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The Comparative Evaluation Of Chemical, Chromatographic And Immunological Tests For The Detection Of Mefloquine And Other Antimalarial Drugs In Body Fluids

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THE COMPARATIVE EVALUATION OF CHEMICAL, CHROMATOGRAPHIC AND IMMUNOLOGICAL TESTS FOR THE DETECTION OF MEFLOQUINE AND OTHER ANTIMALARIAL DRUGS IN BODY FLUIDS

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22 - 26 July 1991

The third Penang workshop in the TDR sponsored antimalarial drug studies series, entitled "Workshop for the Comparative Evaluation of Chemical, Chromatographic and Immunological Tests for the Detection of Mefloquine and Other Antimalarial Drugs in Body Fluids" - Penang III - was held from 22 to 26 July 1991 at the National Drug Research Centre, Universiti Sains Malaysia, Penang, Malaysia.

In the 20 months which had passed since the last workshop - The Validation of Chemical and Immunological Tests for Antimalarials in Body Fluids, (Penang II)* - considerable advances had been made by the TDR collaborating scientists in all three fields of study - Chemical, Chromatographic and Immunological. These findings are detailed in the working papers which appear later in this document but may be summarized as follows. (Where ever indicated these working papers have been updated by the respective author to include the relevant findings of the Penang III Workshop):

Chemical

A simple field test for the detection of sulfadoxine in urine, plasma or serum has been developed by Mr. D.L. Mount of the Centres for Disease Control, Atlanta, USA. The now standard CDC modification of the Saker-Solomons drug assay test - CDC Saker-Solomons CQI - which gives a detection threshold of 1 mg/L for chloroquine, was also included in the programme of work to see if it could also be used for a non-specific identification of quinine and mefloquine by enhancement of the volume of the test sample. Mr. Mount and his CDC colleagues have also done some very important studies on the comparative levels of mefloquine in the urine and the whole blood over time and this highly useful data appears in Mr. Mount's report.

Chromatographic

A large amount of development work has been done by Dr. B. Betschart of the Swiss Tropical Institute, Basel, Switzerland on

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* See published document on the proceedings of "The Validation of Chemical and Immunological Tests for Antimalarials in Body Fluids", International Monograph Series No. 3, (1990). Centre for Drug Research, Universiti Sains Malaysia, Penang, Malaysia.

his thin layer chromatographic technique. His HPTLC field test using the native fluorescence of chloroquine and quinine has been developed into an easily portable field test kit which has been successfully employed in field studies in Africa and Asia. Unfortunately, his work, inspired by the findings of the Penang II Workshop on the use of creatinine levels in the urine as an indicator for the variability of the urine levels of chloroquine, and possibly other antimalarial drugs, did not provide the hoped for correction factor as no explainable relationship could be established between creatinine levels and the dilution factor of the chloroquine in the urine. More success was obtained with a HPTLC mefloquine detection test in urine and plasma samples using an additional extraction step and a changed mode of detection (i.e. fluorescence quenching) to give a reliable quantitative detection method for mefloquine.

Immunological

Dr. T.A. Eggelte of the Royal Tropical Institute, Amsterdam, Holland, had meanwhile taken his ELISA monoclonal based microtitre test plate system, which was successfully demonstrated at Penang II, and, with TDR encouragement, developed it into an entirely new concept: an ELISA dipstick assay. Dipsticks have been developed for individual tests for chloroquine, quinine, mefloquine, and also for a combination test with all three drugs on the same dipstick. The dipsticks are equally applicable to urine, plasma, serum and whole blood (lysed or eluted at a ratio of 1:5).

The principal objective of the Penang III Workshop was, therefore, to comparatively evaluate this virtual armamentarium of potential field test systems for the detection of mefloquine and other antimalarial drugs in body fluids using routine samples of urine, whole blood, plasma and serum to provide the basis for the establishment of standardized field tests and to pave the way for their production and distribution in the endemic countries.

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UPDATE ON THE CURRENT NEED FOR, AND APPLICATION OF, FIELD TESTS FOR THE DETECTION OF MEFLOQUINE AND OTHER ANTIMALARIALS IN BODY FLUIDS

David Payne

Introduction

In October 1989, the National Drug Research Centre, (NDRC), Universiti Sains Malaysia and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases held a workshop in Penang - The Validation of Chemical and Immunological Tests for Antimalarials in Body Fluids - with the objective of evaluating the then available test methods for the detection of antimalarials in body fluids; but principally urine and blood/serum/ plasma. In fact this seminar was a spin-off from the recommendations of an earlier seminar of that year at the same venue - The Workshop on Clinical Pharmacology of Antimalarial Drugs - which emphasized the need for the evaluation of the available chemical, chromatographic and immunological tests for the detection of antimalarial drugs.

Once the second workshop(1) - designated Penang Workshop II for brevity sake - got underway it was quickly apparent that, whilst there were fairly reliable test systems for most of the common antimalarials such as chloroquine, quinine, amodiaquine, and the sulfonamides, the only reliable detection system for mefloquine was that based on High Performance Liquid Chromatography (HPLC).

HPLC technology is highly sophisticated and requires expert trained staff and strict apparatus maintenance to obtain the necessary levels of reliability and reproducibility. Obviously, these are not usually available at any level in the field and it is worth quoting the practical experience of a participant of Penang Workshop II to emphasize just how difficult it is to attain the required level of technical excellence in HPLC technology in any but the optimum working environment. He has written QUOTE ..... The HPLC saga amply demonstrates a problem I mentioned in the project proposal ..... clinical departments like us in the Third World do not and cannot handle machinery such as HPLC's. We do not have the technical expertise and the work load to justify buying such an expensive machine. I have decided not to attempt any more HPLC for malaria drugs ..... UNQUOTE.

It is quite evident, therefore, that the routine detection of mefloquine, or any other antimalarial, in body fluids cannot be dependant on this sophisticated technology and other ways must be sought.

So, even before Penang Workshop II was completed, plans were being formulated for a Penang Workshop III, which would concentrate principally on detection systems for mefloquine. Consequently, Penang III has had to wait whilst collaborating investigators have had the time to develop these alternative technologies. It is these very new techniques which we shall be evaluating in Penang Workshop III and, hopefully, making recommendations for the further evaluation at the field level in Sabah, during 1991 and 1992, of the more promising of them.

The Need for a Mefloquine Detection System

The half life of mefloquine is, comparatively, very long; up to 33 days in healthy persons, but only about half that in patients with P. falciparum infections (2). A recent study in Thailand gave half-life means of 11.7 ± 1.9 days for P. falciparum malaria patients and 15.4 ± 0.9 days for healthy volunteers (3). In either event this means the levels of excretion (e.g. the urine) are very low and even in the plasma the mean level in the Thai study at peak concentration is only about 2700 ug/L.(3), with whole blood having double the level of that found in the plasma or serum (4). Nonetheless mefloquine is a toxic drug and serious complications could occur if patients are overdosed. As an indicator of how important this problem is in the field, it has been reported that in a survey in Nigeria, 50% of the children attending an outpatient clinic for malaria treatment already had levels of an antimalarial equivalent to that required for a therapeutic cure (2).

Other important considerations which make the availability of a simple field level test for mefloquine, and, of course, other antimalarials, so imperative are:

- in the event of a suspected failure of therapy of P. falciparum it is important to know if the patient has indeed taken drugs previously and, if so, and if feasible, to have some idea of how much: i.e. a quantitative test as well as a qualitative test.
- if drugs have been taken previously, just what these drugs are so the risk of drug interactions of an alternative therapy can be assessed, or, otherwise, whether a change in dosage is required to achieve a curative dose.
- to assess the compliance of the patient with the prescribed dose and to verify if side-effects such as vomiting, diarrhoea or diet have affected the absorption of a curative dose of the drug.
- to ascertain the pattern of the use of a particular drug in a community.
- to screen malaria patients involved in drug efficacy studies to ascertain previous drug intake.

- as an incidental monitoring device to indicate a possible loss of efficacy of treatment due to resistance by the malaria parasite to the drug, or preparations which do not contain, or fail to release, the required level of the active constituents of the drug.

It will be seen, therefore, that the availability of a suitable simple and reliable test system for mefloquine is an essential requirement for the proper management of the drug.

Operational Considerations

Plasmodium falciparum is throughout most of its range on the globe resistant to chloroquine to a greater, or lesser, extent and in some areas such as Amazonia in South America, and South East Asia the parasite has shown resistance to alternative second line, third line and even fourth line alternatives, including mefloquine. (In 1989 there were two isolated reports of P. vivax chloroquine resistant strains in patients infected in Papua New Guinea) (5,6).

Accordingly, travellers and indigenes are increasingly turning to alternative drugs for prophylaxis and therapy. The days when clinically diagnosed malaria patients could be given presumptive doses of chloroquine with every confidence of a successful cure, and little likelihood of any serious side effects, are largely passed. Mefloquine, and other relatively toxic drugs such as quinine, sulfadoxine/pyrimethamine, halofantrine are being used in increasing quantities in most parts of the world and with this more widespread use the risk of overdosing or deleterious drug reactions increases respectively.

It is hoped that this Third Penang Workshop will provide the basis for a test system for the detection of antimalarial drugs, and, in particular, mefloquine, in readily obtained body fluids such as urine or blood/serum/plasma (though saliva should not be forgotten) which will permit these tests to be carried to the most peripheral limits of the health care system where usually, in truth, their need is, simultaneously, the greatest and the most difficult to provide.

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UPDATE ON CHEMICAL FIELD TESTS FOR THE DETECTION OF MEFLOQUINE AND OTHER COMMON ANTIMALARIALS IN URINE AND FINGER-PRICK BLOOD SAMPLES

Dwight L. Mount

Often, field malaria studies require methods for on-site detection of antimalarial drugs and their metabolites in body fluids to screen patients for in vivo drug sensitivity studies and to verify ingestion and absorption of drugs. For in vitro field tests, researchers need to know if blood samples contain other drugs that contribute to the antiparasitic effect of a specific added drugs, to avoid false results. Assays that can be conducted on-site permit monitoring of chemoprophylaxis and treatment compliance and are also useful when exploring relationships between drug use practices and the incidence of disease or the development of resistance in a community.

Although other methods, such as thin layer chromatographic (TLC) or immunoanalytical assays, can be used for on-site determination of antimalarials, colorimetric assays for determining antimalarials in urine are usually simpler to conduct, an important consideration. However, specificity and sensitivity are usually compromised when choosing colorimetric over TLC or immunoanalytical assays.

Qualitative colorimetric methods for field assay of chloroquine (CQ) in urine have been available for many years and include the Dill-Glazko test[1], the Haskins test[2] and the Wilson-Edeson test[3]. Of these three, the Dill-Glazko has been most used because of its simplicity; however several research groups have concluded that this test is not sufficiently sensitive or reliable in assessing history of chloroquine use in the field[4-6].

In recent years the Haskins test has been modified to simplify and to incorporate a battery-operated, hand-held portable photometer to give quantitative results[7] (Haskins MMII). Another ion-pairing test similar to the Haskins method was also developed[8]. This approach utilizes bromothymol blue (BTB) as the ion-pairing reagent instead of methyl orange used in the Haskins test. The BTB test is comparable in sensitivity to the Haskins (1 mg/L) and can also be used with the portable photometer to give quantitative results. The method of E.G. Saker and E.T. Solomons, originally used to assay drugs of abuse in urine[9], has been modified and adapted to permit the detection of chloroquine and its metabolites in urine[10]. This test (Saker-Solomons CQI) is as simple to conduct as the Dill-Glazko test, yet is superior to the semiquantitative in the 0-3 mg/L concentration range. Unknowns are visually compared against a standard curve prepared by fortifying blank urine at concentrations of 0,1,2,3 mg/L using an aqueous solution of known chloroquine concentration. The detection limit is 1 mg/L. During the last CHEMAL/RCS workshop (National Drug

Research Centre, Universiti Sains Malaysia, Penang, 16-21 October, 1989) the modified Haskins and Saker-Solomons tests were compared with corresponding high-performance liquid chromatographic results for field samples. A report of this validation process has been prepared.

Field Assays for Drugs Other Than Chloroquine

As parasite resistance to chloroquine becomes more of a problem throughout the world, such drugs as mefloquine (MQ) and Fansidar (sulfadoxine + pyrimethamine) will increasingly be used for prophylaxis and self-treatment of parasite infections. Thus, for future field malaria studies, the availability of simple, sensitive and reliable field assays for these and other drugs is important.

Mefloquine gives a positive response in the Saker-Solomons test, although the intensity of the response was determined to be 62% of the intensity of response of chloroquine with the test reagent[10]. However, comparison of the matched blood and urine assay results of MQ dosed volunteers presented in Tables 1 and 2, illustrates the problems of applying this simple test or any other urine field-test to the monitoring of MQ. First of all, MQ levels in urine are usually in the range of 0.5 to 2.0 mg/L. The intensity of response of MQ to the Saker-Solomons is about 2 mg/L using the Saker-Solomons test in its current configuration. Thus, the usual concentration of MQ in urine is at or below the detection limit of the test. However, the detection limit of the test might be lowered by increasing the volume of the urine sample above the 2 ml volume now used. Secondly, urinary MQ concentrations do not correlate well with those from blood, as illustrated by the results presented in Table 1 for seven volunteers taking a low therapeutic dose of MQ, $r = 0.56$ ($n=38$), and in Table 2 for single volunteer taking a prophylactic dose of MQ, $r = 0.45$ ($n=11$). Note in Table 2, that between 6 and 12 hours post dose MQ blood concentrations and urine specific gravities of the volunteer remained virtually constant, while urine MQ concentrations fluctuated from 487 to 4628 ug/L. In summary, these data suggest that efforts should be directed at developing MQ field assays utilizing blood since urine MQ levels are usually relatively low and blood and urine MQ levels correlate rather poorly.

It has been recently reported[11] that various solutions of MQ can be UV-irradiated, producing a fluorescent photoproduct. Preliminary experiments in our lab indicate that this might be used to measure MQ levels in 0.2 ml volumes of finger-print whole blood. The preliminary procedure involves: (1) extracting the whole blood sample with 1 ml of methyl-t-butyl ether (MTBE), adjusting the pH with 0.5 ml of 20% aqueous trisodium phosphate; (2) centrifugating to break the emulsion; (3) transferring the clear MTBE extract to a quartz cell; (4) UV-irradiating the MTBE extract; and (5) measuring the fluorescence of the photoproduct at 380 nm after excitation at 320 nm. In these preliminary experiments a research-grade,

grating spectrofluorometer was used. Should comparable results be possible with a simpler filter-fluorometer, the method could be made field-applicable. Even so, the need for the filter instrument, and the necessity for centrifugating of samples, would restrict this method to the hospital laboratory setting where field malaria studies are being conducted. Also, the method may not be specific for MQ since the same excitation and emission wavelengths have been used to monitor the elution of CQ in a normal-phase, high-performance liquid chromatographic assay[12]. Thus, the utility of such a method in the scheme of the field analysis of antimalarials remains to be demonstrated.

The Bratton-Marshall test for sulfonamides[13] has been used to screen for sulfadoxine in urine to verify Fansidar use. This test is nonspecific, giving positive tests for a wide variety of sulfonamides which might be encountered in the field setting. We have found that the interpretation of the results can be subjective, because of observed colours which are different shades and tints than the expected purple colour for a positive test. Also, recent unpublished results obtained in our laboratory indicate that the intensity of the background reaction of urine components with the Bratton-Marshall reagents to form the indicating purple colour can be highly variable from sample to sample of blank urine, providing numerous false positives.

We have developed an alternative colorimetric test for detecting sulfonamides in urine. The development of this test was a synthesis from two different methods reported in the literature[14,15]. The quantitative configuration of this alternative test involves: (1) extracting a volume of urine with ethyl acetate with the addition of phosphate buffer, pH = 5.5; (2) breaking the extraction emulsion by adding a drop of silicone de-foamer; and (3) transferring a measured volume of the extract to a measured volume of an acidic, methanolic solution of p-dimethylaminocinnamaldehyde (DMACNA). The primary aromatic amine function of sulfonamides reacts with the aldehyde function of the DMACNA to form a violet-red coloured Schiff base. For quantification, the intensity can be measured at 535 nm using the portable hand-held photometer used in the Haskins test, after 3 ml of methanol has been added to bring the absorption intensity within the linear range of the instrument. A simpler qualitative configuration of the test is similar to the quantitative test except that a smaller volume of ethyl acetate extract, three drops of the DMACNA solution at a higher concentration is added to the top of the ethyl acetate layer without any agitation. The appearance of the violet-red colour at the top of the ethyl acetate layer comprises a positive test for sulfonamides. Since the sulfonamide is extracted from the urine, there is less background interference from the urine than observed with the Bratton-Marshall test. Thus, the new test is more reliable than the Bratton-Marshall test and has a detection limit of 1 mg/L compared with 5 mg/L for the Bratton-Marshall[13]. Since the new test, like the Bratton-Marshall test, reacts with sulfonamides in general, a complimentary, simple TLC urine test is needed to determine if observed colorimetric positives are due to sulfadoxine.

Possible Strategies for On-site Monitoring of Antimalarial Drug Use

As MQ becomes more available in malaria-endemic areas for prophylactic use and self-treatment, strategies will have to be addressed for the on-site monitoring of antimalarial drug use in connection with malaria field studies. Currently there is no colorimetric urine test available for mefloquine that is quick and simple to conduct and has been proven to be reliable at screening for individuals having therapeutically significant blood levels of MQ. For in-vivo and in-vitro field studies where monitoring study populations for prior drug use is essential, one possible strategy for monitoring each study subject would involve the following steps: (1) running a Saker-Solomons test to detect CQ, quinine (Q), or proguanil (PG); (2) running either a Bratton-Marshall or the new alternative test to detect sulfonamides; and (3) running either a blood or immunoanalytical assay to detect MQ. Running a blood-TLC test for each prospective study subject would probably be very cumbersome and time consuming. Running a urine-TLC test might be simpler, but would not demonstrate whether or not the subject had a therapeutically significant MQ blood level. This might not be a problem if the number of prospective study subjects will allow exclusion of all subjects that test positive for MQ using a urine-TLC test. An immunoanalytical blood assay for MQ would provide the needed information if methods and materials/reagents were available, stable and convenient for field use.

Recently in our lab an experiment was conducted where 10 ml volumes of blank urine were fortified with a MQ standard solution to give concentrations of 0.0, 0.2, 0.4 and 1.0 mg/L. A Saker-Solomons test was run on each 10 ml fortified sample. The responses of the 0.2 and 0.4 mg/L fortified samples were slightly distinguishable from the blank sample determination, with the response of the 0.4 mg/L sample appearing stronger. However, much work needs to be done at assessing the realiability of the test for detecting such low levels of MQ when the subject to subject variability of blank urine samples has been determined. If the 10 ml test proved reliable at detecting MQ at such low levels and this volume of urine sample can be routinely collected from each study subject, the test would be a useful addition to the strategy discussed above in eliminating the necessity of running a blood field test for each subject, since it appears that a patient with a therapeutically significant MQ blood level will rarely have a urine level below 0.2 mg/L. Thus, the test would serve as a "flag" to alert the investigator to possible MQ dosing. A TLC or an immunoanalytical test could then be run on a finger-prick blood sample to identify the presence of MQ (or other antimalarials) and determine its concentration in the blood.

