive bacteria), and bacterial resistance is frequent. Use of other antibiotics or anti-fungal agents, such as amphotericin B and nystatin, is not recommended. Investigators should bear in mind that some antibiotics (e.g. tetracycline, macrolides, clindamycin and trimethoprim) inhibit the growth of *Plasmodium* parasites and should therefore be avoided for the culture of malaria parasites.

Gentamicin is added during preparation of liquid RPMI 1640 from powder. To calculate the amount of gentamicin that should be added to 1 litre of RPMI 1640, it is convenient to express the gentamicin concentration as mg/l. The usual concentration of 20–50 µg/ml is equivalent to 20–50 mg/l; if the concentration of the gentamicin solution is 10 mg/ml, 2 ml should be added to every litre of RPMI 1640 to obtain a working concentration of 20 mg/l of gentamicin.

Investigators ordering liquid RPMI 1640 from reliable suppliers can safely assume that the medium is sterile, unless leakage has occurred during delivery and handling. They might nonetheless prefer to add gentamycin the first time a bottle containing RPMI 1640 is opened under the hood.

Addition of antibiotics to a culture medium should not be relied upon to keep cultures free of contamination. The best means of avoiding bacterial contamination is use of careful, aseptic techniques in a clean laboratory. Blood samples and culture reagents should be handled in a disinfected, clean hood or near a gas burner on an open bench. The laboratory room should be closed, and air conditioners near the aseptic handling area should be turned off, at least during manipulation. The hood, bench and other working surfaces should be cleaned daily with disinfectant, before or after manipulation.

The organization of laboratory space is important. The handling area should be clear, with only necessary items within reach. Specific surfaces should be assigned for the basic functions of the laboratory, i.e. aseptic handling, incubation, sterilization (if done in the laboratory), washing and

microscopic examination. If DNA amplification is one of the activities, three widely separated working areas are needed: for DNA extraction, DNA amplification (thermal cycler) and DNA analysis (agarose gel electrophoresis).

All wastes should be set apart and discarded, if necessary after disinfection, outside the working area. Disinfectants are indispensable in biological laboratories. The type of disinfectant used in the laboratory depends partly on the country and on personal choice. Oxidants (sodium hypochlorite, peroxygen) are highly effective disinfectants that kill and destroy bacteria, viruses, fungi and spores. Sodium hypochlorite is bleaching and corrosive and gives off a strong odour (chlorine). Peroxygen is non-bleaching and non-corrosive. Oxidants should be handled with disposable gloves. Alcohol is widely available and is effective against many, but not all, microorganisms; spores are not affected by alcohol. Diluted alcohol (75–85%) can be applied to the hands before aseptic manipulations. Care must be taken when handling alcohol near a gas burner as it is highly flammable. Other disinfectants might not kill the entire range of microorganisms.

Personal hygiene should be observed, including washing the hands before manipulations, wearing gloves and a clean laboratory coat and the use of face masks by personnel with upper respiratory infections. Strict silence during handling is important. Persons who are not directly involved in the activities of the laboratory should stay out.

2.2.3 Serum

In our experience as well as that of Trager and Jensen (1980), human serum is best obtained at blood banks in a malaria-free country. A unit of fresh whole blood (approximately 500 ml) is collected from non-immune donors directly into anticoagulant-free collection bags. Whole blood should be allowed to clot at 4 °C overnight and centrifuged. Serum is transferred under sterile conditions to another collection bag, without anticoagulant, tubes or bottles, for storage at –20 °C. In some laboratories, serum is sterilized by filtration. If blood is collected aseptically and all procedures are performed under aseptic conditions, filtration is unnecessary. Blood banks are routinely required to screen for common bloodborne pathogens, including hepatitis virus and human immunodeficiency virus, and serum samples from blood banks should be both sterile and safe for medical and research use.

Alternative sources include plasma from whole blood freshly collected in citrate, and serum obtained from fresh plasma by the addition of $CaCl_2$ solution (1 ml of a 10% solution for every 100 ml plasma). The quality of these products may be poorer than that of freshly collected serum. Recuperation of old, unfrozen serum is not recommended.

Batches of human serum from commercial suppliers are not always suitable for culture of malaria parasites, for unknown reasons. Several batches might have to be ordered and tested individually for optimal parasite growth.

Type AB+ human serum available from commercial suppliers is expensive (approximate cost without shipping fees, US$ 100–200 per 100 ml). Some suppliers pool serum samples from several donors.

In malaria-endemic countries, serum from persons residing in stable transmission areas and from persons who have recently taken antimalarial drugs or antibiotics with antimalarial action (sulfamethoxazole-trimethoprim, tetracycline, doxycycline, macrolides) are generally unsuitable for culture of malaria parasites. Parasite growth in such serum samples (or uninfected erythrocytes) should be evaluated before they are used for in vitro drug sensitivity assays.

Frozen serum samples from at least five donors (the more, the better) are thawed and pooled. The serum is aliquoted and frozen at –20 °C in a volume appropriate for the needs of each investigator (usually 50-ml aliquots but perhaps smaller) at this step to avoid further freeze-thaw cycles before use. It is also possible to prepare complete RPMI 1640 medium containing 10% pooled serum and to store the complete medium at –20 °C. This method is convenient, especially when the exact volume of complete medium for in vitro assays is known in advance. Once an aliquot of serum is thawed, it can be stored at 4 °C for several weeks. The stock of pooled serum can be stored at –20 °C indefinitely (or at –80 °C if such facilities are available). It is strongly recommended that repeated freezing and thawing of serum samples be avoided.

The recommended final volume of human serum in RPMI 1640 is 10% (v/v), as lower concentrations might not support parasite growth adequately. Heat inactivation of serum is not necessary. All procedures that involve exposure of serum, erythrocytes or culture medium to air (preparation of pooled serum and aliquots, addition of serum to sterile RPMI 1640 medium, washing of erythrocytes) should be performed aseptically under a laminar flow hood or next to a gas burner on a bench.

2.2.4 Serum substitutes

Sera from several animal sources can be used to replace human serum for culture of malaria parasites. Serum from certain animals, notably fetal calves, is widely used for culturing many types of mammalian cells. Abundant supplies of fetal calf serum are available from international commercial outlets at lower prices than human serum; however, as in the case of human serum, there is wide batch-to-batch variation in the capacity of animal sera to support *P. falciparum* growth in vitro. Moreover, the IC_{50} values obtained with animal serum can be significantly different from those obtained with human serum. Although the use of fetal calf (or other animal) serum instead of human serum is promising, further investigations are required before animal sera can be used routinely for antimalarial drug sensitivity assays.

At present, Albumax®, a lipid-enriched bovine albumin preparation, is the only human serum substitute that has been widely tested for culture of malaria parasites and antimalarial drug sensitivity assays. Information on its exact composition is not available. Albumax® I and Albumax® II differ in the concentration of immunoglobulins M and G, but this does not appear to affect in vitro culture of malaria parasites. Some investigators have found poor parasite growth with certain batches of Albumax®. In our experience, there is little batch-to-batch variation in the degree to which this product supports the growth of fresh clinical isolates. Albumax® does not, however, consistently support the growth of clinical parasite isolates that have not been previously adapted to in vitro culture conditions, and some grow much better in serum-supplemented RPMI 1640 than in Albumax®-supplemented medium. The reasons for this difference in the capacity of *P. falciparum* isolates to adapt to Albumax®-supplemented RPMI 1640 are unknown. Moreover, the IC_{50} values of most antimalarial drugs determined with Albumax®-supplemented and serum-supplemented RPMI 1640 are different, possibly due to differences in drug-protein binding affinity. Therefore, IC_{50} values determined with Albumax®-supplemented RPMI 1640 should not be compared directly with those determined with serum-supplemented RPMI 1640 until a correction factor between the two has been established.

Albumax® is cheaper than commercially available type AB^+ human serum. It is available as a lyophilized crystalline powder, and its light weight and compact presentation are convenient for shipping. Albumax® has a shelf-life of several years when stored at 4 °C. Albumax® I or II can be used. A stock solution is also stable for several months when stored at 4 °C. The recommended final concentration is 0.5%. The preparation of stock Albumax® solution (5% w/v) is as follows:

- weigh 5 g of Albumax®;
- dissolve in 100 ml of glass-distilled or demineralized water;
- sterilize by filtration through a 0.2-µm filter;
- make aliquots and store at –20 °C. Aliquots for routine use can be stored at 4 °C;
- add 1 ml of 5% (w/v) Albumax® stock solution to 9 ml of RPMI 1640 to obtain a final concentration of 0.5% Albumax®.

Some investigators prefer a stock of highly concentrated Albumax®. A 20% (w/v) stock solution can be prepared by weighing 20 g of Albumax® and dissolving it in 100 ml of water (or RPMI 1640). It might be necessary to warm the mixture to 37 °C for complete dissolution. The solution should be sterilized by filtration and stored as indicated above. To supplement RPMI 1640, the stock solution is diluted to 1/40 (final concentration of Albumax®, 0.5%).

2.2.5 Uninfected human erythrocytes

Researchers working in well-equipped laboratories and in field laboratories should maintain a stock of donors' erythrocytes, even if continuous culture of *P. falciparum* is not planned. The main use of uninfected human erythrocytes in in vitro drug sensitivity assays is to dilute heavily parasitized blood samples to a range of 0.1–1% starting parasitaemia for [^{3}H]hypoxanthine-based assays and to < 0.1% for ELISA-based assays. Uninfected erythrocytes can also be used as a negative control to determine the background level of [^{3}H]hypoxanthine incorporation and the background OD for ELISA-based assays.

For several obvious reasons, the host erythrocyte of choice for in vitro culture and in vitro drug sensitivity assays of *P. falciparum* (also for *P. vivax, P. ovale* and *P. malariae*) is human. Humans are the natural host of these *Plasmodium* species, and, for practical purposes, human blood is the easiest to obtain. Human erythrocytes can be freshly obtained from a healthy, non-immune donor without a recent history of intake of antimalarial drugs or antibiotics with antimalarial activity. The usual practice is to obtain uninfected erythrocytes from healthy donors from a blood bank at monthly intervals.

Venous blood from healthy donors (about 500 ml) is transferred directly into sterile collecting bags containing an anticoagulant (e.g. acid citrate dextrose, citrate-phosphate-dextrose, saline-adenine-glucose). It is preferable to request packed red cells rather than whole blood, so that the plasma from the donor's blood can be used for medical purposes. Basic laboratory examinations are carried out at the blood bank to characterize the erythrocytes and plasma (blood type) and to exclude blood samples with infectious pathogens.

In non-endemic countries, blood samples that pass the basic laboratory examinations can be safely assumed to be fit for culture of malaria parasites. The growth rate of a laboratory-adapted *P. falciparum* strain can, however, vary from batch to batch of uninfected blood. Whenever possible, it is advisable to compare the growth of a laboratory-adapted strain in old (up to 4–5 weeks after blood collection) and new batches of uninfected erythrocytes for a few cycles before switching entirely to the new batch.

In field laboratories, non-immune human blood might not be easy to obtain from blood banks. One possible solution is to forego use of uninfected human erythrocytes, thus eliminating one of the logistical problems of culture of malaria parasites. If a stock of uninfected blood is not available, investigators must set an upper limit of starting parasitaemia (1% for [^{3}H]hypoxanthine-based assays) and exclude samples of fresh isolates with higher parasitaemia for in vitro studies.

If the main use of uninfected erythrocytes is to dilute freshly collected samples for in vitro determination of drug response, any of the four ABO blood types can be used with type AB$^+$ human serum or serum supplements, such as Albumax®. In laboratories that maintain a continuous culture

or adapt freshly obtained isolates to in vitro culture before performing experiments, including in vitro drug sensitivity assays, it might be preferable to use the predominant blood type of the local human population (for example, type A^+ erythrocytes and A^+ serum).

Uninfected erythrocytes can be used immediately after collection and for up to 4–5 weeks. Venous blood samples should be stored in aliquots (e.g. 6-ml aliquots in sterile 15-ml tubes or 20-ml aliquots in 50-ml tubes) at 4 °C. It is advisable to discard haemolysed blood samples after about 4 weeks. Before use, aliquots should be centrifuged at 500–600 × *g* for 10 min, and the supernatant (plasma), the buffy coat (thin, whitish layer of cells at the interface between the plasma and the red cell pellet) and the upper layer of erythrocytes (approximately 1 mm in depth) be removed. The packed uninfected erythrocytes should be washed in buffered RPMI 1640 medium (without serum) three times by centrifugation. After the final centrifugation, the washed erythrocytes can be stored with the medium at 4 °C. Before addition to on-going cultures or to infected erythrocytes freshly obtained from patients, the medium of the third wash of packed uninfected erythrocytes is removed. The packed erythrocytes without supernatant constitute a suspension of approximately 75% erythrocyte volume fraction.

The composition of commonly used anticoagulants for blood preservation is as follows:

- acid citrate dextrose: 8 g/l citric acid, 22 g/l sodium citrate, 24.5 g/l D-glucose; add 15 ml of acid citrate dextrose to 100 ml of whole blood;
- citrate-phosphate-dextrose: 3.27 g/l citric acid, 26.3 g/l sodium citrate, 25.5 g/l D-glucose, 2.22 g/l monobasic sodium phosphate; add 14 ml of citrate-phosphate-dextrose to 100 ml of whole blood (v/v 14%);
- citrate-phosphate-dextrose-adenine: add 0.2 g/l adenine to citrate-phosphate-dextrose and mix at 14% (v/v) with whole blood;
- saline-adenine-glucose-mannitol: 0.15 mol/l NaCl, 1.25 mmol/l adenine, 45 mmol/l D-glucose, 29 mmol/l mannitol.

2.2.6 Malaria-infected erythrocytes

Reference P. falciparum *strains and clones*

Most field laboratories work directly with freshly collected isolates from patients and do not culture them beyond a few schizogonic cycles. Continuous culture of parasites, notably reference strains, is usually performed in laboratories outside endemic areas, where the only alternative source of living parasites is malaria-infected travellers returning from endemic area (i.e. imported malaria) or infected blood samples transported from an endemic area.

Reference strains of *P. falciparum* (and some rodent malaria parasites) are cryopreserved at the Institute of Cell, Animal and Population Biology, University of Edinburgh (Scotland, United Kingdom) and the Malaria Research and Reference Reagent Resource Center (MR4), American Type Culture Collection (Manassas, Virginia, United States). Before these strains are used for experiments, they should be screened for mycoplasma contamination, and the genotype of the strain should be determined. Several reports have drawn attention to the possibility of mycoplasma contamination (Turrini et al., 1997; Rowe et al., 1998) and of cross-contamination of different reference malaria strains and clones (Robson et al., 1992; Trager, 1993; Saul et al., 1997).

A description of the preparation and maintenance of continuous cultures is beyond the scope of this document. Investigators interested in the technical aspects of culture systems can refer to the original articles of Haynes et al. (1976) and Trager & Jensen (1976), review articles written by W. Trager, original articles on specific aspects of malaria culture (cryopreservation, synchronization, parasite cloning and gametocyte production) and laboratory manuals (see annex 2).

Patients

Both capillary and venous blood samples have been used for in vitro drug sensitivity assays. To establish a new strain from freshly collected *P. falciparum* isolates, venous blood is usually used. Fingerprick capillary blood is also suitable for initiating a continuous culture (Thaithong, Seugorn & Beale, 1994).

The type of malaria-infected patients from whom blood samples are collected varies considerably. Many malaria-infected patients in areas of stable malaria do not consult medical personnel, at least during the first few days of malaria-associated symptoms. Self-medication is a common household practice. Some patients consult traditional healers. When self-medication or traditional medicine fails to relieve the symptoms, many patients with uncomplicated malaria tend to end up consulting at a dispensary or primary health-care centre. These two health structures are the sentinel sites of choice for evaluating therapeutic efficacy, for collecting blood samples for testing the in vitro activity of drugs and for analysing molecular markers in endemic countries.

2.2.7 [^{3}H]Hypoxanthine

[^{3}H]Hypoxanthine can be obtained from several international suppliers. Lyophilized powder was available in the past, but now most [^{3}H]hypoxanthine preparations are available in 50% ethanol solution. [^{3}H]Hypoxanthine solution containing 5 mCi per vial in 5 ml 50% ethanol can be stored in 1-ml

aliquots (1 mCi per aliquot) at –20 °C. Before use, 24 ml of RPMI 1640 medium (without serum or serum substitute) are added to a 1-ml aliquot to obtain a 25-ml solution with 1 mCi [^{3}H]hypoxanthine. To radiolabel parasites with 1 µCi per well in tissue culture plates, 25 µl of this solution are added to each well.

The half-life of tritium is about 11 years. In addition to the natural decay of radioactive compounds, radioisotopes undergo autoradiolysis. Stocks should be maintained in 50% ethanol at –20 °C to minimize degradation of [^{3}H]hypoxanthine, which occurs at a rate of about 1% per year. When stocks are stored in water, the rate of autoradiolysis can be as much as 10% per year. From a practical viewpoint, each laboratory in which [^{3}H]hypoxanthine is used as a growth indicator for in vitro assays should estimate its annual consumption and avoid storage of excessive stocks. It is advisable to order fresh stocks annually.

2.2.8 Monoclonal antibodies

Microtitre plates pre-coated with monoclonal antibodies directed against *P. falciparum* HRP II or malarial LDH are available from international suppliers. The necessary reagents and other items are assembled in a kit. At present, the prices of pre-coated plates are exorbitant for most endemic countries; however, monoclonal antibodies for the HRP II assay are not proprietary and are available from various sources (Noedl et al., 2005).

2.2.9 Saker-Solomons urine test

To ensure successful in vitro growth of field isolates and interpretable assays, systematic use of urine screening to exclude patients with recent intake of antimalarial drugs is highly recommended. Mount et al. (1989) reported a simple, rapid colorimetric method adapted from a technique originally developed by E. Saker and E. Solomons (1979) for screening for phencyclidine and similar drugs in urine. The quantitative version of the test requires a photometer. For field use, the qualitative colorimetric test suffices.

For preparation of a 0.05% solution of tetrabromophenolphthalein ethyl ester (Mount et al., 1989), 10 mg of this compound, 20 ml chloroform and 2 ml 2N HCl are mixed in a clean glass bottle (not plastic). Tetrabromophenolphthalein ethyl ester powder dissolves very slowly in the acid-chloroform mixture, and constant agitation with a magnetic stirrer and slight heating can accelerate the process. When the powder is completely dissolved, the solution is left standing for a few minutes. The aqueous phase (upper, clear, colourless phase) is aspirated with a glass pipette and discarded. The bright-yellow organic phase containing 0.05% tetrabromophenolphthalein ethyl ester is sensitive to direct sunlight and ambient temperature, and the bottle should be covered with aluminium foil and stored in a refrigerator (not freezer). The solution can be used for 3–4 weeks. Beyond this period,

the initial bright-yellow colour fades, and the solution becomes colourless over time owing to decomposition of tetrabromophenolphthalein ethyl ester.

Before screening patients, it is advisable to prepare a sufficient number of 5-ml glass tubes (with caps) containing 1 ml of phosphate buffer (pH 8, prepared with 324 g $K_2HPO_4 \cdot 3H_2O$, 10 g KH_2PO_4 and addition of water to 1 litre) and 0.2 ml of the tetrabromophenolphthalein ethyl ester solution. Before urine is added, the organic phase (lower phase) is yellow-green. A small amount of urine (5–10 ml) is collected from patients in clean (not necessarily sterile) plastic bottles, and 2 ml are transferred into pre-charged tubes with a disposable pipette. The tubes are capped and shaken for a few seconds. The test is positive if there is an immediate change of colour to red or purple (the shade depends on the concentration of drugs in the urine sample). The test is negative if there is no change in colour (the organic phase remains yellow-green).

