Field application of a colorimetric method of assaying chloroquine and desethylchloroquine in urine

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In a study in western Kenya of malaria-infected adult women who had been treated with chloroquine, we compared the level of chloroquine and its principal metabolite, desethylchloroquine, in urine, measured using a newly developed modified Haskins test, with the level of chloroquine in whole blood, determined by high-performance liquid chromatography. Over a 28-day follow-up period, 277 matched urine and blood samples from 81 women were evaluated. A high correlation was observed between the level of chloroquine in whole blood (in µg/l) and that of chloroquine + desethylchloroquine in urine (in mg/l). The test was easily performed and may be useful for monitoring use of chloroquine in a community and determining pre-study or post-treatment ingestion or absorption of the drug in in vivo *studies of parasite sensitivity.*

Information about the use and efficacy of anti-malarial drugs is important for planning and monitoring malaria control programmes. Monitoring the use of chloroquine in a community is best carried out by quantitative determination of its level in a body fluid in conjunction with administration of a questionnaire about recent drug ingestion. Also, investigations of reports of chloroquine-resistant *Plasmodium falciparum* in new areas and assessment of changes in areas with known chloroquine resistance can be aided by measuring the levels of the drug in the body fluids of individuals or populations. Verification of a reported case of chloroquine-resistant *P. falciparum* in a new area requires confirmation that a patient has taken and absorbed the drug. Furthermore, *in vivo* drug-sensitivity studies of *P. falciparum* in a community are best carried out on persons who have been confirmed not to have chloroquine already in their system and can be shown to have absorbed the study dose of the drug.

Chromatographic methods are available for the quantification of chloroquine and its major metabolite, desethylchloroquine, in whole blood, blood fractions, and urine (*1–6*). However, the methods for determining the drug in plasma or whole blood require the use of sophisticated laboratory instruments, and this causes delays for workers in the field. Colorimetric methods for the field assay of chloroquine + desethylchloroquine in urine have been available for many years and include the Dill-Glazko test (*7*), the Haskins test (*8*), and the Wilson-Edeson test (*9*). These tests are, however, less specific and sensitive than analysis of plasma or whole blood and show greater individual variation owing to differences in the rate of drug absorption, tissue distribution, and renal excretion. Such tests are nevertheless, simple to perform in the field, and, to date, have been used qualitatively to determine the presence or absence of chloroquine + desethylchloroquine in a urine sample. Because of its simplicity, the Dill-Glazko test, in particular, has been used most frequently for this purpose, but it has low sensitivity and is unreliable (*10–12*).

Recently developed colorimetric tests do, however, permit quantification of chloroquine + desethylchloroquine in urine in the field (*13, 14*). One such method developed in our laboratory using a modification of the Haskins urine test (Haskins, MMII) can detect chloroquine + desethylchloroquine to a limit of 1 mg/l (*14*). We compare here the results obtained using this test for determining chloroquine + desethylchloroquine levels in urine with those for chloroquine in whole blood determined by high-performance liquid chromatography (HPLC) on samples obtained from malaria-infected adult women (pregnant and non-pregnant) from western Kenya who had been treated with the drug. Possible applications of this test are also discussed.

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MATERIALS AND METHODS

Patient selection

The selection of patients for the study is described elsewhere (15). Consecutive pregnant women who attended a district hospital antenatal clinic with *Plasmodium falciparum* parasitaemia were enrolled and placed into one of two treatment groups: those receiving 25 mg chloroquine per kg body weight over 3 days (10 mg, 10 mg and 5 mg) or those receiving 5 mg chloroquine per kg body weight each week for 4 weeks. All females aged 14–18 years (non-pregnant, as confirmed by urine pregnancy test) attending one large school were screened and enrolled in the study if they exhibited *P. falciparum* parasitaemia; they were assigned to one of the two treatment groups for pregnant women described above.

Collection of specimens

Urine and filter-paper-absorbed fingerstick blood specimens were collected from study participants on enrolment (day 0) and also on days 2, 7, 14, 21, and 28.

Processing of specimens

Filter-paper blood specimens were air dried, individually wrapped, and stored in desiccant until processed. The levels of chloroquine and desethyl-chloroquine in whole blood were determined using HPLC (6). Urine specimens were examined at the study site within 6 hours of collection and their specific gravity was determined with a urinometer.