Conclusion

Now that a reliable and sensitive colorimetric urine test for detecting chloroquine, quinine and/or proguanil use is available (Saker-Solomons), efforts should be concentrated on developing sensitive and reliable tests to detect the use of such antimalarials as mefloquine and Fansidar (sulfadoxine + pyrimethamine). An alternative colorimetric test for determining sulfonamides in urine has been developed that is more sensitive and reliable than the Bratton-Marshall test which is currently used. The limit of detection for the alternative test is 1 mg/L. Due to low mefloquine levels in the urine of mefloquine dosed patients and to the poor correlation between matched blood and urine levels, developing a reliable colorimetric urine test for detecting mefloquine use in a general study population will be difficult. As antimalarials other than chloroquine, such as mefloquine and Fansidar, are increasingly used in malaria-endemic areas for prophylaxis and self treatment, strategies will need to be considered on how to use available tools most conveniently, effectively and reliably in screening populations for previous antimalarial treatment before inclusion of subjects into field malaria studies.

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Table 1: Mefloquine assay results for pair whole blood and urine samples

Vol #	Dosage mg/kg		MQ Drug Levels (ng/ml) Day 1	Day 7	Day 14	Day 21	Day 35	Day 56
1	14.4	WB	1732	1039	768	522	291	102
		UR	946	2287	1036	428	129	68
2	14.7	WB	NA[a]	974	659	386	194	64
		UR	NA	1188	738	1787	313	274
3	15.1	WB	1280	723	448	436	138	52
		UR	NA	1399	184	295	NA	<35
4	13.5	WB	918	622	406	269	158	82
		UR	3326	328	129	187	556	22
5	15.1	WB	1533	971	796	NA	323	146
		UR	1761	1397	1113	NA	1495	177
6	15.4	WB	1056	620	435	321	148	51
		UR	268	1769	2473	383	55	<35
7	14.5	WB	969	652	519	285	153	58
		UR	1426	1862	202	561	121	53

[a] specimen not available for analysis.

Table 2: Mefloquine assay results for paired whole blood and urine samples collected from a volunteer dosed with 250 mg (as base) MQ* HCl

Time of Sample collection	Amt. MQ (ug/L) in whole blood	Amt. MQ (ug/L) in urine	Urine sp.gr.	Urine[a] pH
pre-dose(10:00 am)	139	404	1.022	---
2 hr (12:10 pm)	405	1038	1.025	5.4
4 hr (2:10 pm)	622	3768	1.030	5.5
6 hr (4:10 pm)	590	487	1.022	6.6
8 hr (6:10 pm)	590	1335	1.023	5.8
10 hr (8:25 pm)	567	4435	1.025	5.3
12 hr (10:40 pm)	526	4628	1.023	5.3
24 hr	484	650	1.007	8.5
48 hr	398	2854	-----	5.4
72 hr	448	405	-----	6.5
7 dy	343	752	1.011	5.8

[a] determined after storage in refrigerator.

Annex 1

QUALITATIVE COLORIMETRIC TEST FOR SULFADOXINE IN URINE

This test is based on the extraction of sulfadoxine from urine into ethyl acetate followed by formation of a violet-red Shiff's base upon addition of acidic methanolic p-dimethylamino-cinnamaldehyde. The intensity of the colour is proportional to sulfadoxine concentration in the sample. Estimates can be made by comparing colour intensities in unknowns with those in fortified, drug-free urine standards.

A. Reagents

1. Colour reagent solution: 0.3% (w/v) p-diemthylamino-cinnamaldehyde (DMACNA) and 0.6% (v/v) H_2SO_4 in methanol (300 mg DMACNA + 0.6 ml H_2SO_4 + 100 ml methanol).
2. Buffer solution: 1.5 M phosphate buffer, pH=5.5 (11 g $K_2HPO_4.3H_2O$ + 85 g KH_2PO_4 dissolved and diluted to 500 ml with water).
3. SAG-10 silicone antifoam emulsion diluted ca 50/50 with water.
4. Ethyl acetate.
5. sulfadoxine standard: 1 g/L in ethanol.

B. Equipment

1. Clay-Adams pipette and tips.
2. 1-dram clear glass vials with caps.
3. 50 ul Hamilton syringe for spiking urine standards.
4. Two dropper bottles (one used for adding colour reagent solution, the other used for adding SAG-10 silicone antifoam emulsifier).

C. Procedure

1. Standard curve: A standard curve is prepared by adding appropriate volumes of standard solution to 2 ml of drug free urine according to the following table:

Sulfadoxine (ppm)	Vol. (ul) of 1 ug/ml std. sol. added
0	0
1	2
3	6
7	14

2. Analysis of standards and samples: 0.5 ml of ethyl acetate, 0.5 ml of buffer solution, and 2 ml of urine are added to a 1-dram clear glass screw-cap vial using the Clay-Adams pipetter. The vial is capped and inverted 20 times by hand. After the ethyl acetate phase rises to the top, one drop of SAG-10 defoamer is added to the ethyl acetate layer to break the emulsion formed during inversion. If necessary, the ethyl acetate layer has clarified, add 3 drops of the colour reagent to the top of the layer without any agitation. The formation of a red-purple ring at the top of the ethyl acetate layer indicates the presence of sulfadoxine. Estimations may be made by visual comparison of the intensities of the red-purple colour in samples against those in the standards.

D. Operational notes

1. The red-purple colour will fade immediately upon significant agitation or mixing. The colour will fade slowly with time without any agitation or mixing. Therefore, a fresh set of standards should always be run concurrent with a batch of unknowns to be compared for estimation of sulfadoxine concentration.

2. Vials may be precharged with ethyl acetate and buffer solution prior to beginning the analysis of samples.

3. Occasionally a component may be present in a urine sample which reacts with the colour reagent at a slower rate than sulfadoxine, giving a green-blue colour. Since the reaction with sulfadoxine is immediate, a qualitative indication of sulfadoxine is valid and a later darkening of the solution is of no consequence.

4. The test will give a positive result for sulfonamide drugs and other extracted compounds containing an arylamine moiety.

5. Storing the colour reagent solution in the refrigerator extends its shelf-life. Degradation is indicated by loss of yellow-orange colour of the solution.

Annex 2

QUANTITATIVE COLORIMETRIC TEST FOR SULFADOXINE IN URINE

This test is based on the extraction of sulfadoxine from urine into ethyl acetate followed by formation of a violet-red Shiff's base upon addition of acidic methanolic p-dimethylamino-cinnamaldehyde. The intensity of the colour is proportional to sulfadoxine concentration in the sample and can be quantitated using a portable, hand-held, battery-operated filter photometer. A concentration value in ppm is determined by reference to a predetermined standard curve.

A. Reagents

1. Colour reagent solution: 0.1% (w/v) p-dimethylamino-cinnamaldehyde (DMACNA) and 0.2% (v/v) H_2SO_4 in methanol (100 mg DMACNA + 0.2 ml H_2SO_4 + 100 ml methanol).

2. Buffer solution: 1.5 M phosphate buffer, pH=5.5 (11 g $K_2HPO_4.3H_2O$ ___ 85 g KH_2PO_4 dissolved and diluted to 500 ml with water).

3. SAG-10 silicone antifoam emulsion diluted ca 50/50 with water.

4. Ethyl acetate.

5. Methanol.

6. Sulfadoxine standard: 1 g/L in ethanol.

B. Equipment

1. Clay-Adams pipetter and tips.

2. 1-dram clear glass vials with caps.

3. 13 x 100 mm screw-cap culture tubes.

4. 50 ul Hamilton syringe for spiking urine standards.

5. Dropper bottle (used for adding SAG-10 silicone antifoam emulsifier).

6. Portable, hand-held, battery-operated filter photometer with 535 nm filter.

7. Test tube rack.

C. Procedure

1. Standard curve: A standard curve is prepared by adding appropriate volumes of standard solution to 2 ml of drug free urine according to the following table:

Sulfadoxine (ppm)	Vol. (ul) of 1 g/L std. sol. added
0	0
1	2
3	6
7	14
11	22

2. Analysis of standards and samples: 1.0 ml of ethyl acetate, 0.5 ml of buffer solution, and 2 ml of urine are added to a 1-dram clear glass screw-cap vial using the Clay-Adams pipetter. The vial is capped and inverted 20 times by hand. After the ethyl acetate phase rises to the top, one drop of SAG-1-0 defoamer is added to the ethyl acetate layer to break the emulsion formed during inversion. If necessary, the ethyl acetate layer can be gently swirled in the vial to facilitate breaking of the emulsion. Using the Clay-Adams pipetter, 0.5 ml of the clear ethyl acetate layer is transferred to a clean culture tube containing 0.5 ml of the colour reagent solution. (Alternatively, another 1-dram vial can be used if quantification with the photometer is not desired.) The tube is capped, and the contents are mixed. If sulfadoxine is present, an immediate and distinctive colour change from yellow-orange to red-purple will be observed. If quantification with the photometer is desired, remove the cap, add 3 ml of methanol, replace cap, mix contents and place tube in sample-well of photometer for measurement of absorption intensity at 535 nm. A concentration value in ppm is determined by reference to a predetermined standard curve. (Alternatively, the addition of the methanol and use of the photometer can be omitted and estimations may be made by visual comparison of the intensities in samples against those in the standards.)

D. Operational notes

1. Storing the colour reagent solution in the refrigerator extends its shelf-life. Degradation is indicated by loss of yellow-orange colour of solution.

2. Vials may be precharged with ethyl acetate and buffer solution and the culture tubes precharged with the colour reagent prior to beginning the analysis of samples.

3. Occasionally a component may be present in a urine sample which reacts with the colour reagent at a slower rate than sulfadoxine, giving a green-blue colour. Since the reaction with sulfadoxine is immediate, a qualitative indication of sulfadoxine is valud, but a later darkening of the solution will affect quantification with the photometer.

4. The test will give a positive result for sulfonamide drugs and other extracted compounds containing an arylamine moiety.

Annex 3

DISCUSSION OF RESULTS OF WORKSHOP SAMPLES USING COLORIMETRIC TESTS

For the set of urine samples presented to the workshop participants to the analysed for the presence of antimalarials using the different methods (colourimeteric, TLC, immuno-analytical), a Saker-Solomons test using 2 ml of urine sample was first run on each sample. All samples giving a clearly negative result were re-analysed with the Saker-Solomons test using 10 ml of urine sample. Also, all the samples were analysed using the newly developed alternative (to Bratton-Marshall) test for sulfonamides in urine, dubbed the Mount sulfonamide urine test at the workshop.

Samples M1-7 of the set were artificially spiked samples, fortified with various combinations of chloroquine (CQ), mefloquine (MQ), quinine (QN), quinidine (QD), primaquine (PQ), sulfadoxine (SDX) and pyrimethamine (PY), all at a level of 0.1 mg/L. The Saker-Solomons test using 10 ml of sample (SS10) seemed to be picking up these low level combinations quite well. Only one of this series of samples was missed by the test which consisted of CQ and PQ. This is not disturbing, since for CQ a person with this level in th urine would likely have a blood level that was far below the therapeutically significant level and should be considered negative for the purpose of inclusion into a field study. This statement is probably true for all the drugs in this series at these levels, except for MQ. The Mount sulfonamide assay missed the 0.1 mg/L levels of SDX. The stated reliable detection limit for the method is 1.0 mg/L. Again, persons with a 0.1 mg/L level of SDX in the urine are likely to have therapeutically insignificant blood levels. However, after the key to the samples was revealed, one of the SDX samples was re-analysed. After allowing extra time for the reaction (about 30 seconds) and with a closer inspection of the test, a faint response was observed. However, reliability at this level is to be determined.

Samples C1-4 of the series were samples collected from healthy volunteers with CQ levels of 0.1-0.3 mg/L, according to the key. The Saker-Solomons test using 2 ml of sample (SS2) indicated a strong positive for these samples. The TLC and immunoanalytical assays indicated strong positives for CQ also for these samples. The limit of detection for the SS2 test is 1 mg/L. Thus, there seems to be some sort of discrepancy between the key and the responses that were observed for all three types of assays. Samples D1 and D3 were also from healthy volunteers having QN concentrations of 5.0 and 3.2 mg/L respectively. The SS2 test gave strongly positive results for these two samples.

Sample B1-4 were taken from patients dosed with Fansimef. The SS10 test adequately indicated the combined concentrations of MQ and PY. Also the Mount sulfonamide assay adequately indicated the presence of SDX.

Samples F1-3, H1-8, K1-3 and I5 were from patients having severe malaria. The SS10 test gave false positives for samples F2 and H7 and false negatives for samples H5 and I5 having CQ concentration of 3.4 and 0.5 mg/L respectively. The SS2 test also indicated a false negative for sample H5 and indicated a slightly positive response for sample I5. Many of the samples of this group were brown in colour with much precipitates. Following the workshop, the SS10 test was run on a sample of blank urine that had been sitting at ambient temperature on a laboratory bench for several months and was brown in colour. Some the brown colour extracted into the chloroform layer of the test mixture, giving the test a positive appearance. Thus, the Saker-Solomons tests may not be applicable to samples that have decomposed to a brown colour, which would not be the case for freshly collected samples, collected as part of a field study. The Mount sulfonamide test gave positive indications for samples H5 and K2 for which the sample key only indicated the presence of CQ. Perhaps the samples contained another commonly used sulfonamide other than SDX that was not analysed for by the analysts that prepared the sample key.

In order to further evaluate the SS10 test at detecting low levels of MQ in urine, a set of artificially spiked samples were prepared on request that they be fortified at levels of 0-1.0 mg/L MQ, using several different samples of blank urine. Sample number 1 of this series contained a combination of MQ, CQ and QN all at a level of 1 mg/L. The test gave a strongly positive indication for this sample. Samples 2 and 3 were blank samples. After discussion among the workshop participants using this test, it was decided that sample 2 was positive, but such is the problem of trying to detect such low levels of MQ in urine. Samples 4, 5 and 6 were fortified at levels of 0.2, 0.5 and 1.0 mg/L using the same blank urine sample in their preparation. The test gave a strongly positive indication for all three samples, much stronger than a test at the 1.0 mg/L level. Perhaps some other previously undetected drug was present in this supposedly blank urine sample. For samples 7, 8 and 9 prepared at the same respective levels as the previous samples but with a different sample of blank, the test gave the proper responses. The same pattern was again followed in the preparation of samples 10, 11 and 12, again with a different blank urine sample. The test failed to indicate the 0.2 mg/L level for sample 10, indicated a positive response for sample 11 with a level of 0.5 mg/L and failed to indicate a positive response for sample 12 having a level of 1.0 mg/L. Being that these three samples were prepared from the same blank urine sample, and thus, influences by urine on the test should be the same, it seems logical that if the test indicated a 0.5 mg/L level it should have indicated the 1.0 mg/L level even stronger. A possible explanation is that the preparer of the sample failed to add the MQ for sample 12.

For the SS10 test to be an adequate screen for detecting possible MQ dosing, the test needs to be adequately reliable at the 0.2 mg/L level. for the four credible samples in this set

of samples in the 0-0.2 mg/L range (2, 3, 7 and 10), the test indicated 1 false positive and 1 false negative. Thus this set of data suggests that the test has a 50% chance of accurately detecting the presence of MQ at the 0.2 mg/L level. The reliability of the test at this level needs to be further evaluated. If upon further evaluation the 50% reliability factor appeared to be the case for the test, this is probably better than the reliability factor for the Dill-Glazko test for CQ that is still used to some extent. If the SS10 test was the only test available to an investigator for screening in an in-vivo drug sensitivity field study where the incidence of prior MQ dosing was likely, at present, we would have to suggest that he collect finger-prick blood samples for those individuals testing negative with the SS10 test to be analysed later for MQ by HPLC to confirm that the individuals had not been previously dosed with MQ.

UPDATE OF THIN LAYER CHROMATOGRAPHIC FIELD TESTS FOR THE DETECTION OF MEFLOQUINE AND OTHER COMMON ANTIMALARIALS IN URINE AND FINGER PRICK BLOOD SAMPLES

B. Betschart, C. Ong & J. Koella

Introduction

During the Workshop on "The validation of chemical and immunological tests for antimalarials in body fluids"(1) a laboratory based comparison of the chemical, immunological and chromatographic methods to determine antimalarial drugs in urine samples was carried out by using 20 samples.

The thin layer chromatographic test involves the direct application of 25 ul urine onto the silica layer and the plates are developed in a solvent mixture of toluene/diethylamine/methanol(2). After the chromatographic separation the plates are directly evaluated under excitation with a UV-light. The evaluation of the 20 urine samples by three persons independently indicated the high reliability, specificity and sensitivity of the technique (Table 1).

Based on these results and the recommendations of the working group further developments were carried out on the basis of the thin layer chromatographic technique in order to facilitate the introduction of appropriate field tests:

1. A complete prototype testkit including all necessary items to determine chloroquine and quinine in urine samples under field conditions was prepared.

2. Creatinine as an internal standard for correcting drug levels in different urine samples was evaluated.

3. The relationship of chloroquine levels in whole blood and urine samples was studied in a limited set of samples.

4. The correctness of the categories used in semiquantitative, visual evaluations to group urine samples according to their chloroquine levels was examined.

5. The HPTLC method to determine mefloquine in urine and blood samples was adapted to include the method in the test kit for field applications.

6. The degree of correlation of mefloquine concentrations in plasma and urine samples was determined.

1. Development of a Prototype Test Kit

A ready-to-be-used prototype test kit was developed for the determination of chloroquine and quinine in urine samples(2). The test kit contains all necessary items to perform a semiquantitative determination of these antimalarial drugs on the spot. Sufficient consumables are included to carry out more than 1000 determinations without replenishment. A detailed self-explaining operating manual was compiled (see Annex). The approximate weight of the whole kit is 15 kg., a weight comparable with that of a regular suitcase. The kit was assembled in an aluminium container, lined with shock absorbing plastic foam to withstand rough roads and wet and/or hot environmental conditions. The tests can be carried out at any place. No electricity or air conditioning is needed.

In order to comply with international regulations for air transportation, the test kit was evaluated by air transportation authorities. According to these rules it is not possible to transport the kit with its solvents as regular luggage, but it can easily be transported as air freight, if the necessary safety requirements are followed and the declarations correctly filled out. All chemical solvents in the kit can be packed together in the commercially available aluminium box, approved for air freight transportation.

The simplicity of the test system was demonstrated in a workshop on new tools in the study and diagnosis of parasitic diseases held at the IVIC in Caracas, Venezuela, November 26 - December 14, 1990. The students were able to run chromatograms and to interpret the data correctly within one and a half days.

In its present form chloroquine and quinine in urine samples can be determined qualitatively for a fast response, semiquantitatively to obtain an information on the amount of the drug(s) in the sample, as well as quantitatively by using the included luminance meter or an appropriate scanner in a central setting. The quantitation is only possible if appropriate standards are spotted onto the plates. To avoid the cumbersome preparation of standards, three different concentrations of chloroquine and quinine were added to blank urine samples. One ml volumes of the drug-spiked urine samples were lyophilized and can be kept for at least six months without degradation. Before use, one ml of distilled water is added to one tube of each standard. Once they have been reconstituted and are stored without refrigeration they have to be used within two to three days. If the standards are kept frozen they can be reused for several weeks.