The change in colour indicates the presence of chloroquine at concentrations that inhibit parasite growth in vitro. The lowest limit of detection of chloroquine is 1 μg/ml. By cross-reaction, quinine and, to a lesser extent, mefloquine in urine also yield positive results in the Saker-Solomons test. Likewise, the presence of proguanil and cycloguanil (a biologically active metabolite of proguanil) also gives positive results (with cycloguanil, the colour changes to amber rather than purple). Sulfadoxine is not detectable in this test. Other common drugs, such as caffeine, nicotine, aspirin, paracetamol and antibiotics, give negative results (Mount et al., 1989). One of the possible drawbacks of the test is a slight change in colour, usually to dark yellow, but not red or violet, for patients who have taken herbal medicines. As most of these plant extracts are not highly active against the parasites, blood samples from such patients can be used for in vitro drug assays.

The results obtained with the Saker-Solomons test are in good agreement with those of the modification II (MMII) of the Haskins test, but the Dill-Glazko test is much less sensitive than the other two (Mount et al., 1987b). For the purpose of screening malaria-infected patients in the field for venous blood collection and in vitro drug sensitivity assays, the results of the qualitative Saker-Solomons urine colorimetric test are largely sufficient to ensure a high probability of obtaining an interpretable in vitro assay. Preparation of the solutions is straightforward, although some of the chemicals might have to be ordered from an international supplier. The test can be performed by the personnel of a health-care centre after a few minutes of training and demonstration. This simple, rapid test is a valuable adjunct for in vitro drug assays.

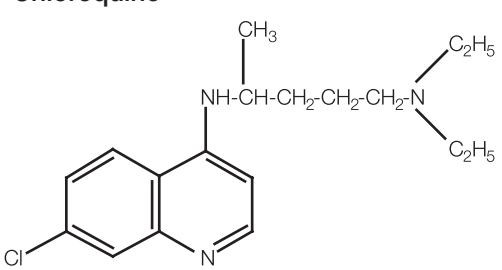
2.2.10 Antimalarial drugs

This section provides succinct information about the commonly used antimalarial drugs, as well as some drugs in advanced phases of clinical

development, and proposes a protocol for preparing drug solutions. Readers interested in obtaining more information on the current clinical use of antimalarial drugs in endemic countries can consult recent WHO documents, which are available free of charge (WHO, 2001a,b). Drugs for which inconsistent in vitro results have been obtained (e.g. sulfadoxine and dapsone), antibiotics with moderate antimalarial activity (e.g. tetracycline, doxycycline, clindamycin and azithromycin), drugs that are not commonly used for the treatment of uncomplicated malaria (e.g. quinidine), drugs that are not commonly used as blood schizontocides (e.g. primaquine) and experimental drugs are not covered. In vitro assays are not recommended for pyrimethamine and cycloguanil for epidemiological purposes, and molecular assays should be used instead. The active principles described in this section can be obtained from commercial suppliers, pharmaceutical companies, or by courtesy of the Special Programme for Research and Training in Tropical Diseases (TDR), WHO. Quality-controlled test plates of the WHO Mark III assay system are still available for orders from the WHO collaborating centre in Penang, Malaysia.

The proposed protocols were designed for the particular needs of field workers, who find it more practical to prepare pre-dosed test plates at a central laboratory before going to the field for blood collection and on-site in vitro assays. Drug solutions prepared at the central laboratory should be stocked in class A, borosilicate-coated volumetric flasks. The concentration ranges of each drug described below were adapted from our experience with freshly collected *P. falciparum*-infected erythrocytes suspended in RPMI medium-10% non-immune human serum and tested with a radioisotope assay. Investigators using RPMI medium-Albumax® supplement, acute-phase autologous serum or animal serum might have to adjust the concentration ranges. It is emphasized that these are only proposed protocols. Some researchers may prefer alternative solvents (in particular 70% ethanol) for certain active principles. The use of absolute ethanol or pure methanol to prepare solutions may pose some technical difficulties, in particular in obtaining consistent small volumes. These problems can usually be overcome with experience.

Chloroquine



7-Chloro-4-(4´-diethylamino-1´-methylbutylamino)quinoline

Chloroquine base, $C_{18}H_{26}ClN_3$ (FW, 319.9)
Chloroquine sulfate monohydrate, $C_{18}H_{26}ClN_3 \bullet H_2SO_4 \bullet H_2O$ (FW, 436.0)
Chloroquine diphosphate, $C_{18}H_{26}ClN_3 \bullet 2H_3PO_4$ (FW, 515.9)

Chloroquine is a 4-aminoquinoline derivative. It occurs as a white crystalline powder, and the oral formulations are the sulfate or phosphate salt. The parenteral formulation (rarely used) is the hydrochloride salt. Both the sulfate and phosphate salts of chloroquine are readily soluble in water (1 g dissolves in 3–4 ml of water [1:3–4]), but they are practically insoluble in alcohol. Stock and working solutions, as well as pre-dosed microtitre plates, remain stable over several years; they should be stored at 4 °C. The active principle and drug solutions should be stored in airtight containers and protected from light. Microtitre plates pre-coated with chloroquine can be stored at 4 °C or room temperature (even in tropical countries) for > 1 year (Rieckmann, 1982).

The distribution of chloroquine in uninfected blood has been determined in vitro (Bergqvist et al., 1983): 36% in plasma, 12% in erythrocytes, 24% in platelets and 28% in leukocytes. In the presence of malaria-infected erythrocytes, chloroquine is highly concentrated in the erythrocytes. Although chloroquine-resistant *P. falciparum* is now widespread, regular monitoring of its in vitro activity is important. Chloroquine is still an essential drug, which is widely available and used in malaria-endemic countries. From the ethical viewpoint, chloroquine monotherapy can be used only in the few countries where the drug remains relatively effective. In vitro drug sensitivity assays and molecular analysis of the *pfcrt* gene have become indispensable alternative methods for monitoring chloroquine efficacy.

The proposed protocol for preparing chloroquine solutions to test seven twofold concentrations in microtitre plates (rows B–H; row A consists of drug-free control wells) involves preparation of a stock solution of chloroquine diphosphate (FW, 515.9) by dissolving 66.0 mg in 100 ml sterile distilled water. A high-precision balance is required to weigh exactly 66.0 mg of the active principle, which should be placed in an autoclaved volumetric flask (class A quality, made of borosilicate glass) and sterile distilled water added for precise measurement of the required volume. Filtration (or autoclaving) of the solution is not required (and not recommended) if the materials used are clean or sterile (autoclaved volumetric flasks and spatula, sterile distilled or demineralized water, clean weighing paper, closed laboratory, properly stored active principle). The definition of a molar concentration, mol/l, is concentration expressed as g/l divided by FW, to obtain in the case of chloroquine 0.066 g/100 ml, or 0.66 g/l divided by 515.9 g/mol = 0.001279 mol/l = 1.279 mmol/l = 1279 µmol/l. This is the molar concentration of the stock solution of chloroquine diphosphate.

The stock is diluted to 1/100 in distilled water to obtain a working solution at a concentration of 12.8 µmol/l. A precise volume of 1 ml of stock can be obtained with 1-ml graduated, sterile plastic pipettes or 1000 µl volume-adjustable precision pipettes. The latter should be calibrated by pipetting 1000 µl of water and weighing it on a precision balance. The stock solution (1 ml) is placed in a 100-ml volumetric flask, and sterile water is added to the 100-ml level.

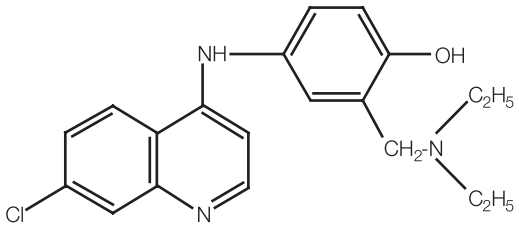
Twofold serial dilutions of the working solution (12.8 µmol/l for well H) are prepared in distilled water. For example, 1 ml of working solution is diluted in 1 ml of water to obtain a concentration of 6.4 µmol/l (for well G). To obtain the dilution for well F (3.2 µmol/l), 1 ml of the solution at 6.4 µmol/l is diluted in 1 ml of water. The other dilutions are obtained in similarly.

When 25 µl of the working solution at 12.8 µmol/l are distributed to well H and air-dried, and 200 µl of blood-medium-serum supplement mixture are subsequently placed in the well, the final concentration in well H will be 1.6 µmol/l or 1600 nmol/l (use the formula, initial concentration x initial volume = final concentration x final volume: 12.8 µmol/l x 25 µl = final concentration x 200 µl; final concentration = 1.6 µmol/l or 1600 nmol/l). The final drug concentrations of the serial dilutions will be 25 nmol/l (row B), 50 nmol/l (row C), 100 nmol/l (row D), 200 nmol/l (row E), 400 nmol/l (row F), 800 nmol/l (row G) and 1600 nmol/l (row H). Row A consists of drug-free control wells. "Final concentration" refers to the concentration obtained when 200 µl of blood-medium mixture are added to the wells after thorough air-drying; 25 µl of drug solution distributed in the test plates should be completely evaporated before use. For aqueous solutions, complete air-drying in humid tropical countries may take several days.

In our experience with the radioisotope method, the IC_{50} values of chloroquine in most freshly collected *P. falciparum* isolates can be classified as those responding at 25–100 nmol/l (usually interpreted as "chloroquine-sensitive") and those responding within the 100–400 nmol/l range (sometimes at higher values; interpreted as "chloroquine-resistant").

Some investigators prefer to dilute the working solution in buffered RPMI medium, with or without serum supplement, if the test plates are to be used immediately, i.e. without air-drying the drug solutions. In such cases, the final volume will be 225 µl (25 µl drug solution plus 200 µl blood-medium mixture), slightly modifying the final drug concentrations.

Amodiaquine and monodesethylamodiaquine



4-(7-Chloro-4-quinolylamino)-2-(diethylaminomethyl)phenol;
7-chloro-4-(3´-diethylaminomethyl-4´-hydroxyanilino)-quinoline

Amodiaquine dihydrochloride dihydrate, $C_{20}H_{22}ClN_3O \bullet 2HCl \bullet 2H_2O$ (FW, 464.8)

Monodesethylamodiaquine dihydrochloride, $C_{18}H_{18}ClN_3O \bullet 2HCl$ (FW, 400.7)

Monodesethylamodiaquine diphosphate dihydrate, $C_{18}H_{18}ClN_3O \bullet 2H_3PO_4 \bullet 2H_2O$ (FW, 559.8)

Amodiaquine is a Mannich-base type 4-aminoquinoline derivative. Monodesethylamodiaquine, the main biologically active metabolite of amodiaquine, lacks a single ethyl group in the side-chain of the Mannich base. Amodiaquine dihydrochloride dihydrate is a yellow crystalline powder that is soluble in water (1:22) and in alcohol (1:70). The colour of the crystalline powder of monodesethylamodiaquine depends on its salt form, being yellowish for dihydrochloride and brownish for diphosphate. Monodesethylamodiaquine salt is soluble in water (no precise data available). Water is the preferred solvent for these two compounds. Amodiaquine and monodesethylamodiaquine aqueous solutions and pre-dosed plates are stable for > 1 year, even at ambient temperature. They should be stored at 4 °C.

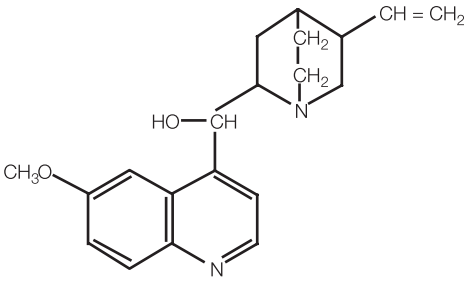
Like chloroquine, amodiaquine is highly concentrated in malaria-infected erythrocytes (Hawley et al., 1996). Although amodiaquine exerts greater schizontocidal activity in vitro than its metabolite (Churchill et al., 1985; Childs et al., 1989; Mariga et al., 2005), monodesethylamodiaquine may be the more appropriate active principle for in vitro testing. Pharmacokinetics studies have shown that, after administration of the parent compound by the oral route, it is rapidly transformed to monodesethylamodiaquine, the biologically active, main human metabolite of amodiaquine, which has a much longer elimination half-life (Churchill et al., 1985).

The proposed protocol for preparing solutions of amodiaquine dihydrochloride dihydrate (FW, 464.8) first involves preparing a stock solution by dissolving 11.9 mg in 100 ml sterile distilled water (0.0119 g/100 ml = 0.119 g/l; molar concentration = 0.119 g/l divided by 464.8 g/mol =

0.000256 mol/l = 0.256 mmol/l = 256 µmol/l). The stock is diluted to 1/100 in sterile distilled water to obtain the working solution at a concentration of 2.560 µmol/l or 2560 nmol/l. Seven twofold serial dilutions of the working solution are prepared in distilled water. To prepare the test plates, 25 µl of the working solution are distributed in well H (initial concentration x initial volume = final concentration x final volume: 2560 nmol/l x 25 µl = final concentration x 200 µl; final concentration in well H = 320 nmol/l). The initial volume of 25 µl of drug solution is distributed in the corresponding wells to obtain the following final concentration range: 5 nmol/l (row B), 10 nmol/l (row C), 20 nmol/l (row D), 40 nmol/l (row E), 80 nmol/l (row F), 160 nmol/l (row G) and 320 nmol/l (row H). Row A consists of drug-free control wells. These final concentrations are the concentrations obtained when 200 µl of blood-medium mixture are added to the wells after thorough air-drying. Drug solution (25 µl) distributed in the test plates is completely evaporated before use; complete air-drying may take several days in tropical countries.

The concentration range of the proposed protocol for monodesethylamodiaquine includes 60 nmol/l, which was suggested to be the threshold value for in vitro resistance (Basco et al., 2002). The results of in vitro assays with monodesethylamodiaquine dihydrochloride and monodesethylamodiaquine diphosphate dihydrate are identical (LK Basco, unpublished data, 2003). To prepare the stock solution, 43.0 mg of monodesethylamodiaquine diphosphate dihydrate are dissolved in 100 ml of sterile distilled water (0.043 g/100 ml = 0.43 g/l; molar concentration = 0.43 g/l divided by 559.8 g/mol = 0.000768 mol/l = 0.768 mmol/l = 768 µmol/l). The working solution is prepared by diluting the stock solution 1/100 in sterile distilled water (i.e. 7.68 µmol/l = 7680 nmol/l). When 25 µl of the working solution are distributed in the microtitre plate and air-dried, and 200 µl of blood-medium mixture are subsequently added, the final drug concentration will be 960 nmol/l (initial concentration x initial volume = final concentration x final volume: 7680 nmol/l x 25 µl = final concentration x 200 µl; final concentration = 960 nmol/l). Twofold dilutions are prepared in sterile distilled water to obtain the following final concentrations: 7.5 nmol/l (row B), 15 nmol/l (row C), 30 nmol/l (row D), 60 nmol/l (row E), 120 nmol/l (row F), 240 nmol/l (row G) and 480 nmol/l (row H). Row A consists of drug-free control wells.

Quinine



(α*R*)-α-(6-Methoxy-4-quinolyl)-α-[(2*S*,4*S*,5*R*)-(5-vinylquinuclidin-2-yl)] methanol; 6-methoxy-α-(5-vinyl-2-quinuclidinyl)-4-quinolinemethanol

Quinine free base, $C_{20}H_{24}N_2O_2$ (FW, 324.4)

Quinine hydrochloride, $C_{20}H_{24}N_2O_2 \bullet HCl$ (FW, 360.9)

Quinine dihydrochloride, $C_{20}H_{24}N_2O_2 \bullet 2HCl$ (FW, 397.3)

Quinine hydrochloride dihydrate, $C_{20}H_{24}N_2O_2 \bullet HCl \bullet 2H_2O$ (FW, 396.9)

Quinine sulfate dihydrate, $(C_{20}H_{24}N_2O_2)_2 \bullet H_2SO_4 \bullet 2H_2O$ (FW, 782.9)

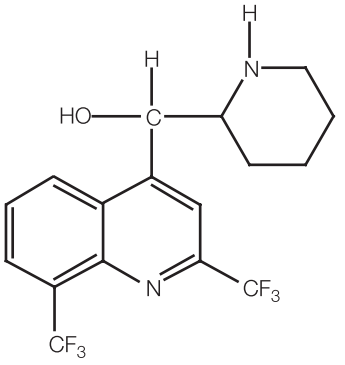
Quinine is one of the natural alkaloids (amino alcohol) extracted from cinchona tree bark. Quinidine is a stereoisomer of quinine and has similar antimalarial action, although it is more commonly used as an antiarrhythmic agent. A number of quinine salts are available from commercial sources, including the sulfate, bisulfate, hemisulfate, hydrochloride, hydrochloride dihydrate, dihydrochloride, formate, salicylate, hydrobromide and dihydrobromide. Quinine hydrochloride dihydrate occurs as colourless, fine crystals that can turn yellow on exposure to light. The powder is soluble in water (1:23) and alcohol (1:0.9). Quinine dihydrochloride is a white powder that is readily soluble in water (1:0.5) and in alcohol (1:14). Quinine sulfate is a white crystalline powder that is soluble in alcohol (1:95) and slightly soluble in water (1:810). Quinine sulfate dihydrate occurs as fine, needle-like, white crystals which are soluble in water (2 mg/ml) and alcohol (8.3 mg/ml) at 25 °C. Solutions in alcohol are stable. The active principles should be stored at 4 °C in airtight containers and protected from light. Quinine-coated plates can be stored for several months up to 2 years at 4 °C.

The active principle is available from commercial suppliers. Investigators are advised to check the salt form and the corresponding chemical formula before preparing drug solutions. The following calculations are based on quinine hydrochloride salt. For other quinine salts, investigators should adjust the calculations on the basis of the formula weight.

The stock solution is prepared from 46.2 mg of quinine hydrochloride dissolved in 50 ml pure methanol (0.0462 g/0.05 l = 0.924 g/l; molar concen-

tration = 0.924 g/l divided by 360.9 g/mol = 0.00256 mol/l = 2.56 mmol/l). The working solution is obtained by diluting the stock solution 1/100 in pure methanol (i.e. 25.6 µmol/l = 25 600 nmol/l). The final concentration of the working solution in test plates with 25 µl is 3200 nmol/l (initial concentration × initial volume = final concentration × final volume: 25 600 nmol/l × 25 µl = final concentration × 200 µl; final concentration = 3200 nmol/l). Seven twofold dilutions are prepared from the working solution in pure methanol to obtain the following final concentrations in the test plate (25 µl of each dilution are distributed in the wells and air-dried before use to eliminate methanol completely): 50 nmol/l (row B), 100 nmol/l (row C), 200 nmol/l (row D), 400 nmol/l (row E), 800 nmol/l (row F), 1600 nmol/l (row G) and 3200 nmol/l (row H). Row A consists of drug-free control wells. It may take several hours for alcoholic solutions to dry completely. Test plates with a cover can be left overnight for drying.

