

Adaptations of the Saker–Solomons test: simple, reliable colorimetric field assays for chloroquine and its metabolites in urine*

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Two field-adapted colorimetric methods for measuring the antimalarial drug chloroquine in urine are described. Both are modifications of the method of Saker and Solomons for screening urine for phencyclidine and other drugs of abuse, using the colour reagent tetrabromophenolphthalein ethyl ester. One method is semiquantitative, detecting the presence of chloroquine (Cq) and its metabolites in urine with a 1 µg/ml detection limit; it is more sensitive and reliable than the commonly used Dill–Glazko method and is as easy to apply in the field. The second method uses a hand-held, battery-operated filter photometer to quantify Cq and its metabolites with a 2 µg/ml detection limit and a linear range up to 8 µg/ml. The first method was validated in the field using a published quantitative colorimetric method and samples from a malaria study in Nigeria. The second method was validated in the laboratory against high-performance liquid chromatographic results on paired samples from the Nigerian study. Both methods may be used in remote locations where malaria is endemic and no electricity is available.

A number of colorimetric methods for detecting and estimating the concentrations of chloroquine (Cq) and its metabolites (primarily desethylchloroquine (DECq)) in urine have been published in the past 40 years for use in malaria field studies (1–5). Because of its simplicity the Dill–Glazko method (1) is most commonly used, but recent publications have concluded that the test is neither sufficiently sensitive nor reliable to assess effectively the history of Cq use (6–8). A simple, more sensitive and reliable field assay than the Dill–Glazko method is therefore needed for monitoring chemoprophylaxis and treatment compliance, investigating drug-use patterns, and screening prospective subjects for *in-vivo* and *in-vitro* Cq resistance studies based on evidence of recent drug use.

Quantitative measurements of Cq + metabolites in urine can be a useful adjunct to drug sensitivity studies in the field. Three methods (1–3) are semiquantitative at best, while two recently published methods (4, 5) permit quantitation of Cq + metabolites. The latter methods require more equipment than the former, but may be performed

without mains electricity using a hand-held battery-operated, filter photometer (5).

This paper describes an adaptation of a method developed by E. G. Saker and E. T. Solomons for assay of drugs of abuse in urine (9) which permits facile qualitative or quantitative determination of Cq + metabolites in the field. Cq was partitioned from buffered urine (pH 8) into a chloroform solution of tetrabromophenolphthalein ethyl ester (TBPEE) to give a purple colour, the intensity of which is proportional to the sum of the concentrations of Cq and its metabolites. We quantified Cq + metabolites by comparing the colour intensity between samples and standards either by eye (Saker–Solomons, Cq Method I) or by using a hand-held, battery-operated filter photometer (Saker–Solomons, Cq Method II). These two methods are abbreviated S–S/CqI and S–S/CqII, respectively. We validated S–S/CqI with the Haskins MMII assay (5) during a malaria study in Nigeria and S–S/CqII with a reference high-performance liquid chromatographic (HPLC) method (10) in the laboratory using urine samples from the same study. Both Saker–Solomons tests may be applied without mains electricity.

Materials and methods^a

A hand-held, battery-operated filter photometer^b equipped with a 565-nm filter was used for colorimetric quantification.

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^a Use of trade names and commercial sources is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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Reagents and standards

Chloroquine and desethylchloroquine standards available from earlier studies were donated.^c Chloroform,^d TBPEE,^e $K_2HPO_4 \cdot 3H_2O$,^f and KH_2PO_4 ^f were obtained commercially. All other chemicals were of reagent grade or better. The pH-8 buffer contained 324 g of $K_2HPO_4 \cdot 3H_2O$ and 10 g of KH_2PO_4 in 1 litre of water. The solution of 50 mg TBPEE dissolved in 100 ml of chloroform was shaken with 10 ml of 2 mol/l aqueous HCl. After the separation of phases, the aqueous phase was aspirated by Pasteur pipette, leaving 0.05% TBPEE-in-chloroform reagent solution. Sunlight and high ambient temperatures promote decomposition of the reagent in solution, which may be monitored by noting the decrease in reagent colour intensity with time. To minimize decomposition the solution should be stored in the dark and refrigerated, using a fresh solution every 3 weeks. If the solution is stored at room temperature, fresh solutions should be made every 3–4 days.

Procedure for the S–S/CqI assay

Pre-field preparations. Screw-cap conical centrifuge tubes (15 ml) were charged with 1 ml of pH-8 phosphate buffer and 0.2 ml of the TBPEE solution.