The performance of the luminance meter and the solar-powered UV-lamp, both included in the test kit, were evaluated. The intensity of the lamp is sufficient to give an accurate reading, if a few basic rules are followed (see operating manual, Annex 2). This combination of equipment can be used to quantify chloroquine and quinine samples, but due to its limited capacity should only be used if no other scanning device is available.

The prototype test kit with the luminance meter costs around US$5000. The price can be reduced to US$2000, if the luminance meter is omitted. The replenishment of consumables, once they are used up, amount to US$150 per 1000 samples.

2. The Effect of Using Creatinine Concentrations as an Internal Standard for Drug Levels in Different Urine Samples

In the last workshop it was recommended to study the predictive value of creatinine-normalized chloroquine concentrations in urine samples. One of the reasons for initiating this study was the finding that large fluctuations were encountered in individual chloroquine levels in urine samples of subjects, receiving a single dose of CQ (2) and Fig. 1. The influence of using creatinine as a normalization factor was studied in the 20 urine samples of the Penang Workshop (Table 2) as well as in urine samples from a study carried out at the Swiss Tropical Institute (Fig. 1). The difference were eliminated after adjustment to normalized creatinine levels. The observed variations in the urine levels are therefore not simply due to general fluctuations in the density of the different urine samples. A simple conversion of urine values to the blood levels will be questionable. This finding is in agreement with a similar study carried out on the mefloquine levels in urine and blood samples (see Mount D., this document and paragraph 6). It is too preliminary to conclude that urine values cannot be used, since it is possible that the observed fluctuations only occur at lower drug levels (< 5 mg/l) at a time point, when the blood levels are anyway of no therapeutic relevance (see below).

3. The Relationship of Chloroquine Levels in Whole Blood and Urine Samples

During a five week study 44 volunteers from the Swiss Tropical Institute Field Station in Ifakara, Tanzania provided blood and urine samples once weekly. Blood samples were spotted onto filter papers and all samples were quantitatively analysed at the STI (Table 3) to study the relationship between the CQ levels in whole blood and urine. As shown in some representative samples (Fig. 2) large individual variations in the <u>total amounts</u> of CQ were found, a situation expected to occur in endemic areas. In spite of that fact a correlation between urine and blood levels could be detected: high blood levels correlated with high urine levels. Yet, the individual variations, on the basis of this data set, do not allow an

accurate estimation of the exact blood levels and further studies involving people under therapeutic regiment have to be carried out.

The urine values are a good indicator whether or not somebody has consumed a specific drug recently. The determination of the exact level of a drug in the plasma or blood to verify the presence of a therapeutically relevant concentration has presently to be done on blood samples directly if needed at all. Probably a large number of situations occur where it is only necessary to monitor the presence of relevant amounts of a specific drug.

4. The Value of Categories Used in Semiquantitative, Visual Evaluations to Group Urine Samples According to their Chloroquine Levels

If large scale screenings for antimalarial drugs are made, it is important to have appropriate criteria for a correct interpretation of the data. Wh ch chloroquine levels in urine indicate a significant, recent d' ; consumption? At which level has the presence of a drug to regarded as of no therapeutic relevance? The specific objectives of the studies will influence the type of data needed, but these questions become especially important if very sensitive detection methods are used e.g. like the immunological tests.

Since 1989 annual surveys were carried out among school children in two villages in the Kilombero river area, Tanzania. At each survey urine samples were collected and analysed directly in Ifakara for the presence of chloroqouine by using TLC-technique. The samples were semiquantitatively evaluated and grouped into five categories by visually comparing the intensity of the fluorescent chloroquine spots with the intensity of spiked urine standards. The same plates were later quantitatively evaluated in our laboratory by using a TLC-scanner.

During this study 50 ul urine samples were directly applied onto the plates (the application volume had been reduced to 25 ul urine in the meantime). During the first survey the standard concentrations had to be adjusted several times from initially 0.5, 4 and 12 mg CQ/L to 1, 10 and 100 mg CQ/L urine to cope with unexpected high CQ concentrations in some of the samples. Nevertheless, it was possible to compare the semiquantitative and quantitative data by using five standard categories (see Table 4).

Sixty percent of the samples contained no chloroquine or only traces of it. The distribution of the samples within some of the categories is indicated in Fig. 3. The correctness of the visual estimation was excellent since 84% of the negative samples were scored correctly (see Table 5). Of the samples, which were estimated to be negative, 15.4% belonged in category 1 and only 0.6% belonged into category 2 and were misplaced. No samples which contained chloroquine were missed. Only 5 samples (0.12%) were judged to contain chloroquine in large quantities (between 20-50 mg/L) whereas they actually contained no drugs. This small number of samples has obviously been misinterpreted. In the categories 2-4 the number of samples put in wrong categories increased and was most significant in category 3, which comprised 6% of all the samples. This indicates that it is difficult to estimate visually whether a positive sample is close to 20 or to 40 mg/L. Since concentrations above 5 mg/L urine have to be regarded as significant (see chapter 2 & 3) it is proposed that three to four categories (see data sheet in operating manual) are sufficient for epidemiological studies. A grouping of the study samples according to this new criteria would result in 64% of the samples in category 1 (> 1 mg/L), 16% in category 2 (1-5 mg CQ/L) and 20% in category 3 (5-50 mg CQ/L urine). Further studies are required to verify the appropriateness of these categories. The relevance of the categories has also to be critically analysed with respect to their relationship with the actual blood values discussed in section 3.

The study clearly showed that TLC of urine samples followed by visual estimation of the intensity of the chloroquine spots is a reliable, specific and fast method to discriminate between low and high levels of chloroquine in urine samples under field conditions. The method can be used perfectly on an individual level to analyse single samples as well as on an epidemiological level where large numbers have to be processed.

5. Determination of Mefloquine in Urine and Blood Samples in Order to Include them in a Test Kit for Field Applications

The determination of mefloquine in urine and plasma samples causes special problems due to the lack of a sufficiently strong native fluorescence as well as due to the low concentrations of the drug in urine samples. These facts require an initial extraction step and a changed mode of detection to allow the reliable detection of mefloquine. An instrumental HPTLC - method for the determination of mefloquine in urine and plasma samples was first established(3). Briefly, the samples are extracted by using 1000 ul of whole blood or urine with 3 ml H_2O, 1 ml 5 N NaOH and 5 ml heptane/isoamylalcohol (100/1.5). After extraction for 20 minutes 4 ml of the organic layer are evaporated and the residue is taken up in 50 ul methanol. The samples are separated on HPTLC silica gel 60 plates using a solvent of toluene- diethylamine-methanol (8/1/1). The plates with the samples are densitometrically evaluated by using UV

absorption at 290 nm and a CAMAG TLC Scanner II in reflectance mode. The method has a good sensitivity and a sufficient extraction efficiency (Table 6). The HPTLC method was compared with an established HPLC technique(4) in an interlaboratory study and a good agreement between the data of the paired samples of the two methods was obtained (r = 0.9867).

To allow the determination of mefloquine under field conditions the quantitative HPTLC method was modified: 1 ml urine (or 0.5 ml plasma and 0.5 ml H_2O) are mixed with 0.25 ml 5 N NaOH and 0.75 ml heptane/isoamylalcohol (100 + 1.5) in an Eppendorf tube. The extraction is carried out for 20 minutes at room temperature and the 0.75 ml of the organic layer are aspirated with a Pasteur pipette and transferred into an Eppendorf tube for passive evaporation or for evaporation under a stream of nitrogen, if available. The residue is taken up in 25 ul of methanol and applied manually as a spot onto a silica layer with a 254 nm fluorescent indicator incorporated in the layer. The plates are developed in the solvent toluene/diethylamine/methanol (8/1/1). Visual detection is carried out under excitation at 254 nm by using the mefloquine induced quenching of the fluorescence indicator.

The absolute lower visual detection limit is 100 ng on the plate corresponding to 350 ug/L plasma or 175 ug/L urine. The test can be carried out in simple laboratory settings with an appropriate infrastructure (e.g. with a simple hairdryer) for the evaporation step.

According to published pharmacokinetic data (e.g. Riviere et al.,(5) plasma levels above 100 ug/L plasma can be taken as a significant indicator for a recent mefloquine consumption. Among five cases only in one case a decline of the plasma to around 100 ug/L was found already after four days. The others showed these levels only after a period of more than 16 days. Levels above 100 ug/L could only be detected with the field adapted TLC-method, if around 1 ml plasma would be used. The data of Lobel et al. (6) on the effectiveness of malaria prophylaxis with mefloquine levels of roughly 400 ug/L whole blood. If these data can be confirmed, then it would be necessary to detect levels above 500 ug/L and 0.5 ml blood would then be sufficient to detect these values.

6. The Degree of Correlation of Mefloquine Levels in Plasma and Urine Samples

Plasma and urine samples from patients under prophylaxis were used to determine the actual mefloquine levels and to study the relationship between the mefloquine concentrations in the two body fluids (Fig. 4). The patients showed plasma levels of 300 - 1800 ug/L and high plasma levels correlate with high urine levels. In general the urine samples show higher concentrations than the plasma samples. It is therefore possible to use urine samples, in places where the collection of urine samples is feasible to determine the presence of mefloquine by using the

TLC method, since a sufficient sensitivity can easily be reached. The degree of correlation in this set of samples is similar to the one described by D. Mount (this document) and also described for chloroquine in this chapter. Again, it is obvious that a direct conversion of urine levels to blood levels will not be possible and the same arguments have to be applied as discussed above with chloroquine levels. The TLC method has the advantage that it can be used to detect a potential consumption of other antimalarial drugs simultaneously and provides also informations on the presence of several drug metabolites.

Conclusion

The prototype test kit allows a simple, fast, sensitive and specific semiquantitative or even quantitative determination of different antimalarial drugs of up to 25 urine samples in one run under field conditions. A variety of data can be obtained on an individual as well as on an epidemiological level. The method can also be used to determine antimalarial drugs in blood samples, if an extraction step prior to analysis is included. At present, the extraction step should be carried out in a simple laboratory environment. The method has its potential in a variety of settings. Additional field studies should show its practical value.

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Table 1: Results of a semiquantitative visual evaluation of the presence of chloroquine in urine samples** by three independent persons using thin layer chromatography

Conc.	< 1 mg/L			1 - 5 mg/L			5 - 50 mg/L			> 50 mg/L		
Person	1	2	3	1	2	3	1	2	3	1	2	3
Sample No.												
B1	+	+	+									
B2							+	+	+			
B3										+	+	+
B4				+	+	+						
B5	+	+	+									
B6	+	+	+									
B7	+	+	+									
B8	+	+	+									
B9	+	+	+									
B10	+	+	+									
B11	+	+	+									
B12	+	+	+									
B13	+	+	+									
B14										+	+	+
B15				+	+	+						
B16	+	+	+									
B17				+		+		+				
B18	+	+	+									
B19				+	+	+						
B20			+	+	+							

** The samples are the same as indicated in Table 2, where the actual levels of chloroquine are also shown.

Table 2: Effect of creatinine concentrations on CQ levels in the urine samples B1-B20 from the Penang workshop 1989

Nr.	Creatinine	CQ*	QN	MQ	Corrected values **
1	no urine	-			
2	1.43	18.4			--
3	3.04	100			23
4	0.31	4			59
5	2.17	<1			23
6	0.56	<1			--
7	1.44	<1	12.4		--
8	1.96	<1	62		15
9	2.04	<1	124		57
10	2.5	<1		<1	109
11	1.5	<1		3	--
12	0.31	<1		1	4
13	0.54	<1			6
14	no urine				--
15	3.97	6.8			--
16	1.64	<1			3
17	0.6	10.6			--
18	2.19	<1			32
19	0.89	2.4			--
20	2.74	0.5			5
					0.3

*
**mg/L
1.8 g creatinine/100 ml is used as standard concentration

Table 3: Comparison of chloroquine concentrations in matched urine and plasma samples of 44 volunteers of Ifakara, Tanzania

	Urine	(%)	Blood
Samples collected	194	100	182
Samples determined	194	100	105*
Nr. positive	119	61	90

The samples were collected weekly during five weeks.

* The urine samples were first analysed and from the 119 positive samples it was possible to select 95 matched blood samples. Ten blood samples where included as controls from people which contained no CQ in the urine.

Table 4: Distribution of 1524 urine samples in the five concentrations categories estimated by visual comparison of the CQ fluorescence with the fluorscence intensity of the included CG standards

	Categories	N	%
0	(<= 0.5 mg CQ/L)	922	60
1	(0.5 - 4 mg CQ/L)	228	15
2	(4 - 20 mg CQ/L)	219	14
3	(20 - 40 mg CQ/L)	89	6
4	(>= 42 mg CQ/L)	66	5

Table 5: Comparison of the sample distribution of the visual estimation with the distribution obtained after quantitative evaluation

Categ.	Measured					
observ.	0	1	2	3	4	Total
0	772	144	6	0	0	922
1	22	168	37	1	0	228
2	11	57	109	25	17	219
3	3	1	26	6	53	89
4	2	0	8	12	44	66
Total	810	370	186	44	114	1524

The criteria for the categories are given in Table 4

Table 6: Sensitivity of the quantitative HPTLC method for mefloquine determinations in urine and plasma samples

Limits of detection	
absolute	10 ng
in urine and plasma	20 ug/L
Linearity	10 - 200 ng
Extraction efficiency	
urine	78% +/- 10 (13)
plasma	76% +/- 14 (29)

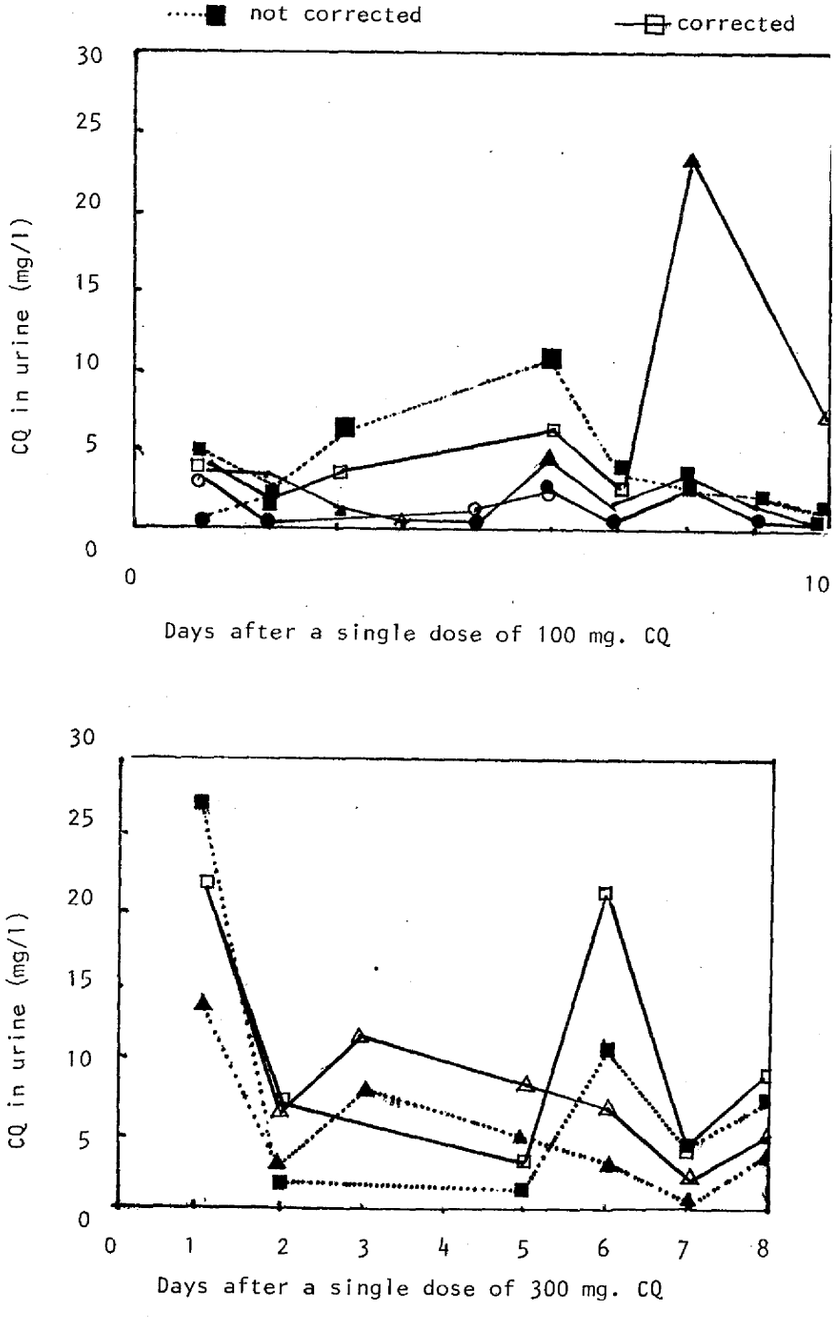


Fig. 1: Comparison of chloroquine levels in urine samples of volunteers which obtained either 100 mg CQ or 300 mg CQ (.....). The creatinine concentrations in these urine samples were determined and the chloroquine concentrations were corrected to correspond to a normalized creatinine amount of 1.8 g/100 ml (------).

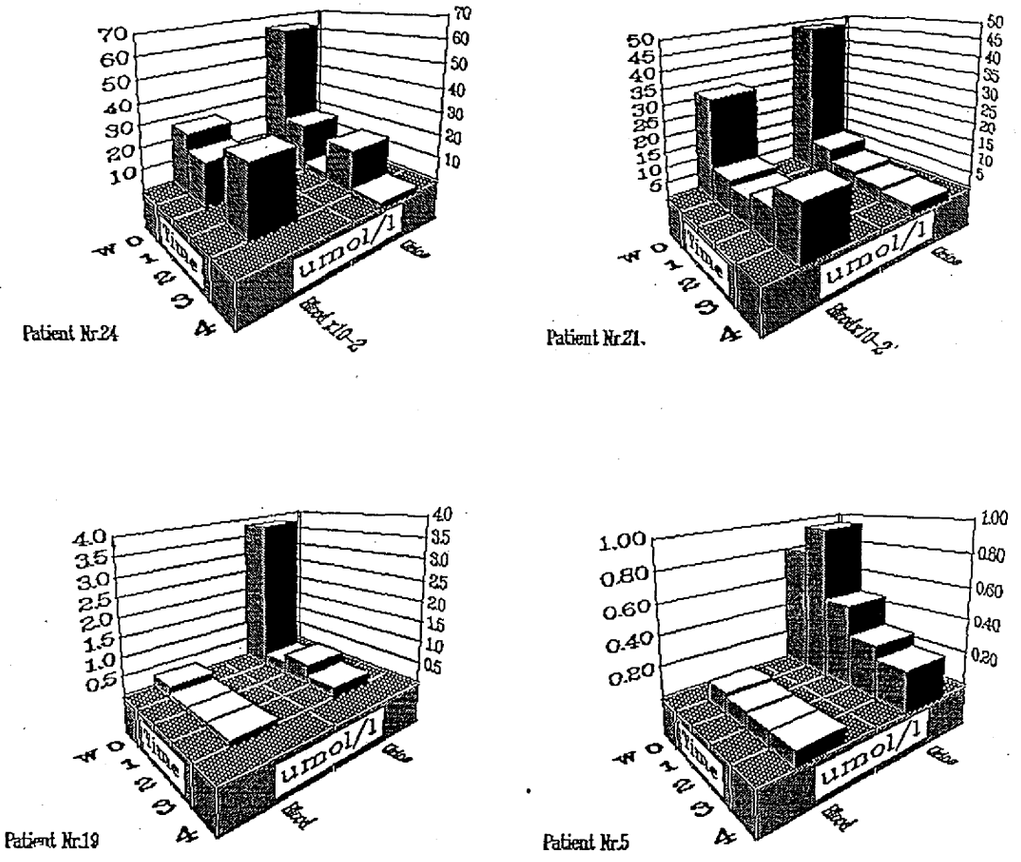


Fig. 2 The relation of chloroquine concentrations between whole blood and urine samples. Four representative samples with decreasing concentrations of chloroquine were selected and compared. Blood and urine levels are given in umol/L.