Mefloquine



DL-*erythro*-α-2-Piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol; (*R,*S**)-(±)-α-2-piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol; (±)-α-[2,8-bis(trifluoromethyl)-4-quinolyl]-α-(2-piperidyl)methanol**

Mefloquine base, $C_{17}H_{16}F_6N_2O$ (FW, 378.3)

Mefloquine hydrochloride, $C_{17}H_{16}F_6N_2O \bullet HCl$ (FW, 414.8)

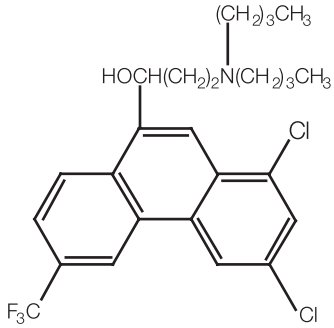
Mefloquine (code given by Walter Reed Army Institute of Research, Washington DC, USA, WR 142 490) is a 4-quinolinemethanol-type amino alcohol. Mefloquine hydrochloride is a white crystalline powder and is slightly soluble in water (3.8 mg/ml), soluble in ethanol (52 mg/ml) and dimethyl sulfoxide (32 mg/ml) and readily soluble in methanol (165 mg/ml) (data provided by Hoffmann-La Roche, Basel, Switzerland, by courtesy of WHO). According to *The International Pharmacopoeia* (WHO, 2003a), mefloquine hydrochloride is slightly soluble in water, soluble in ethanol

(750 g/l) and freely soluble in methanol. Solutions in methanol can be stored at 4 °C for several months. Mefloquine-pre-dosed plates are not stable, and the molecule is thought to be degraded to its 4-carboxylic acid derivative, which is practically devoid of antimalarial activity (Franssen et al., 1989; Hakanson, Landberg-Lindgren & Björkman, 1990; Basco et al., 1991). The shelf-life of pre-dosed mefloquine plates is 2 months at 4 °C.

Mefloquine is extensively bound (> 95%) to plasma proteins. Unlike chloroquine and amodiaquine, the drug does not appear to concentrate selectively or to a high degree in malaria-infected erythrocytes. Mefloquine occupies an important place in current antimalarial chemotherapy, for both chemoprophylaxis and treatment, usually in combination therapy. It is an essential drug. It is widely used in Asian countries, where clinical resistance has been reported, but its use on the African and American continents has been limited. Surveillance of the in vitro activity of mefloquine is important from the epidemiological viewpoint.

The stock solution is prepared in pure methanol (26.5 mg mefloquine hydrochloride in 100 ml methanol; 0.0265 g/100 ml = 0.265 g/l; molar concentration = 0.265 g/l divided by 414.8 g/mol = 0.000639 mol/l = 640 µmol/l [rounded]). The intermediate solution is obtained by diluting the stock solution 1/50 in pure methanol (to 12.8 µmol/l). The working solution is obtained by diluting the intermediate solution 1/10 in pure methanol (concentration = 1.28 µmol/l = 1280 nmol/l). When the working solution (25 µl) is distributed in the test plate, the final concentration is 160 nmol/l after addition of 200 µl of blood-medium mixture (initial concentration × initial volume = final concentration × final volume: 1280 nmol/l × 25 µl = final concentration × 200 µl; final concentration = 160 nmol/l). Seven two-fold dilutions are prepared from the working solution in pure methanol to obtain the following final concentrations in the test plate: 2.5 nmol/l (row B), 5 nmol/l (row C), 10 nmol/l (row D), 20 nmol/l (row E), 40 nmol/l (row F), 80 nmol/l (row G) and 160 nmol/l (row H). Row A consists of drug-free control wells. The test plates should be air-dried completely before use to avoid a toxic effect of methanol on the parasites. It may take several hours for alcoholic solutions to dry completely. Test plates with a cover can be left overnight for drying.

Halofantrine



1,3-Dichloro-α-[2-(dibutylamino)-ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol

Halofantrine base, $C_{26}H_{30}Cl_2F_3NO$ (FW, 500.4)

Halofantrine hydrochloride, $C_{26}H_{30}Cl_2F_3NO \bullet HCl$ (FW, 536.8)

Halofantrine (code given by Walter Reed Army Institute of Research, Washington DC, USA, WR 171 669) is a 9-phenanthrenemethanol-type amino alcohol. Its main human metabolite, *N*-desbutylhalofantrine, is as potent as the parent compound against malaria parasites. Halofantrine hydrochloride is a white powder, which is soluble in alcohol but poorly soluble in water (< 1 g in 100 ml). Solutions in pure methanol remain stable for years, but solutions containing even a small percentage of water can produce invisible microprecipitations that result in abnormally elevated IC_{50} values due to drug loss. The stock solution, working solution and dilutions should be prepared in pure methanol. Test plates with pre-dosed halofantrine can be stored for several months up to 1 year at 4 °C.

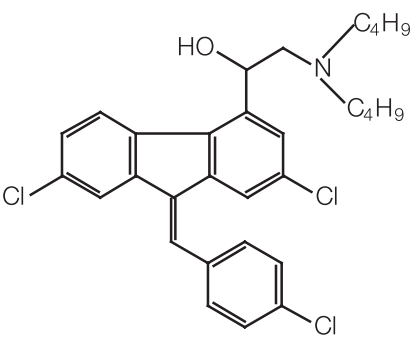
A study on the in vitro distribution of [^{14}C]halofantrine in various blood components showed that drug uptake into parasitized erythrocytes is 50–60-fold higher than that in uninfected erythrocytes (Cenni & Betschart, 1995). In uninfected blood, most halofantrine is associated with the plasma fraction (85%) and much less with erythrocytes (11%), platelets (2%) and leukocytes (1.3%). Halofantrine uptake is stage-dependent, increasing with higher proportions of mature trophozoites and schizonts. Halofantrine binds extensively (> 99.5%) to serum proteins, but mostly to low-density and high-density lipoproteins (Cenni et al., 1995); only a small fraction of halofantrine is bound to α_1-acid glycoprotein and albumin.

At present, the role of halofantrine in antimalarial chemotherapy is limited owing to its cardiotoxic effect, which can lead to the death of patients with pre-existing cardiac disease (Nosten et al., 1993; Monlun et al., 1995; Matson et al., 1996; Touze et al., 1996; Sowunmi et al., 2000; Wesche et al., 2000). Its metabolite, *N*-desbutylhalofantrine, is not cardiotoxic, and two

treatment courses at a 1-week interval are recommended by the pharmaceutical company to ensure adequate drug absorption in non-immune patients. This requirement and its high cost are the main reasons why halofantrine is not an essential antimalarial drug. Several studies have demonstrated a strong correlation between the in vitro responses to mefloquine and halofantrine, suggesting in vitro cross-resistance (Gay et al., 1990; Ringwald et al., 1990; Basco & Le Bras, 1992a; Rojas-Rivero et al., 1992; Cowman, Galatis & Thompson, 1994). The existence of cross-resistance in vitro implies that monitoring of the in vitro activity of mefloquine alone, and not that of both mefloquine and halofantrine, provides sufficient information. Therefore, in vitro monitoring of the activity of halofantrine against field isolates has little impact on malaria control.

In our experience, halofantrine solutions are best prepared by making a stock solution in pure methanol (34.2 mg halofantrine hydrochloride [FW, 536.8] in 100 ml methanol), for a concentration of 637 µmol/l (34.2 mg/100 ml = 0.342 g/l; molar concentration = 0.342 g/l divided by 536.8 g/mol = 0.000637 mol/l = 0.637 mmol/l = 637 µmol/l). The intermediate solution is prepared by diluting the stock to 1/50 in pure methanol (i.e. 637 µmol/l divided by 50 = 12.74 µmol/l). The working solution is obtained by diluting the intermediate solution in pure methanol to 1/50 (i.e. 12.74 µmol/l divided by 50 = 0.2548 µmol/l = 255 nmol/l). Twofold serial dilutions are prepared in pure methanol to obtain the following final concentrations: 0.5 nmol/l (row B), 1 nmol/l (row C), 2 nmol/l (row D), 4 nmol/l (row E), 8 nmol/l (row F), 16 nmol/l (row G) and 32 nmol/l (row H). Row A consists of drug-free control wells. The test plates should be air-dried completely before use. Drying of alcoholic solutions takes several hours, especially if the plate cover is left closed to avoid microbial contamination. Test plates with a cover can be left overnight for drying.

Lumefantrine



2-Dibutylamino-1-[2,7-dichloro-9-(4-chlorobenzylidene)-9*H*-fluoren-4-yl]ethanol

Lumefantrine base, $C_{30}H_{32}Cl_3NO$ (FW, 528.95)

Lumefantrine (formerly known as benflumetol; code given by Ciba-Geigy Pharma [now Novartis Pharma] CGP 56 695) is a synthetic fluorene derivative (2,3-benzindene) belonging to the group of amino alcohols. It occurs as a pale-yellowish powder that is soluble in ethanol (0.46–0.56 g/l at 25 °C) and practically insoluble in water (< 0.04 mg/l) (data provided by Novartis Pharma Ltd, Basel, Switzerland). W. Wernsdorfer has prepared stock solutions in ethanol-linoleic acid-Tween 80 mixture (v/v/v 1:1:1) (Tween 80 is polyoxyethylenesorbitan monooleate), which remain stable for several years (Alin, Björkman & Wernsdorfer, 1999). The shelf-life of lumefantrine-dosed test plates stored at 4 °C is 4 months (Kaddouri et al., 2006). More data on plate stability are required.

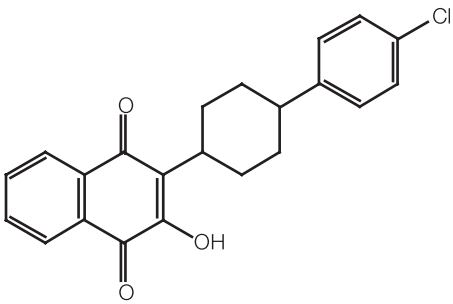
Lumefantrine is a lipophilic compound, like halofantrine, characterized by erratic absorption after oral administration. It binds extensively to plasma proteins (> 99%). Lumefantrine-artemether fixed combination is being used as an alternative therapy for multidrug-resistant *P. falciparum* infections. The in vitro activity of lumefantrine is positively correlated with that of other amino alcohols, mefloquine and halofantrine (Basco, Bickii & Ringwald, 1998). For these reasons, baseline and regular monitoring of its in vitro activity may be important.

The stock solution is prepared by dissolving 33.8 mg of lumefantrine in 100 ml absolute ethanol (0.0338 g/100 ml = 0.338 g/l; molar concentration = 0.338 g/l divided by 528.95 g/mol = 0.00064 mol/l = 0.64 mmol/l = 640 µmol/l). The intermediate solution is obtained by diluting the stock 1/100 in absolute ethanol (i.e. 6.4 µmol/l = 6,400 nmol/l). The working solution is obtained from the intermediate solution by diluting it to 1/10 in absolute ethanol (i.e. 640 nmol/l). When 25 µl of the working solution (640 nmol/l) are distributed in well H of the microtitre plate and air-dried, the final concentration is 80 nmol/l after addition of 200 µl of blood-medium mixture (initial concentration × initial volume = final concentration × final volume: 640 nmol/l × 25 µl = final concentration × 200 µl; final concentration = 80 nmol/l). Twofold dilutions are prepared in absolute ethanol from the working solution to obtain the following final concentrations: 1.25 nmol/l (row B), 2.5 nmol/l (row C), 5 nmol/l (row D), 10 nmol/l (row E), 20 nmol/l (row F), 40 nmol/l (row G) and 80 nmol/l (row H). Row A consists of drug-free control wells. The test plates should be air-dried completely before use. Drying of alcoholic solutions takes several hours, especially if the plate cover is left closed to avoid microbial contamination. Test plates with a cover can be left overnight for drying.

In an alternative protocol, an absolute ethanol-linoleic acid-Tween 80 mixture (v/v/v 1:1:1) is used to prepare the stock solution (Alin, Björkman & Wernsdorfer, 1999). The mixture is viscous and yellowish, but the active principle dissolves well in this solvent. The stock solution is diluted in sterile distilled water, as described above, to obtain the intermediate and working solutions. The intermediate solution becomes turbid and should be mixed well before preparation of the working solution. These solutions are stable

for several years. In our experience, absolute ethanol-linoleic acid-Tween 80 solvent is toxic to cell cultures, inducing haemolysis in cultures of malaria parasites, even when it is completely air-dried, unless it is diluted to more than 1/1000 in water, as described above.

Atovaquone



2-[*trans*-4-(4´-Chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone

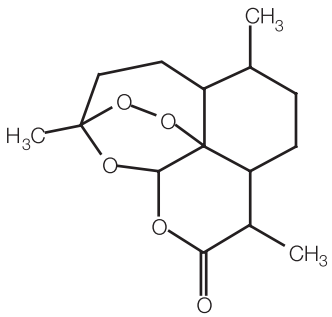
Atovaquone, $C_{22}H_{19}ClO_3$ (FW, 366.8)

Atovaquone (code given by Burroughs Wellcome Inc. [now Glaxo SmithKline], BW566C80) is a hydroxynaphthoquinone derivative and a ubiquinone analogue. Atovaquone hydrochloride is a micronized, yellowish powder that is moderately soluble in alcohol and practically insoluble in water. Its solubility in methanol-dimethylformamide (v/v 99:1) is 0.3 mg/ml. The stock solution in alcohol remains stable for years, but the shelf-life of atovaquone-dosed test plates has not been determined.

Atovaquone binds extensively (> 95%) to plasma proteins. Its in vitro activity varies widely, depending on the serum (human or animal) and serum substitute (e.g. Albumax®) used (Basco, 2003c). Regular monitoring of its in vitro activity was justified in parts of the African continent recently when the pharmaceutical company sponsored a drug donation programme of 1 million free doses per year. The programme has since been suspended indefinitely. At present, atovaquone-proguanil combination is essentially used as a chemoprophylactic agent for non-immune temporary visitors in endemic areas and for outpatient treatment of cases of imported malaria in industrialized countries. It is not available for sale at most pharmacies in endemic countries. The drug combination is not intended for the treatment of indigenous populations owing to its excessively high cost. Because of its wide antiprotozoal spectrum, atovaquone is also used to treat other parasitic infections. In the opinion of the author, regular in vitro monitoring of its activity in endemic countries is not a priority. From the academic viewpoint, however, it would be interesting to determine the correlation between the in vitro-in vivo response and point mutations in the *P. falciparum* cytochrome *b* gene.

The stock solution, at a concentration of 640 µmol/l, is obtained by dissolving 23.55 mg in 100 ml pure methanol (0.0236 g/100 ml = 0.236 g/l; molar concentration = 0.236 g/l divided by 366.8 g/mol = 0.00064 mol/l = 0.642 mmol/l = 642 µmol/l). The intermediate solution is prepared by diluting the stock solution 1/50 (i.e. 12.8 µmol/l). The working solution is obtained by diluting the intermediate solution 1/100 (i.e. 128 nmol/l). When 25 µl of the working solution are distributed and air-dried in well H and 200 µl of blood-medium mixture are subsequently added, the final concentration in the well is 16 nmol/l (initial concentration × initial volume = final concentration × final volume: 128 nmol/l × 25 µl = final concentration × 200 µl; final concentration = 16 nmol/l). Twofold serial dilutions are prepared in pure methanol from the working solution to obtain the following final concentrations: 0.25 nmol/l (row B), 0.5 nmol/l (row C), 1 nmol/l (row D), 2 nmol/l (row E), 4 nmol/l (row F), 8 nmol/l (row G) and 16 nmol/l (row H). Row A consists of drug-free control wells. Test plates must be thoroughly air-dried before use.

Artemisinin or artemisinin derivatives



Artemisinin: (3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,12a*R*)-octahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4.3-*j*]-1,2-benzodioxepin-10(3*H*)-one; 3,6,9-trimethyl-9,10*b*-epi-dioxyperhydropyranol[4,3,2-*jk*] benzoxepin-2-one

Artesunate: (3R,5aS,6R,8aS,9R,10S,12R,12aR)-decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10-ol, hydrogen succinate

Artemether: (3R,5aS,6R,8aS,9R,10S,12R,12aR)]-decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin

Arteether: (3R,5aS,6R,8aS,9R,10S,12R,12aR)]-decahydro-10-ethoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin

Dihydroartemisinin: (3R,5aS,6R,8aS,9R,10S,12R,12aR)]-decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10-ol

Artemisinin, $C_{15}H_{22}O_5$ (FW, 282.3)

Artesunate, $C_{19}H_{28}O_8$ (FW, 384.4)

Artemether, $C_{16}H_{26}O_5$ (FW, 298.4)

Arteether, $C_{17}H_{28}O_5$ (FW, 312.4)

Dihydroartemisinin, $C_{15}H_{24}O_5$ (FW, 284.3)

Artemisinin (also called *qinghaosu*) is a sesquiterpene lactone peroxide extracted and purified from the herb *Artemisia annua*. The carbonyl group at C-10 of the parent compound is reduced (from =O to –OH) to obtain dihydroartemisinin. Addition of a methyl ($–CH_3$) or ethyl ($–C_2H_5$) substituent to the hydroxyl group of dihydroartemisinin results in formation of artemether (methyl ether derivative) and arteether (ethyl ether derivative), respectively. Artesunate (ester derivative) is the sodium hemisuccinate salt of artemisinin. Artemisinin, artesunate, artemether, arteether and dihydroartemisinin are white crystalline powders.

Artemisinin derivatives, in particular the parent compound, are poorly soluble in water, and sodium artesunate hydrolyses to dihydroartemisinin in aqueous solutions. Artemisinin, artesunate, artemether, arteether and dihydroartemisinin are freely soluble in ethanol (750 g/l) and methanol (WHO, 2003a). Stock solutions in pure alcohol remain stable for years. In the experience of the group of W. Wernsdorfer, artemether binds irreversibly to plastic materials (Tanariya et al., 2000). The most stable compounds, which are suitable for preparing pre-dosed test plates, are artemisinin and dihydroartemisinin. The shelf-life of pre-dosed artemisinin, dihydroartemisinin, and artesunate plates is 6 months at 4 °C (J. Le Bras, unpublished data, 2004).

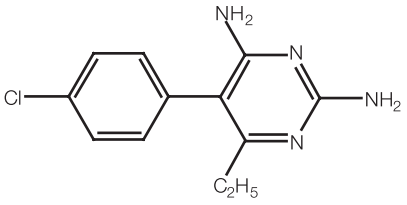
Artemisinin drugs undergo hepatic biotransformation to dihydroartemisinin, a biologically active, major human metabolite, and minor inactive metabolites (Lee & Hufford, 1990). On the basis of these findings, some investigators consider artemisinin derivatives, in particular artesunate, to be "pro-drugs" of dihydroartemisinin. Nevertheless, all artemisinin derivatives are not only highly active against *P. falciparum* in vitro at a low nanomolar range but are also almost equipotent: artemisinin is usually two to three times less active than its derivatives but is active within a low nanomolar range. Dihydroartemisinin concentrates in malaria-infected erythrocytes (Gu, Warhurst & Peters, 1984). Depending on the derivative, binding to plasma proteins varies from 43% to 82%, binding being mostly to albumin and α_1-acid glycoprotein (Wanwimolruk et al., 1992). The implications of the latter finding require further investigations in acute-phase serum from malaria-infected patients.

Artemisinin derivatives have become the key component of current antimalarial drug policy, in the combination therapy advocated by most malaria experts. Clinical resistance to artemisinin derivatives has not been

reported, despite their massive use in recent years in many parts of the world. Recrudescence is known to occur, however, particularly after monotherapy. Recrudescence is usually related to rapid elimination of the drug. As the current therapeutic approach is based almost entirely on artemisinin combination therapy, in vivo and in vitro surveillance of artemisinin efficacy is of prime importance.

