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FIELD-ADAPTED METHOD FOR HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC DETECTION AND ESTIMATION OF CHLOROQUINE AND DESETHYLCHLOROQUINE IN URINE

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SUMMARY

A high-performance thin-layer chromatographic (HPTLC) method which can be used in remote field locations lacking electricity has been developed for selective and sensitive detection and estimation of chloroquine (Cq), desethylchloroquine (DECq) and two other antimalarial compounds in urine. The method requires a single extraction step and has a detection limit of 0.25 μg/ml for both Cq and DECq. The HPTLC method was coupled with a colorimetric field assay for Cq and metabolites in urine and a field-interfaced, laboratory-based, high-performance liquid chromatographic assay of Cq and DECq in finger-stick blood to survey antimalarial drug use practices in Esmeraldas Province in northwestern Ecuador. Fourteen of 66 patients from whom urine samples were obtained were found to have detectable Cq and DECq. Results of the three assays are compared for these individuals, and the role of the HPTLC method in field studies is assessed.

INTRODUCTION

It is often important in malaria field studies to establish the presence and measure the concentrations of antimalarial drugs and their metabolites in blood and urine. Such information is vital in surveys of drug use practices, chemoprophylaxis compliance, and, in concert with in vivo drug sensitivity testing, presence of drug resistance in strains of *Plasmodium falciparum*. Selective and sensitive assay of chloroquine (Cq) and its major metabolite, desethylchloroquine (DECq) (Fig. 1), is possible using high-performance liquid chromatography (HPLC) with fluorescence detection [1,2]. These methods require the use of sophisticated instruments, although interfacing with field studies is possible by approaches such as preservation of blood samples on filter paper for transport to the laboratory

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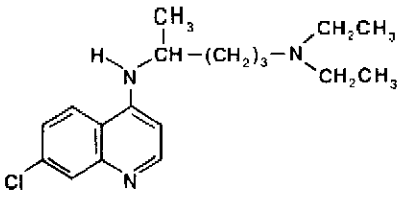
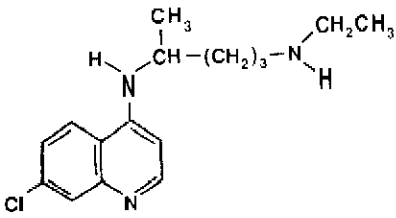


Chloroquine



Desethylchloroquine

Fig. 1. Structures of chloroquine and desethylchloroquine.

[3,4]. Improved colorimetric methods have been introduced to permit quantification of Cq and metabolites in urine in the field [5,6]. Obtaining such results in the field can provide important information which can guide the study and screen samples for possible additional determinations later at a central laboratory. The colorimetric methods are less specific than chromatographic methods and are not sufficiently sensitive to quantify Cq and DECq in blood.

A thin-layer chromatographic (TLC) method has been paired with a colorimetric method to provide confirmation of Cq and DECq presence [6]. High-performance thin-layer chromatography (HPTLC) has been used to quantify Cq and DECq in the laboratory using fluorescence densitometry with a purported detection limit of 0.01 μmol/l (0.003 μg/ml [7]; adaptation to the field precludes use of the apparatus for quantitative sample application and use of the fluorescence densitometer [7].

In the present study we describe a field-adapted HPTLC method suitable for detecting and quantifying Cq, DECq, and other antimalarials and metabolites in urine. Sample addition to the HPTLC plates is in 500-nl increments, which is at the upper end of the range of incremental volumes used in HPTLC. Addition of the samples in this incremental volume permits a useful balance among convenience of sample application, time of analysis, fraction of total extract spotted, chromatographic efficiency, and method sensitivity. The method can be used under primitive conditions and requires no electricity. An application of the method under field conditions is described, and the results are compared and correlated with two reference methods, the Haskins MMII colorimetric method [5] and an assay in which finger-stick blood preserved on filter paper is quantified using HPLC [3].