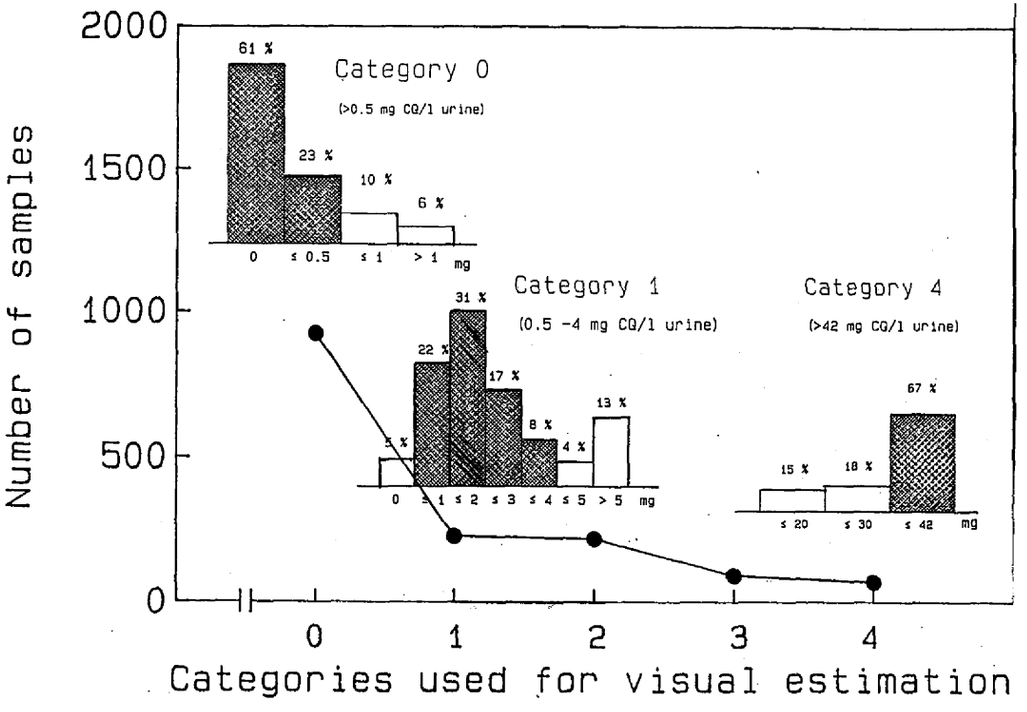


Fig. 3: Comparison of the number of samples categorized visually with the ones of the instrumental, quantitative evaluation (insets). The dark bars show the percentage of samples correctly estimated. The distribution of the samples in categories 2 and 3 is not shown.

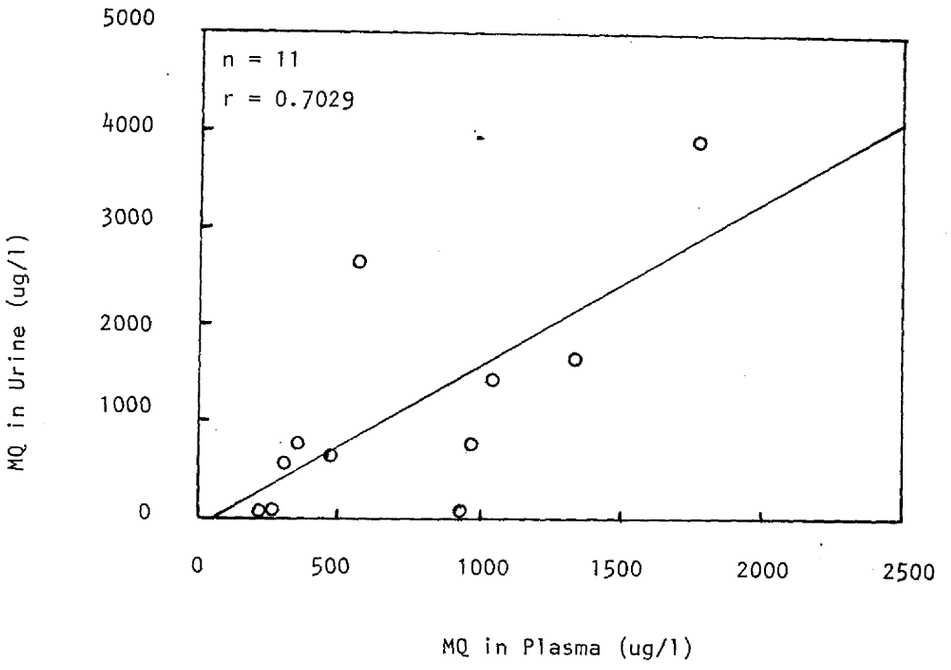


Fig. 4 Regression analysis of mefloquine levels in urine and plasma samples determined by using the quantitative HPTLC technique.

ANNEX: Considerations on the problems of using urine samples of severe malaria patients and the concommitant failure to detect chloroquine in these samples

A surprising result of the study of urine samples during this workshop was that neither the TLC nor the immunological (ELISA and DIPSTICK) or the colorimetric tests were able to reliably detect chloroquine in urine samples of severe malaria patients (see Results and Conclusion section, Table 1). the HPLC values indicated the presence of significant amounts of chloroquine (between 0.3 - 10.2 mg/L). Most of these samples should have given a clear positive result with all three test systems.

Some of the samples were reanalysed by TLC and 50 ul urine was applied which increased the sensitivity of detection twice. Visual as well as densitometrical evaluation of the samples failed to identify any chloroquine on the plates. These urine samples had a brown-reddish colour and on the places of the TLC plates, where such samples have been spotted a brown band was visible after chromatographic development.

A brown colour of urine samples might be an indication for a renal failure in these malaria patients with a concomittant excretion of porphyrines. It is suggested that chloroquine might be complexed in a way similar to or even identical with the well-known ferriprotoporphyrin IX-chloroquine complex. A complexation of quinine and mefloquine with ferriprotoporphyrin IX was recently reported [Sugioka and Suzuki BBA, 1074, 19-24 (1991)] and should also be considered. Such a complex would behave differently than the free drug and might not be detected with the three methods.

The phenomenon was so far only detected in these urine samples containing chloroquine. If the hypothesis of the complexation can be confirmed, it would be necessary to extend the study also to blood samples of severe malarial patients. It might be necessary that samples of severe malaria patients have first to be pretreated to dissociate the drug complex, if a complexation is occurring. A possible way would be to use the extract procedure of the HPLC technique used for the analysis of these samples, since the HPLC method was capable to chromatographically detect the drugs.

Annex 2

O P E R A T I N G M A N U A L

FOR

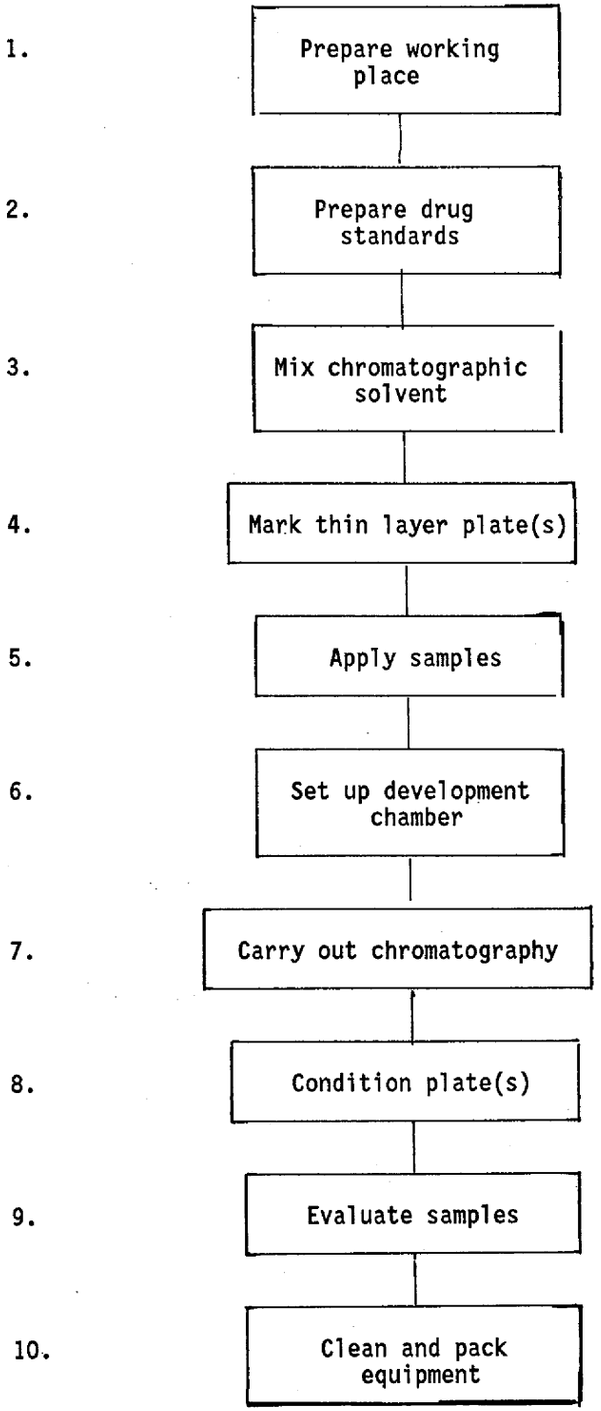
PROTOTYPE FIELD TEST KIT FOR THE DETERMINATION OF ANTIMALARIAL DRUGS

Prepared by Dr. B. Betschart, Swiss Tropical Institute, Basel, Switzerland on behalf of the WHO Steering Committee FIELDMAL.

CONTENTS OF MANUAL

1. FLOW SHEET

The major steps involved in the determination of antimalarial drugs in urine samples:



General Remarks

The field assay is especially designed for the determination of chloroquine, quinine and their metabolites in urine.

If you are not familiar with the assay, you will need to start by having a good look at the test kit and checking the contents, and also reading through the manual so that you understand the procedure.

2. MATERIAL IN THE TEST KIT

The components of the test kit are listed here as they are arranged in the aluminium suitcase (with 2 keys). The letters refer to the photographs inside the lid.

Some of the equipment is described in greater detail in the annexes at the end of the manual.

Lid of the suitcase with:

1 black cloth hood, permanently fixed to the inside of the lid (A)

Top layer in the suitcase with:

1 operating manual with four annexes

Chromatographic solutions: (B)
- 2 bottles with 80 ml toluene
- 2 bottles with 100 ml hexane-paraffin mixture
- 2 bottles with 10 ml diethylamine
- 2 bottles with 10 ml methanol absolute

Standards: (C)

For chloroquine (CQ):
- 4 bottles with 1 ml lyophilized urine (1 ug CQ/ml)
- 4 bottles with 1 ml lyophilized urine (5 ug CQ/ml)
- 4 bottles with 1 ml lyophilized urine (50 ug CQ/ml)

For quinine (QN):
- 4 bottles with 1 ml lyophilized urine (5 ug QN/ml)
- 4 bottles with 1 ml lyophilized urine (25 ug QN/ml)
- 4 bottles with 1 ml lyophilized urine (100 ug QN/ml)

Distilled water:
- 6 bottles with 10 ml distilled water each

HPTLC plates: (D)
1 box with 50 HPTLC silica gel plates (10 x20 cm)
1 empty box for storage of the chromatographed plates

Auxiliary equipment: (E)
1 variable Eppendorf pipette 10 - 100 ul (black with yellow top)
1 fixed Eppendorf pipette 1000 ul (blue)
1 30 cm ruler
1 soft pencil
1 pencil sharpener

Solar powered UV-lamp: (F)
1 lamp case with solar panel and a precharged battery pack in place
1 clear plastic lamp protection
1 bulm (366 nm excitation) installed
1 replacement bulb
1 package of 3 replacement batteries (precharged)

Bottom layer with:

Horizontal development chamber: (G)
1 Teflon chamber
1 glass counterplate
1 package with glass strips for solvent troughs
4 glass cover strips
1 spirit level
12 blue Eppendorf tips

Luminance meter LS-110: (H)
1 case with luminance meter
1 luminance meter
1 replacement battery
1 close up filter
1 photographic stand

Diverse items: (J)
1 box with paper towels
2 plastic bags with Eppendorf tips
1 big glass pipette with a large rubber bulb

METHOD

1. Preparation of the Working Place and Lamp

The test kit can be used in any suitable place with a table big enough to allow the handling of the urine samples.

As a first step, take out the "Sunlite" lamp, and put it in a place where the blue solar panels are in a good light, so that the batteries will be well charged.

2. Preparation of Drug Standards

Two different antimalarial drugs are provided as lyophilized standards in urine. For each drug three suitable standard concentrations are provided.

There are four complete sets of standards. The standards can be used for at least a week if the bottles are closed after use.

For each drug to be tested, standard solutions at the three different concentrations have to be reconstituted.

Reconstitution of Standards:

- Use the distilled water from one of the aluminium-capped bottles.
- Using the 1 ml Eppendorf pipette and a blue tip, add 1 ml distilled water to each of the three standard bottles of the drug to be used.
- After capping the bottle, dissolve the contents by gentle shaking.
- Write the date on the label.

This ready-to-be-used standard solution is stable for at least one week if properly sealed after each use.

3. Chromatographic Solvent

- Take one 100 ml bottle with 80 ml toluene, and pour the contents (10 ml each) of one bottle of methanol and one bottle of diethylamine into the toluene bottle to give a final proportion of 8/1/1.
- Close the bottle and mix the solvents by gentle shaking.
- Write the date of mixing on the label.

This mixture is stable for at least one month, if the bottle is closed properly.

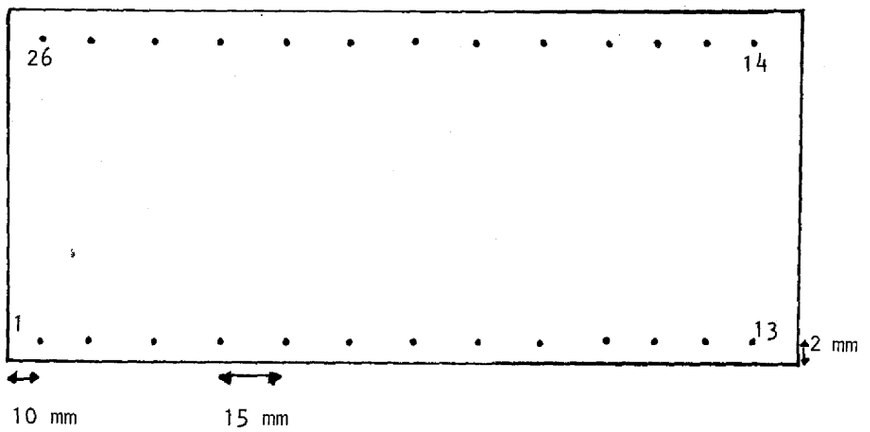
4. Preparation of the Thin Layer Plates:

- Remove one silica plate from the cardboard box. Do not touch the white silica layer and do not bend the plate.
- With the soft pencil and the ruler apply marks along the two long sides of the plate (see scheme below):
  - The first mark is made at a distance of 1 cm from the left-hand edge of the plate.
  - The marks should be made 2 mm from the lower edge at intervals of 15 mm. This allows you to apply a total of 26 samples per plate.
  - Number the first and the last application spots for identification purposes (1 and 13, and 14 and 26).
  - Number each plate and put the date on it.

If more than twenty samples have to be assayed, several plates have to be prepared.

If only a few samples have to be assayed the silica plate can be cut into two halves (10 x 10 cm) with scissors.

DIAGRAM OF PREPARED PLATE



5. Sample Application:

- Assemble the samples and standards in the order in which they are to be applied.
  The three standard dilutions of the drug(s) should be applied first beginning with the smallest concentration.
- Use the black Eppendorf pipette with a yellow tip to apply the urine samples.
  Apply the samples directly onto the predrawn marks, beginning with Nr. 1. The pipette tip should not touch the coating on the plate.
- Use a separate pipette tip for each sample.
- A total volume of 25 ul of each sample has to be applied. The diameter of the spot must not be more than 1.5 cm (slight overlapping of the spots does not matter).
- It is normally necessary to apply two portions of 12.5 ul to each spot, to prevent the spots becoming too big. A suitable procedure is as follows:
  - In a first round, apply 12.5 ul of each sample.
  - By the time all samples have been applied, the first spots will be dry.
  - In a second round, apply another 12.5 ul on top of the first spot, giving a total volume of 25 ul.
- Let the applied spots dry completely.

The Eppendorf tips can be re-used after washing three times with 70% ethanol.

Notes:
1. To avoid using too many pipette tips, you can leave the tips removed after the first round in the sample tubes, and re-use them for the same sample in the second round!
2. You may find that you can apply 25 ul at once, slowly, without the spots becoming too big, if you are working in a warm dry climate. If a fan or hairdryer is available a gentle stream of air can speed the drying process.

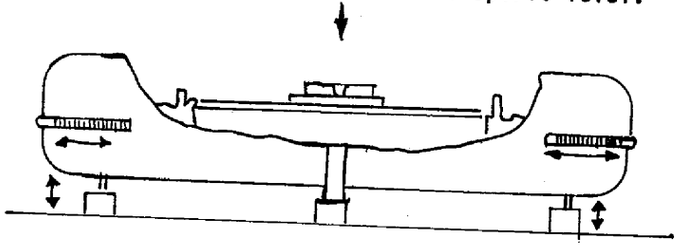
6. Horizontal Development Chamber

The Development Chamber (Part G of the testkit) is described in Annex 1, with a diagram. The numbers in the following description refer to that diagram.

- Remove the white development chamber and its parts from the box. There should be one glass plate, four glass strips and a package with thin glass strips.
- Place the development chamber on a horizontal surface.

Leveling the Chamber

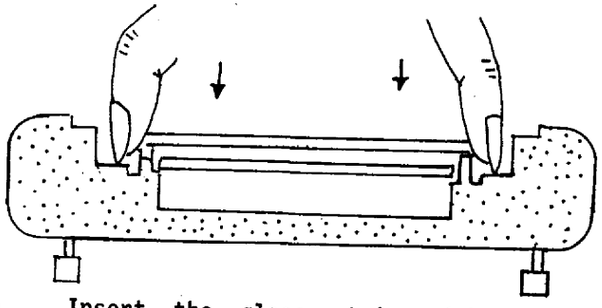
- Insert the glass counterplate (2). Place the spirit level on it.
- Level the chamber by turning the wheels on the corners (6), which adjust the feet. Remove the spirit level.