The stock solution of artemisinin is prepared by dissolving 36.1 mg in 100 ml pure methanol (0.0361 g/100 ml = 0.361 g/l; molar concentration = 0.361 g/l divided by 282.3 g/mol = 0.001 279 mol/l = 1.28 mmol/l = 1280 µmol/l) (for dihydroartemisinin, 36.4 mg in 100 ml pure methanol). The intermediate solution is prepared by diluting the stock solution 1/100 in pure methanol (i.e. 12.8 µmol/l). The working solution is obtained by further diluting the intermediate solution by 1/100 in pure methanol (i.e. 0.128 µmol/l = 128 nmol/l). When 25 µl of the working solution are distributed and air-dried in well H and blood-medium mixture (200 µl) is subsequently added, the final concentration in the well is 16 nmol/l (initial concentration × initial volume = final concentration × final volume: 128 nmol/l × 25 µl = final concentration × 200 µl; final concentration = 16 nmol/l). Twofold serial dilutions are prepared from the working solution in pure methanol to obtain the following final concentrations ("final concentration" refers to the concentration obtained when 200 µl of blood-medium mixture are added to the wells): 0.25 nmol/l (row B), 0.5 nmol/l (row C), 1 nmol/l (row D), 2 nmol/l (row E), 4 nmol/l (row F), 8 nmol/l (row G) and 16 nmol/l (row H). Row A consists of drug-free control wells. The test plates should be thoroughly air-dried before use.

Pyrimethamine



5-(4-Chlorophenyl)-6-ethyl-2,4-pyrimidinediamine; 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine

Pyrimethamine base, $C_{12}H_{13}ClN_4$ (FW, 248.7)

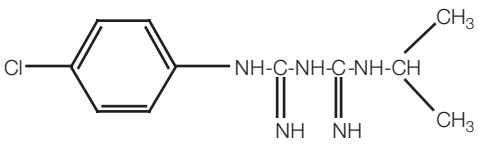
Pyrimethamine is an aminopyrimidine-derivative DHFR inhibitor. Pyrimethamine base (not available as salts) is a white crystalline powder that is slightly soluble in ethanol (1:200) but practically insoluble in water. The stock solution in alcohol remains stable for years. The shelf-life of predosed plates extends to several months up to 1 year at 4 °C.

Sulfadoxine-pyrimethamine combination is the usual replacement for treating chloroquine-resistant *P. falciparum*. Resistance to this combination has been widespread in South-East Asia for 30 years and is spreading in East Africa and, to a lesser extent, in central and West Africa. In the opinion of the author, in vitro monitoring of pyrimethamine activity is not a priority, since equivalent information can be derived more easily from an analysis of the molecular markers, *P. falciparum dhfr* and *dhps*. Field studies have shown that *dhfr* mutations are highly correlated with the in vitro response to pyrimethamine (Reeder et al., 1996; Basco & Ringwald, 2000a; Basco et al., 2000; Basco, 2003b). Moreover, monitoring of pyrimethamine activity requires specific medium and incubation period.

The protocol given below was designed to cover the range of possible in vitro responses of field isolates to pyrimethamine. The difference in IC_{50} values between wild-type parasites and those with multiple mutations in *dhfr* can attain > 1000-fold. Testing drug concentrations at twofold dilutions would require too many wells, and testing at 10-fold dilutions would result in inaccurate IC_{50} values or MICs. As a compromise, use of fourfold dilutions is proposed.

The stock solution, which is also the working solution, is prepared by dissolving 10.2 mg pyrimethamine base in 100 ml absolute ethanol (10.2 mg/100 ml = 0.102 g/l; molar concentration = 0.102 g/l divided by 248.7 g/mol = 0.000410 mol/l = 0.410 mmol/l = 410 µmol/l). The final concentration of this solution in the pre-dosed plate is 51 200 nmol/l (initial concentration × initial volume = final concentration × final volume: 410 µmol/l × 25 µl = final concentration × 200 µl; final concentration = 51.25 µmol/l rounded to 51.2 µmol/l = 51 200 nmol/l). The stock solution is diluted fourfold (e.g. 1 ml of stock solution plus 3 ml of absolute ethanol) in series to obtain the following final concentrations in the test plate: 0.05 nmol/l (column 2), 0.2 nmol/l (column 3), 0.8 nmol/l (column 4), 3.1 nmol/l (column 5), 12.5 nmol/l (column 6), 50 nmol/l (column 7), 200 nmol/l (column 8), 800 nmol/l (column 9), 3200 nmol/l (column 10), 12 800 nmol/l (column 11) and 51 200 nmol/l (column 12). The drug dilutions are distributed horizontally across the microtitre plates. Column 1 consists of drug-free control wells. The test plates must be thoroughly air-dried before use (for several hours to overnight in tropical countries). It is recommended that in vitro drug sensitivity assays for pyrimethamine be performed with PABA- and folic acid-free RPMI (low-PABA, low-folic acid RPMI can also be used) for an incubation period of 66–72 h.

Proguanil and chlorproguanil



Proguanil, 1-(4-chlorophenyl)-5-isopropylbiguanide
Chlorproguanil, 1-(3,4-dichlorophenyl)-5-isopropylbiguanide

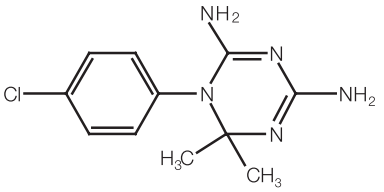
Proguanil hydrochloride, $C_{11}H_{16}ClN_5 \bullet HCl$ (FW, 290.2)
Chlorproguanil hydrochloride, $C_{11}H_{15}Cl_2N_5 \bullet HCl$ (FW, 324.6)

Proguanil and chlorproguanil (with an additional chlorine atom at position 3) are biguanide-type DHFR inhibitors. Proguanil hydrochloride is a white crystalline powder that is soluble in water (1:110) and alcohol (1:40) at 20 °C. Chlorproguanil hydrochloride is a white crystalline powder that is also soluble in water (1:140) and alcohol (1:50). Aqueous solutions remain stable for at least several months. The stability of pre-dosed test plates has not been evaluated.

Proguanil and chlorproguanil administered orally undergo biotransformation in the liver to cycloguanil and chlorcycloguanil, respectively. The parent compounds are pro-drugs. The in vitro schizontocidal activity of cycloguanil is about 1000-fold higher than that of proguanil (Ndounga, Basco & Ringwald, 1999). Testing the activity of proguanil and chlorproguanil is not relevant for epidemiological studies, but experimental studies could be conducted with these pro-drugs.

The stock solution of proguanil hydrochloride is prepared at a concentration of 1.64 mmol/l (47.6 mg in 100 ml sterile distilled water; molar concentration = 0.476 g/l divided by 290.2 g/mol = 0.00164 mol/l = 1.64 mmol/l = 1640 µmol/l). The stock solution is also the working solution. When 25 µl of the stock solution are distributed in the microtitre well and air-dried, the final concentration is 205 µmol/l (initial concentration × initial volume = final concentration × final volume: 1640 µmol/l × 25 µl = final concentration × 200 µl; final concentration = 205 µmol/l). Twofold dilutions of the stock are prepared in water. The following final concentrations are recommended: 1600 nmol/l (row B), 3200 nmol/l (row C), 6400 nmol/l (row D), 12 800 nmol/l (row E), 25 600 nmol/l (row F), 51 200 nmol/l (row G) and 102 400 nmol/l (row H). Row A consists of drug-free control wells. In vitro drug sensitivity assays for proguanil can be performed with RPMI containing the standard quantities of PABA and folic acid or in low-PABA, low-folic acid RPMI (Ndounga, Basco & Ringwald, 1999).

Cycloguanil



Bis-[1-(4-chlorophenyl)-1,2-dihydro-2,2-dimethyl-1,3,5-triazine-4,6-diamine]; 4,6-diamino-1-(*p*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine

Cycloguanil base, $C_{11}H_{14}ClN_5$ (FW, 251.7)

Chlorcycloguanil, a metabolite of chlorproguanil, has an extra Cl at the 3 position.

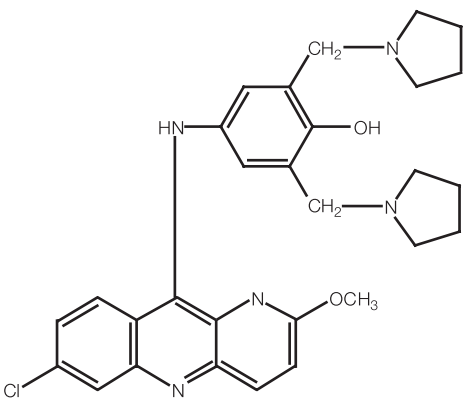
Cycloguanil is the major biologically active metabolite of proguanil and acts as a specific DHFR inhibitor of malaria parasites. It occurs as a white crystalline powder that is soluble in water but is practically insoluble in alcohol. The aqueous solution remains stable for months, and the shelf-life of pre-dosed plates also extends to months.

Cycloguanil is currently the second or third choice as a prophylactic agent (chloroquine-proguanil combination) in antimalarial chemotherapy. This combination is sometimes prescribed to pregnant women (including those residing permanently in malaria-endemic areas) at risk of malarial infection and to non-immune travellers staying in areas of low-level or high-level chloroquine resistance but who cannot tolerate more effective prophylactic drugs. Proguanil is also used to enhance the activity of atovaquone through a mitochondrion-specific mechanism (not as a pro-drug or DHFR inhibitor). In this context, testing the in vitro activity of cycloguanil for epidemiological studies is not a priority; epidemiological studies can focus on analysis of a reliable molecular marker, *P. falciparum dhfr*. Point mutations in *dhfr* are highly correlated with the in vitro response to cycloguanil (Basco et al., 1995b; Basco & Ringwald, 2000a; Basco, 2002a).

The stock solution, which is also the working solution, is prepared by dissolving 10.3 mg cycloguanil base in 100 ml sterile distilled water (10.3 mg/100 ml = 0.103 g/l; molar concentration = 0.103 g/l divided by 251.7 g/mol = 0.000409 mol/l = 0.409 mmol/l = 409.22 µmol/l). The final concentration of this solution in the pre-dosed plate is 51 200 nmol/l (initial concentration × initial volume = final concentration × final volume: 409.22 µmol/l × 25 µl = final concentration × 200 µl; final concentration = 51.15 µmol/l rounded to 51.2 µmol/l = 51 200 nmol/l). The stock solution is diluted fourfold (e.g. 1 ml of stock solution plus 3 ml of sterile distilled water) in series to obtain the following final concentrations in the test plate: 0.05 nmol/l (column 2),

0.2 nmol/l (column 3), 0.8 nmol/l (column 4), 3.1 nmol/l (column 5), 12.5 nmol/l (column 6), 50 nmol/l (column 7), 200 nmol/l (column 8), 800 nmol/l (column 9), 3200 nmol/l (column 10), 12 800 nmol/l (column 11) and 51 200 nmol/l (column 12). The drug dilutions are distributed horizontally across the microtitre plates. Column 1 consists of drug-free control wells. It is recommended that in vitro drug sensitivity assays for cycloguanil be performed with PABA- and folic acid-free RPMI (low-PABA, low-folic acid RPMI can also be used) for an incubation period of 66–72 h.

Pyronaridine



2-Methoxy-6-chloro-9[3,5-bis(1-pyrrolidinylmethyl)-4-hydroxy] anilino-1-aza-acridine

Pyronaridine tetraphosphate, $C_{29}H_{33}ClN_5O_2 \bullet 4H_3PO_4$ (FW, 910.0)

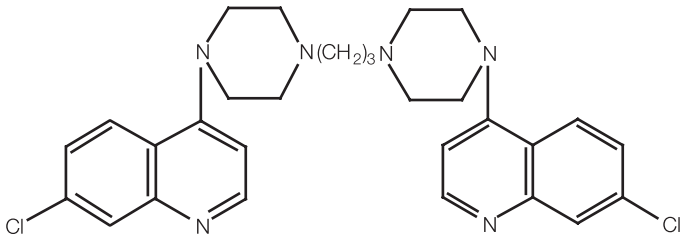
Pyronaridine is an acridine-type Mannich base. It is a yellow-to-orange crystalline powder that is freely soluble in water. Aqueous solutions remain stable for years in borosilicate-coated volumetric flasks.

Pyronaridine has been registered and used clinically in China for more than a decade. Monotherapy with this drug has been shown to be highly effective in areas of chloroquine resistance (Cameroon) and multidrug resistance (Thailand) (Looareesuwan et al., 1996b; Ringwald, Bickii & Basco, 1996, 1998). In vitro studies have shown high activity of pyronaridine against chloroquine-resistant *P. falciparum* isolates (Childs et al., 1988; Warsame et al., 1991; Basco & Le Bras, 1992b, 1994c; Pradines et al., 1998c, 1999a; Ringwald et al., 1999b). The clinical efficacy of a pyronaridine–artesunate combination is being evaluated.

A tentative protocol using fresh drug solutions is proposed here. The stock solution is prepared in sterile distilled water (32.8 mg/100 ml; molar concentration = 0.328 g/l divided by 910 g/mol = 360 µmol/l). The interme-

diate solution is obtained by diluting the stock 1/100 in sterile distilled water (i.e. 3.6 µmol/l = 3600 nmol/l). The working solution is obtained by diluting the intermediate solution 1/5 in sterile RPMI medium (i.e. 720 nmol/l). Fresh aqueous solutions should be distributed in the test plate just before the assay. The final concentration of the working solution corresponds to 80 nmol/l (initial concentration × initial volume = final concentration × final volume: 720 nmol/l × 25 µl = final concentration × 225 µl; final concentration = 80 nmol/l; note that the final volume is 225 µl because fresh solutions are used without air-drying). Twofold serial dilutions are prepared just before assays to obtain the following final concentrations: 1.25 nmol/l (row B), 2.5 nmol/l (row C), 5 nmol/l (row D), 10 nmol/l (row E), 20 nmol/l (row F), 40 nmol/l (row G) and 80 nmol/l (row H). Row A consists of drug-free control wells. It is advisable to add 25 µl of drug-free, fresh solvent to row A. To avoid changes in osmolarity in plates with fresh drug solutions, the working solution and twofold serial dilutions should be prepared in RPMI medium. An alternative protocol for the preparation of pre-dosed pyronaridine plate (shelf-life of 6 months at 4 °C) is awaiting further validation in field studies (Liu et al., 2006).

Piperaquine



1,3-Bis[1-(7-chloro-4´-quinolyl)-4´-piperazinyl]-propane

Piperaquine tetraphosphate tetrahydrate, $C_{29}H_{32}Cl_2N_6 \bullet 4H_3PO_4 \bullet 4H_2O$ (FW, 999.6)

Piperaquine is a bisquinoline, i.e. it is composed of two covalently bound quinoline moieties. Piperaquine tetraphosphate tetrahydrate is a white crystalline powder that is slightly soluble in water at neutral pH and practically insoluble in absolute ethanol. To dissolve piperaquine in a culture-compatible solvent, a stock solution of piperaquine tetraphosphate is prepared in 0.5% lactic acid, and further dilutions are made in sterile water (protocol suggested by Dr Yang Henglin, Yunnan Institute of Malaria Control, Simao, Yunnan, China). The aqueous solution remains stable for at least 1 month, and the shelf-life of pre-dosed test plates stored at 4 °C has been reported to be at least 6 months (Yang Henglin, personal communication, 2001).

Piperaquine was synthesized independently in the 1960s by Chinese investigators and by the French pharmaceutical company, Rhone-Poulenc (now Sanofi-Aventis, Antony, France; under the code name 13228 RP). The latter abandoned research on this product several decades ago, but development of piperaquine was pursued in China, where it is registered for clinical use (Chen, Qu & Zhou, 1982). Because of increasing numbers of cases of therapeutic failure with piperaquine monotherapy, Chinese investigators have been evaluating suitable drug partners for combination therapy. Piperaquine-dihydroartemisinin combination is in an advanced phase of clinical development (Denis et al., 2002; Giao et al., 2004; Karunajeewa et al., 2004; Tran et al., 2004) and is marketed in some countries.

Piperaquine remains highly active in vitro against chloroquine-resistant *P. falciparum* strains (Le Bras, Deloron & Charmot, 1983; Deloron et al., 1985; Vennerstrom et al., 1992; Basco & Ringwald, 2003). Some degree of in vitro cross-resistance between chloroquine and piperaquine has been suggested in Chinese studies (Zhang et al., 1987; Liu et al., 1996; Yang et al., 1999). In our study in central Africa, a significant but low correlation of response was found between chloroquine and piperaquine (Basco & Ringwald, 2003).

Protocols on pre-dosed plate preparation, shelf-life, and drug stability are currently being evaluated. While waiting for the results of these studies, the following protocol was designed to test the activity of fresh drug solutions. In contrast to the protocols for other drugs described above, the final volume in the wells is 225 µl (i.e. 25 µl fresh drug solution plus 200 µl blood-medium mixture). The stock solution is prepared by dissolving 143.9 mg of piperaquine in 100 ml 0.5% lactic acid (0.1439 g/100 ml = 1.439 g/l; molar concentration = 1.439 g/l divided by 999.6 g/mol = 0.00144 mol/l = 1.44 mmol/l = 1440 µmol/l). To avoid any toxic effect of the acidic solvent, the working solution is prepared by diluting the stock 1/100 in RPMI medium (i.e. 14.4 µmol/l = 14 400 nmol/l). When the working solution (25 µl per well) is distributed in the test plate and blood-medium mixture (200 µl) is added to the freshly prepared test plate, the final concentration is 1600 nmol/l (initial concentration × initial volume = final concentration × final volume: 14 400 nmol/l × 25 µl = final concentration × 225 µl; final concentration = 1600 nmol/l). Twofold dilutions are prepared from the working solution to obtain the following final concentrations in the test plate: 12.5 nmol/l (row B), 25 nmol/l (row C), 50 nmol/l (row D), 100 nmol/l (row E), 200 nmol/l (row F), 400 nmol/l (row G) and 800 nmol/l (row H). Row A consists of drug-free control wells. In this protocol, the working solution itself (final concentration, 1600 nmol/l) is not distributed in the test plate. It is advisable to add 25 µl of drug-free, fresh solvent to row A. To avoid changes in osmolarity in plates with fresh drug solutions, the working solution and twofold serial dilutions should be prepared in RPMI medium.

2.3 Routine procedures

2.3.1 Washing blood samples

The WHO protocol does not include a washing step to remove autologous plasma (i.e. the patient's own plasma) and replace it with non-immune human plasma (or non-immune human serum or serum substitutes). The erythrocyte volume fraction of whole blood can be as low as 15% in anaemic patients or can be within the normal range, i.e. as high as 50%. Thus, the final erythrocyte volume fraction, after dilution of whole blood in culture medium (v/v 1:9), can range from 1.5% to 5%. When the starting parasitaemia is kept constant and the erythrocyte volume fraction is varied, parasite growth is affected over a 42-h incubation period. Furthermore, the IC_{50} values for chloroquine increase significantly at higher erythrocyte volume fractions (Basco, 2004b). The absence of a washing step in the WHO microtest system is thought to contribute to the generally poor in vitro growth rate. Washing infected blood in RPMI 1640 medium and using 10% non-immune human serum, instead of the patient's own plasma, result in higher rates of success in in vitro assays.