EXPERIMENTAl

Standards

Cq diphosphate was purchased from Sigma (St. Louis, MO, U.S.A.). DECq base was supplied by Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). Quinine hydrochloride was purchased from Merck, Sharp and Dohme (Rahway, NJ, U.S.A.). Desethylamodiaquine was provided by Parke-Davis Division of Warner-Lambert (Ann Arbor, MI, U.S.A.).

Reagents and materials

Spectroscopic-quality methyl *tert.*-butyl ether (MTBE) and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were glass-distilled. Sodium phosphate, tribasic dodecahydrate was from Aldrich (Milwaukee, WI, U.S.A.) and SAG-10 silicone antifoam emulsion was from Union Carbide (Danbury, CT, U.S.A.). Silica-gel 60 HPTLC plates (10 cm × 10 cm) with impregnated fluorophore were from Merck (Darmstadt, F.R.G.).

Equipment

HPTLC was performed by using a linear developing chamber for 10 cm × 10 cm plates from CAMAG (Wrightsville Beach, NC, U.S.A.). Samples were transferred to the plate by using a 25-μl Hamilton (Reno, NV, U.S.A.) blunt-tipped syringe secured in a Hamilton PB-600-1 repeating dispenser. Plates were visualized with the aid of a Mineralight lamp, Model G-14, which provides short-wave ultraviolet (254 nm) illumination and is battery-operated (UVP, San Gabriel, CA, U.S.A.).

HPTLC method

A stock standard was prepared that contained 1.0 mg/ml (expressed as base) each of Cq and DECq dissolved in 0.001 *M* aqueous hydrochloric acid in a screw-cap vial. Accurately measured volumes of this solution were added with a 50-μl syringe to 15-ml screw-cap centrifuge tubes to yield 0.0, 1.0 and 5.0 μg/ml standards for use to quantify 5-ml urine samples (0.0 μg/ml, 0.0 μl; 1.0 μg/ml, 5.0 μl; 5.0 μg/ml, 25.0 μl). Centrifuge tubes fortified with standards and those labeled for analysis of samples were each charged with about 1 ml of dry sodium phosphate and with exactly 0.8 ml of MTBE. A 5-ml quantity of control (blank) urine was then added to each standard tube, and 5 ml of sample urine were added to the labeled sample tubes. All tubes were capped and inverted twenty times (right side up to right side up is counted as one inversion). Many tubes were grasped and inverted simultaneously. The tubes were allowed to stand for about 1 min to permit the foamy MTBE layer to separate. One drop of SAG-10 silicone antifoam emulsion was added to each MTBE layer and the tube gently agitated. The samples and standards were allowed to stand until the MTBE layer clarified; 5 min was usually sufficient. Occasionally a second drop of SAG-10 was required to

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clarify the MTBE layer. The 25-μl syringe mounted in the repeating dispenser was used to transfer (spot) 10-μl quantities in 0.5-μl increments of each standard and sample in turn onto the HPTLC plate. The solvent evaporated in a few seconds after the addition of each 0.5-μl increment, so that each sample or standard was applied in twenty increments over a period of about 1 min. The cumulative addition of 10 μl gave a spot about 2 mm in diameter for each sample and standard. Opposite edges of the plate were spotted and accomodated a total of 28 standards and samples. The plate was placed in the linear development chamber and eluted from the two edges to the middle of the plate with a solvent of 18% methanol–8% concentrated ammonia in MTBE (18 ml of methanol and 8 ml of ammonia were diluted to 100 ml with MTBE in a graduate cylinder). After elution (about 10 min) the plate was air-dried and viewed under the ultraviolet lamp. In cases where the presence of quinine or desethylamodiaquine [8] in urine was suspected, the standards used were also fortified with these compounds.

Field study

The HPTLC method was evaluated in San Lorenzo (Esmeraldas Province, Ecuador) in connection with a survey of patterns of antimalarial drug use. This was part of a larger survey conducted to prepare for a proposed in vivo drug sensitivity study. Persons who came to the Servicio Nacional de la Eradicacion de Malaria (SNEM) headquarters in San Lorenzo because they believed they had malaria were asked to provide information for a short questionnaire on history of drug use and provide a urine sample and finger-stick blood; the latter was used to yield a 100-μl sample on filter paper for laboratory analysis of Cq and DECq and to give a thick smear on a microscope slide which was examined for parasites. Patients with smears positive for either *P. falciparum* or *P. vivax* were given a curative regimen of chloroquine diphosphate.