A modification of the Haskins test based on color-imetric measurement of ion-pair formation between chloroquine and methyl orange in chloroform (Haskins, MMII) (14) was used to determine the level of chloroquine + desethylchloroquine in urine samples. The percentage transmittance of the chloroform layer was read using a hand-held, battery-operated, filter photometer (filter wavelength, 420 nm). For samples with transmittance < 0.02 (absorbance > 1.70), specimens were diluted twofold sequentially until a transmittance of 0.10–0.80 was obtained. Absorbances were recorded and compared with a prepared standard curve to convert them into concentrations (in mg/l). The standard curve is given by:

$$\text{Chloroquine concentration (mg/l)} = -0.348 + 8.64 \times \text{absorbance}; R^2 = 0.99.$$

The concentrations obtained were corrected for specific gravity (SG) using a standard curve determined in our laboratory to give an adjusted concentration in mg/l. The following adjusted-SG standard curve was used:

$$\text{Chloroquine concentration (adjusted-mg/l)} = -89.34 + 89.49 \times \text{SG}; R^2 = 0.99.$$

Measurement units

In the test described, the combined level of chloroquine and desethylchloroquine in urine is expressed in mg/l or adjusted mg/l. Whole-blood determinations distinguished between the levels of chloroquine and desethylchloroquine and, here, we report the measured level of chloroquine in μg/l, where for chloroquine, 1 μg/l = 0.003 μmol/l.

Data analysis

For persons with < 100 μg/l of chloroquine in whole blood on day 0, the correlation between the level of chloroquine in urine and whole blood was analysed in corresponding sample pairs by day of collection. Urine specimens whose volume was insufficient for the required dilutions or specific-gravity measurements were not included in the analysis. The levels of chloroquine in urine and whole blood are plotted in Fig. 1 for days 0, 2, 7, 14, 21, and 28 and fitted with a regression line using a least-squares method. Intervals, which included 80% of observed whole-blood chloroquine levels (80%-prediction intervals), were calculated from the regression line for urine values (16).
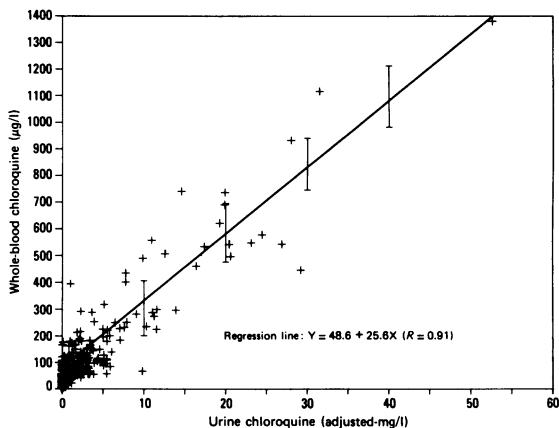

Fig. 1. Comparison of the level of chloroquine in whole blood and the specific-gravity-adjusted level of chloroquine + desethylchloroquine in urine from malaria-infected Kenyan women on days 0, 2, and 7 after weekly (5 mg chloroquine per kg body weight) or therapeutic (25 mg chloroquine per kg body weight) doses. Bars represent the 80%-prediction interval.

Table 1. Mean level of chloroquine in whole blood and urine, by days after treatment for 81 women who received 25 mg of the drug per kg body weight (10 mg, 10 mg, and 5 mg on days 0, 1, and 2, respectively) or 5 mg per kg weekly

| | Day after enrolment | | | | | |
	Day 0	Day 2	Day 7	Day 14	Day 21	Day 28
25 mg/kg dose						
Level in whole blood (μg/l)	26 (31)[a]	626 (215)	262 (97)	96 (58)	50 (22)	49 (27)
Urine[b]						
Absorbance	0.01 (0.02)	NA[c]	0.8 (0.4)	0.4 (0.3)	0.1 (0.1)	0.1 (0.3)
Level (mg/l)	0.03 (0.10)	34 (23)	9.2 (7.7)	3.3 (2.1)	0.6 (0.8)	0.8 (2.9)
Adjusted level (mg/l)	0.02 (0.06)	27 (17)	8.0 (6.0)	3.2 (2.1)	0.6 (0.7)	0.5 (1.8)
5 mg/kg dose						
Level in whole blood (μg/l)	30 (21)	112 (55)	65 (25)	92 (41)	98 (40)	112 (91)
Urine[b]						
Absorbance	0.01 (0.02)	0.3 (0.25)	0.2 (0.23)	0.4 (0.30)	0.2 (0.27)	0.3 (0.23)
Level (mg/l)	0.01 (0.05)	4.5 (6.5)	1.4 (1.9)	3.2 (5.3)	2.6 (8.7)	1.8 (2.0)
Adjusted level (mg/l)	0.01 (0.02)	2.8 (3.8)	1.1 (1.3)	2.9 (3.6)	2.0 (5.3)	1.5 (1.7)

[a] Figures in parentheses are standard deviations.