Field assay. Urine (2 ml) from each subject was transferred to a pre-charged centrifuge tube. The tubes were capped, vigorously hand-shaken for about 15 seconds, and kept standing for about 15 minutes to allow phase separation before the result was read. A yellow-green colour of the chloroform layer indicates a negative test for Cq + metabolites; a red to purple colour of the organic layer indicates a positive result with the shade depending on the concentration of Cq + metabolites. We estimated Cq concentrations by comparing the sample tubes with tubes of control urine fortified with 0, 1, 2, or 3 $\mu\text{g/ml}$ Cq.

Procedure for the S–S/CqII assay

Pre-field preparations. The 0.05% TBPEE-in-chloroform solution was diluted to 0.02% with chloroform; screw-cap culture tubes (13 × 100 mm) were charged with 2 ml of the diluted (0.02%) TBPEE solution and 1 ml of pH-8 phosphate buffer.

Field assay. Urine (2 ml) from each subject was transferred to a pre-charged culture tube. The tubes were capped and inverted 30 times by hand; many tubes

may be grasped and inverted simultaneously. The tubes were kept standing for about 15 minutes and the chloroform phase was clarified using a glass-wool swab (5). The percentage transmittance of the chloroform layer was read using the filter photometer (565-nm filter). Each value was recorded, converted to absorbance, and compared with a freshly prepared standard curve to yield the concentration values. In practice, the filter photometer was zeroed with the blank, and measurements on standards and samples were made relative to this value. The absorbance of the blank was 0.21 when measured relative to chloroform and is due largely to the colour of the TBPEE-in-chloroform solution.

Procedure for liquid chromatographic assay

The liquid chromatographic method has been previously described (10); 100- μl samples of urine were taken for HPLC analysis.

Determination of cross-reactivity of other antimalarials to S–S/CqII

Blank urine was spiked to give individual 5 $\mu\text{g/ml}$ concentration standards for each of 9 antimalarial drugs or metabolites. We analysed each standard by S–S/CqII, subtracting the blank absorbance, and compared the resulting value with that from a 5 $\mu\text{g/ml}$ Cq standard.

Comparison of the S–S/CqI, Dill–Glazko, and Haskins MMII tests during field trials

The S–S/CqI method was evaluated in Oban (Cross River State) and Igbo Ora (Oyo State), Nigeria, in connection with *in-vivo* and *in-vitro* drug sensitivity studies during July and August of 1987. Thick blood smears from consecutive children <5 years of age who were brought to the local health clinics at the two study sites were examined for the presence of *Plasmodium* parasites. Children whose blood smears were positive for *P. falciparum* were treated with 25 mg/kg Cq in three doses (10, 10 and 5 mg/kg) over 3 days. A urine sample was collected from each study patient before treatment on day 0 and at follow-up visits on days 2, 7, and 14.

Urine samples were analysed by the S–S/CqI, Dill–Glazko, and Haskins MMII assays at the local health clinic laboratories in Oban and Igbo Ora. Urine specific gravities were measured and recorded. The quantitative Haskins MMII test was interpreted according to the percentage of light transmittance using a filter photometer. A urine sample was considered positive by S–S/CqI if the colour changed to purple in the chloroform layer, by Dill–Glazko if the reagent colour changed to rose, and by Haskins

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MMII if there was <70% transmittance (corresponding to a concentration of >1 µg/ml Cq + metabolites (5)).

Comparison of the S-S/CqII and HPLC assay results

A 3-ml sample from each urine collection in the study in Nigeria was refrigerated and transported to the Centers for Disease Control laboratories in Atlanta for later analysis by S-S/CqII and HPLC.

Results

Comparison of the S-S/CqI, Dill-Glazko, and Haskins MMII tests in the field

Table 1 shows the results of 115 urine samples tested by both S-S/CqI and Haskins MMII. The sensitivity of the S-S/CqI test is 95% and its specificity 99% relative to Haskins MMII. There is no significant difference in results obtained by the S-S/CqI and the Haskins MMII tests. The detection limit for S-S/CqI is 1 µg/ml. Table 2 shows poor agreement of results from the Dill-Glazko test and the Haskins MMII test; 19 out of 119 samples gave negative results for the former and positive results for the latter.