Seventy-six patients were seen during five consecutive weekdays. Blood samples were obtained from all 76 of these and urine samples from 66. Urine samples were analyzed at SNEM headquarters for Cq, DECq, and quinine by HPTLC. The Haskins MMII method [6] was used at SNEM to quantify Cq and DECq in urine. Urine specific gravities were measured and recorded. Filter paper blood samples were transported to the laboratory for analysis by a published method [3].

RESULTS AND DISCUSSION

Characterization of the HPTLC method

Partitioning between the 5 ml of basic urine and 0.8 ml of MTBE results in the concentration of Cq and DECq into the organic phase. The final volume of MTBE after the extraction step is about 0.5 ml due to the solubility of this solvent in water. The 10-μl quantity of organic extract transferred to the TLC plate represents roughly a 1/50 aliquot of the total extract; thus, the amount of analyte spotted on the plate is equivalent to that in 100 μl of urine. The detection limit under these conditions is about 0.25 μg/ml (ppm) each for Cq and DECq.

The use of adapted HPTLC (10-μl sample addition in 0.5-μl increments) rather

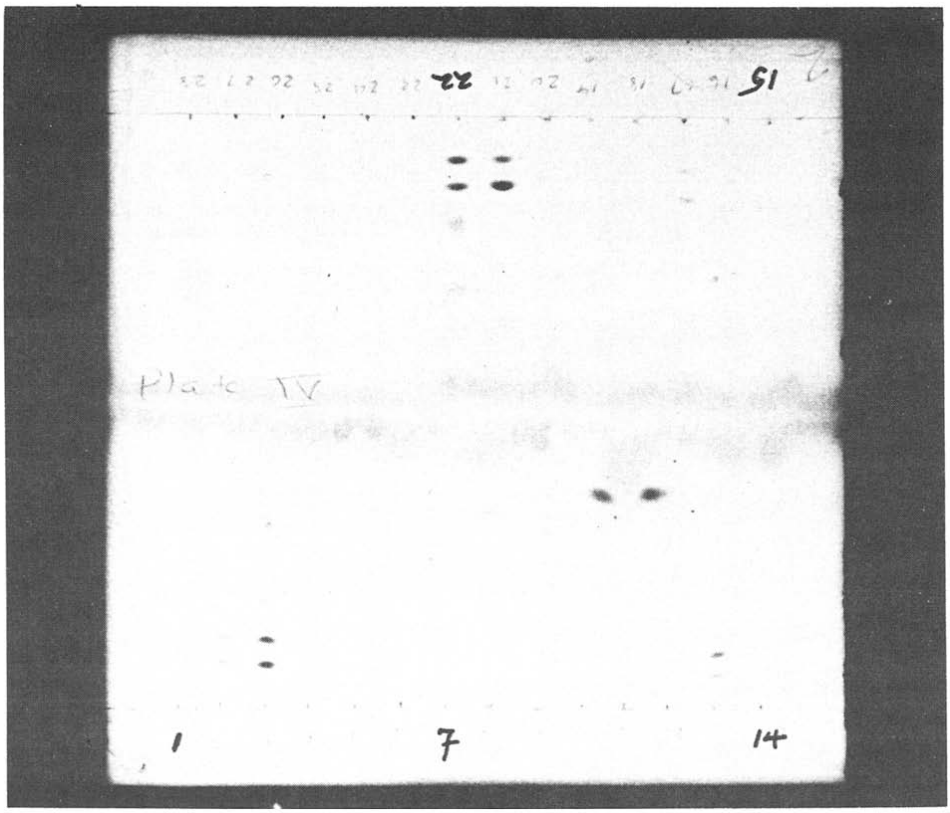

Fig. 2. HPTLC profile of standards and samples from a field study in Ecuador. The photograph of the plate, impregnated with a fluorophore, was taken under ultraviolet light with emission centered at 254 nm. Cq has an R_F of 0.14, DECq 0.23, and quinine 0.36 for these HPTLC conditions. The number in the first column of each set corresponds to the position of the spot on the plate; the second column identifies the standard or sample. (1) Blank; (2) standard, 1 μg/ml; (3) standard, 5 μg/ml; (4) 25; (5) 26; (6) 27; (7) 28; (8) 29; (9) 30; (10) 31; (11) 32; (12) 33; (13) PCV; (14) 35; (15) 36; (16) 37; (17) 38; (18) 41; (19) 42; (20) 43; (21) 44; (22) standard, 5 μg/ml.

than conventional TLC [6] contributes to the sensitivity and selectivity of the method. The size of the spots are small when transferred using the hand-held dispenser, and dispersion during elution is minimized by HPTLC elution conditions. The small size of the spots upon viewing under the ultraviolet light results in good sensitivity and good resolution of Cq and DECq. The two-spot pattern with Cq eluted just above the smaller, less intense DECq spot selectively, indeed perhaps specifically, confirms a history of Cq use (Fig. 2).