[b] Results are for chloroquine + desethylchloroquine. Absorbances were measured at λ = 420 nm using a filter photometer. The level was determined from a standard curve, and the adjusted level obtained from this by taking also specific gravity measurements into consideration.

[c] NA = not applicable because specimens were diluted.

RESULTS

The chloroquine levels in matched samples of urine and blood were obtained for 81 women and 277 matched samples for the 28-day follow-up period. Mean levels of chloroquine in whole blood and of chloroquine + desethylchloroquine in urine for each day of the follow-up and for the two different drug doses are shown in Table 1. A high degree of correlation was observed between a plot of the level of chloroquine in blood (μg/l) and that of chloroquine + desethylchloroquine in urine (adjusted-mg/l) for the 277 samples (Fig. 1) ($R=0.91$, $P<0.0001$). The least-squares regression line is given by:

Blood-chloroquine level (μg/l) = 48.6 + 25.6 (urine chloroquine + desethylchloroquine [adjusted-mg/l]).

The correlation between blood chloroquine levels and urine chloroquine + desethylchloroquine levels without adjusting for specific gravity was only slightly lower ($R=0.88$, $P<0.0001$).

The 80%-prediction intervals for whole blood chloroquine estimates based on the least-squares regression line are given for specific values of the adjusted level of chloroquine + desethylchloroquine in urine in Table 2. From these prediction intervals, an estimated range for the level of chloroquine in whole blood (μg/l) can be determined for an individual urine chloroquine + desethylchloroquine (adjusted-mg/l) value. The plot of the mean level of

Table 2. Level of chloroquine in whole blood and estimated number of days since last ingestion of the drug predicted from the adjusted level of chloroquine (CQ) + desethylchloroquine (DES) in urine using the modified Haskins procedure

| Adjusted level of CQ + DES in urine (mg/l)[a] | Associated mean level of CQ in whole blood (μg/l): | No. of days after taking CQ in a dose of: | |
		5 mg/kg	25 mg/kg
1	74 (0–163)[b]	≥7	≥12
2	100 (11–189)	≥7	11–16
3	125 (36–214)	5–8	10–14
4	151 (62–240)	4–7	9–13
5	177 (87–266)	4–6	8–12
6	202 (113–291)	3–5	7–11
7	228 (139–317)	2–4	6–10
8	253 (164–343)	2–4	5–10
9	279 (190–368)	1–3	4–8
10	305 (215–394)	1–2	3–7
12	356 (266–445)	1	2–5
15	432 (343–522)	1	2–4
17	484 (394–574)	—	1–3
20	561 (470–651)	—	1–3

[a] Level of chloroquine + desethylchloroquine in mg/l determined by modified Haskins procedure and adjusted for specific gravity.

[b] Figures in parentheses are the 80%-prediction intervals for an individual's whole-blood chloroquine level for a given level of the drug in urine.

chloroquine in whole blood against number of days since last dose for therapeutic (25 mg chloroquine per kg) or prophylactic (5 mg chloroquine per kg per week) doses (Fig. 2) together with the 80%-prediction intervals from Table 2 can be used to determine that an individual's urine-chloroquine + desethylchloroquine level is consistent with administration of a known dose of the drug within a given time period. For example, a urine level of chloroquine + desethylchloroquine (adjusted-mg/l) of 7 mg/l is consistent with a chloroquine level in whole blood of 228 μg/l (80%-prediction interval = 139–317 μg/l) and with the ingestion of 5 mg chloroquine per kg body weight within 2–4 days or of 25 mg chloroquine per kg within 6–10 days previously.

The sensitivity and predictive value for a cut-off of ≤ 1 mg/l in urine for the adjusted level of chloroquine + desethylchloroquine and of < 100 μg/l for chloroquine in whole blood were 84% and 89%, respectively. Since levels of chloroquine in whole blood that are < 100 μg/l are sub-therapeutic, adjusted concentrations of chloroquine + desethylchloroquine in urine that are ≤ 1 mg/l could be used to determine that an individual has small amounts of chloroquine in his or her system and could be an eligible subject for an *in vivo* study to assess the therapeutic treatment of malaria infection.

Determination of chloroquine + desethylchloroquine in urine showed an intra-test error of 0.04 of transmittance units for 20 repeated photometric measurements. When the standard curve is corrected by this amount, the error in the predicted concentration of chloroquine + desethylchloroquine is ± 0.30 mg/l. Comparable errors in the specific gravity measurements were not determined; however, a specific gravity error of only 0.001 would introduce an error of ± 0.35 mg/l to the adjusted level of chloroquine + desethylchloroquine in urine.