Comparison of the S-S/CqII and HPLC assays in the laboratory

Fig. 1 shows a standard curve for the S-S/CqII assay prepared by fortifying the control urines at 0, 2, 4, 6, and 8 µg/ml with Cq. A result from assay of a 12 µg/ml standard is also plotted to illustrate the limits of linearity of the method. The standard curve is essentially linear to a concentration of approximately 8 µg/ml; at higher concentrations the absorbance response per unit of concentration falls. The correlation coefficient for the curve shown in Fig. 1 is 0.9896 over the range of 0 to 8 µg/ml but is 0.977 if the 12 µg/ml data point is included in the

Table 1: S-S/CqI urine test compared with the Haskins MMII (n = 115)

	Haskins MMII	
	Positive ^a	Negative
S-S/CqI:		
Positive	41	1
Negative	2	71
Sign Test: ^b P = 0.50	Sensitivity = 0.95 Specificity = 0.99 Pos. predictive value = 0.98 Neg. predictive value = 0.97	

^a Positive Haskins MMII result corresponds to ≤70% transmittance.

^b Non-parametric test for related samples.

Table 2: Dill-Glazko urine test compared with the Haskins MMII (n = 119)

	Haskins MMII	
	Positive	Negative
Dill-Glazko:		
Positive ^a	28	4
Negative	19	68
Sign Test: ^b P = 0.001	Sensitivity = 0.60 Specificity = 0.94 Pos. predictive value = 0.88 Neg. predictive value = 0.78	

^a Positive Haskins MMII result corresponds to ≤70% transmittance.

^b Non-parametric test for related samples.

calculation. The deviation from linearity above 8 µg/ml is apparent. The detection limit for this test is 2 µg/ml.

Table 3 compares the results from urine samples by the S-S/CqII assay with those tested by the HPLC assay. The HPLC values represent the sum of concentrations found for Cq and its metabolites

Fig. 1. Calibration curve for the S-S/CqII assay. The standard curve is for the range 0-8 µg/ml (see text).

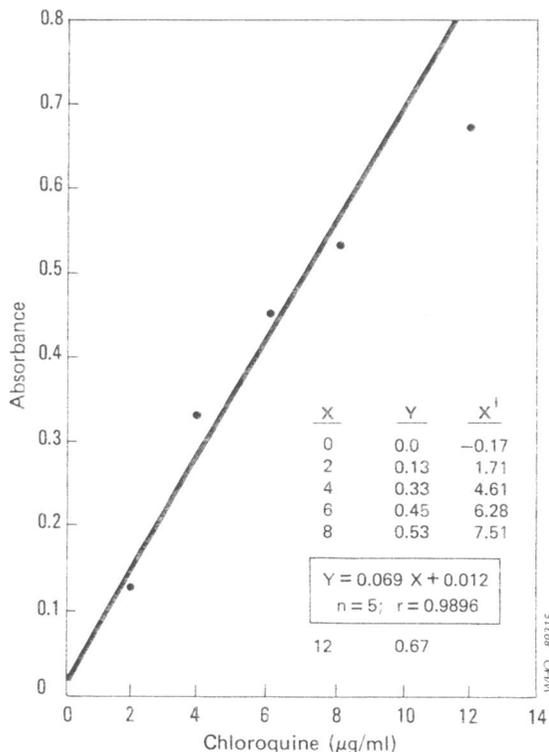


Fig. 2. S-S/CqII results compared with high-performance liquid chromatographic results (data from Table 3). S-S/CqII results combine the contributions from DECq and Cq; for HPLC values of Cq + DECq above 8 µg/ml the corresponding S-S/CqII values are low, as expected from the nonlinearity of the S-S/CqII method above 8 µg/ml (see text).

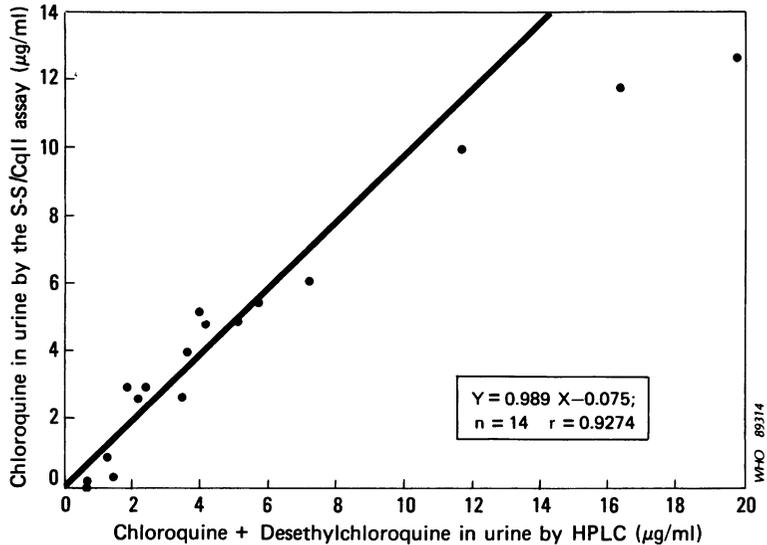


Table 3: Concentrations of chloroquine and metabolite in urine analysed by Saker-Solomons test and by high-performance liquid chromatographic (HPLC) assay