During the first centrifugation, fresh whole blood is separated into plasma and cellular elements. The plasma can either be stored for further studies (e.g. to search for antimalarial drugs) or discarded. Packed cells are suspended in buffered RPMI medium (without serum supplement) in sterile tubes. For effective washes, it is recommended that packed cells be suspended in the wash medium at a ratio of 1:5 or higher. The cells are mixed with the medium several times before low-speed centrifugation. After centrifugation, the supernatant is discarded, and the procedure is repeated for three washes. At the end of the third wash, the packed cells are resuspended in complete RPMI medium-10% human serum mixture (or serum substitute) for culture or drug assays.

In a comparative study by Long et al. (1987), twice as many isolates grew satisfactorily in vitro with washed blood samples than with unwashed blood. The washing steps, when tubes containing packed cells are exposed to air, require aseptic techniques, and washing is best performed under a laminar flow hood or next to a gas burner in a closed room. Washing blood samples is strongly recommended in order to ensure the highest possible success rate of culture, even though it may pose logistical problems in the field. An alternative field-applicable procedure suggested by Noedl et al. (2004a) requires the addition of 0.5% Albumax® to complement unwashed, malaria-infected whole blood samples.

2.3.2 Transport of blood samples

For epidemiological studies, in vitro assays are ideally performed directly in the field, immediately after blood collection. The WHO microtest kit was

specifically designed for this purpose. Depending on the infrastructure, organization and strategy adopted by a research team in an endemic country, on-site assays might not be feasible. In such cases, alternative strategies have to be adopted to minimize the delay between blood sample collection and performance of in vitro assays. A delay of over 24 h between blood collection and culture is associated with lower rates of parasite growth and poorer results in in vitro assays.

Investigators might prefer to conduct in vitro assays in a central laboratory with malaria culture facilities. In this case, a means for transporting blood samples rapidly from the field must be devised. When blood is collected by venepuncture, only single-use, sterile syringes and needles should be used. A 5–7-ml vacuum-tube system is convenient. Venepuncture should be performed by qualified health personnel. As most blood collection tubes are made of glass, the samples must be protected from physical shock during handling and transport, unless some initial procedures, such as washing blood with RPMI medium and transferring it to another tube, are carried out in the field before transport.

Venous blood samples can be transported to a central laboratory in their original collection tubes, without any further treatment. They can also be suspended in RPMI 1640, HEPES and $NaHCO_3$ medium (2.5 ml of blood plus 12.5 ml of medium) or in complete RPMI 1640 medium supplemented with human serum (or serum substitutes) in filled 15-ml or 50-ml sterile tubes (1% erythrocyte volume fraction) (Divo et al., 1985b; Childs et al., 1987, 1989). If fingerprick capillary blood is collected in capillary tubes, the blood should be transferred in RPMI medium (v/v 1:9), and the blood-medium mixture should be transported either in a shirt pocket (close to body temperature) for a short trip not exceeding 6 h or on wet ice for longer trips (up to 72 h) (Kan et al., 1982; WHO, 1984; Wernsdorfer & Payne, 1988). The 1:9 blood-medium mixture can be transferred directly to either pre-dosed test plates for drug sensitivity assay or to other vessels for culture. Washing infected erythrocytes in RPMI 1640 before transport is optional.

It is common practice to transport samples on wet ice or its equivalent, such as coolant packs. Direct contact of the tube with the ice or coolant should be avoided. According to Divo et al. (1985b), transport at ambient temperature (21–35 °C) resulted in better recuperation of parasites than transport on wet ice. The temperature at which samples are best transported depends on the expected delay between blood collection and in vitro culture or assay. If the assay is conducted on the same day (i.e. within < 6 h), blood samples can be left at ambient temperature or kept close to the body, especially if wet ice is not readily available in the field. If the ambient temperature exceeds 37 °C, however, samples should be placed on wet ice even if the delay is expected to be a few hours. If the expected delay is at least overnight, the samples should be placed on wet ice.

EDTA- or heparin-coated collection tubes can be used if the expected delay between blood collection and in vitro assay is less than 12 h. An acid citrate dextrose-containing tube is recommended if the expected delay exceeds 12 h (LK Basco, unpublished data, 2004). If whole blood is transferred to RPMI (v/v 1:9) and stored in a refrigerator or on wet ice, successful assays can be performed for up to 72 h. Nevertheless, the shortest possible delay between blood collection and culture is associated with high success rates of in vitro assays.

Another option is to cryopreserve malaria-infected blood in the field and transport the samples in liquid nitrogen (Webster et al., 1985). This option should be avoided, as the procedure is labour-intensive and costly (liquid nitrogen, special cannister for transport), and culture for at least a few cycles is necessary before drug assays are performed. It is also likely that parasite populations will be selected, owing to the losses due to cryopreservation and further selection during adaptation to continuous culture. Moreover, if transport by air is planned, investigators should check with the airline company whether such items are allowed on a regular passenger flight. Special authorization might be required to send fresh or frozen blood samples to another country, depending on the country of destination, for customs clearance of infectious biological material.

2.3.3 Quality control of pre-dosed plates

Experience has shown that quality control of pre-dosed plates, as well as plates that are prepared from fresh solutions, is a major factor in meaningful monitoring of antimalarial drug resistance (Borrmann et al., 2002). Several methods exist to ensure the quality of test plates.

Sensitivity of reference clones

In a central laboratory where facilities for continuous culture are available, the sensitivity pattern of reference clones can be determined with different batches of pre-dosed plates and plates prepared from fresh solutions. This is especially important when a new batch of pre-dosed plates is prepared. A highly sensitive method, such as the radioisotope assay and ELISA-based assays, should be used for quality control. The morphological assay is less accurate and is not suitable for this purpose. Field workers can also submit several plates from different batches for quality control at a collaborating reference centre with facilities for continuous culture.

Testing different batches of plates with the same isolates

In laboratories where continuous culture is not practised or in the field, the sensitivity of several freshly collected isolates is determined simultane-

ously on pre-dosed plates from old and new batches. The dose-response curves generated for the two batches should be close to identical for each isolate. The old batch should have been controlled in parallel with previous batches in the same way.

HPLC measurement of concentrations on pre-dosed plates

In well-equipped laboratories, the actual concentration on pre-dosed test plates can be determined for each well by HPLC. Because of the small volume and, for many antimalarial drugs, the low concentration (in the nanomolar range), this control method can produce erratic results. A less satisfying alternative is to determine the actual drug concentrations in stock and working solutions.

Any unusual or unexpected observation in consecutive isolates, such as a sudden increase in IC_{50} values, insufficient inhibition of parasite growth even at the highest drug concentration, total inhibition of growth even at the lowest drug concentration or results that are discordant with those from molecular markers, should arouse suspicion of wrongly dosed plates or drug solutions, drug degradation or microbial contamination (mycoplasma, bacteria or fungus) of the medium, serum or serum substitutes or blood samples. When the cause is identified, the entire new batch of pre-dosed plates might have to be discarded.

3. Theoretical aspects of data interpretation

3.1 IC_{50} vs MIC

As in other dose-effect studies on living organisms, the in vitro response of malaria parasites to various drug concentrations is generally expressed as the IC_{50} value (also sometimes referred to as the 50% inhibitory dose [ID_{50}] or the 50% effective dose [ED_{50}]) or the MIC (synonymous with IC_{100}). Use of the IC_{95} value instead of the MIC is quite feasible, as this gives a result that closely approximates a reliable MIC, which is based on an actual count and is therefore more precise. In many biomedical and pharmacological fields, the IC_{50} value is the most widely used parameter. In studies on antimalarial drug activity in vitro, the IC_{50} value is defined as:

- the concentration of an antimalarial drug that inhibits 50% of schizont maturation as compared with the development in drug-free control wells (for the morphology method), or
- the drug concentration at which [^{3}H]hypoxanthine incorporation or parasite metabolism, measured by the quantity of LDH or HRP II production, is inhibited by 50%, as compared with the corresponding values in drug-free control wells (for radioisotope and ELISA-based methods).

The MIC is generally defined as the lowest drug concentration that inhibits the development of rings to schizonts. In the "48-h test", which allows sufficient incubation time for erythrocyte re-invasion, the MIC is defined as the lowest drug concentration at which no rings are found in a thin blood film (Spencer et al., 1984). The MIC is determined from direct observation of parasite growth under a microscope. For assays based on indirect measures of parasite growth inhibition ([^{3}H]hypoxanthine, LDH, HRP II), the IC_{50} value is the common measure. The results of morphological assays are expressed as either IC_{50} or MIC.

The IC_{50} value obtained by the radioisotope method is generally more than twice as high as that determined by microscopic examination of blood films (Schapira et al., 1986; Wongsrichanalai et al., 1992a; Kosaisavee et al., 2006). Petersen (1987) found an opposite trend, i.e. lower pyrimethamine IC_{50} values with the radioisotope method (2% erythrocyte volume fraction, 0.05–0.2% starting parasitaemia, 200 µl per well) than with microscopy (5% erythrocyte volume fraction, 0.2–0.9% starting parasitaemia, 100 µl per well), but the conditions of the two assays were not identical. Furthermore, in his study, an asynchronous laboratory-adapted strain was cultured, and parasites were counted after an incubation period of 48 h or 72 h, i.e. after re-invasion, instead of the number of schizonts of synchronized parasites.

Because of the differences in IC_{50} values derived by radioisotope and morphological assays, the results from two or more studies in which different assay methods were used cannot be compared. As an illustration, Tanariya et al. (2000) determined the in vitro activity of lumefantrine by the 48-h test of Nguyen-Dinh, Hobbs & Campbell (1981), rather than by a modification of the method of Richards & Maples (1979), as stated in their paper (see "microscopic or visual examination" in the sub-section "48-h test and its variants"). The authors compared their results for lumefantrine activity with that in various geographical regions determined by other investigators who had used radioisotope methods. The comparison is not valid for the reasons given above. Valid comparisons of in vitro responses can be made by research groups conducting in vitro studies in various geographical regions, provided that they use the same assay method (Gay et al., 1997; Pradines et al., 1998a,b,c, 1999a,b).

The IC_{50} values and the MIC can refer to either an individual isolate or a group of isolates with some common points, usually collected at a single study site within a defined period. In the latter case, common practice is to report the geometric mean IC_{50} value, which is obtained by transforming the IC_{50} values to logarithmic values, calculating the mean of the logarithmic values and taking the anti-logarithm of the mean. If data on individual isolates are stored in a computer, many statistics softwares and even the basic spreadsheet can be used to calculate the geometric (and arithmetic) mean. The geometric mean IC_{50} value is a more precise measure of the central tendency than the arithmetic mean because IC_{50} values are calculated from the logarithmic values of drug concentrations along the *X* axis to obtain the normal distribution. When the geometric mean IC_{50} value is used to express the central tendency, it is also useful to calculate the 95% confidence interval or to present the range (minimum and maximum individual values) as measures of dispersion. If the arithmetic mean is used, the appropriate measure of dispersion, in many cases, would be the standard deviation and not the standard error of the mean.

The MIC is a less precise measure in malariology (although perhaps not in microbiology), for several reasons. First, in contrast to the antibiotic susceptibility test in which there is an objective measure of growth inhibition (i.e. the diameter of area cleared of bacterial growth on an agar plate), appreciation of whether an individual parasite is dead or alive after being exposed to a drug in vitro is subjective. After a 24–42-h incubation, the ring forms that were present before assay are either still present but somewhat morphologically deformed or have matured into trophozoites or schizonts. Some parasites may require a longer incubation period to develop into schizonts. It is often uncertain that, at time 0, the parasites are indeed viable. "Growth inhibition" assessed by microscopy is subject to error.

Secondly, in contrast to IC_{50} values, the MIC is a discrete, discontinuous variable with no intermediate value between the actual concentrations used to test the in vitro response. For example, if the concentrations tested are 1,

2, 4, 8, 16, 32 and 64 nmol/l, the MIC must be one of those seven values (it cannot, for example, be 4.3 nmol/l); hence the value of estimating the IC_{95}. This implies that, unless the same concentration range is used, two laboratories evaluating the response of the same isolate or laboratory-adapted strain to a given drug would necessarily find different MIC values, even if all the other factors in the assays were strictly identical. A second laboratory using the concentrations 1.5, 3, 6, 12, 24, 48 and 96 nmol/l might report a MIC of 6 nmol/l, while the laboratory using the first concentration range might report a MIC of 4 nmol/l. Both laboratories have the "right" MIC value.

Thirdly, because of the uncertainty and subjective interpretation of complete inhibition of parasite growth, the precision range of MIC values for a given isolate is wide. One microscopist might report the presence of one or two schizonts at a certain drug concentration (e.g. 182, 182, 132, 27, 7, 2, 1 and 0 schizonts with more than two nuclei for 200 asexual parasites), while a second microscopist might report the absence of schizonts at the same concentrations (e.g. 180, 180, 130, 25, 5, 0, 0 and 0 schizonts with more than two nuclei for 200 asexual parasites). These interpretations can lead to large discrepancies in the MIC, while the IC_{50} value would not be much affected. This means that a two- to fourfold variation (e.g. a MIC of 2–8 nmol/l or 24–96 nmol/l) for a given isolate or laboratory-adapted strain must be accepted as valid to take into account the uncertainty factor.

Nonetheless, the MIC is a valuable measure for expressing the results of morphological assays. Some malaria researchers advocate use of narrow intervals between drug concentrations in order to increase the accuracy of MICs, rather than the twofold dilutions that are commonly used, as in the WHO microtest system (Russell et al., 2003). In their opinion, MICs derived from narrow ranges of drug concentrations might be more clinically relevant than mathematically derived IC_{50} values.

As part of the standardized procedures of the WHO microtest system, the in vitro response of individual isolates is recorded in a table that shows schizont maturation in the presence of various drug concentrations and for drug-free controls. The concentration at which there is no schizont maturation, called the "cut-off point" or MIC, is noted and compared with the arbitrarily fixed threshold for the in vitro resistance of the corresponding drug. Grouped data are also presented in a table showing the number and percentage of isolates with complete inhibition of schizont formation at each drug concentration (Wernsdorfer & Wernsdorfer, 1995). Grouped data collected from the same study site over at least two different periods can be further analysed in a 2 × 2 contingency table to determine whether there is a significant change in the proportions of sensitive and resistant isolates by the χ^2 test.

3.2 Linear regression analysis

To calculate the IC_{50} value, raw data are plotted on a graph, with the "effect" or "response" (i.e. parasite growth inhibition) on the *Y* axis and drug concentrations on the *X* axis. As in most pharmacological calculations, drug concentrations are transformed into logarithmic values. One of two mathematical analyses is applied to calculate the IC_{50} values: linear regression and non-linear regression.

Experimental data are fitted to a mathematical model that describes the relation between the doses or drug concentrations (independent regressor variables that are controlled in the experiment), which are plotted on the abscissa (*X* axis), and the effects (dependent variables), which are plotted on the ordinate (*Y* axis). A common method of presenting dose-effect relations is normalization. The ordinate is expressed as the percentage of maximum effect (or maximum growth of the parasite, which is the reciprocal of the maximum drug effect) by dividing each effect (or growth) by the maximal effect (or maximal growth) and multiplying by 100. In a typical experiment, there is a single response, *y*, for every *x*, i.e. a simple regression of *y* on *x*. The fitted regression line is defined by:

$$y = mx + b$$

where *y* is the predicted value given by the regression line for a given *x*, and *b* and *m* are the estimated values of the y intercept and the slope, respectively.

In a linear regression analysis, linearity between two variables is assumed. The *x* variables are logarithmically transformed because drug concentrations are usually determined in a geometric progression. The *y* variables can also be transformed into logarithmic values (probability values approximating logarithmic) if the drug effects are expressed as probit, i.e. probability unit, defined as the standardized normal variate $z + 5$, in which the constant 5 is added to avoid negative values of the variate *z*; *z* is the area under the standard normal curve. These transformations usually enhance the fit of the linear model. Other possible transformations, such as exponential, reciprocal and hyperbolic functions, are rarely used in studies on drug effects. The log-log model after data transformation is still a linear regression model because the regression coefficients *b* and *m* are linear.

These two parameters are estimated by the method of least squares, in which the sum of the squares of the residuals (also called the sum of squares of the errors around the regression line) is kept to a minimum. A residual is the difference between an observed *y* value and a predicted *y* value from the fitted line, i.e. the vertical distance of each experimental point from the fitted regression line. It describes the error in the fit of the linear model at a particular point along the *X* axis. The least square method generates a single, unique line for a given set of data.

Once the estimated linear relationship is defined by a fitted regression line and if normal distribution of the *y* variable and residuals is assumed, several inferences can be drawn, including confidence intervals for the slope and the *y* intercept. If repeated observations on the response *y* for each value of *x* are obtained by testing each drug concentration in triplicate or more with the same isolate, a significance test for linearity of regression can be performed. The test for linearity is not statistically valid for grouped data from different isolates. The most important reason for fitting a regression line is to predict the drug concentration *x* that would yield the response value, IC_{50}. The linear regression model is useful for prediction within the range of *x* values actually used in the experiment.

It must be emphasized that the dose-response relation is not linear but that a straight line is fitted to a given set of data. A linear regression line can be fitted after transformation of *x*, or both *x* and *y*, to estimate the IC_{50} value. The best estimate from a fitted regression line is obtained at the IC_{50}, i.e. near the mid-point of the regression line where variation is least. Calculations of predicted values at two extremes, i.e. the IC_{10} and below and the IC_{90} and above (IC_{95} and IC_{99} values are often calculated in the WHO microtest protocol), are less accurate estimates. It is not mathematically sound to extrapolate the IC_{99} or IC_{100} (i.e. MIC) which lies beyond the measured *x* value.

W. Wernsdorfer developed a distinct system of linear regression based on probit analysis of the dose-response relation to interpret the response of all field isolates from a single study site in the WHO microtest (Grab & Wernsdorfer, 1983; Wernsdorfer & Wernsdorfer, 1995). The mean relative frequency of schizonts at each drug concentration is calculated from the raw data. The drug effect, i.e. percentage inhibition of schizont maturation, is transformed into probits and plotted on the *Y* axis. Drug concentrations in µmol/l of blood-medium mixture or blood are expressed in a logarithmic scale on the *X* axis, as in most pharmacological calculations for normal distribution. A single, grouped straight regression line is either traced visually on log-probit graph paper or fitted to the data points by the least squares method with specific software on a computer. The IC_{50}, IC_{90}, IC_{95} and IC_{99} values are calculated from the fitted line. This procedure can also be used to calculate the parameters associated with the drug response of individual isolates, but the collective data are considered to be more important for epidemiological studies.

3.3 Non-linear regression analysis

Linear regression analysis is an obsolete method today, at least for interpretation of dose-response relations in pharmacology. Non-linear regression conforms more closely to classical theories of drug action involving agonist- or antagonist-receptor interaction. Plotting *x*-*y* data points on rectangular

coordinates with linear *x* and *y* scales often yields a hyperbolic curve. If the abscissa is a logarithmic scale, the curve becomes sigmoid (i.e. S-shaped). Pharmacologists have shown that logistic equations, which yield a sigmoidal curve when plotted on a logarithmic abscissa, are useful models for describing dose-response relations mathematically.

Non-linear regression analysis is based on a logistic, hyperbolic tangent or polynomial function. The logistic model assumes that data points are normally and symmetrically distributed after logarithmic transformation and that a sigmoidal curve with variable slope can be fitted. The logistic function also assumes that the data on the *Y* axis range from 0 to 1 (or 0 to 100%) as minimum and maximum values. The mathematical formulae of the functions are found in several published papers (Desjardins et al., 1979; Milhous et al., 1985; Childs & Pang, 1988). Two examples of logistic equations are (Childs & Pang, 1988):

$$Y = -A/(1 + [X/IC_{50}]^k) + A \text{ and}$$

$$Y = A-D/(1 + [X/IC_{50}]^k) + D$$

where X is the concentration, Y is the drug effect, A is the upper asymptote (minimal response when $X = 0$), D is the lower asymptote (maximal response when X is infinite) and k is a slope parameter. A hyperbolic tangent function has also been used (Desjardins et al., 1979).