DISCUSSION

In the field study of the described modification of the Haskins test, we found a highly significant correlation between the level of chloroquine + desethylchloroquine in urine and that of chloroquine in whole blood for 28 days after treatment with the drug. Correlations were slightly improved when the estimated urine level of chloroquine + desethylchloroquine was adjusted for specific gravity, and the use of this adjustment may be important for quantitative measurements.

The experimental error in comparisons of the urine chloroquine + desethylchloroquine and whole blood chloroquine tests is low. The method of determining chloroquine in whole blood has a very low intra- and inter-test error and a sensitivity of 5 μg/l (6), and thus

does not contribute significantly to such errors. Likewise, the intra-test error for the urine test and for the adjusted-mg/l values were acceptably low and well below the range of individual biological variation in chloroquine metabolism. The concentration in whole blood of chloroquine varies in individuals administered the same dose per kg body weight due to differences in absorption and tissue distribution (17), and measurements of chloroquine in urine introduce additional biological variability due to renal excretion. Because of such biological variations and the time lag in renal excretion and urinary bladder collection and storage, simultaneous determinations of the concentration of chloroquine in whole blood and urine can be expected to correlate imperfectly. None the less, the ability to rapidly estimate chloroquine + desethylchloroquine levels outweighs the disadvantages of the lower sensitivity and greater biological variation in the urine measurements made in the field.

The Haskins-MMII test for determining chloroquine + desethylchloroquine in urine was easily performed and readily taught to field technicians. On the average, 30–50 urine specimens could be collected and processed over a 2-hour period. Much of that time was spent in labelling specimens and recording data, operations which are part of any test procedure. The use of a hand-held, battery-operated filter photometer permitted precise quantification of the results; however, the test could also be performed quantitatively by visual comparison of photometer readings with those of known spiked standards. Use of a urinometer or a hand-held refractometer to measure urine specific gravity required an additional 20–30 minutes for 30–50 specimens.

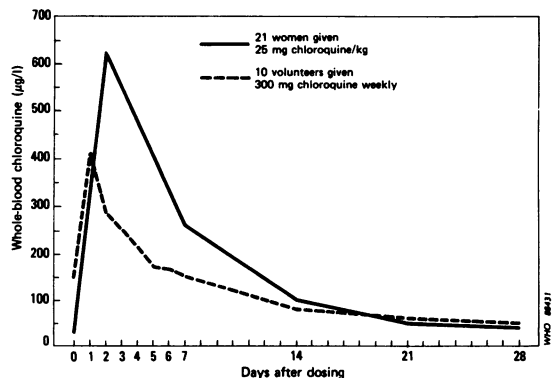The modified Haskins test had a sensitivity of 1 mg/l, which permits estimations of chloroquine +

Fig. 2. Level of chloroquine in whole blood after a therapeutic dose (25 mg chloroquine per kg body weight) or a weekly prophylactic dose (300 mg per week) for at least 10 weeks in adults (18).

desethylchloroquine in urine or whole blood following ingestion of the drug in the previous seven days. The specificity of the test was not evaluated during our field studies; however, other investigators (13) have reported that it can detect similar micromolar concentrations of desethylchloroquine, hydroxychloroquine, quinine, mefloquine, and proguanil. At much higher levels, pyrimethamine and amodiaquine cross-react under the test conditions; neither of these drugs was, however, available in our study. Other medications that are commonly found in rural areas of developing countries, including analgesics, antipyretics, and antibiotics, do not cross-react in the test (13).

Determination of the concentration of chloroquine + desethylchloroquine in urine can assist in assessing drug use practices in a population by establishing whether individuals are responding to febrile illnesses by taking therapeutic doses of chloroquine. Since doses of 25 mg chloroquine per kg body weight would produce blood levels of the drug > 200 μg/l for approximately 10 days, measurement of the level in urine of chloroquine and its principal metabolite during this period would show positive results. Similarly, pregnant women could be assessed for compliance with a chloroquine chemoprophylaxis programme. For a pregnant woman taking a weekly dose of 300 mg chloroquine base, whole-blood levels of the drug would be expected to exceed 150 μg/l within 5 days of the most recent dose. Such a level in blood would be expected to produce a urine concentration > 1 mg/l, and thus, urine measurements of the type described could verify recent ingestion of the drug. The test could also be used to determine the background rate of use of chloroquine in a population where the drug is widely available and taken for a variety of health problems.