Sample identifier ^a	S-S/CqII value (µg/ml)	HPLC results (µg/ml)		
		Cq	DECq	Cq + DECq
22-D14	2.58	2.58	0.92	3.50
49-D14	2.87	1.49	0.86	2.35
108-D14	0.00	0.54	0.24	0.78
122-D14	2.89	1.27	0.58	1.85
134-D14	5.19	3.27	0.68	3.95
144-D14	0.26	0.88	0.53	1.41
148-D14	0.11	0.71	<0.2	0.71
232-D14	0.84	1.01	0.24	1.25
174-D07	4.75	3.02	1.14	4.16
201-D07	4.03	2.87	0.82	3.69
241-D07	5.48	4.02	1.75	5.77
321-D07	2.58	1.90	0.34	2.24
337-D07	6.06	6.15	1.13	7.28
342-D07	4.90	3.90	1.16	5.06
21-D02 ^b	9.86	8.09	3.59	11.7
112-D02 ^b	11.7	12.9	3.46	16.4
127-D02 ^b	12.6	18.3	1.47	19.8

^a The second portion of the sample identifier refers to the number of days (14, 7 and 2) after treatment when the sample was taken.

^b When measured by HPLC, these samples contained a sum of Cq and DECq beyond the range of linearity for S-S/CqII.

(primarily DECq) since Cq and DECq were shown to provide essentially equivalent responses to both Saker-Solomons tests. A standard curve over the range of 0 to 8 µg/ml Cq was used to calculate the

S-S/CqII results. Least-squares comparison of S-S/CqII test results to the summed HPLC data for the range of 0 to 8 µg/ml of Cq (Fig. 2) yields the statistical result $y = 0.989x - 0.075$; $n = 14$, $r = 0.9274$.

Cross-reactivity of other antimalarials to S-S/CqII

The results of the cross-reactivity study are shown in Table 4.

Table 4. Relative sensitivities of S-S/CqII to some common antimalarial drugs and metabolites

Drug (X)	Absorbance _X /Absorbance _{Cq}
Chloroquine	1.0
Quinine	0.74
Proguanil	0.98
Cycloguanil	1.15*
Mefloquine	0.62
Desethylamodiaquine	0.12
Sulfadoxine	0.0
Primaquine	0.0
Pyrimethamine	0.31

* The test resulted in an amber colour rather than the characteristic purple colour.

Discussion

The S-S/CqII method meets all the criteria of an acceptable field test—sensitivity, reliability, simplicity, rapidity, and low cost—and proved to be as easily taught to and performed by field technicians as the Dill-Glazko test. An average of 50 specimens

were processed in 1 hour, including time for labelling and recording. S-S/CqI is more sensitive than the Dill-Glazko test and therefore more effectively excludes patients who recently ingested Cq. It has been argued that Cq concentrations in urine of less than 1 µg/ml (the detection limit of S-S/CqI) indicate corresponding blood concentrations of less than 100 ng/ml, the therapeutic threshold concentration for Cq-sensitive strains of *P. falciparum* (11). The negative predictive value (97%) for the S-S/CqI test indicates that this test is useful during *in-vivo* drug sensitivity studies for including only persons with a urine Cq concentration of <1 µg/ml (Table 1). The high positive predictive value (98%) indicates that this test is acceptable for documenting drug absorption in patients on day 2 or day 7 after Cq treatment. Table 2 shows that both positive and negative predictive values of the Dill-Glazko test are substantially lower than those of S-S/CqI relative to the Haskins MMII test used for validation.

The S-S/CqI test does not detect drugs such as caffeine, nicotine, aspirin, paracetamol (acetaminophen), or antibiotics which might be in common use and give false positive results (9, 12). However, the Saker-Solomons Cq methods, like the bromthymol blue (4) and Haskins tests (4, 5), give positive results for the antimalarials quinine and proguanil; relative sensitivities of the S-S/Cq tests to these and other antimalarials are shown in Table 4. Even so, in applications in *in-vivo* and *in-vitro* Cq sensitivity trials, such imperfect specificity for Cq would serve the designated purpose of excluding patients with appreciable concentrations of antimalarial drugs in their bodies that could adversely affect the outcome of the studies.