An equation for a sigmoidal curve is an appropriate model for most dose-effect relations in pharmacological experiments. Curves are fitted by iterative adjustment of the variables in small steps until the residuals are reduced to a minimum and the best-fitting sigmoidal curve is derived. The Levenberg-Marquardt algorithm is often used to find the curve that best fits the data points (Levenberg, 1944; Marquardt, 1963). This algorithm includes the maximum and minimum plateaux of the non-linear curve, and the IC_{50} value on the steepest part of the curve bisects the distance on the *Y* axis between these plateaux.

Another function that is sometimes used in non-linear regression is the polynomial equation. Strictly speaking, polynomials are not truly non-linear functions. Furthermore, the dose-response relation does not follow the models described by polynomial equations. It is, however, easier to programme a polynomial equation than a sigmoidal function. Thus, from a pragmatic viewpoint, use of a polynomial model to calculate IC_{50} values is an acceptable compromise if field laboratories agree to its use.

When the WHO microtest kit was developed in the 1980s, personal computers were not readily available to field researchers, and there were no suitable, easy-to-use statistical software packages. Rough estimates of IC_{50}, IC_{90} and IC_{95} values were made by visual inspection and by tracing a straight line with a ruler on log-log graph paper. A computer program was subsequently written to analyse the results of morphological tests by linear and

non-linear regression (Grab & Wernsdorfer, 1983; Childs & Pang, 1988; Wernsdorfer & Wernsdorfer, 1995). A linear interpolation was also proposed as an alternative, simple method that does not require a statistical program (Huber & Koella, 1993).

Today, most central laboratories in countries where malaria is endemic have desktop and laptop computers, as a computer has become a basic necessity for interpretation of in vitro assays. Owing to wide application of non-linear regression analysis in many areas of health science, several versatile, user-friendly statistical software programs that include non-linear regression analysis and a choice of built-in equations are available from commercial sources. Calculation of regression parameters, in particular the IC_{50} value, and graphical representation of the fitted sigmoidal (or polynomial) curve are best performed with curve-fitting software. Although such programmes are generally expensive, they provide a pre-programmed data analysis system with reliable output, regular updated versions and graphs of publication quality. Many are available for sale on the internet and can be downloaded directly. The free software R (The R Foundation for Statistical Computing, version 2.4.1 updated in December 2006; copyright 2006) is a versatile statistical package. This software calculates the IC_{50} by non-linear regression.

For laboratories with limited financial resources, a pre-programmed curve-fitting spreadsheet called HN-NonLin is available for free downloading at <http://malaria.farch.net>. It requires a commercial spreadsheet that is found in most computers. The software performs non-linear regression analysis with a polynomial model. Raw data from any of the major types of drug sensitivity assays (radioisotope method, ELISA-based assays or morphological assay) in 96-well plate format, and the twofold drug concentrations used in the assays are entered manually. The inhibitory concentrations and dose-response curves for individual isolates or strains are obtained instantaneously. Although the spreadsheet lacks some of the sophisticated features offered by commercially available statistical software programs, it is easy and ready to use and is conceived specifically for in vitro antimalarial drug assays. The programme works on PC computers and not on Macintosh computers. This free software was developed by malaria experts at the Armed Forces Research Institute for Medical Sciences (Bangkok, Thailand) and the University of Vienna (Austria). The copyright is held by H. Noedl (harald.noedl@univie.ac.at), and credit should be given to this investigator if the software is used in scientific work submitted for publication.

From a practical viewpoint, most of the commercially available and free software programmes are expected to yield similar IC_{50} values for the same data input. The variation in IC_{50} values calculated with different mathematical models will become negligible if the mean value is calculated for a large number (≥ 30) of field isolates collected at a single study site. The choice of mathematical model and software to be recommended to researchers worldwide for standardized data interpretation remains to be decided.

3.4 Units of drug concentration

Two concentration units are generally used in publications on malaria: weight per volume (e.g. ng/ml) and molar concentration (e.g., nmol/l). In the WHO microtest system, drug quantity or concentration is expressed in three different units: picomoles per well, µmol/l of blood or nmol/l or µmol/l of blood-medium mixture. All drug quantities in the test wells of 96-well plates are expressed as picomoles. The corresponding drug concentrations are further expressed as µmol/l of blood or µmol/l of blood-medium mixture, depending on the drug. The triple unit system of the WHO microtest can be a source of confusion.

In accordance with the *Système international d'unités* (International System of Units; SI), drug concentrations are expressed as molar units, i.e. in terms of the drug quantity per given volume of erythrocyte suspension in RPMI 1640 (blood-medium mixture), e.g. nmol/l. This expression allows direct comparison of results from different laboratories. If the results are expressed in weights, the details of the salt form, including the number of molecules of water of crystallization and other information (e.g. whether a hemisulfate was used in quinine salts), should be provided. When new drugs or natural products that have not been fully characterized are screened, the results should be expressed in weight-to-volume units.

4. Proposed protocol for in vitro assays in the field

The present document does not recommend any particular assay method over others. It presents many of the basic procedures that are common to all in vitro assays, attempts to reconcile different ways to perform the assays, and describes in detail, as an example, the procedures that are used for the morphological assay. The protocol may be adapted to perform the radioisotope assay (add [^{3}H]hypoxanthine to the blood-medium mixture at the beginning of the incubation and, at the end of the incubation, freeze the test plates until analysis) or non-isotope assays (perform the assay, as for the morphological assay, and keep the test plates frozen at the end of the incubation until analysis by ELISA or fluorimetry at the central laboratory). Because there is no common protocol for all in vitro drug sensitivity assays, some restrictions apply, and each investigator is expected to adjust the parameters for the particular assay method that is being used (e.g., initial parasitaemia for radioisotope method and ELISA-based assays).

The following protocol is not only designed for central laboratories but also and essentially for a mobile team who have received training at and materials from a central laboratory, except for the isotope test which should be limited to central laboratories. It is assumed that sentinel sites in the country are not staffed by personnel trained to perform in vitro assays. The protocol allows for several variations, in order to satisfy the particular needs of each research team and to allow its adaptation to different field situations. The "minimalist" version is emphasized, which is based on collection of 100 µl of fingerprick capillary blood and microscopic examination of blood smears after culture.

Investigators are strongly encouraged to identify and enroll symptomatic older children and adults, rather than young children, so that venous blood, rather than capillary blood, can be collected with relative ease and without worrying the parents. The availability of 5–7 ml of whole venous blood has several advantages: performance of assays in duplicate or triplicate, testing several different antimalarial drugs, including new and experimental drugs, easier handling of collected samples (rather than handling the fragile capillary tubes), and complementary laboratory procedures (e.g., PCR, measurement of plasmatic drug concentration).

4.1 Preparation of studies

4.1.1 Initial contacts

It is important to contact local political and health authorities to inform them of the objectives of any planned studies. A visit from the study coordinator is preferable to a letter, fax, electronic message or telephone call. An official letter from the competent authorities at the ministry of health and ethical clearance from the national ethics committee are usually required in order to obtain rapidly tacit or official acceptance by local authorities. An initial contact with and survey of various health centres in the locality, if possible accompanied by the local health officer, could be organized at the same time.

4.1.2 Preparation of reagents and materials

The reagents and materials for in vitro assays must be ordered individually, assembled, prepared and stored at the central laboratory. The number of malaria-infected patients who will be examined during the screening phase varies, depending on the transmission period and pattern at the study site. In a typical situation in areas of stable transmission, a minimum of 100–200 patients would require screening. Initial contacts and visits to local health centres, including close examination of patient records and rapid evaluation of the quality of health care and laboratory examinations, are helpful for estimating not only the quantity of materials that needs to be prepared and transported but also the duration of the study required to enroll an adequate number of patients. The quantity of materials for blood films and urine tests to be transported to the field should be estimated accordingly.

Before materials are taken to the field, their quality should be checked at the central laboratory, including a rapid check of microscopes, Giemsa staining, urine tests and all culture reagents (sterility, pH and parasite growth of reference strains or fresh clinical isolates). An acceptable minimum number of in vitro assays to be performed at a given sentinel site is 50. The aim is to obtain interpretable assay results for at least 30 isolates within a short time (3–4 weeks).

Below is a list of materials required for all phases of in vitro assays. The list is not exhaustive. Although some materials might already be available at the study site, it is advisable to transport all necessary materials from the central laboratory.

Material for screening blood films

- microscope (at least two microscopes should be taken, even if there is a microscope in good working condition at the sentinel site);

- immersion oil;
- clean glass slides with frosted end;
- pencil and pen;
- absorbent cotton-wool imbibed with 70% alcohol and dry absorbent cotton-wool (or single-use alcohol swabs);
- single-use, sterile lancets;
- concentrated Giemsa stain of good quality;
- Field stain (alternative to Giemsa stain; optional);
- phosphate-buffered water, pH 6.8 or 7.2, for diluting Giemsa stain;
- graduated cylinders, preferably plastic;
- staining jars or dishes;
- small hair dryer (optional; for rapid drying of stained slides);
- slide boxes;
- hand-held counter;
- toluene for cleaning used slides (optional);
- human chorionic gonadotropin pregnancy test strips (optional; to exclude women in the early first trimester of pregnancy);
- timer (optional); and
- malaria antigen detection dipsticks (optional).

Materials for urine test

See section 2.2.9, "Saker-Solomons test".

Materials for blood collection for in vitro assays

- absorbent cotton-wool imbibed with 70% alcohol or single-use alcohol swabs;
- single-use, sterile lancets;
- 100-µl heparin-coated capillary tubes;
- 5- or 7-ml tubes (Vacutainer®) with EDTA, heparin or acid citrate dextrose anticoagulant;
- Vacutainer® tube adaptors, disposable syringe needles, portable needle collection container;
- examination gloves;
- tube racks;
- Whatman® 3MM filter papers for DNA collection and storage or other commercially available special filter papers for DNA storage (optional); and

- informed consent forms written in the language or dialect understood by the study populations.

Materials for drug assay

- RPMI 1640 buffered with 25 mmol/l HEPES and 25 mmol/l $NaHCO_3$ and supplemented with either 10% (v/v) pooled non-immune AB^+ human serum or 0.5% (w/v) Albumax® aliquoted in 3-ml sterile plastic tubes, ready to use (i.e. the tube contains 1960 µl of RPMI, HEPES and $NaHCO_3$ mixture with serum or serum substitute);
- freshly collected erythrocytes from a healthy donor; a single tube of 7–10 ml of whole venous blood is sufficient for the entire study, planned for 50 assays over 1 month; collected in anticoagulant (e.g. EDTA, acid citrate dextrose); washed by centrifugation three times in RPMI, HEPES and $NaHCO_3$ medium without serum or serum supplement, and aliquot the washed blood pellet in sterile microfuge tubes filled with RPMI medium;
- 1.5-ml microfuge tubes (autoclaved before being taken to the field);
- 10-µl or 20-µl adjustable-volume pipette;
- 200-µl adjustable-volume pipette;
- 1000-µl adjustable-volume pipette;
- 8- or 12-channel adjustable-volume (20–200 µl) pipette (optional);
- 2-ml sterile, graduated serological plastic pipettes;
- sterile (autoclaved) disposable pipette tips;
- benchtop microcentrifuge (6000 × *g*)
- portable centrifuge, maximum capacity (6–8 tubes × 15 ml), maximum relative centrifugal force (11.18×10^{-7} × radius in mm × [speed in rotations per min]^2), 2900 × *g*;
- 96-well culture plates pre-dosed with test drugs;
- incubator or water-bath;
- extra undosed microtitre plates (optional);
- working drug solutions (optional);
- portable generators (for microscope, centrifuge and incubator or water-bath; optional but useful in remote areas);
- 96-well PCR plates and plate seal or cap seals (optional); and
- freezing vials, cryoprotectant, liquid nitrogen, canister, liquid nitrogen vessel (optional).

4.1.3 Transport of materials to the field

Unless the planned study is to take place in an urban site with well-developed commercial activity, it is best to assume that the basic necessities for in vitro assays will not be available on-site. All the required reagents and materials should be transported from the central laboratory to the field, usually in a land vehicle.

Some precautions are necessary during transport of laboratory materials and reagents. Microscopes and centrifuges should be protected from physical shock. Likewise, all glassware should be carefully arranged in protective cases. Plasticware should be preferred over glassware. Some reagents and materials should be transported and stored at 4 °C to extend their useful shelf-life for the duration of the in vitro study, including tetrabromophenolphthalein ethyl ester solution (for the Saker-Solomons urine test), culture media, pre-dosed plates and drug solutions. These items can be transported in ice boxes with coolant packs. To save space, they can be transported at ambient temperature, in particular if the study site is within a day's travel. Organic solvents (ethanol for skin disinfection, methanol in concentrated Giemsa stain, toluene for cleaning slides, chloroform for Saker-Solomons urine test) should be packed separately in safe, leak-proof bottles and protective boxes. Special care is needed in transporting flammable substances (e.g., alcohol).

4.2 Step-by-step protocol

4.2.1 Screening of malaria-infected symptomatic patients

The usual screening procedure at health centres includes:

- a clinical examination (for fever or recent history of fever, anaemia, signs of severe and complicated malaria) by an experienced nurse or medical practitioner;
- a basic laboratory examination, with microscopic examination of blood films; and
- a urine test for antimalarial drugs (to be performed only if microscopic examination shows a positive blood film).

4.2.2 Enrollment of infected patients

The following criteria should be fulfilled before choosing patients from whom blood is to be collected for in vitro assays (and PCR analysis):

- a blood film with *P. falciparum*, with no other *Plasmodium* species. In case of doubt, as in mixed infections, the microscopic diagnosis should be confirmed with a species-specific dipstick. If doubt persists, the

patient should be enrolled anyway. *Plasmodium* species can be determined retrospectively by careful re-examination of slides or PCR at the central laboratory. Blood samples should not be selected on the basis of developmental stage (small, medium or large rings) or personal appreciation of parasite viability (e.g., "healthy looking" rings);

- parasitaemia ≥ 0.02% (≥ 1000 asexual parasites per µl of blood). Studies with ELISA-based assays suggest that the minimum parasitaemia might be even lower, i.e. ≥ 0.002% or ≥ 100 asexual parasites per µl of blood. There is no upper limit because in this protocol blood with a high parasitaemia is diluted with uninfected blood. A higher parasitaemia (≥ 0.1%) is required for some growth indicators, such as [^{3}H]hypoxanthine;
- a negative Saker-Solomons urine test; and
- explanation of the purpose of the study and different uses of the blood sample (e.g., in vitro drug sensitivity assay, PCR, measurement of plasmatic concentration of antimalarial drugs) and written informed consent for blood collection, signed by the patient or, for children, by the accompanying guardian; different consent forms are needed for adolescents.

Basic information on the selected patients, including age, sex, weight, body temperature at the time of consultation, laboratory results and prescribed treatment, is recorded on individual files, with codes instead of patients' names. For practical reasons related to the duration of incubation, patient screening should begin as early as possible in the morning and terminated before noon.

The maximum workload for a mobile team with one or two laboratory technicians is three to four isolates per day in areas of stable transmission. At this accelerated pace, assays accumulate every day and there may be as many as 12 assays to be conducted simultaneously in 3 days: four from the first day, another four from the second day under incubation and four more to perform on the third day. Furthermore, the space in a candle jar or incubator is usually limited. Therefore, even if there are many patients to enroll on a particular day, it is advisable to limit enrollment to a maximum of four per day. Assuming a 5-day work week, an entire in vitro study on at least 50 isolates can be terminated within 3–4 weeks.

4.2.3 Collection of blood

In obtaining capillary blood, a blood lancet should be used only once and discarded in a special container. One 100-µl capillary tube is filled for each antimalarial drug to be tested in vitro. If two drugs are being evaluated, two capillary tubes should be filled. It is always preferable to collect blood in at least two capillary tubes, even if an assay for only one drug is planned. As

the blood volume will be adjusted later, it is not necessary to collect exactly 100 µl of capillary blood.

The second capillary tube can serve as a "reserve". If a patient is anaemic and the first tube contains insufficient red cells, the second tube can be added; or, a parallel assay (e.g., DELI, HRP II, fluoroassay) can be run after the sample has been transported to the central laboratory; or, the second sample can be imbibed onto appropriate filter paper for DNA analysis.

For children ≥ 12 years old and adults, 5–7 ml of venous blood should be obtained. This quantity of blood allows in vitro tests with several antimalarial drugs and assessment of each drug concentration in duplicate or triplicate. The blood remaining after in vitro assays can be cryopreserved or used to extract a large quantity of parasite DNA.

4.2.4 Treatment of patients

Before in vitro assays are begun but after blood collection, an antimalarial drug (or combination of drugs) and antipyretics should be prescribed to all patients with a positive blood film, in accordance with the current national policy for treating uncomplicated falciparum malaria, whether they were included in or excluded from the in vitro drug sensitivity study. This important medical responsibility and obligation can be delegated to local health staff familiar with the patients. It must be remembered at all times that the welfare of the patients is much more important than the performance of in vitro assays.

4.2.5 Preparation of the working area

If there is no field laboratory or station nearby, a clean working area should be prepared on a table (or the laboratory bench of the dispensary after closing hours) in a closed space (to minimize the risk of microbial contamination). Only persons working directly on in vitro assays should be allowed to enter and stay in the improvised laboratory. The working area can be delimited with aluminium foil, an absorbent bench sheet or even clean, ordinary paper. A Bunsen burner, alcohol lamp or camping gas burner can be lit to ensure a rising current of air in the workplace and help keep it sterile. If there are air conditioners in the working room, they should be turned off to avoid microbial contamination.

4.2.6 Washes

The collected blood samples are washed with RPMI medium containing buffers (25 mmol/l HEPES and 25 mmol/l $NaHCO_3$) but without serum or serum substitutes. With some experience, the procedure takes less than 5 min (for two to three tubes). This step is important for obtaining consistent

results and maximizing the success rate of the in vitro assay. (In the WHO microtest protocol, there is no washing step: 0.1 ml of unwashed capillary whole blood is mixed with 0.9 ml of RPMI, and the blood-medium mixture is distributed in the test plates.)

Fingerprick capillary blood (0.1 ml) is transferred from the capillary tube to a sterile (autoclaved) 1.5-ml microfuge tube. As an optional step, the sample (whole blood) is centrifuged for 10 s in a benchtop microcentrifuge. Plasma is removed, imbibed onto a piece of Whatman® 3MM filter paper and dried thoroughly. The filter paper is stored in a sealed plastic bag with desiccant. These plasma samples can be sent to specialized laboratories for drug measurement.

If plasma collection is not planned, 1 ml of RPMI medium is added directly to the tube with a 1000-µl pipette and mixed gently several times. The mixture is centrifuged for 10 s in a benchtop microcentrifuge (first wash), the supernatant is discarded with a 200-µl pipette, and fresh RPMI medium (1 ml) is added. The tube is mixed gently several times and centrifuged for 10 s in a benchtop microcentrifuge (second wash), and the supernatant is discarded with the 200-µl pipette. The third wash is performed by adding 1 ml of RPMI medium, shaking the tube gently several times and centrifuging for 20 s (not 10 s for the third wash) in a benchtop microcentrifuge; the supernatant is discarded with the 200-µl pipette. Unless the patient is severely anaemic, there should be > 40 µl of blood pellet. Note that all washes are done in the same microfuge tube.