In in vivo drug sensitivity studies, the test could be used to screen potential study subjects to ensure that only persons who have not recently ingested chloroquine, as indicated by a chloroquine + desethylchloroquine level in urine of ≤ 1 mg/l are included. The high negative predictive value of 89% found for the study population suggests that a cut-off of ≤ 1 mg/l is useful in establishing that the whole-blood level of the drug is < 100 μg/l. Additionally, the test could be used to monitor enrolled subjects on day 2 or day 7 after ingesting chloroquine in order to assess proper drug absorption. Also, study subjects who failed to clear malaria parasites could be selected for measurement of blood chloroquine based on the results of the urine test.

During surveillance for in vivo drug resistance of P. falciparum to chloroquine the test could be used to verify recent ingestion of the drug and to help in estimating the amount taken. From observations of whole-blood chloroquine levels after a standard therapeutic dose of 25 mg chloroquine per kg body weight or a weekly prophylaxis regimen of 300 mg chloroquine (Fig. 2), the whole-blood level of chloroquine estimated from the urine test result can be used to establish whether an individual has ingested a certain mg/kg-dose of chloroquine within a given period. For example, if a patient has a P. falciparum infection and has taken a therapeutic dose of chloroquine five days previously, the expected level of the drug in urine could be compared with that measured using the test. If the two values are widely disparate, inadequate drug ingestion, poor drug absorption, or recent additional drug ingestion could explain the observed differences.

Finally, the test could also be beneficial in hospitals and clinics in malarious areas. For example, in such areas, where patients often present with malaria and an uncertain history of prior medication, a reliable, quantitative test for urine chloroquine would permit clinical personnel to make more informed decisions about further treatment, allowing them to avoid overdosing patients or to choose alternative drugs, if appropriate.

ACKNOWLEDGEMENTS

RÉSUMÉ

APPLICATION SUR LE TERRAIN D'UNE MÉTHODE COLORIMÉTRIQUE DE DOSAGE DE LA CHLOROQUINE ET DE LA DÉSÉTHYLCHLOROQUINE DANS L'URINE

Des méthodes colorimétriques de dosage de la chloroquine et de ses métabolites dans l'urine seraient nécessaires pour évaluer l'utilisation thérapeutique et chimioprophylactique de ce médicament; l'épreuve actuellement la plus

utilisée (Dill-Glazko) manque à la fois de sensibilité et de fiabilité. Une méthode de terrain mise au point dans notre laboratoire d'après une modification de l'épreuve urinaire d'Haskins (Haskins-MMII) est capable de détecter la présence de chloroquine et de déséthylchloroquine à partir de 1 mg/l dans les urines. Lors d'une étude portant sur des femmes présentant une parasitémie palustre dans l'ouest du Kenya, nous avons comparé les taux de chloroquine+déséthylchloroquine dans les urines, mesurés par l'épreuve d'Haskins-MMII et le taux de chloroquine dans le sang total, déterminé par chromatographie liquide haute performance (HPLC). Pendant la période de suivi de 28 jours, on a ainsi examiné 277 échantillons appariés d'urine et de sang obtenus chez 81 femmes. On a observé une forte corrélation ($R=0,91$; $P<0,0001$) entre le taux de chloroquine dans le sang total (en $\mu g/l$) et le taux de chloroquine+déséthylchloroquine dans l'urine (en mg/l après correction de la densité). La droite de régression obtenue par la méthode des moindres carrés correspondait à la formule: chloroquine dans le sang ($\mu g/l$)=48,6+25,6× (chloroquine+déséthyl-chloroquine dans l'urine [valeur corrigée en mg/l]).

En prenant comme seuil 1 mg/l pour la chloroquine+déséthylchloroquine dans l'urine et 100 $\mu g/l$ pour la chloroquine dans le sang total, on a obtenu pour l'épreuve une sensibilité de 84% et une valeur prédictive positive de 89%. On peut savoir si le taux de chloroquine+déséthylchloroquine obtenu dans l'urine est compatible avec la prise d'une dose connue de médicament au cours d'une période donnée d'après le taux prévu de chloroquine dans le sang total d'un sujet un nombre donné de jours après la prise d'une dose connue et d'après les intervalles de valeur prédictive 80% correspondant au taux de chloroquine dans le sang total obtenu à partir de l'équation de régression. Cette épreuve quantitative de dosage de la chloroquine dans les urines s'est révélée facile à exécuter et peut être utilisée pour surveiller l'utilisation de la chloroquine dans une communauté et déterminer quelle a été la prise de chloroquine avant l'étude ou après le traitement lors d'études in vivo de la sensibilité des parasites.

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