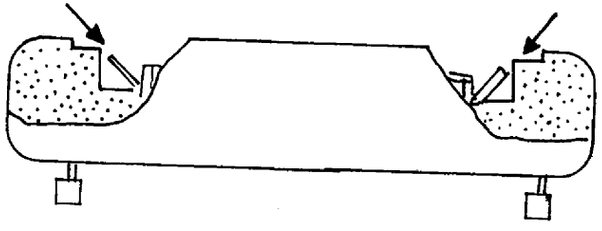
7. Chromatography

This step should be carried out in a place with a good access of fresh air, e.g. at an open window or out of doors, but direct wind should be avoided.

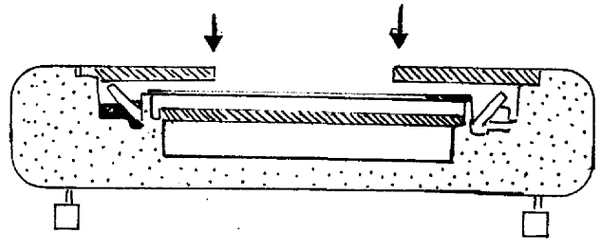
- Position the HPTLC silica plate with the applied samples facing upwards on the bridges on each side of the glass counterplate, holding the silica plate between the thumb and the index finger.



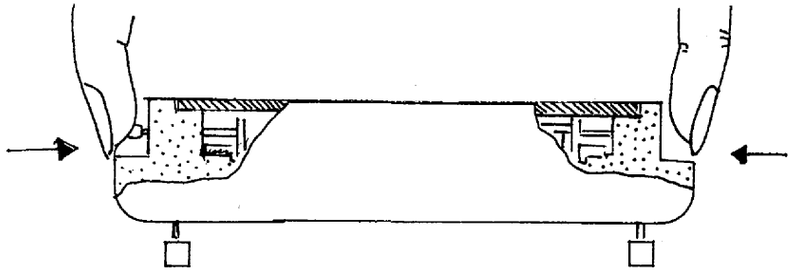
- Insert the glass strips (4) into the troughs that run along the long sides of the counterplate. The strips must be sloping outwards (see diagram).



- Add 5 ml solvent mixture (diethylamine/methanol/toluene) to each trough (3) using a blue Eppendorf tip. Cover the solvent troughs with the two thick glass strips (5).



- Bring both the two thin glass strips into an upright position at the same time, by pushing the two rods (6). The strips should then be in contact with the edges of the silica plate.



The solvent instantaneously rises in the capillary slit and enters the silica layer:

DEVELOPMENT STARTS

Should this not happen, or should development occur unevenly, the plate was not correctly positioned. Tilt the glass strips outwards again, and position the plate correctly. Continue chromatography by pushing the rods again.

Stopping Development

- Stop chromatography when the two solvent fronts have met in the center (after approximately 20 minutes).
- Take off the cover glass strips (5), tilt the thin glass strips (4) outwards.
- Lift the HPTLC plate by means of of lever (8) and take it out.

The plate should never be left longer than 5 minutes more than the regular developing time. If it is left longer, the separation zones diffuse.

If a new plate has to be developed start again with point 4.

EVALUATION OF THE RESULTS

8. Conditioning the plates

(If an immediate evaluation of chloroquine is desirable than this step can be omitted until later. However, the plate must then be examined between five and twenty minutes after the chromatography was stopped. The native of fluorescence of chloroquine fades after this time. Quinine cannot be seen without conditioning.

The plates should first dry for at least 1 hour at room temperature before further processing. (After drying, the plates can easily be stored in the empty cardboard box for up to a month without further processing.)

The conditioning step enhances and stabilizes the fluorescence of the drugs. It is carried out in the horizontal development chamber after the chromatography is finished.

- Remove the glass support (20 in the middle the fluorescence.
- Take one bottle with a mixture of 100 ml hexane-paraffin, and using the big glass pipette transfer about 40 ml (two fillings of the pipette) into the central part of the chamber (7).
- Soak each developed plate in this solution by pulling the plate, with the white silica layer facing up, slowly through this solution. (The plate will have to be gently curved in order to wet it evenly.)
- Put the plate with the white layer facing upwards on a paper towel (paraffin is sticky!).
- After conditioning of all plates the rest of the paraffin-hexane solution should immediately be replaced in the bottle, again using the glass pipette.

  The solution can be repeatedly used.
- When the silica layer is dry, the aluminum side of the plate can be wiped clean with a paper towel.

9. SAMPLE EVALUATION

Samples are evaluated by looking for bands which are fluorescent in UV light, and comparing them with the standards. This can only be done satisfactorily in a really dark place. If you do not have a room available that can be made completely dark, you can use the black cloth attached to the lid of the suitcase, with the lid upright.

- Take the solar-powered UV lamp with the blue bulb, and remove the plastic cover from the bulb. The switch for the lamp is on the back of the case.
- The plate can be positioned upright against the suitcase lid.
- Place the black cloth over your head and shoulders so that outside light is eliminated.
- Hold the lamp a few centimeters in front of the plate and move it from sample to sample.
- Positive samples show a blue fluorescent line (see photograph below).
- Compare the brightness with that of the standards to obtain an assessment of the quantity of drug present.
- Fill out the data sheet.

Table showing original drug concentrations in urine samples

Standard Nr.	CQ in urine (ug/ml)	QN in urine (ug/ml)
1	1	5
2	5	25
3	50	100

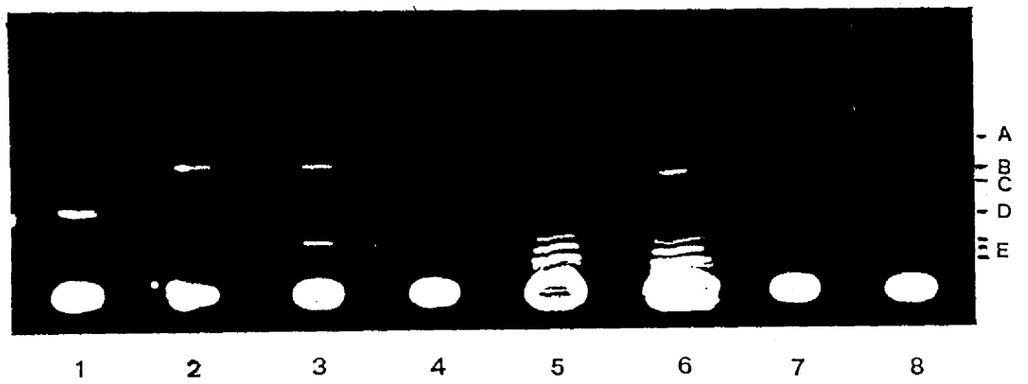
SEMIQUANTITATIVE EVALUATION EXAMPLE:

If a sample is found with a fluorscence between standard Nr. 1 and 2, then it can be concluded that it contains between 1 to 5 ug CQ/ml.

This visual estimation allows a fast and easy categorization of the antimalarial drugs in the samples.

A QUANTITATIVE DETERMINATION USING A LUMINANCE METER IS DESCRIBED IN ANNEX 4. THIS CAN BE USED IF APPROPRIATE LABORATORY CONDITIONS ARE AVAILABLE.

A typical photograph which demonstrates the relative migration distances of different antimalarial drugs separated on HPTLC-silica plates with toluene:diethylamine:methanol (8/1/1) as the chromatographic solvent.



Sample composition:

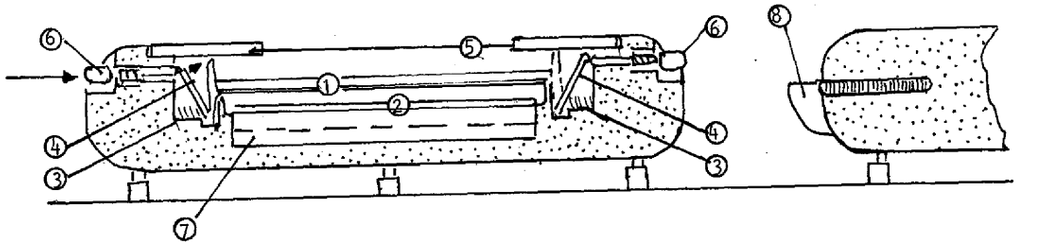
1. Desethylchloroquine added to normal urine
2. Chloroquine added to normal urine
3. Urine sample with metabolized chloroquine, desethylchloroquine and bidesethylchloroquine
4. Quinine added to normal urine
5. Urine sample with metabolized quinine
6. A 1:6 mixture of urine samples 3 and 5 to indicate a composite picture of metabolized quinine and chloroquine
7. Primaquine added to normal urine
8. Amodiaquine added to normal urine

A = Amodiaquine; B = Chloroquine; C = Primaquine; D = Quinine or desethylchloroquine; E = other metabolites.

Annex 1 CAMAG Horizontal developing chamber

Components

(1) HPTLC plate (silica layer facing up)
(2) glass counterplate as a support
(3) solvent troughs (each holding 5 ml)
(4) thin glass strips
(5) covering glass strips (thick)
(6) push rods to tilt the glass strips
(7) conditioning tray for postchromatographic treatment (approximately 40 ml)
(8) lever (to raise the chromatogram and the counterplate)



Annex 2A

Operating instructions for the solar fluorescent handlight (Sunlite)

The Sunlite runs off rechargeable batteries. They can be charged by means of light falling on the blue solar panel.

When the batteries are fully charged, the light should function for more than an hour.

The Sunlite is provided precharged, but if it has been kept for a longer period (several days) in the box it should be exposed to full sunlight.

CAUTION

Do not use alkaline batteries in your lamp; they may cause damage. If you have to replace the batteries use 3 rechargeable nickel-cadmiun AA batteries.

Using the Sunlite:

The sunlite works simply by sliding the yellow switch on the back up toward the fluorescent bulb. To check the presence of antimalarial drugs on chromatographic plates the plastic shield over the bulb has to be removed.

Care of the Sunlite:

Batteries: The three rechargeable nickel cadmium batteries in the Sunlite are designed to function for about 1,000 charging cycles. For the best performance keep the Sunlite indoors in a sunny place when not in use (e.g. hang it up near a window).

Fluorescent lamps: For most antimalarials the blue blub (366 nm) should be used.

Solar panel: The solar panel needs no maintenance, other than an occasional cleaning to help it operate at a higher efficiency. Be careful! The solar panel is made of glass.

Trouble Shooting

If the Sunlite fails to operate, please take the following steps:

a. Examine the bulb to make sure that it has not been broken or loosened from its oscket; if it is loose, insert it properly into the socket. If necessary, tighten the socket holders. If the lamp is broken or has been in use for a very long period, replace it.

b. Examine the solar panel to see if it is broken. If so, it has to be replaced.

c. If the lamp will not work after a two-day charging period, carefully open the battery case with a coin in the slot, gently lifting the cover. Check to make sure the battery cells are all making good contact.

d. If the battery contacts are loose, bend them a little to make good contact and then charge for two days from sunup to sundown.

Annex 2B

Handling of Eppendorf pipettes

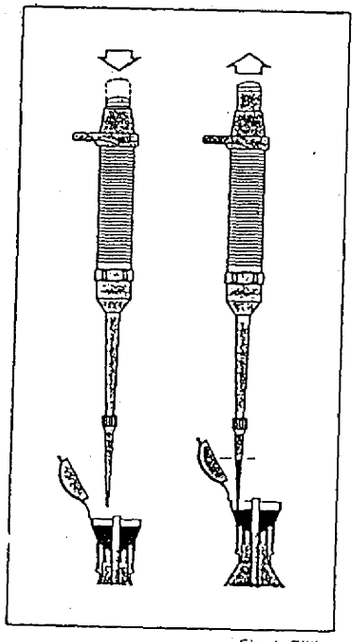


Fig. 4: Filling

Fig. 3:
Holding the pipette properly

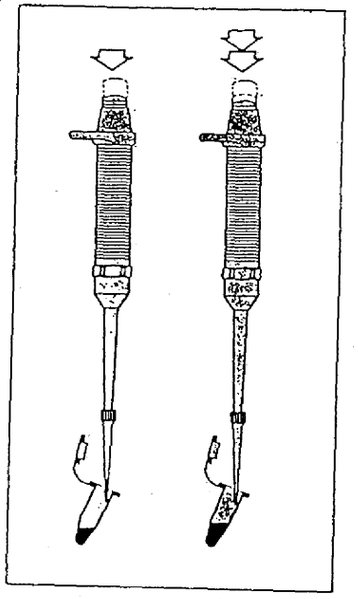


Fig. 5: Emptying

Filling
Never aspirate liquid without attached tip!

- Hold pipette acc. to Fig. 3

Caution: Pipettes with filled tips must never be laid down!

- Press the button downwards to the first stop
- Immerse pipette tip about 2 - 3 mm into the liquid
- Allow the button to glide back to the top slowly

Caution: Never let it snap back!

- Slide the tip out along the inside of the vessel
- *Wipe off pipette tip carefully with a lint-free tissue*

Remark: When pipetting serum, rinse the tip 2 - 3 times and reject serum.

Emptying (Fig. 5)

- Hold pipette tip against the inside of the vessel
- Slowly press the button downward to the first stop
- Hold for 2 - 3 sec
- Press button to the final stop
- Hold button in this position and slide the pipette out of the vessel
- Let the knob slide back slowly to the stop

eppendorf

Fig. 5: Volume adjustment

volume µl		counter display
0.5 – 10		00.50 00.60 00.70 00.80 10.00
10 – 100		010.0 010.1 010.2 100.0
100 – 1,000		0100 0101 0102 1000

2. Operation

2.1 Volume selection

After selecting the volume range (Variopette 0.5 - 10, 10 - 100 or 100 - 1,000 µl), the volume is adjusted by turning the control button (Fig. 5).

Note:

When using the 0.5 - 10 µl Variopette, care must be taken that a zero ("0") appears in the second place after the decimal point. The following volume settings are possible:

2.2 Filling (Fig. 7)

IMPORTANT:
Never pipette liquid without pipette tip attached!

- Choose the most functional working position.
- Hold the pipette housing according to Fig. 6, using the flange around the digital display as a rest for your index finger.
- Attach pipette tip.
- Keep pipette almost vertical.
- Press button to the first stop.
- Immerse tip 2 – 3 mm into the liquid.
- *Allow the button to glide back slowly (never let it snap back).*
- Slide tip out along the inside of the vessel.
- Wipe off any droplets with lint-free tissue.

IMPORTANT:
Never lay Variopettes down with filled tips!

When pipetting viscous liquids or liquids with a low surface tension (e. g. serum, organic solvents):

- Rinse yellow and blue tips two or three times.

Note: Pipetting serum cristal tips:

When pipetting viscous liquids or liquids with a low surface tension, the pipette tip must not be wetted!

NOTE
When pipetting different liquids, always change the tips before using a new liquid.

When pipetting serum, rinse each yellow or blue tip. If small bubbles collect in the tip, the tip must be changed.

Filling with serum:

- Attach cristal tip.
- Press button to the first stop.
- Immerse pipette tip 2 – 3 mm into the serum.
- Slowly allow the button to glide back.
- Wipe off tip with lint-free tissue.

Dispensing serum:

- Immerse tip into the liquid to be dispensed.
- Take up sample by pressing the button two times to the first stop.
- Actuate blow-out and slide the tip out along the inside of the vessel.
- Eject tip.

Dispensing precision and accuracy can only be guaranteed when these notes are observed.

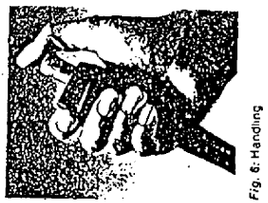


Fig. 6: Handling

2.3 Dispensing (Fig. 7)

There are two possible ways of emptying the tip:

1. Vessel
2. Chromatographic paper.

- Hold tip against the inside of the vessel or directly to the surface of the liquid.
- Press button down to the first stop and wait 1 – 3 sec.
- Press button on to the second stop.
- Slide tip out along the inside of the vessel.

When pipetting small volumes into a larger amount of liquid the remaining sample should first be rinsed out. Then press blow-out in order to empty remaining liquid.

2.4 Tip Ejection (Fig

Used pipette tips are ejected pressing the control button to final stop

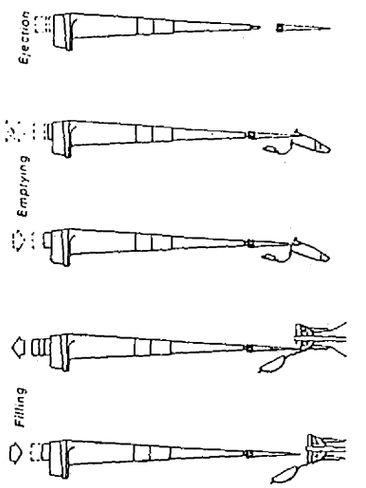


Fig. 7: Operation

Annex 2C

Quantitative evaluation with the Luminance meter (LS-110) from Minolta

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General remarks:

If the semiquantitative visual evaluation is not sufficiently precise, it is possible to obtain quantitative values for the concentrations of the antimalarials in the urine by using an appropriate densitometer or a luminance meter.

The luminance meter provided in the test kit has been tested for its functionality. It was found that, with practice, it could provide good standard values which made it possible to determine the amounts of the drugs present in the samples. However, the luminance meter is a highly sophisticated instrument, and it is strongly recommended that it be used only in laboratory surroundings, where a UV-lamp is available. Since the thin-layer chromatograms are stable they can easily be stored and evaluated later. If it is possible to do the evaluation with a densitometer, this is still the method of choice at present.

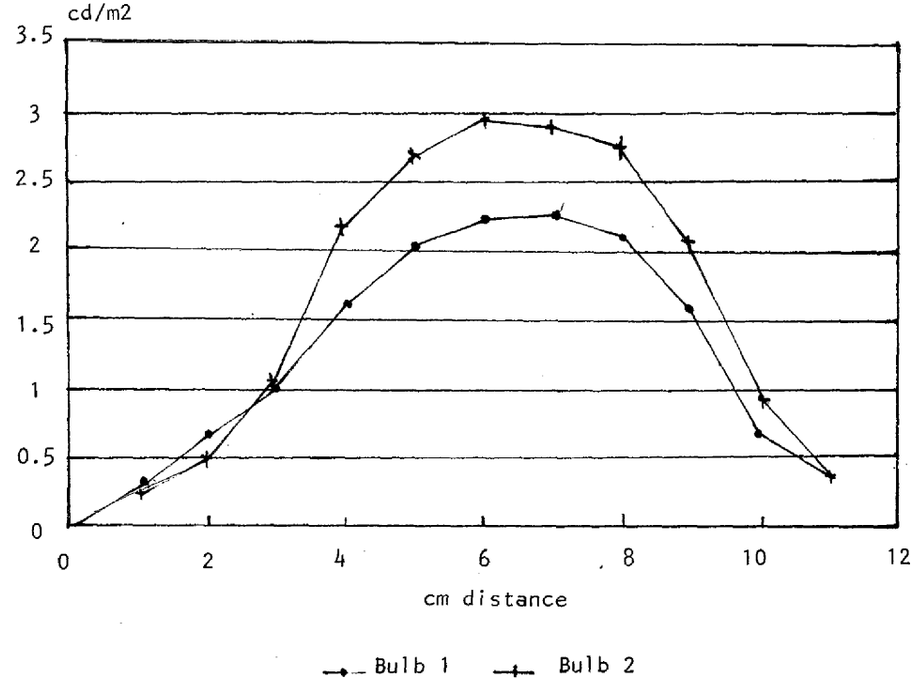
It is very difficult to obtain reproducible results with the luminance meter under field conditions, with the hand-held solar lamp, since these bulbs do not provide an uniform light intensity over their length (see Fig. 1). This can lead to large variations in the measurement unless thoroughly standardized procedures are used. On average the results received with the semiquantitative, visual evaluation are sufficient, and are obtained much faster than with the luminance meter.