Urine-Cq tests are also useful for community investigations of drug-use practices. For example, an individual taking 25 mg/kg Cq in response to a febrile episode will be found positive for approximately 10 days using a urine-Cq method with a 1 µg/ml detection limit; similarly, individuals taking chemoprophylactic Cq (300 mg weekly) will remain positive for up to 5 days after the most recent dose (13). The S-S/CqI assay is adequately sensitive and reliable to determine compliance with therapeutic or chemoprophylactic regimens.

Although the results of a qualitative urine test are sufficient for the purposes of many field studies, quantitative tests can help to estimate the Cq concentration in blood as well (4, 5, 13). Finger-stick blood taken at the time of the urine sample can be examined later in a field laboratory (14) or a more fully equipped laboratory (10). Selection of blood samples for analysis would depend on the parasitological and urine assay results. S-S/CqII is a reliable method for quantifying Cq + metabolites in urine in

the range of 0 to 8 µg/ml, the test results correlating highly with the sum of Cq and DECq concentration in urine as determined by HPLC.

In summary, for measuring Cq in urine, the S-S/CqI assay yields results which correlate excellently with the Haskins MMII test, one of the most sensitive colorimetric tests to date, and is as simple to run as the Dill-Glazko test, but is more sensitive and reliable. The average cost of reagents per urine test in our experience is US\$ 0.02 for the S-S/CqI test, \$0.005 for the Dill-Glazko test, and \$0.05 for the Haskins MMII test. The companion method, S-S/CqII, provides the option of quantifying Cq + metabolites but demands slightly greater effort and with a modest compromise in sensitivity. As demonstrated by our study, the S-S/Cq methods can be used in field studies instead of the Dill-Glazko method.

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Résumé

Adaptations du test de Saker-Solomons : méthodes colorimétriques simples et fiables pour le dosage sur le terrain de la chloroquine et de ses métabolites dans les urines

Deux méthodes de détection de la chloroquine (Cq), un antipaludique, et de ses métabolites (principalement la déséthylchloroquine (DECq)) dans les urines, adaptées au travail sur le terrain, ont été mises au point et validées. Ces méthodes sont des variantes du test de Saker-Solomons (S-S) qui sert au dépistage dans les urines de certains médicaments susceptibles de donner lieu à des abus. A partir de l'urine tamponnée (pH 8), la chloroquine est extraite dans une solution d'ester éthylique de tétrabromophénolphtaléine (TBPEE)

dans le chloroforme, qui vire au violet. L'intensité de la coloration est proportionnelle à la somme des concentrations de la Cq et de ses métabolites. Dans la première méthode (S-S/CqI), on compare à l'oeil nu les échantillons et les témoins; c'est une méthode aussi simple à appliquer que le test de Dill-Glazko, mais elle est plus fiable et plus sensible, avec une concentration limite décelable de 1,0 µg/ml. La seconde méthode (S-S/CqII) permet au moyen d'un photomètre à filtre (565 nm) alimenté par piles de quantifier sur le terrain la Cq et ses métabolites avec une concentration limite de 2,0 µg/ml. Le proguanil, la quinine et la méfloquine donnent également des résultats positifs avec ces épreuves.

La méthode S-S/CqI a été validée au cours d'une étude effectuée sur le terrain au Nigéria en comparant ses résultats et ceux du test de Dill-Glazko aux résultats obtenus avec le dosage de Haskins MMII, une méthode colorimétrique classique. Les résultats de la S-S/CqI concordaient avec ceux de la méthode de référence, contrairement à ceux de l'épreuve de Dill-Glazko. La seconde méthode a été validée au laboratoire en comparant ses résultats avec ceux obtenus pour des échantillons identiques analysés par chromatographie liquide à haute performance (HPLC). Jusqu'à 8 µg/ml les valeurs obtenues avec la S-S/CqII, comparées à la somme des concentrations de Cq et de DECq obtenues par HPLC, suivent une droite de régression linéaire donnée par l'équation $y = 0,989x - 0,075$ ($n = 14$), avec un coefficient de corrélation de 0,9274.

On avait besoin d'une méthode de terrain pour rechercher la présence de la Cq et de ses métabolites chez les sujets susceptibles de participer à des essais *in vitro* de pharmacosensibilité et pour vérifier l'observance du traitement au cours de ces essais et en chimioprophylaxie. La méthode S-S/CqI répond à ces critères et, bien qu'un peu plus complexe et légèrement moins sensible, la méthode S-S/CqII permet de doser la Cq et ses métabolites. On a démontré l'utilité de ces variantes de la méthode de Saker-Solomons pour le dosage de la chloroquine lors de l'analyse d'échantillons d'urine provenant d'un essai réalisé sur le terrain au Nigéria. Ces deux épreuves peuvent être pratiquées en l'absence de raccordement au secteur.

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