If 5–7 ml of venous blood is collected, the whole blood is separated into plasma and cellular elements by low-speed centrifugation, and the plasma is either stored in a tube for further analysis (drug measurement) or discarded. Packed red cells are washed three times with RPMI medium directly in the collection tube.

A recent study suggested that washing (step 6 of this protocol) might be unnecessary if unwashed whole blood samples are added directly to RPMI–0.5% Albumax® mixture (Noedl et al., 2004a). This alternative procedure simplifies sample handling in the field. However, an important advantage of washing blood cells is the possibility to adjust haematocrit and dilute high parasitaemia in a more consistent way, and to avoid interference with binding proteins present in autologous serum.

4.2.7 Preparation of the blood-medium mixture

It is recommended that aliquots of pre-distributed, ready-to-use, complete culture medium be prepared at the central laboratory. This avoids the extra step of preparing the medium-serum (or serum supplement) mixture in the field before addition of the washed blood pellet. The following reagents are mixed in sterile 3-ml plastic (polystyrene) tubes for testing one drug (one well per drug concentration for the "minimalist" version):

RPMI medium (with 25 mmol/l HEPES and 25 mmol/l $NaHCO_3$) *distribute 2 × 880 µl with a 1000-µl pipette or measure the volume with a sterile 2-ml graduated pipette*	1760 µl
Serum or serum substitute *non-immune human serum (final concentration 10%) or 5% Albumax® (final concentration, 0.5%)*	200 µl
Washed blood pellet from the previous step	40 µl
Total volume	2000 µl

Volume of pellet = (total volume × erythrocyte volume fraction) ÷ 75%
= (2000 µl × 1.5%) ÷ 75%
= 40 µl

With the 200-µl volumetric pipette adjusted to 40 µl, the tips should be placed at the bottom of the tube containing washed red cells, and the required volume of blood pellet should be aspirated gently. The blood-medium-serum suspension is then mixed. If the patient is anaemic and a single 100-µl capillary tube does not yield 40 µl of blood pellet after the third wash, the washed pellet from the second capillary tube should be added to obtain 40 µl of blood pellet.

If the patient's parasitaemia is ≥ 1.0% (i.e. ≥ 50 000 asexual parasites per µl of blood), the blood pellet should be mixed with uninfected erythrocytes to adjust the starting parasitaemia to 0.5–0.6% (i.e. 25 000–30 000 asexual parasites per µl of blood) to avoid the inoculum effect. For ELISA-based assays, high parasitaemia can be adjusted to 0.1%. Before the blood-medium mixture is prepared, the required volumes of patient's erythrocytes and uninfected erythrocytes are calculated. For example, for a patient who has a parasitaemia of 4.0%, his or her erythrocytes will have to be diluted by 1/8 to obtain a starting parasitaemia of 0.5%. Of the total volume of blood pellet (40 µl), 1/8 will be the patient's erythrocytes, and 7/8 will be uninfected erythrocytes. Thus, 5 µl of the patient's blood pellet (taken with a 10-µl or 20-µl adjustable-volume pipette) should be mixed with 35 µl of uninfected erythrocytes in the culture medium.

If the study is designed to assess two drugs per patient, the second erythrocyte suspension should be prepared in a 3-ml tube. If three or four capillary tube samples are obtained per patient, the contents of up to two capillary tubes can be washed in a microfuge tube. If four capillary tubes are obtained per patient, preparation of the erythrocyte suspension will be simplified if samples from the same patient are pooled and the blood-medium mixture is prepared in a single 10-ml tube. In such cases, the central laboratory should prepare aliquots containing 7920 µl of RPMI, HEPES and $NaHCO_3$ and 900 µl of human serum (10% final concentration) or 5%

Albumax® (0.5 % final concentration). With the addition of pooled infected erythrocytes (180 µl), up to five drugs can be tested (or fewer drugs in duplicate).

Readers are reminded here that a more preferred method is to collect venous blood in a single 5–7-ml tube and adjust the above-mentioned calculations so that each drug concentration can be tested in triplicate.

4.2.8 Distribution of blood–medium mixture in microtitre plates

The blood-medium-serum suspension (200 µl per well) is distributed in the test plates, starting from the drug-free control well in increasing drug concentrations. The erythrocytes should be suspended homogeneously in the blood-medium mixture by mixing the tube just before distributing the contents into the wells. The tips should be changed before the mixture prepared from the same patient is distributed into wells with different antimalarial drugs. There is no need to shake the plates to dissolve the drugs, and shaking the plates manually can result in projection of small volumes of distributed suspension onto the microplate lid. Note that 4 µl of erythrocytes settle at the bottom of each well.

The remaining blood-medium mixture is distributed into drug-free wells, either in the same microtitre plate as the assay plate if drug-free control wells are available or into another microtitre plate. Samples from these wells are used to prepare blood slides after ≥ 24 h of incubation to assess parasite growth and stages.

Because the incubation period of individual isolates for morphological assays varies from 24 h to 42 h, all assays of a given isolate should be performed in a single microtitre plate containing all the test compounds (i.e. one plate per sample as opposed to one plate per drug). Re-using pre-dosed plates (i.e. the unused pre-dosed wells of the plate) for subsequent assays is not recommended. Some drugs (e.g., artemisinin) can undergo degradation when pre-dosed plates are exposed repeatedly to the standard incubation conditions, resulting in higher IC_{50} values after each assay with the same pre-dosed plate.

4.2.9 Preparation of the candle jar

The microplates are placed in a candle jar, a candle is lit, the candle jar is tightly sealed with silicon gel sealant and the vent tap is closed as the candle light goes out. To obtain a consistent gas mixture in the candle jar, two candles should be lit on opposite sides of the inside of the jar, the vent tap should be closed when first candle goes out, and the remaining candle goes out quickly (DC Warhurst, personal communication, 2004). Alternative field-applicable methods include flushing gas mixtures into a culture vessel and tightly sealing it or placing a special CO_2-releasing sachet in an airtight

vessel. High relative humidity should be maintained in the candle jar to minimize evaporation of blood-medium mixture from the wells.

4.2.10 Incubation at 37 °C

A standard incubator or water-bath can be used. Once the plates have been placed in an incubator, blood slides from the patients can be re-examined to determine the predominant ring form (small, medium or large), and this information is recorded on the patient's file. The initial size of the rings is a helpful guide for predicting the incubation time necessary for morphological assays based on schizont counts (Table 5).

4.2.11 Determination of the end-point

For the morphological assay

After 18–24 h of incubation (i.e. before noon the following day), the developmental stage of each isolate is checked rapidly by microscopic examination of Field-stained or Giemsa-stained blood smears prepared from the supplementary drug-free control wells. Field staining is more rapid (less than 30 s to stain, followed by air-drying) and is adequate for this step. If > 25% of asexual parasites have initiated schizogony, the morphological assay can be terminated. Otherwise, incubation is continued for an additional 6 h. At this time, another blood smear is prepared from the second drug-free well to determine the developmental stage. The assay is terminated if enough schizonts have developed. Otherwise, for practical reasons, incubation can be pursued for an additional 10–12 h to avoid working during the night. In this case, the total incubation period is 40–42 h. The assay is terminated at this time, whether or not sufficient numbers of parasites have developed into schizonts.

If the proportion of schizonts is still < 25% after incubation for 40–42 h, the assay is considered to be uninterpretable and unsuccessful. The common causes of slow in vitro development are recent intake of antimalarial drugs, antibiotics or some traditional medicines, fluctuations in temperature during incubation, an inappropriate gas mixture or a gas leak from the culture vessel. Other causes include suboptimal pH of the medium, poor nutritional quality of human serum and microbial contamination, but these potential causes of poor parasite growth should have been eliminated after quality control at the central laboratory.

After an incubation of 40–42 h, some parasites with a predominance of large rings at 0 h might commence the re-invasion phase. If > 50% of asexual parasites are still schizonts, the assay might still be interpretable by the morphological method. If > 50% of the parasites are young rings (in the second schizogonic cycle), the assay is excluded from further analysis by microscopic examination because the common end-point for assessing anti-

malarial drug activity is inhibition of schizont maturation and not inhibition of re-invasion. Assay plates with parasites entering and initiating the second schizogonic cycle should, however, be frozen, if the facilities allow, for alternative readings (HRP II or LDH assays) at the central laboratory.

For other assays

As a general rule, the suggested incubation period for non-morphological assays based on LDH production or fluoroassay (and [^{3}H]hypoxanthine incorporation, if applicable) is 42–48 h, regardless of the proportion of parasites that develop into schizonts. At present, a 72-h incubation period is recommended for the HRP II-based assay. As this assay depends on accumulation of HRP II, there would seem to be no reason to restrict its duration. Further studies and comparisons with standard tests are required for this assay. At the end of the incubation period of 42–48 h (72 h for the HRP II-based assay), the test plates that are to be interpreted by ELISA reading or fluoroassay (also for [^{3}H]hypoxanthine assay) are frozen at –20 °C.

In case of field studies the plates should also preferably be frozen for transportation to the central laboratory for further processing. The blood-medium-serum mixture in the test plate should be transferred before to the corresponding wells of 96-well PCR plates immediately after incubation, even though this requires extra manipulation. The PCR plate with deeper wells is hermetically sealed with a plastic seal or, even better, with cap seals. Plastic seals form an air- and water-tight seal on each well and can be opened and re-sealed several times as assays are terminated and the series of eight wells are filled. Cap seals allow storage by eight wells (rows A–H). Blood-medium-serum mixture stored and frozen in seal PCR plates is transported back to the central laboratory. An alternative method that requires less handling would be to freeze and seal with a plastic seal the test plate and transport it, without transferring its contents to another plate. An extra precaution would be needed in case of plastic sealing to ensure that the plates would not be thawed during transport and the contents would not leak out of the plate. Transportation of isotopic material is not recommended even if the plate is frozen and tightly sealed.

The next two steps pertain only to morphological assays.

4.2.12 Cell harvesting and staining for morphological examination

At the end of the variable incubation period, the supernatant is carefully removed from each test well, leaving a small volume to allow resuspension of settled erythrocytes. There are three alternative methods for counting by microscopic examination: (i) the number of pre-schizonts and schizonts per 10 000 erythrocytes in thin blood films, (ii) the number of pre-schizonts and schizonts per 200 leukocytes in thick blood films and (iii) the number of pre-schizonts and schizonts per 200 asexual parasites in thick blood films.

With each method, differential counts (rings, trophozoites, pre-schizonts, schizonts) should be determined. Microscopic examination of the slides should start with the drug-free control, followed by test wells in ascending drug concentrations.

Thin blood films are fixed with methanol; thick films are not fixed. In both cases, the blood films should be completely dry before staining (leaving overnight on a bench not accessible to insects is suitable for thick films, or 1 h in an incubator at 37 °C). Blood films are best stained with 3% Giemsa for 45 min or 10% Giemsa for 10–15 min. Giemsa solution should be prepared in buffered water (pH 7.2) (WHO, 1991). Field stain is an alternative rapid staining method. After staining, the slides are gently rinsed with water. At this stage, the slides can be air-dried or dried with a low-power hair dryer for rapid processing.

4.2.13 Microscopic examination and parasite count

Thin blood films (when the initial parasitaemia is 0.2–1.0%)

A series of eight thin blood films is prepared from each column of the microtitre plate. Two (or more) thin films can be prepared on the same slide. Preparation of thin blood films from the contents of each well requires practice and skill because of the small volume (Schapira & Schwalbach, 1988; Tanariya et al., 2000). The advantage of thin films is easy recognition of developmental stages and accurate parasite counts against the total of uninfected and infected erythrocytes, rather than relative counts based on 200 asexual parasites.

If two or more parasites are present in a single erythrocyte, one infected erythrocyte is recorded. Some parasites transform into gametocytes, and early stages of gametocytes can be difficult to distinguish from trophozoites; with experience, however, the oval or typical D-shaped gametocytes can be recognized. Gametocytes are not counted, but their presence should be recorded. The parasite count of each well is expressed as:

(number of infected erythrocytes ÷ total number of uninfected and infected erythrocytes, i.e. 10 000 erythrocytes) × 100%.

Thick blood films (when the initial parasitaemia is < 0.2%), counting against leukocytes

Thin film counts are tedious and less accurate for cases of low initial parasitaemia, and it is more convenient to use thick films. Counts are made with one hand tally counter for the leukocyte count and another for schizonts. The parasite count in each well is expressed as:

number of schizonts per 200 leukocytes × 8000 leukocytes per µl of blood/200 = schizonts per µl of blood.

Thick blood films (when the initial parasitaemia is < 0.2 %), counting against asexual parasites

In the WHO microtest protocol, to facilitate counting, the number of schizonts (defined as asexual parasites with three or more [i.e. more than two] nuclei) is determined in a count of 200 asexual parasites. The results are expressed as percentage of schizont maturation compared with the control well:

(number of schizonts in 200 asexual parasites in a test well ÷ number of schizonts in 200 asexual parasites in a control well) × 100 %.

4.2.14 Data analysis and validity of assays

The following criteria have been proposed for evaluating the validity of assays:

- morphological test (WHO Mark III system): ≥ 10% schizont maturation (i.e. ≥ 20 schizonts with three or more nuclei per 200 asexual parasites);
- radioisotope method: incorporation of > 1000 cpm in drug-free control wells (0.8–1.0 μCi of [^{3}H]hypoxanthine added per well); cpm in control well five or more times the cpm in the well with the highest drug concentration;
- plasmodial LDH assay: $OD_{max}/OD_{min} \geq 1.7$, where OD_{max} is the OD in the drug-free control well and OD_{min} is the OD in the well containing the highest drug concentration;
- HRP II assay: $OD_{max}/OD_{background} \geq 2$, where OD_{max} is the OD in the drug-free control well and $OD_{background}$ is the OD in the well with the highest drug concentration at 72 h, or $OD_{max} - OD_{background} \geq 0.4$ at 72 h;
- SYBR® Green assay: still undetermined.

Data derived for individual isolates should be analysed separately. For each isolate or drug tested, the MIC or IC_{50} values are calculated and expressed as nmol/l of blood-medium mixture (i.e. no conversion to nmol/l of blood or picomoles).

The terms "sensitive" and "resistant" based on threshold values do not in any way imply or predict the response that would have been obtained had the drug tested in vitro been given to the patient from whom the isolate was obtained. It is best to avoid these terms if the result is based solely on in vitro assays.

5. Recommendations for monitoring drug resistance

1. National and regional antimalarial drug policies for the treatment of uncomplicated *P. falciparum* infections are based on studies of the clinical and parasitological responses of symptomatic patients, as determined with the latest WHO protocol for the evaluation of therapeutic efficacy (WHO, 2003b). Malaria control programmes should give high priority to regular monitoring of the clinical efficacy of combinations of drugs in the country.

2. In vitro drug sensitivity assays on field isolates and molecular markers for antimalarial drug resistance are laboratory tools that can be used to measure the parasite's response to drugs and to characterize the genetic composition that might determine its response to drugs outside the context of host-parasite-drug interactions, including acquired immunity, pharmacodynamics, pharmacokinetics and social behaviour. At present, these two research methods cannot be used to predict with accuracy an individual patient's response to drug therapy in various epidemiological contexts. Retrospective studies also suggest that the results of in vitro assays and of molecular markers are not highly correlated with those of clinical studies. The two research tools cannot substitute for clinical studies on therapeutic efficacy. Furthermore, with the exception of chloroquine, in vitro thresholds used to distinguish between drug-susceptible and drug-resistant isolates have not been validated. A cut-off point that has been validated or is currently being validated for a particular in vitro assay procedure is only applicable for that procedure.

3. The performance of in vitro drug sensitivity assays and analysis of molecular markers require highly trained personnel, well-equipped central laboratories and financial investment. Malaria control programmes cannot be expected to finance or undertake field research with these tools, in particular when they are faced with financial contraints and shortages of personnel.

4. In malaria-endemic countries that have attained adequate research capacity, research structures, financial resources, political commitment and strong partnerships with other research organizations and programmes, in vitro drug sensitivity assays and molecular markers are important but ancillary laboratory methods that contribute to overall monitoring of drug-resistant malaria. These tools are best used in collaborative work to provide complementary data on the epidemiology of drug-resistant malaria. Such programmes would involve universities, national and international research organizations, the ministry of health, nongovernmental organizations or specialized research units of the armed forces.

5. There is no single, universally-accepted protocol for in vitro drug sensitivity assays. The need for a standardized assay that would allow direct comparison of results from different laboratories should be evaluated. At present, a researcher may choose one of the several assay systems that satisfies best his or her particular requirements. Once the choice is made, it is important to maintain the same protocol over time so that significant findings (e.g., trends, emergence of drug-resistant isolates) can be deduced.

6. The radioisotope method is not appropriate for most endemic countries. Unless the country already has a complete set of the costly equipment necessary for radioisotope assays, together with trained personnel, it is recommended that they invest in ELISA-based assays, as the basic equipment and training are also useful for a variety of other medical purposes. At present, the LDH- and HRP II-based ELISA tests and fluoroassays with SYBR® Green I and other DNA-binding fluorophore dyes are being developed. It is too early at this stage, however, to recommend any current ELISA-based assay or fluoroassay as a long-term monitoring strategy for drug-resistant malaria. These new methods should progressively replace the microscopic tests to improve quality of the results.

7. In vitro drug sensitivity studies and molecular analysis of resistance markers would have more effect if the investigations were conducted in parallel with clinical studies, including pharmacokinetics. A simultaneous analysis of in vitro response, in vivo outcome, molecular characteristics, and pharmacokinetics would help establish the threshold for the in vitro assay being used in such studies. However, in vitro data alone, without any accompanying molecular or in vivo data, are also of value if carried out in the long term provided that quality control of data is ensured. The most reliable existing method for quality control is the determination of in vitro responses of reference clones of *P. falciparum* using old and new batches of pre-dosed test plates. It is one of the important tasks that the central laboratory is responsible for in order to produce reliable and reproducible results in the field.

8. The role of in vitro drug sensitivity assays in malaria control programmes and in the epidemiology of drug-resistant malaria has been, and will probably continue to be, limited. This laboratory tool has many other applications in deriving important data on drug resistance. The technical know-how involved in malaria culture and the in vitro drug sensitivity assay is an asset for any country. It is recommended that training and expertise in this specialized laboratory technique be maintained in a proportion of countries where malaria is endemic.

9. In vitro drug sensitivity assay is a versatile laboratory tool that has several applications in both advanced research laboratories and in the

field. In vitro assay is indispensable for primary drug screening. It is useful for experimental studies to determine drug interactions (synergy, additivity or antagonism), to assess the degree of cross-resistance, and compare the response of pre-treatment and post-treatment isolates. In the field, bioassays may prove to be useful to estimate plasmatic drug concentrations without resorting to more sophisticated methods. For epidemiological studies, in vitro assay may be used to establish the baseline level of response of local strains of *P. falciparum* and/or *P. vivax* to new drugs before their introduction to a country or region, monitor the trends of in vitro response over time, detect the emergence and spread of drug-resistant malaria parasites, and validate candidate molecular markers for drug resistance in field isolates.