Better results are obtained by combining the luminance meter with a commercially-available, pretested UV-lamp, which illuminates the whole silica plate uniformly.

Material

1 container with
- 1 Luminance meter LS 110
- 1 Close-Up lens Nr. 135
- 1 Hama stand for placing the meter on a table
- 1 key
- 1 battery
- 1 ocular protection

Fig. 1 Intensity of the UV light along the length of UV bulbs in the solar lamp as measured with a luminance meter.



Operation

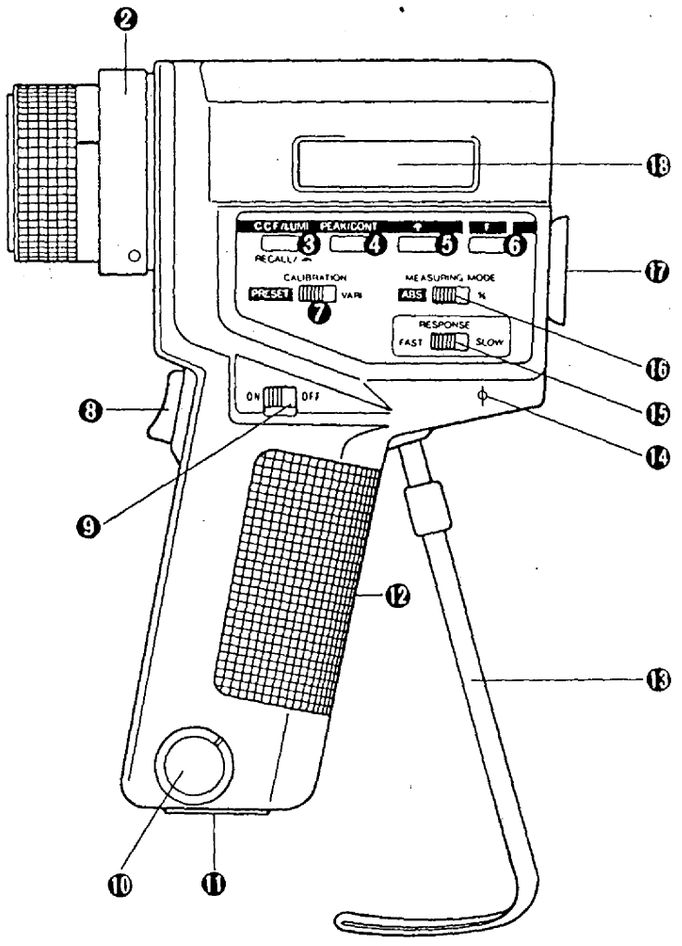
As with the visual method, you must work in a dark place, for example using the cloth hood attached to the suitcase lid.

- The luminance meter is taken out of its box and attached to the stand, which can be fixed to a table. Alternatively the stand contains in its handle two metal rods, which serve as a tripod-like fixation, if the rods are screwed into the holes of the stand.
- Turn the main switch on the luminance meter on (see Fig. 2). In the liquid crystal display window cd/m2 should appear.
- Set up the silica plate with the separated samples vertically, e.g. against the lid of the suitcase.
- Take the solar UV-lamp with the 366 nm lamp and position it directly in front of the plate with the middle part of the lamp at the sample to be assayed.
- Focus through the ocular the spot of the standard with the lowest concentration.
- Press the measuring switch, wait for a few seconds and read the value, which will also be indicated in the LCD window.
- Proceed to the next standards. Then read the unknown values and note all the readings on a separate sheet of paper.

Evaluation

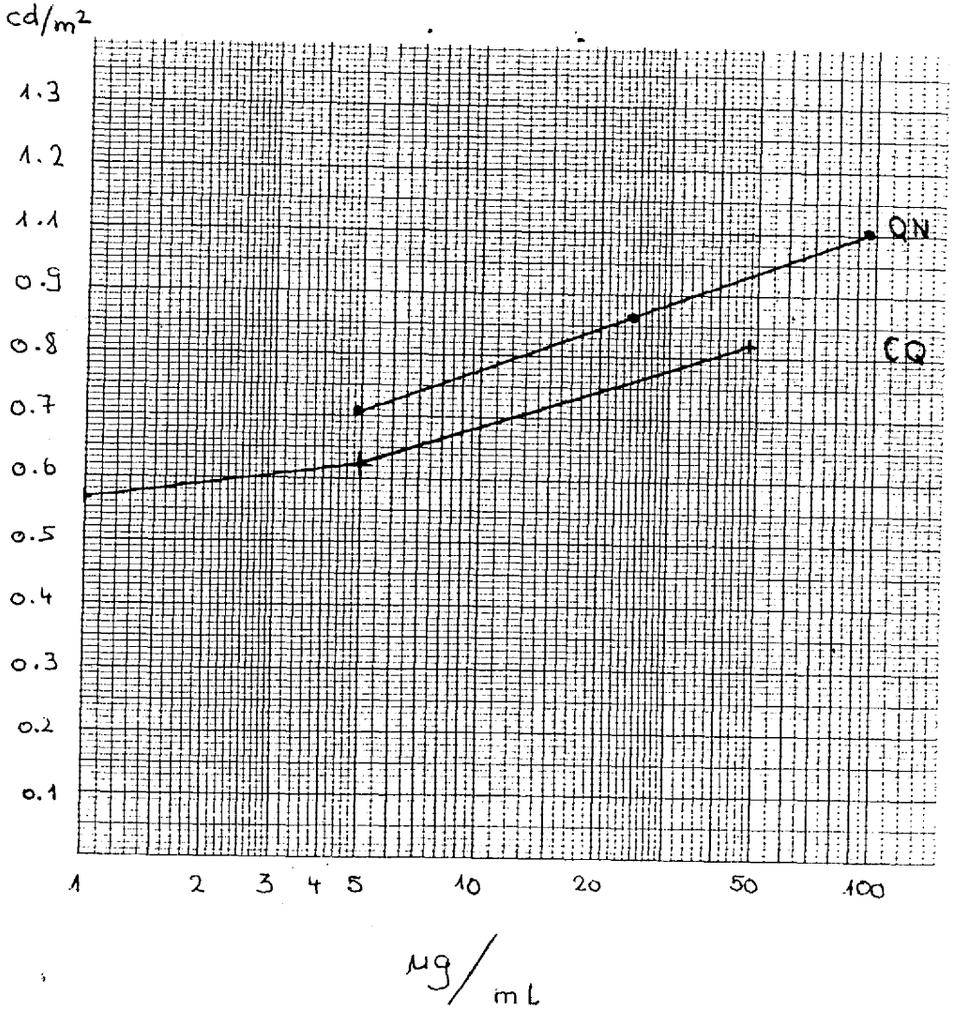
- Plot the three standard values on semilogarithmic paper. On the x-axis plot the actual concentrations of the standard drugs in ug/ml urine.
  The y-axis corresponds to the actual values read from the luminance meter.
- Connect the points with the ruler to draw a standard curve (see Fig. 3).
- Read the unknowns from the graph by connecting the luminance value with the line of the standard concentrations and then reading the actual concentration in ug/ml urine.

Fig.2. Description of the main parts of the luminance meter LS-110



2. Distance scale
8. Measuring switch
9. On-/Off-switch
11. Connection for stativ
17. Ocular
18. LCD window

Fig.3 A typical standard **curve of quinine** and chloroquine standards as measured with the luminance meter and by using a solar UV-lamp



DATA SHEET

Plate No:

Date:

		Category			ug/ml
		CQ	QN	MF	
1	Standard				
2	Standard				
3	Standard				
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23					
24					
25					
26					

Categories	Chloroquine (ug/ml)	Quinine(ug/ml)
1 = smaller than	1	5
2 = between	1 - 5	5 - 25
3 = between	5 - 50	25 - 100
4 = bigger or equal	50	100

DEVELOPMENT OF IMMUNOASSAYS FOR ANTIMALARIAL DRUGS

T.A. Eggelte

Introduction

With the increasing resistance of P. falciparum to the currently available antimalarial drugs there is a great need for tests for antimalarial drugs in body fluids. Such tests will be used in connection with:

1. in vivo and in vitro drug resistance studies;
2. chemoprophylaxis compliance studies; and
3. drug utilisation studies.

The tests that are required for such studies need to be:

1. sensitive;
2. specific; and
3. simple.

There is a variety of methods available for detection and determination of drugs in body fluids:

1. chemical tests (colorimetric test);
2. spectrophotometric tests (UV);
3. chromatographic assays (HPLC, TLC, HPTLC, GLC);
4. microbiological tests; and
5. immunoassays.

In principle all of the above listed tests can be used for antimalarial drugs and have in fact been applied to some of these.

There is no doubt that assays be like the HPLC and GLC are the tests with which other methods will compare as these are very specific and, often, sensitive tests. This does not imply that HPLC assays are without its problems especially when dealing with drugs that do not have a good chromophores and when high sensitivity is required. Each of these methods has its advantages and disadvantages.

Points that also have to be taken into account for the various applications of tests for antimalarial drugs are:

- application in a field situation (equipment)
- can large number of samples be handled (labour)
- sample (blood or urine)
- requirement of trained personnel
- quick results
- costs

If we take these factors into account, then a different picture may emerge for the suitability of the different methods for various applications.

Immunoassays

We have selected the approach of developing immunoassays for antimalarial drugs as it is our belief that many of the requirements can be met using this type of assay. It is our intention to do so for the whole range of antimalarial drugs.

Many types of immunoassays do exist and can be used at a whole range of available technology.

Immunoassays can be very sensitive but the sensitivty is dependant on the properties of the antibodies. Specificity of the immunoassays is that no special pre-treatment of the samples is required and small sample volumes are sufficient. A blood capillary provides enough sample for several assays, which is especially important when blood is collected from infants.

A wide variety of immunoassays have been developed for analyses.

The crucial point in all immunoassay techniques is the way that formation antibody-antigen complex is detected. We have concentrated our efforts on those immunoassay techniques which will require either no equipment or equipment that is most likely to be found in a research laboratory in a malaria endemic era.

We have chosen for the development of enzyme immunoassays (EIA).

We have developed two types of EIA:

1. ELISA's using 96 wells microtitre plates.
2. ELISA's using dipsticks.

The ELISA's using polystyrene microtitre plates are intended for:

1. quantitative measurement using a multiscan reader;
2. qualitative assessment of results by the naked eye; and
3. handling large numbers of samples.

The dipstick assays are for simple qualitative detection of drugs in body fluids and intended for testing single or only a few specimens, whereby results can be obtained in about 10 minutes.

They may also be used to screen samples for further quantitative measurement of the antimalarials.

Production of antibodies against antimalarial drugs

Central to the development of immunoassays for antimalarial drugs are the antibodies that recognise these drugs.

So far we have produced polyclonal and/or monoclonal antibodies against the following antimalarials:

chloroquine	dapsone
quinine	(chlor)proguanil
mefloquine	(chlor)cycloguanil
halofantrine	pyrimethamine
artelinic acid	sulfadoxine

A number of these antibodies have been used to provide assays for these antimalarial drugs.

ELISA's

Using the specific antibodies against the antimalarial drugs various types of ELISA's have been developed.

1. Drug-antigen, either a drug-protein conjugate or a poly-acrylamide-drug antigen is coated onto a 96-well microtitre plate, and free drug in solution competes with solid phase bound drug antigen for binding to the specific antibody. Binding of the specific antibodies is detected using an enzyme labelled second antibody, followed by addition of the enzyme substrate.
2. The ELISA procedure in para 1 can be shortened by using directly labelled antibodies.
3. Specific antibody is coated on the microtitre plate and drug and enzyme labelled drug competes for binding to solid phase bound antibody.

The last type of ELISA is selected for development of test kits for antimalarial drugs.

Chloroquine

Two types of monoclonal antibodies against chloroquine have been produced. The first type was produced against a chloroquine-protein conjugate in which hydroxychloroquine was coupled to the protein. The monoclonal antibodies F73-1 and F73-8 are directed against the quinoline moiety of the chloroquine molecule and these antibodies not only recognise chloroquine, and its metabolites but also amodiaquine.

A second type of antibody was produced against a chloroquine conjugate in which chloroquine was coupled through a 1,3-diaminopropyl link at the 7-position of the quinoline ring. These antibodies (e.g. F149012) are very specific and do not react with amodiaquine and only to some extent with desethyl-chloroquine.

Recently a new series of monoclonal antibodies (F157) have been produced against chloroquine, which have improved characteristics over the F149-12.

Different types of ELISA's have been set up using these antibodies and with the F73-8 antibody a test can be set up showing a 50% inhibition at 1-2 ug/L. For F149-12 this 50% inhibition point will be around 5-10 ng, whereas with F157 we hope to reach also 1-2 ug/L 50% inhibition.

The ELISA's for chloroquine have been used in many studies of chloroquine utilisation. From these studies carried out in several African countries e.g. Kenya, Tanzania, Ghana, Malawi it was clear that many patients seeking treatment for malaria from hospitals had already taken antimalarials e.g. chloroquine.

Chloroquine is readily available from shops, markets or through primary health care. The wide use of chloroquine may have been a contributing factor in the rapid spread of drug resistant P. falciparum in Africa.

Quinine

Antibodies against quinine were obtained by coupling the 9-O-hemisuccinate to BSA and producing monoclonal antibodies against this quinine conjugate. A series of monoclonal antibodies was obtained and F125-1 selected for further use. This antibody did not react with quinidine (< 0.05%), but recognised dihydroquinine and showed about 10% cross-reactivity with cinchonidine.

A very good correlation was obtained between results obtained with HPLC results and ELISA on quinine concentrations in sera from volunteers and malaria patients.

Mefloquine

In the case of mefloquine, specificity of the monoclonal antibodies is of utmost importance as mefloquine is extensively metabolised to 4-carboxy-2,8-di-trifluoromethylquinoline. The concentration of this inactive metabolite in the blood is much higher than that of mefloquine. Concentrations of mefloquine in urine are low and a test for demonstration of mefloquine in urine has also to be a very sensitive one.

We have produced monoclonal antibodies against mefloquine, which are very specific for mefloquine and which do not recognise the major metabolite. The ELISA shows a 50% inhibition around 5-10 ug/L, which under optimal conditions can even be lower.

So far we have produced only monoclonal antibodies against mefloquine, which may have also some serious drawbacks in this particular case. Polyclonal antibodies against mefloquine will also be produced.

For the demonstration of mefloquine intake one also may look at the metabolite instead of mefloquine itself. It is likely that antibody against the metabolite will cross-react with mefloquine.

An assay for the detection of the main mefloquine metabolite is under development.

DIPSTICK TESTS

Although ELISA's can be performed in a simple field laboratory it still requires well trained personnel and the assays are not really very suitable for testing single specimens. We have therefore started on the development of dipstick assays for antimalarial drugs, based upon our experience with ELISA's.

We have transferred our ELISA technology of tests using antibody solid phase coated microtitre plate to nitrocellulose membrances on a solid support.

The specific antibody is spotted as dots or coated as bands onto the nitrocellulose membrane. The cellulose membrane is then glued to a solid support (dipstick).

As in the ELISA drug and enzyme labelled drug compete for binding with antibody on the dipstick. Similarly, to the ELISA in microtitre plates, antibody and drug-enzyme concentrations are the important factors which determine the sensitivity of the assay. Various assay procedures can be used. In some of these procedures one is actually dealing with a blocking assay than with an inhibition assay, when incubation with the test sample is separated from incubation with the drug-conjugate.

In the microtitre plate ELISA's enzyme substrates are being used which gives the enzyme catalyzed conversion of the colourless substrate into a soluble chromogenic product. With the dipstick assay special substrates are used which give rise to the formation of a water insoluble product.

For this substrate we initially selected diaminobenzidine (DAB) which is one of the best substrates for this purpose but having the drawback that is potentially carcinogenic. As no colour will be formed on the dipstick when a high concentration of the drug is present, since total hibition will be obtained, an internal control is incorporated into the dipstick as a check that the test has been conducted properly and that also can be used as a reference.

The colour intensity of the control band is matched with that of the drug band when no drug is present. Several solutions for such an internal control have been studied.

In its present format the test for detecting antimalarial drugs in urine consists of a dipstick with two bands of immobilized antibodies (drug + control), and two vials; one vial containing a drug-enzyme conjugate and the other the enzyme substrate for developing the colour on the dipstick.

A dipstick is dipped into the urine sample or into a dilution of the urine sample for 1 minute. It is then briefly rinsed in tap water before being transferred to a vial containing the drug-enzyme reagent and incubated for 5 minutes. After a brief wash the dipstick is transferred to the second vial and within seconds colour development starts. The dipstick is rinsed in tap water and the colour of the bands compared with each other. If the colour of the drug band is less than that of the control band it indicates that the drug was present in the urine.

In principle not only urine but also plasma or whole blood samples can be used in the dipstick assay, depending on the drug levels reached in blood and the sensitivity of the dipstick assay. The sensitivities of the dipstick assays will always be less than those obtained in the ELISA. It is estimated that the present sensitivity of the dipstick is about 10 times less than that of the ELISA. This is probably due to the factor that results have to be assessed visually and that due to precipitation of the substrate onto the antibody-drug-enzyme immune complex, the enzyme activity is not used optimally and thus requires higher concentrations of antibody and drug-enzyme conjugate to obtain a visible precipitate.

Preliminary studies have shown that the dipsticks can be used for detection of chloroquine in whole blood samples. The dipstick tests have already been used on over 300 field samples and showed a 100% correlation with the ELISA in microtitre plates.

In principle it is also possible to test for more than one drug simultaneously by including bands for several drugs on the same dipstick and using a mixture of different drug-enzyme bands for several drugs and a mixture of different drug-enzyme conjugates. As the interaction between antibody and drug or drug-enzyme conjugate is very specific the presence of more than one drug-enzyme conjugate does not pose any problem. We have already shown that a dipstick can be made that can be used for the simultaneous detection of chloroquine, quinine and mefloquine.

The further development of dipstick assays for antimalarial drugs look promising if we can fulfill the requirements of:

1. sensitivity
2. specificity
3. stability
4. simplicity

The first two conditions can be met using the assay in its present form. The assay can probably simplified using the same materials by devising an assay set up which would require only one vial.

A possible problem area may be stability of the reagents. We have already enough experience to state that the antibody coated dipstick are very stable. The main problem will be the stability of the reagent which are necessary for the development of the colour e.g. the drug-enzyme conjugate and the enzyme substrates.

Furthermore, when enzymes are to be used as labels substrates for these should stable, preferably non-toxic and easy to prepare. At the moment a hydrogen peroxide has to be added to the substrate solution. Ideally one should only have to add water to the vials needed in the assay. When blood samples are to be used it will be necessary to incorporate into the kit a vial containing a lysis buffer for the erythrocytes.

The development of next generation of dipstick assays for antimalarial drug has to find answers to following problems:

1. stability of reagents, can we replace the enzyme labels by other more stable labels?
2. quantification, can we develop dipsticks which can quantify antimalarial drugs in blood samples?

Literature

Eggelte, T.A.: Production of monoclonal antibodies against antimalarial drugs for use in immunoassays. Centre for Drug Research International Monograph Series No. 3. In: Navaratnam, V. and Payne, D. (1990): The validation of chemical and immunochemical tests for antimalarials in body fluids.

Annex 1

ELISA TEST FOR ANTIMALARIAL DRUGS

The ELISA test selected for test kit development is based on the competition of free drug in solution (sample) and enzyme-labelled drug for binding with solid phase bound antibody (see Figure 1). Inhibition of colour formation obtained with a blanco sample indicates the presence of drug in the sample.

Materials

Microtitre plate coated with monoclonal antibody

Phosphate/saline buffer tablets

vial drug-enzyme conjugate

vial drug-enzyme diluent

vial OPD enzyme substrate

vial substrate buffer

vial Tween-20

Test procedure

1. Prepare PBS/Tween-20 buffer by dissolving 9 PBS tablet in 200 ml water and adding 100 ul Tween-20.

2. Prepare sample dilutions in PBS.

3. Take microtitre plate out of cover and wash the microtitre plate with two quick rinses with PBS/0.05% Tween-20 followed by incubating the plate for 5 minutes with PBS/Tween to remove protective coating from the wells.

4. Pipette 50 ul of sample dilution into wells of the plate together with a series of calibration samples.

5. Add 10 ml of PBS/0.05% Tween-20 to drug-enzyme diluent vial and dissolve contents of drug-conjugate vial in 6 ml diluent.

6. Add 50 ul of drug-conjugate dilution to the wells of the microtitre plate and mix the contents of the wells by careful shaking of the plate. Incubate the plate for 1 hour at 37°C.

7. Empty the plate and wash with PBS/Tween with 2 quick washes and two 2-minutes incubations.

8. Dissolve a substrate buffer capsule in water and dissolve contents of a substrate vial into 12 ml substrate buffer.

9. Add 100 ul of substrate solution to each well of the microtitre plate.

10. Incubate at room temperature for 30 minutes in the dark and stop the colour reaction with 50 ul of 1 M sulphuric acid.

11. Read the plate in a multiscan reader at 492 nm or assess the results visually.

12. Compare the results of the test samples with those of the calibration series.

DIPSTICK TEST

The dipstick test for antimalarial drugs is based on the same principles as the ELISA for antimalarial drugs. On the dipstick two different monoclonals are coated as bands. The upper band is an internal reference and the lower band is a monoclonal directed against an antimalarial drug e.g. chloroquine, quinine or mefloquine. When no drug is present in the sample two coloured bands will appear on the dipstick (Figure 2). However, when the samples contains the drug, the lower band will not be visible or has reduced intensity.

In the multi-band dipstick four bands of monoclonal are coated onto the dipstick. The upper band is the internal reference, whereas the three others are monoclonals directed against quinine, mefloquine and chloroquine (Figure 3).

Materials

dipstick

vial drug enzyme conjugate

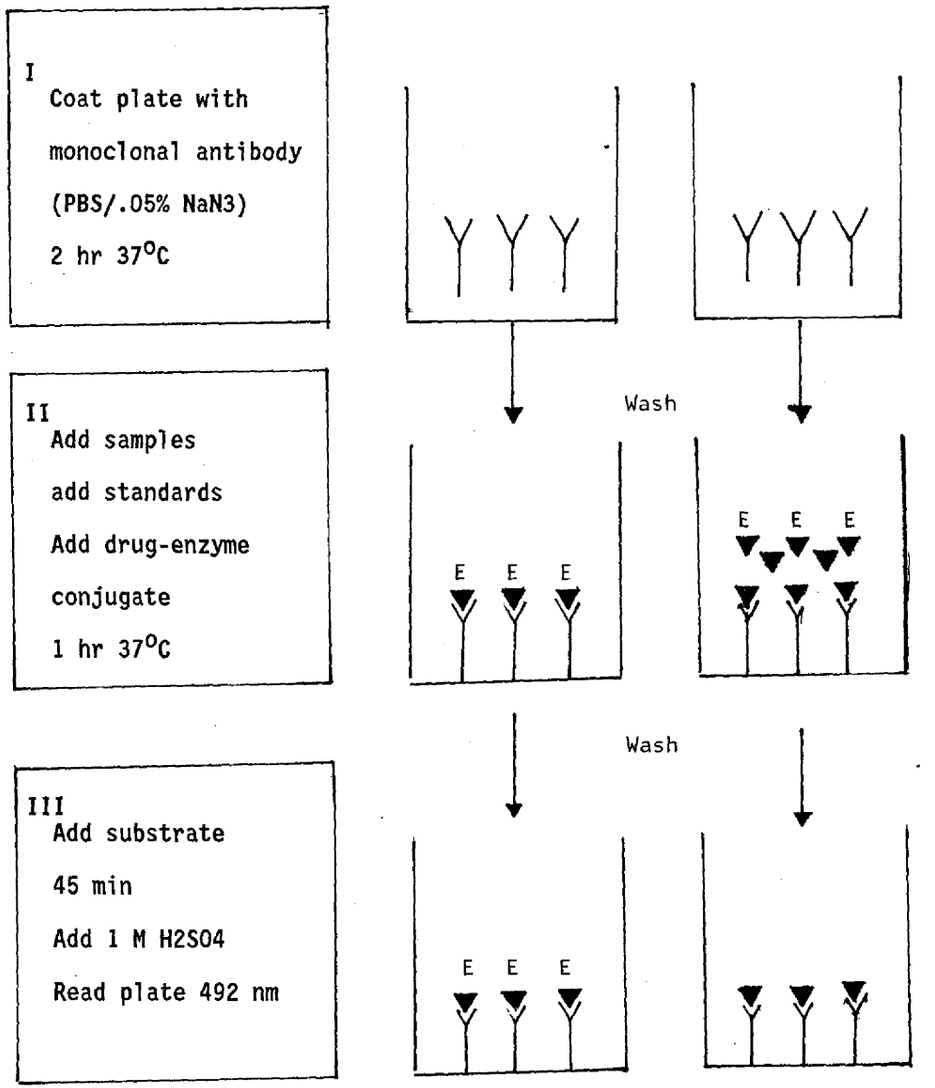
vial enzyme substrate

hydrogenperoxide solution

Procedure (Figure 4)

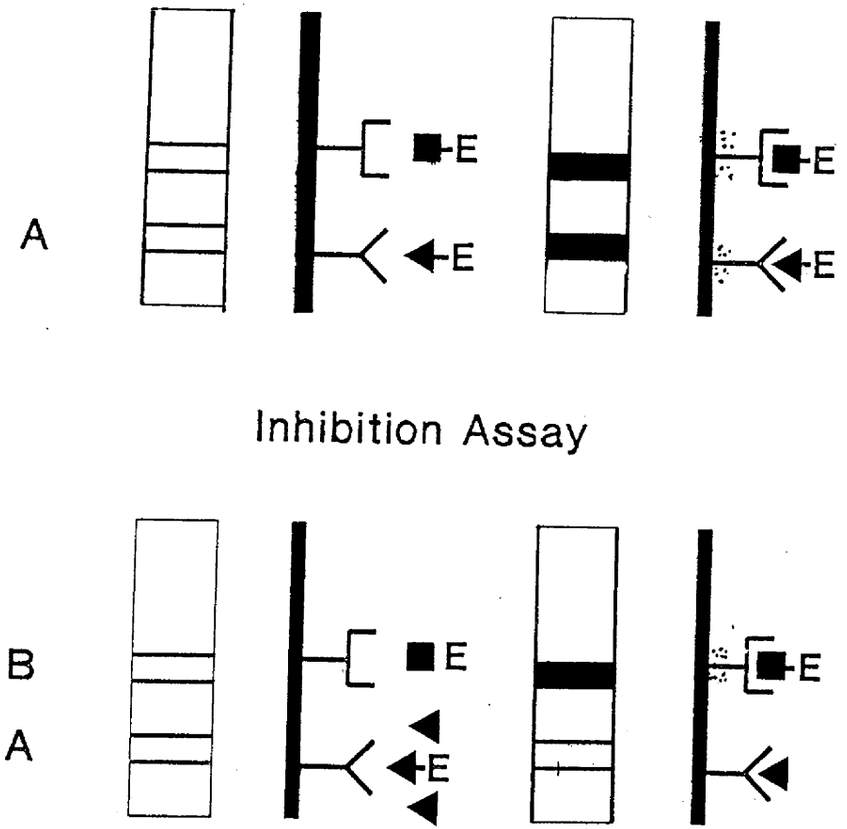
1. Incubate the dipstick for 1 minute in sample (urine or blood lysate).
2. Rinse the dipstick in water to remove sample traces.
3. Add 1 ml of water to drug-conjugate vial.
4. Put dipstick into drug-conjugate vial and agitate the dipstick in the solution.
5. Incubate for 5 minutes.
6. Rinse the dipstick in water.
7. Add 1 ml of water to substrate vial together with 10 ul .3% H202.
8. Take the dipstick out of the substrate vial and rinse the dipstick in water.
9. Compare the colour of the drug bands with that of the internal reference.

Figure 1



DIPSTICK ELISA

Dual drug-conjugate



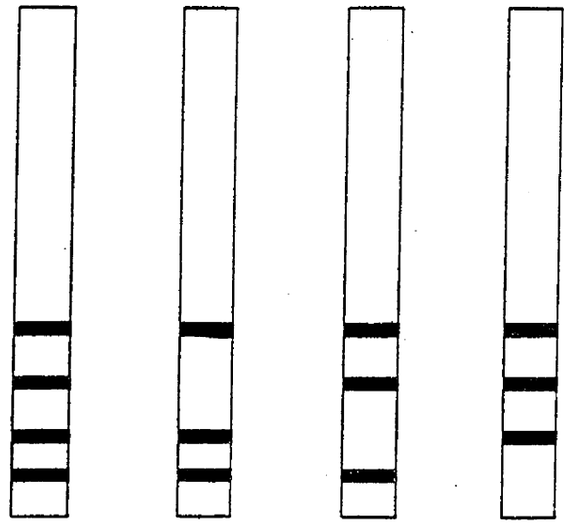
A -Drug antibody

B - Hapten antibody

◀ Drug

■ Hapten

Figure 3



CONTROL

QUININE Q

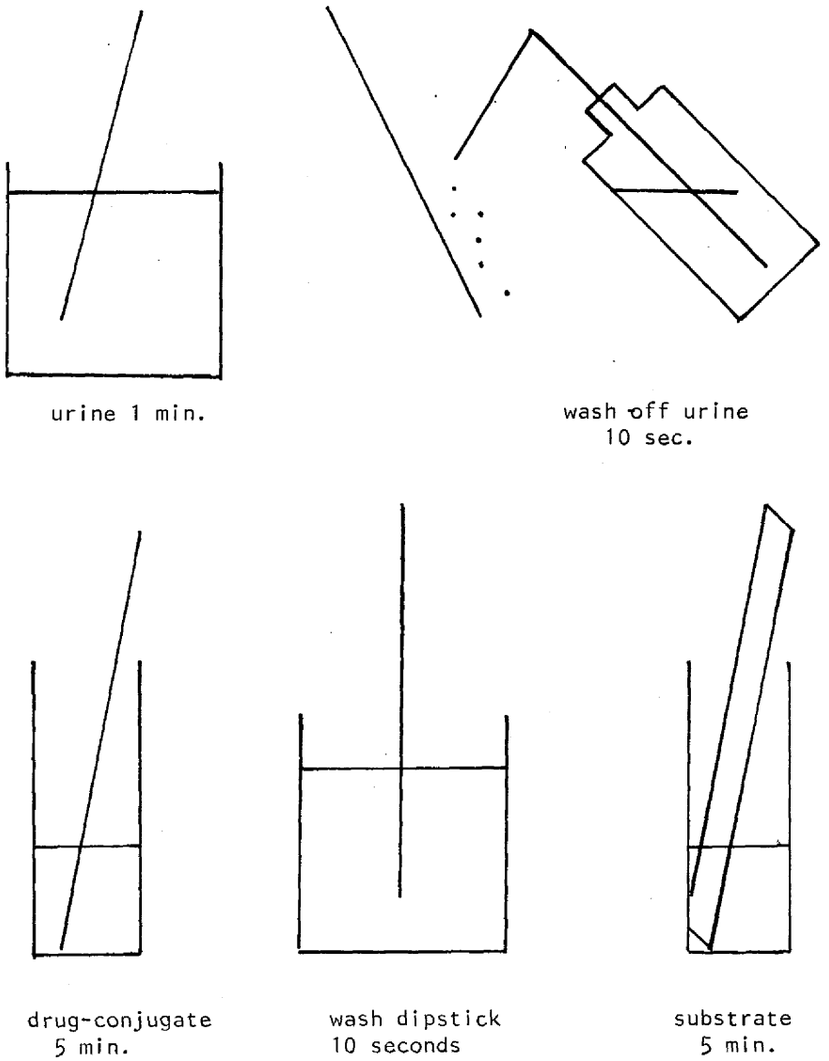
MEFLOQUINE MQ

CHLOROQUINE CQ

Q -	Q +	Q -	Q -
MQ -	MQ -	MQ +	MQ -
CQ -	CQ -	CQ -	CQ +

MULTIPLE DIPSTICK TEST FOR ANTIMALARIAL DRUGS

Figure 4



DIPSTICK TEST FOR ANTIMALARIAL DRUGS

TESTING FOR ANTIMALARIAL DRUGS IN BLOOD AND URINE: AN UPDATE COVERING COUNTRIES IN THE WHO WESTERN PACIFIC REGION

Kevin L. Palmer

Introduction

Within the nine malarious countries of the Western Pacific Region there are currently no malaria control programmes that routinely test urine or blood for the presence of chloroquine, mefloquine or other anti-malarial drugs. Testing is done as part of special studies such as in the screening process for in-vivo or in-vitro drug sensitivity testing, as part of pharmacological evaluations of antimalarials or as part of other studies that require measurement of antimalarial drugs in body fluids.

Screening for Drug Sensitivity Testing

The basic field test for chloroquine in urine, the Dill & Glazko test and the Lignin test for sulfonamides are a recommended part of the screening process for both in-vivo and in-vitro drug sensitivity testing. The urine tests are however so unreliable that it is now common practice to rely totally on the patients' recall of whether they have or have not taken drugs. Patients unfortunately often forget, or are reluctant to say that they have taken drugs, especially if they have treated themselves. Many tests are therefore carried out using patients or samples from patients who have significant levels of antimalarials in their blood. This is one factor that has contributed to the high rate of test failures with the standard micro in-vitro test. Other factors such as contamination, problems with the field incubator, and outdated or improperly stored reagents also raise the rate of test failures to as high as 60%. The low success rate and the high cost of the test materials themselves have been the primary reasons that many countries have cut down or totally stopped in-vitro testing.

Some of those programmes that have stopped in-vitro testing have shifted to in-vivo testing. A variety of formats are being used including the standard fourteen or twenty-eight day test and the shorter seven or three day tests. The success rate is higher than with the micro in-vitro test, expensive test kits are not required and the tests can be carried out by personnel with a minimum amount of training. Screening urine for antimalarials should be part of the process of selecting cases for in-vivo studies but as with the in-vitro tests the currently available field tests are so unreliable that they are not routinely used.

Figures 1 and 2 show the total number of successful micro in-vitro test carried out in the Region through 1987. They show that the number of micro in-vitro tests has dropped while the number of in-vivo cases has steadily risen. The numbers are however still small considering the number of countries where malaria is endemic and the size of the population at risk. More information is needed on the distribution and severity of drug resistance so that rational decisions can be made on malaria treatment regimens.

The availability of an easy to use field test for detecting chloroquine, mefloquine or any of the other common antimalarial drugs should make both the in-vitro and in-vivo tests easier to carry out and at the same time increase the success rate so that more information can be gathered.

Pharmacological Studies

There are two laboratories in the Region that collaborate with WHO to do High Performance Liquid Chromatography (HPLC) assays for antimalarials in blood and urine: The Australian Army Malaria Research Unit (AMRU) located in Ingleburn, Australia and the National Drug Research Centre at Universiti Sains Malaysia in Penang, Malaysia. Assays are carried out in these two centres as part of clinical trials of new drugs, for confirmation of drug levels in specific clinical cases where resistance is suspected or as part of other ongoing projects. The number of specimens that can be processed by the two centres is limited.

HPLC assays are expensive, they require careful handling of specimens, and highly trained personnel are needed to run and maintain the equipment. This severely limits the practicality of using HPLC assays in field studies. The assay is however still considered to be the most authoritative. For example, the recent reports of chloroquine resistant P. vivax from Papua New Guinea were confirmed based on clear demonstrations of parasites in blood films together with the measurement of chloroquine levels in the patients' blood. Any future reports of P. vivax parasites resistant to chloroquine or other antimalarial drugs will require the same level of confirmation.

Compliance Surveys

Mass drug administration (MDA) is used for controlling malaria outbreaks or epidemics in small, well defined areas. The major problem associated with MDA is low compliance. A quick way of checking for the presence of an antimalarial would be a valuable tool both in terms of determining accurate compliance rates and as a signal to the population that there is a way of telling who is and is not taking their drugs. The fact that the people know that there is a way of checking on them should significantly increase their cooperation and level of compliance.

Currently available field tests are not accurate enough and HPLC, though sometimes used for this purpose, is troublesome and expensive. A simple field test would greatly enhance future MDA activities.

The Perfect Test

The sensitivity, selectivity and other characteristics of currently available tests is discussed in the other papers contained in this volume. On the more practical side, from the viewpoint of the user, the nurse, the medical assistant or the malaria worker the important factors are:

1. the test can be done on a small table placed under a tree somewhere in the central highlands of Vietnam or in a small island in Vanuatu;

2. the test materials should not be sensitive to exposure to temperatures up to 37^{o} and should have a shelf life of up to one year;

3. the tests should be self contained and not require special instruments to read the results nor should it require disposable plasticware or glassware other than what comes with the test;

4. personnel should not need extensive technical training to carry out or interpret the test; and

5. most important, the test should be inexpensive, in the range of 20 to 50 cents (US$) per test.

The development of a field test for chloroquine, mefloquine and other antimalarials in blood and urine samples will provide malaria control programmes and national malaria research with another tool that can be used to more fully delineate and understand the drug resistance problem. We in the Western Pacific Region look forward to having field test in the near future.