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List of tables

Table 1. Major types of in vitro drug sensitivity assays

Assay	Characteristics	Present status	References
Morphological assays[1]			
Macrotest	Culture without buffer or medium; not based on culture methods of Trager and Jensen	Outmoded, based on methods of Bass and Johns (1912); abandoned	Rieckmann et al. (1968); Rieckmann (1971)
Microtest	Based on culture methods of Trager and Jensen; adapted for field studies	Adopted by WHO	Rieckmann et al. (1978)
48-h test	For comparison of pre-culture and post-culture parasitaemia; for field isolates and laboratory-adapted strains	Still useful in laboratories with limited resources	Nguyen-Dinh & Trager (1978, 1980)
Radioisotope assay			
[^{3}H]hypoxanthine	For field isolates and laboratory-adapted strains; can be automated for mass drug screening	Current "gold standard"	Desjardins et al. (1979)
Non-radioactive assays			
Colorimetry	Non-ELISA-based reactions; low sensitivity; not adapted for field isolates	Used in some laboratories for initial drug screening because of low cost	Makler et al. (1993)
Flow cytometry	Requires expensive, sophisticated equipment; not adapted for field studies	Being developed	Franklin, Brun & Grieder (1986); van Vianen et al. (1990); Reinders et al. (1995); Pattanapanyasat et al. (1997); Saito-Ito et al. (2001); Contreras et al. (2004)
Fluoroassay	Requires expensive equipment; less sophisticated than flow cytometry	Being developed	Waki et al. (1986); Smeijsters et al. (1996); Bennett et al. (2004); Corbett et al. (2004); Smilkstein et al. (2004)
ELISA	Requires ELISA plate reader; high sensitivity and specificity; candidates to replace radioisotope methods	In advanced phases of development; at present, two versions exist (LDH and HRP II detection)	Druilhe et al. (2001); Noedl et al. (2002a)

Only assays that have been or were widely used are mentioned, with non-radioactive methods being developed in several laboratories. See text for more comprehensive review of drug assays.
ELISA, enzyme-linked immunosorbent assay; LDH, lactate dehydrogenase; HRP II, histidine-rich protein II
[1] Methods that require counting of parasites under a microscope

Table 2. Comparison of factors in various assays

Factor	Selected assays			
	48-h test	WHO microtest	Semi-microtest[1]	Radioisotope test
Blood samples	Venous and capillary	Capillary	Venous	Venous, laboratory strains
Washing of blood samples	NO	NO	YES	YES
Erythrocyte volume fraction (%)	2.0–2.5	5	2.5 – 5.0 (1.5–2.5)	1.5
Culture plate	24-well or 96-well	96-well	24-well	96-well
Volume per well (µl)	100 or 500	50–55	700	225
Serum supplement	Autologous plasma	Autologous plasma	Non-immune serum	Non-immune serum
Incubation period (h)	48	24–30	24–48 (42)	42
Atmosphere	Candle jar	Candle jar	Candle jar or incubator set at 5% O_2 – 6% CO_2	Incubator set at 5% O_2 – 5% CO_2
End-point	Thin smear, % infected erythrocytes	Thick smear, % schizont maturation	Thin smear, % schizont maturation ([^{3}H]hypoxanthine)	[^{3}H]Hypoxanthine incorporation
Results	MIC	MIC	IC_{50}	IC_{50}
Reference	Nguyen-Dinh & Trager (1980)	Rieckmann et al. (1978)	Le Bras & Deloron (1983)	Desjardins et al. (1979)

MIC, minimal inhibitory concentration ; IC_{50}, 50% inhibitory concentration

[1] Existed in two versions: morphological and radioisotope methods; factors pertaining to the radioisotope version are shown in parentheses.

Table 3. Cross-reactions in the Saker-Solomons urine test

Drug	Concentration			
	1 mg/ml	100 µg/ml	10 µg/ml	1 µg/ml
Amodiaquine dihydrochloride	+	+	–	–
Artemether	nd	–	–	–
Artesunate	nd	–	–	–
Atovaquone	nd	–	–	–
Chloroquine diphosphate	+	+	+	–
Cycloguanil	+	+	±	–
Desbutylhalofantrine hydrochloride	nd	+	±	–
Dihydroartemisinin	nd	–	–	–
Halofantrine hydrochloride	nd	±	–	–
Lumefantrine	nd	±	–	–
Mefloquine hydrochloride	nd	+	±	–
Monodesethylamodiaquine dihydro-chloride	+	+	–	–
Piperaquine phosphate	+	±	–	–
Primaquine diphosphate	+	+	±	–
Proguanil hydrochloride	+	+	+	±
Pyrimethamine	nd	+	±	–
Pyronaridine tetraphosphate	+	+	±	–
Quinine sulfate	+	+	+	±
Trimethoprim	nd	+	±	–

LK Basco, unpublished data, 2004

+, violet-to-red colour change; ±, slight colour change to amber or light-brown ; –, no colour change ;

nd, not done owing to solvent incompatibility (absolute ethanol) with chloroform-containing reaction solution. Drugs with a positive reaction at 10 µg/ml are expected to be cross-reactive with chloroquine. None of the test compounds gave a positive result at 0.1 µg/ml.

Table 4. Examples of in vitro threshold values for drug resistance

Drug	Threshold value according to different laboratories					
	WHO microtest (MIC), microscopy	AFRIMS[1] (IC_{99}, nmol/l), radioisotope	IMTSSA[2] (IC_{50}, nmol/l), radioisotope	Pasteur Institute[3] (IC_{50}, nmol/l), radioisotope	CNRCP[4] (IC_{50}, nmol/l), radioisotope	WRAIR[5] (IC_{50}, ng/ml) radioisotope
Chloroquine	8 pmol = 160 nmol/l	20	100	80 or 100	100	R: 30–100, S: 2–10
Amodiaquine	4 pmol = 80 nmol/l	–	80	80	–	–
Monodesethylamodiaquine	–	–	–	–	60	–
Pyronaridine	–	–	15	–	–	–
Quinine	256 pmol = 5.12 µmol/l	1800	500	300	500	R: 50–300, S:10–30
Mefloquine	32 pmol = 640 nmol/l	140 (IC_{50} 20 nmol/l)	20	30	20	R: 10–80, S:1–7
Halofantrine	5 pmol = 100 nmol/l	50	6	–	6	R: 5–10, S:0.1–3
Pyrimethamine[6]	–	–	100	–	100	R: 15–40, S: < 0.1

The values given should not be taken as established thresholds for distinguishing between sensitive and resistant parasites; they are all arbitrary values. Note that thresholds are expressed as IC_{50} values for some assay systems and IC_{99} or MIC for others. Most authors do not quote threshold values for artemisinin derivatives, pyronaridine and atovaquone, principally because of the scarcity of corresponding in vivo data to validate the in vitro result; likewise, the thresholds for sulfonamides, sulfones and antibiotics are not known, as these drugs are never administered alone in antimalarial chemotherapy.

[1] Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand (Wongsrichanalai et al., 1997); threshold IC_{50} for mefloquine resistance determined by Childs et al. (1991)

[2] Institut de Médecine Tropicale du Service de Santé des Armées, Marseille, France (Pradines et al., 1999a,b)

[3] Pasteur Institute, Paris, France (Brasseur et al., 1986, 1988, 1992a,b, 1995)

[4] Centre National de Référence de la Chimiosensibilité du Paludisme, Laboratoire de Parasitologie, Hôpital Bichat, Paris, France (Le Bras & Ringwald, 1990)

[5] Walter Reed Army Institute of Research (Milhous et al., 1989). R, resistance range in ng/ml; S, sensitive range in ng/ml.

[6] Smalley & Brown (1982) suggested that, on the basis of in vitro and in vivo studies conducted in parallel, pyrimethamine-sensitive parasites are completely inhibited (MIC) at ≤40 nmol/l and that parasites that grow in ≥ 100 nmol/l pyrimethamine are likely to be resistant.

Table 5. Expected developmental stages of *P. falciparum* ring forms after 18 h and 42 h of cultivation

Predominant ring stages at 0 h	Expected stages after 16–18 h	Expected stages after 40–42 h
Small	Ring, trophozoite	Trophozoite, pre-schizont
Medium	Trophozoite, pre-schizont	Pre-schizont, schizont
Large	Pre-schizont	Schizont, new rings

Pre-schizont refers to schizonts (i.e. parasites that have undergone at least one complete nuclear division) with 2–8 nuclei. New rings are rings of the second schizogonic cycle. It is assumed that the patients from whom fresh *P. falciparum* isolates were collected had not taken any antimalarial drug during the previous 2–3 weeks.

List of annexes

Annex 1. Check-list of basic reagents for drug assays

Culture medium

- culture medium
  - — RPMI 1640 with L-glutamine, without sodium bicarbonate, 10.4 g/l or
  - — RPMI 1640 with L-glutamine and HEPES, without sodium bicarbonate, 16.4 g/l
- HEPES (if using RPMI 1640 without HEPES), 5.94 g/l
  - — FW 238.3 for free acid, $C_8H_{18}N_2O_4S$, water-soluble, store powder at room temperature
- sodium bicarbonate ($NaHCO_3$), 5 g/100 ml (5%) solution or add 2.1 g/l
  - — FW 84, water soluble (solubility, 1 mol/l at 20 °C), store crystalline powder at room temperature; may take time to dissolve ; might be preferable to prepare $NaHCO_3$ solution and store it at 4 °C until use
- gentamicin (optional)
  - — water-soluble, preferable to order ready-to-use solution (store solution at 4 °C)
- sterile glass-redistilled or demineralized water
- serum supplement or substitute
  - — pooled type AB^+ human serum from non-immune donors
  - — Albumax® I or II

Antimalarial drugs

- active principles
- solvents: sterile water, absolute ethanol, pure methanol, dimethyl sulfoxide, lactic acid, Tween 80, linoleic acid

Urine screening test

- tetrabromophenolphthalein ethyl ester
- hydrochloric acid (2 N)
- chloroform

Buffers for urine screening test and Giemsa or Field staining

- $K_2HPO_4 \bullet 3H_2O$
- KH_2PO_4
- Na_2HPO_4

Annex 2. Selected bibliography on related subjects

Reviews on Trager and Jensen's cultivation method

Druilhe P, Gentilini M. Culture in vitro de *Plasmodium falciparum.* Intérêt et limites – méthodologie. [In vitro cultivation of *Plasmodium falciparum.* Applications and limits, methodology]. *Médecine Tropicale (Marseille),* 1982, 42:437–462.

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Reviews on applications of in vitro assays

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Noedl H, Wongsrichanalai C, Wernsdorfer WH. Malaria drug-sensitivity testing: new assays, new perspectives. *Trends in Parasitology,* 2003, 19:175–181.

Laboratory manuals and books on malaria culture

Deharo E et al. *Técnicas de laboratorio para la selección de sustancias antimaláricas* [Laboratory techniques for the selection of antimalarial substances]. La Paz, Imprenta Perez, 2000.

Schlichtherle M et al., ed. *Methods in malaria research.* 4th ed. Malaria Research and Reference Reagent Resource Center, American Type Culture Collection, Manassas, Virginia, 2004 (available free of charge on the Malaria Research and Reference Reagent Resource Center web site).

Thaithong S, Beale G. *Malaria parasites. A report on 15 years research at Chulalongkorn University.* Bangkok, Chulalongkorn University Printing House, 1992.

Tanabe K et al., ed. *Malariology laboratory manual* [in Japanese]. Tokyo, Saine Press, 2000.

Trigg PI. Plasmodiidae. In: Taylor AER, Baker JR, eds. *Methods of cultivating parasites in vitro.* London, Academic Press, 1978:89–109.

Annex 3. Composition of culture media that support the growth of *P. falciparum*

Composition	Medium (mg/l)						
	RPMI 1640	Medium 199[1]	F-12 Ham	DMEM	IMDM	Waymouth MB 752/1	NCTC 135
Inorganic salts							
$CaCl_2$ (anhydrous)	0	200	33.22	200	165	0	0
$CaCl_2 \bullet 2H_2O$	0	0	0	0	0	120	264
$Ca(NO_3)_2 \bullet 4H_2O$	100	0	0	0	0	0	0
$CuSO_4 \bullet 5H_2O$	0	0	0.003	0	0	0	0
$Fe(NO_3) \bullet 9H_2O$	0	0.72	0	0.1	0	0	0
$Fe(NO_3)_3 \bullet 9H_2O$	0	0	0	0	0	0	0.1
$FeSO_4 \bullet 7H_2O$	0	0	0.834	0	0	0	0
KCl	400	400	223.6	400	330	150	400
KH_2PO_4	0	0	0	0	0	80	0
KNO_3	0	0	0	0	0.076	0	0
$MgCl_2$ (anhydrous)	0	0	57.22	0	0	0	0
$MgCl_2 \bullet 6H_2O$	0	0	0	0	0	240	0
$MgSO_4$ (anhydrous)	48.84	97.67	0	97.67	0	0	0
$MgSO_4 \bullet 2H_2O$	0	0	0	0	97.67	0	0
$MgSO_4 \bullet 7H_2O$	0	0	0	0	0	200	200
NaCl	5500	6800	7599	6400	4500	6000	6800
$NaHCO_3$	0	0	0	0	0	2240	2200
Na_2HPO_4 (anhydrous)	800	0	142.04	0	0	300	0
$NaH_2PO_4 \bullet H_2O$	0	140	0	125	125	0	0
$NaH_2PO_4 \bullet 2H_2O$	0	0	0	0	0	0	158
$Na_2SeO_3 \bullet 5H_2O$	0	0	0	0	0.017	0	0
$ZnSO_4 \bullet 7H_2O$	0	0	0.863	0	0	0	0

RPMI, Roswell Park Memorial Institute; DMEM, Dulbecco modified Eagle medium; IMDM, Iscove modified Dulbecco medium; HEPES, N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid); Tween 80, polyoxyethylenesorbitan monooleate

[1] The composition of Medium 199 with Earle salts is given, whereas it is also used with Hank salts (composition not shown).

Composition	Medium (mg/l)						
	RPMI 1640	Medium 199	F-12 Ham	DMEM	IMDM	Waymouth MB 752/1	NCTC 135
Amino acids							
L-Alanine	0	25	8.9	0	25	0	31.48
L-α-Amino-*n*-butyric acid	0	0	0	0	0	0	5.5
L-Arginine	241.86	70	211	84	84	75	31.2
L-Asparagine	50	0	15.01	0	25	0	8.1
L-Aspartic acid	20	30	13.3	0	30	60	9.91
L-Cysteine	0	0.11	35.12	0	0	90	0
L-Cystine	65.15	26.06	0	62.77	91.24	15	10.5
D-Glucosamine • HCl	0	0	0	0	0	0	3.9
L-Glutamic acid	20	66.82	14.7	0	75	150	8.3
L-Glutamine	300	100	146	584	584	350	135.7
Glycine	10	50	7.5	30	30	50	13.5
L-Histidine	15	21.88	20.96	42	42	128	26.5
L-Hydroxyproline	20	10	0	0	0	0	4.1
L-Isoleucine	50	40	3.94	105	105	25	18
L-Leucine	50	60	13.1	105	105	50	20.4
L-Lysine	40	70	36.5	146	146	240	38.4
L-Methionine	15	15	4.48	30	30	50	4.4
L-Ornithine • HCl	0	0	0	0	0	0	9.4
L-Phenylalanine	15	25	4.96	66	66	50	16.5
L-Proline	20	40	34.5	0	40	50	6.1
L-Serine	30	25	10.5	42	42	0	10.8
L-Taurine	0	0	0	0	0	0	4.2
L-Threonine	20	30	11.9	95	95	75	18.9
L-Tryptophan	5	10	2.04	16	16	40	17.5
L-Tyrosine	28.94	57.88	7.81	104.18	104.2	40	16.4
L-Valine	20	25	11.7	94	94	65	25

Composition	Medium (mg/l)						
	RPMI 1640	Medium 199	F-12 Ham	DMEM	IMDM	Waymouth MB 752/1	NCTC 135
Vitamins							
para-Aminobenzoic acid	1	0.05	0	0	0	0	0.125
Ascorbic acid	0	0.05	0	0	0	17.5	50
D-Biotin	0.2	0.01	0.007	0	0.013	0.02	0.025
Calciferol (vitamin D_2)	0	0.1	0	0	0	0	0.25
D-Calcium pantothenate	0.25	0.01	0.48	4	4	1	0.025
Choline chloride	3	0.5	13.96	4	4	250	1.25
Folic acid	1	0.01	1.3	4	4	0.4	0.025
Inositol	35	0.05	18	7.2	7.2	0.9	0.125
Menadione (vitamin K)	0	0.01	0	0	0	0	0.25
Niacin	0	0.025	0	0	0	0	0.0625
Niacinamide (nicotinamide)	1	0.025	0.037	4	4	1	0.003
Pyridoxal HCl	1	0.025	0	0	4	0	0.0625
Pyridoxine HCl	0	0.025	0.062	4	0	1	0.0625
Riboflavin	0.2	0.01	0.038	0.4	0.4	1	0.025
Thiamine HCl	1	0.01	0.34	4	4	10	0.025
α-Tocopherol	0	0.01	0	0	0	0	0.025
Vitamin A (acetate)	0	0.14	0	0	0	0	0.25
Vitamin B_{12}	0.005	0	1.36	0	0.013	0.2	10
Coenzymes							
Cocarboxylase	0	0	0	0	0	0	1
Coenzyme A	0	0	0	0	0	0	2.5
Diphosphopyridine nucleotide	0	0	0	0	0	0	7
Flavin adenine dinucleotide	0	0	0	0	0	0	1
Triphosphopyridine nucleotide	0	0	0	0	0	0	1
Uridine triphosphate	0	0	0	0	0	0	1

Composition	Medium (mg/l)						
	RPMI 1640	Medium 199	F-12 Ham	DMEM	IMDM	Waymouth MB 752/1	NCTC 135
Others							
Adenine sulfate	0	10	0	0	0	0	0
Adenosine-5-triphosphate	0	1	0	0	0	0	0
Adenosine-5-phosphate	0	0.2	0	0	0	0	0
Cholesterol	0	0.2	0	0	0	0	0
Deoxyadenosine	0	0	0	0	0	0	10
Deoxycytidine HCl	0	0	0	0	0	0	10
Deoxyguanosine	0	0	0	0	0	0	10
2-Deoxy-D-ribose	0	0.5	0	0	0	0	0
Ethyl alcohol	0	0	0	0	0	0	40
D-Glucose	2000	1000	1802	1000	4500	5000	1000
D-Glucuronolactone	0	0	0	0	0	0	1.8
Glutathione	1	0.05	0	0	0	15	10
Guanine HCl	0	0.3	0	0	0	0	0
HEPES	5958	0	0	0	5958	0	0
Hypoxanthine (Na salt)	0	0.354	4.77	0	0	25	0
Linoleic acid	0	0	0.084	0	0	0	0
5-Methylcytosine	0	0	0	0	0	0	0.1
Phenol red	5	20	1.2	15	15	10	20
Ribose	0	0.5	0	0	0	0	0
Sodium acetate	0	0.3	0	0	0	0	0
Sodium acetate • $3H_2O$	0	50	0	0	0	0	50
Sodium glucuronate • H_2O	0	0	0	0	0	0	1.8
Sodium putrescine • 2HCl	0	0	0.161	0	0	0	0
Sodium pyruvate	0	0	110	110	110	0	0
DL-68-Thioctic acid	0	0	0.21	0	0	0	0
Thymidine	0	0	0.73	0	0	0	10
Tween 80	0	20	0	0	0	0	12.5
Uracil	0	0.3	0	0	0	0	0
Xanthine (Na salt)	0	0.344	0	0	0	0	0


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