Figure 1 - In-Vivo Tests
(Chloroquine) WHO Western Pacific Region

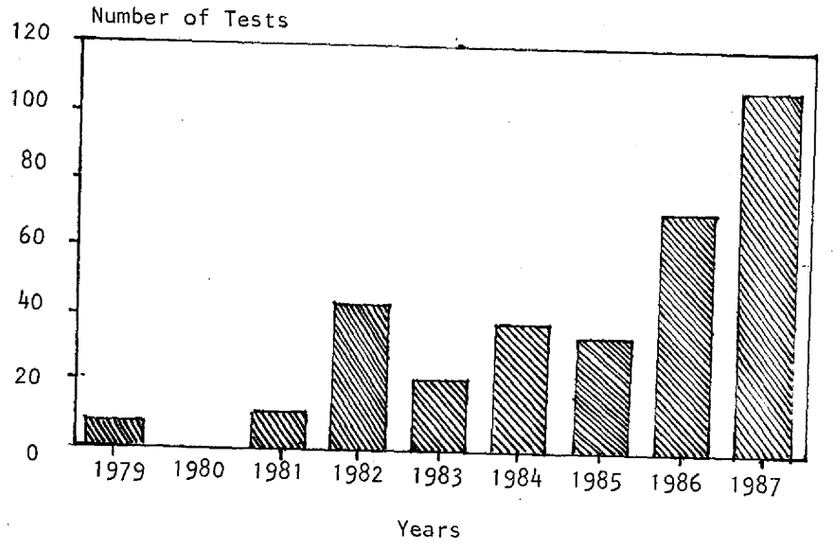
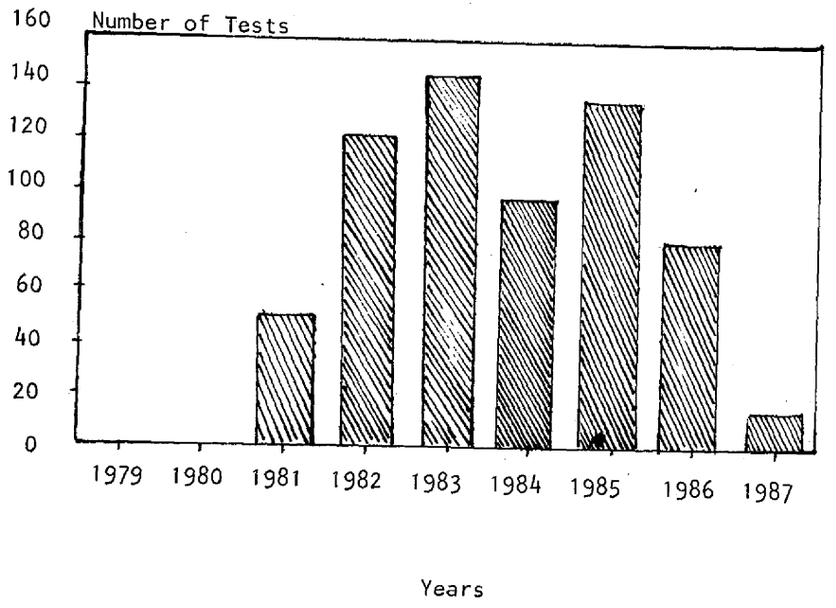


Figure 2 - Micro In-Vitro Tests
(Chloroquine) WHO Western Pacific Region



RESULTS AND CONCLUSIONS

One of the primary objectives of the Penang III Workshop, in common with all such endeavours supported by TDR, was the transfer of technology to the malaria endemic countries. Accordingly, the Workshop was organized to promote this exchange in the most efficacious means possible whilst completing the other prime objective of evaluating the comparative efficacy of the three test system: chemical, chromatographic and immunological.

The comparative evaluation was based on urine, whole blood and plasma samples provided by the NDRC which were of the following types: (1) deliberately spiked with known amounts of the parent drug - i.e. no metabolites present; (2) deliberately spiked with known amounts of the parent drug with known comparative HPLC levels; (3) treated healthy human volunteers with known comparative HPLC levels; (4) treated malaria patients with known comparative HPLC values; (5) treated severe malaria cases with known comparative HPLC values, Table 1.

Two additional series were run. The first compared the ELISA dipstick and plate assays in the detection of mefloquine and other antimalarials in lysed whole blood and plasma samples, Table 2. The second a semi-quantitative test of urines spiked with mefloquine comparing the Saker-Solomons CQI enhanced assay (10 ml samples in lieu of the standard 2 ml) with the ELISA dipstick, Table 3.

Tables 1 - 3 set out the results obtained in these studies.

In view of the known variability of the pH values of urine, and their possible affect on the various test systems, all the urine samples used in the study were tested for pH and gave values in the range pH = 6.0 + 9.0. Where ever relevant these pH results are included in the tables.

The general conclusions which can be drawn from these results are:

1. All three test systems are easily learned - 3 or 4 hours of hands on experience was quite sufficient for all of the participants of the workshop to be able to perform and read the tests.

2. All can be readily carried out at the field level.

3. The sensitivity of the Saker-Solomons CQI chemical assay in urine test can be considerably enhanced by increasing the sample size to 10 ml (from the standard 2 ml) and whilst it is nonspecific,

responding as it does to the 4-aminoquinolines, quinine and its diastereoisomer quinidine, as well as mefloquine and primaquine, these drugs can be detected in the urine at 1 mg/L. Moreover, there is a noticeable differentiation in the colour rection produced by quinine and mefloquine (more reddish) when compared to that of the 4-aminoquinolines and it may be that this colour coding can be used to separate these two groups of drugs.

In comparative studies with the ELISA dipstick assay in spiked urine samples levels in the range 0.5 mg to 1.0 mg/L of mefloquine were routinely detected by the enhanced CDC Saker-Solomons CQI assay, whereas 0.2 mg/L was detected, in one sample but was undetected in another.

4. The Mount sulfadoxine urine assay proved to be reliable at the relatively high concentrations of this drug which occurs in the urine of treated malaria patients: all four patients with SDX HPLC values in the range 16.7 to 38.4 mg/L were detected. However, the Mount SDX assay could not detect any of the three SDX 0.1 mg/L samples in the spiked urine series, but, individuals with such low levels in the urine are most likely to have therapeutically insignificant levels in the blood. The stated limit of detection for the method is 1.0 [illegible] levels that would represent, therapeutically, significant levels in the blood.

5. HPTLC was fairly reliable at detecting chloroquine at 0.1 mg/L in the urine: 3 out of 5 spiked samples and 4 out of 4 in the treated healthy volunteers with HPLC chloroquine values ranging between 0.1 mg/L and 0.3 mg/L. Similar sensitive results were obtained with quinine with very clear fluorescence bands being recorded at 3.2 mg/L. Mefloquine was positively identified at between 0.4 and 0.6 mg/L and queried at 0.3 mg/L. Mefloquine was also strongly identified in whole blood from malaria patients at 0.5 mg/L.

6. Unexpectedly, the ELISA microtitre test plate assay proved to be somewhat less reliable than the ELISA dipstick assay, marginally detecting two quinine spiked urine samples at 0.1 mg/L missed by the dipstick but completely wrongly identifying the drug in 4 urine samples from health volunteers treated only with chloroquine. In the same series it completely missed two quinine positive urines of 3.2 and 5.0 mg/L. Serious consideration must be given, therefore, to the possibility of experimental error in the plate ELISA here: either due to incorrect manipulation of the ELISA plate or, more likely a mix up in the test samples since at the confirmatory stage of the evaluation it became necessary

to have a certain amount of interchange of the samples between different groups.

Both the dipstick and the plate assays routinely detected chloroquine at 0.1 mg/L and mefloquine in the range 0.1 to 0.3 mg/L even when the drug occurred in combination with sulfadoxine and pyrimethamine.

7. Perhaps the most astounding finding was the reliability of the dipstick and plate ELISA in detecting mefloquine in lysed blood samples (1:5) from malaria patients: HPLC mefloquine values of 0.4 to 0.9 mg/L were readily identified. In the same series chloroquine was reliably detected by both systems in the range 0.1 to 0.6 mg/L and quinine at 1.0 mg/L.

8. An incidental study of urine samples from severe malaria patients with chloroquine produced another inexplicable result. Whilst HPLC assays provided values in the range 0.3 and 10.2 mg/L of chloroquine none of the three detection assays detected chloroquine. The CDC Saker-Solomons CQI assay recorded low levels of quinine in the three samples assayed and the Mount SDX assay gave also three positive readings: two were same as the Saker-Solomonss CQI samples the third was a different sample. The dipstick ELISA gave negative results in all 7 of the chloroquine positive samples and the plate ELISA recorded one as weakly chloroquine positive. Due to the blinded nature of the trial these findings were not apparent until the code was broken on the last day. However, a complete new series of the CDC Saker-Solomons CQI, Mount SDX and the dipstick and plate ELISAs assays was run, using more of the original samples, with exactly the same results. Therefore, the question which remains unanswered is whether relatively high levels of a drug, in this case chloroquine, can cause these assays to malfunction or whether some as yet unidentified factor in severe malaria patients interferes with the assays. It will be noted that the pH of all the urines tested was neutral - pH = 7.0.

9. The Penang III Workshop has produced some very interesting data showing that all three of the test systems assayed has a potential application for the detection of antimalarial drugs in body fluids.

These may be summarised as:

CDC Saker-Solomons CQI. A non-specific test for urine giving detection levels of the 4-aminoquinolines, mefloquine, quinine and primaquine at about 1 mg/L. The colour differences observed which were observed between mefloquine/quinine and the d-aminoquinolines should receive further investigation to determine whether this difference may permit the differentiation of mefloquine/quinine and the d-aminoquinolines.

The various reagents used in the test system are stable and no serious problems are anticipated in transportation and storage.

Mount Sulfonamide Urine Assay. This new assay lived up to expectations and the normal ranges of SDX in the urine should fall easily within its sensitivity frame. No particular problem is expected with stability and, it is anticipated, that this test has a great potential as a field tool. (The stated detection limit for both methods at 2 ml of urine is 1 mg/L).

HPTLC Now that the extraction and detection problems with mefloquine have been solved the HPTLC offers a highly sensitive, and with appropriate instrumentation, a quantitative, detection system for a wide range of antimalarial drugs in urine and plasma/serum with detection levels of well below 1 mg/L for CHL, QNN and MEF. TLC is not only a research tool, but can be used for routine applications under a variety of different laboratory conditions with very simple infrastructures up to highly sophisticated settings where the quantitative aspect can be given full utilization.

Plate ELISA Given the comparatively high levels of manipulative and interpretative skills which are required for the implementation of this test, and the apparent little difference demonstrated in this workshop in the sensitivity between it and the dipstick test, any application of this test would appear to be more properly restricted to a strictly laboratory based setting. However, large scale field surveys organized from a central facility with access to the necessary resources would also seem to be a logical application.

In this laboratory setting any potential and as yet undefined problemss with the stability and transportation of the test reagents would be readily resolved.

Dipstick ELISA Considering that this Workshop was the first time that this series of CHL, QNN and MEF dipstick ELISAs were comparatively evaluated the results were extremely encouraging. Particularly so were the results with lysed whole blood samples since these would be the most easily accessible (from a finger prick) and most standardized of the samples available for routine field testing. All three drugs tested, CHL, QNN and MEF, were readily detected at levels considerably less than 1.0 mg/L. Also of note was the success of the multi-test dipstick on which CHL, QNN and MEF detection bands were successfully combined.

However, it should be clearly appreciated that these tests were conducted under laboratory setting with prepared test samples and these results have to be confirmed with routine fresh material collected from malaria positive and negative patients. Additionally, it is not at all clear how stable the various elements of the test system (particularly the drug conjugate and enzyme substrate) would be under all possible variable conditions of transportation and storage. However, the stability of the dipsticks themselves is not in doubt as dipsticks prepared in October 1991 gave comparable results with those prepared in July 1991.

Table 1: Results of Blinded Study of Drug Detection in Urine Samples as Determined by Chemical Chromatographic and Immunological Assays At Penang III Workshop - July 1991

Sample I.D. Number	Assay type and results by drugs tested (for abbreviations used for drugs see Legend next page)												Values							Urine pH
	Sake-Solomons and Mount SNM			HPLTC			DIPSTICK ELISA			ELISA PLATE			HPLC VALUES mg/L							
	SS 2 ml	SS 10 ml	Mount SNM																	
	CHL/QNN/MEF	CHL/QNN/MEF		CHL	QNN	MEF	CHL	QNN	MEF	CHL	QNN	MEF	CHL	QNN	QND	MEF	SDX	PYR	PQN	
SPIKED SAMPLES																				
M1	-	+	-	+	-	-	+	-	-	±	±	-	0.1	0.1						6.0
M2	-	+	-	-	-	-	-	-	-	-	-	-	0.1		0.1					7.5
M3	-	-	-	+	-	-	+	-	-	±	±	-	0.1						0.1	7.0
M4	-	+	-	-	-	-	-	-	-	-	-	-					0.1	0.1		7.0
M5	-	±	-	-	-	-	-	-	+	-	-	±				0.1	0.1	0.1		6.5
M6	-	±	-	+	-	-	+	-	+	±	±	±	0.1	0.1		0.1				7.0
M7	-	-	-	-	-	-	-	-	-	-	-	-					0.1	0.1		7.0
TREATED HEALTHY VOLUNTEERS																				
C1	+++		-	++	-	-	+	-	-	+++	+++	-	0.3							6.0
C2	+++		-	++	-	-	+	-	-	+++	+++	-	0.2							9.0
C3	+++		-	+	-	-	+	-	-	+++	+++	-	0.1							5.0
C4	+++		-	+	-	-	+	-	-	+++	+++	-	0.1							6.5
D1	+++		-	-	++	-	-	+	-	-	-	-		0.5						6.0
D2	+++		-	-	+++	-	-	+	-	-	-	-		3.2						6.5

Continuation

Table 1: Results of Blinded Study of Drug Detection in Urine Samples as Determined by Chemical Chromatographic and Immunological Assays At Penang III Workshop - July 1991

Sample I.D. Number	Assay type and results by drugs tested (for abbreviations used for drugs see Legend next page)												Values							Urine pH
	Sake-Solomons and Mount SNM			HPLTC			DIPSTICK ELISA			ELISA PLATE			HPLC VALUES mg/L							
	SS 2 ml	SS 10 ml	Mount SNM																	
	CHL/QNN/MEF	CHL/QNN/MEF		CHL	QNN	MEF	CHL	QNN	MEF	CHL	QNN	MEF	CHL	QNN	QND	MEF	SDX	PYR	PQN	
TREATED MALARIA PATIENTS																				
B1	-	+	+	-	-	+	-	-	+	-	-	+++				0.6	38.4	0.4		6.0
B2	-	+	+	-	-	+	-	-	+	-	-	+++				0.5	25.7	0.3		6.5
B3	-	+	+	±	-	+	-	-	+	-	-	+++				0.4	23.5	0.2		6.0
B4	-	+	+	+	-	±	-	-	+	++	++	++				0.3	16.7	0.1		6.0
SEVERE MALARIA PATIENTS AND CONTROLS																				
H2				-	-	-							C	O	N	T	R	O	L	-
H3							-	-	-	-	-	-	C	O	N	T	R	O	L	7.0
H5	-	-	±										3.4							-
H6				-	-	-	-	-	-	-	-	-	0.3							-
H7	±	+	-										C	O	N	T	R	O	L	7.0
H8							-	-	-	-	-	-	2.1							7.0
K1				-	-	-							10.2							7.0
K2	+	+	+				-	-	-	-	-	-	8.1							-
K3							-	-	-	-	-	-	10.1							7.0
15	±	-	+				-	-	-	-	-	-	0.5							-

Table 2: Results of Blinded Study of Drug Detection in whole blood (Lysed) and Plasma Samples by Chemical Chromatographic and Immunological Assays at Penang III Workshop - July 1991

Sample I.D. Number	Assay type and results by drugs tested									HPLC VALUES mg/L				
	ELISA DIPSTICK			ELISA PLATE			HPTLC							
	CHL	QNN	MEF	CHL	QNN	MEF	CHL	QNN	MEF	CHL	QNN	MEF	SDX	PYR
WHOLE BLOOD FROM MALARIA PATIENTS/CONTROLS														
WW1	-	-	+	-	-	+++	-	-	++			0.5	0.3	0.2
WW2	+	+	-	++	+	-				0.4	1.0			
BB2	-	-	-	-	-	±						0.4	0.7	0.7
BB4	-	-	-	-	-	±						0.4	12.9	0.1
CC1	-	-	-	-	-	-				C O N T R O L				
CC4	+	-	-	+	-	-				0.6				
PLASMA FROM MALARIA PATIENTS/CONTROLS														
RR1	-	-	+	-	-	+++						0.5	1.5	0.6
RR2	+	+	-	++	+++	-				0.3	1.0			
RR3	+	-	-	+++	-	-				0.3				
RR4	-	-	+	-	-	+++						0.9		
RR5	-	-	-	-	-	-					C O N T R O L			
CC8	+	-	-	±	-	-				0.1				

Legend: CHL - Chloroquine; QNN - Quinine; QND - Quinidine; MEF - Mefloquine; SDX - Sulfadozine; SNM - Sulfonamide; PYR - Pyrimethamine; PQN - Primaquine

Table 3: Semi-Quantitative Comparison Between The CDC Saker-Solomons CQ-I and ELISA DIPSTICK Assays for the detection of Mefloquine in spiked urine samples at Penang Workshop III-July 1991

Sample I.D. Number	Type of Assay				SPIKING DRUG mg/L		
	S-S CQ - I 10 ml	ELISA DIPSTICK					
	CHL/QNN/MEF	CHL	QNN	MEF	CHL	QNN	MEF
Q1	+++	-	-	-	1.0	1.0	0.2
Q2	+	-	-	+			0.5
Q3	-	-	-	-			1.0
Q4	+++	-	-	+			0.2
Q5	+++	-	-	+			0.5
Q6	+++	-	-	+			0.2
Q7	+	-	-	+			0.5
Q8	++	-	-	+			1.0
Q9	+++	-	-	+			0.2
Q10	-	-	-	+			0.5
Q11	+	-	-	+			1.0
Q12	+	-	-	+			

RECOMMENDATIONS

The participants of the Workshop on the Comparative Evaluation of chemical, Chromatographic and Immunological Tests for the Detection Mefloquine and Other Antimalarial Drugs in Body Fluids RECOMMENDS:

1. A comparative field evaluation trial should be carried out in Sabah, Malaysia, for the detection of antimalarial drugs in whole (finger prick) blood from subjects suffering from P. falciparum malaria with, where so indicated, backup urine samples. The NDRC, Universiti Sains Malaysia will coordinate the preparation of research grant protocol for this study to be submitted to FIELDMAL TDR by late 1991.

   The evaluation should be based on the Eggelte multiband dipstick ELISA detectors for chloroquine, quinine and, if possible, sulfadoxine, with whole (finger prick) blood supplemented by the Mount chemical test for sulfonamides in urine.

   Additional samples should be taken for comparative validation tests at the central laboratory level using the Betschart HPTLC test system and standard HPLC procedures.

2. The proposed WHO supported antimalarial drug trials planned for Cambodia and Vietnam should include the multiband ELISA dipstick test for the detection of chloroquine, quinine, mefloquine and, if possible, sulfadoxine, in whole (finger prick) blood samples supplemented by the Mount sulfonamide test in urine. Additional finger prick blood and urine samples should be taken for comparative HPLC validation studies at the NDRC, Penang.

3. Both the Sabah, Malaysia and the Cambodia/Vietnam studies should have a strong training component to train key personnel in the relevant test techniques.

4. The proceedings of the Penang III Workshop should be published as soon as possible to reach the widest possible audience.

5. The field investigators involved in these field investigations should be brought together at the end of 1992 for a critical review of their findings with the aim of facilitating the introduction of standardised test procedures for the routine operational use of the appropriate detection tests in the malaria endemic countries.

6. WHO/TDR should explore with the WHO Regional Offices the possibility of extending these evaluation studies to other endemic countries with drug utilisation programmes (e.g. Vanuatu).