Useful information regarding the presence or absence of Cq (and DECq) may be gained by viewing the transferred sample spots at the origin before elution. Standards will show small fluorescence-quenched spots under ultraviolet light which are well inside the circle resulting at the point of maximum radial solvent travel for the spot. The same is true of sample spots where Cq (and DECq) are present in the sample. Elution confirms that Cq and DECq are responsible for this effect and not some other compound(s) which is (are) similarly tightly held by the silica gel plate under spotting conditions.

TABLE I

EVALUATION OF HPTLC METHOD: MEASUREMENT OF Cq AND DECq IN URINE COMPARED TO TWO COMPLEMENTARY METHODS

Patient No.	Urine analysis for Cq (μg/ml)		Blood analysis (ng/ml)	
	HPTLC*	Haskins MMII**	Cq	DECq
8	>5	> 12.2	61	28
20	0-1	< 1	<5	<5
28	0-1	< 1	7	<5
32	0-1	< 1	100	<5
38	~1	< 1	19	<5
39	—***	7.16	64	25
44	~5	7.37	139	45
45§	0-1	1.96	66	7
54	~5	7.96	167	29
57	>5	> 12.2	135	31
67§	0-1	1.75	49	43
68	~5	4.6	51	19
72	>5	5.3	248	22
75	>5	226	566	179

*The amount of Cq found is estimated within the ranges of 0-1, ~1, 1-5, ~5, and >5 μg/ml. Confirmation of Cq presence required the appearance of a spot at the R_F corresponding to Cq and a less intense spot at the R_F indicative of DECq.

**Result reflects Cq concentration plus 1/2 DECq concentration.

***Too little sample for a 5-ml urine HPTLC determination.

§In these samples, the Cq and DECq were barely detectable by HPTLC and higher R_F spots were observed. It is likely that the higher R_F components contributed to the Haskins MMII result.

The R_F values observed for a representative experiment under the described HPTLC elution conditions for DECq, Cq, desethylamodiaquine [8], and quinine were 0.14, 0.23, 0.27, and 0.36, respectively. This order of elution was preserved in all analyses run, although the exact R_F value varied slightly depending on such factors as the activity of the plate and whether appreciable ammonia had evaporated from the solvent. For best results the stock of eluting solvent should be made fresh daily or precautions taken to minimize evaporation of ammonia. The few milliliters of solvent used for each run are discarded after the run and the developing chamber disassembled and dried between chromatographic runs. Standards containing analytes of interest for a given group of samples were routinely spotted to characterize the sample both qualitatively and semiquantitatively. The eluted HPTLC plates may be retained as a permanent record of each analysis, to be reviewed at a later date if desired.

Field application and evaluation of the HPTLC method

Those patients for which Cq and DECq were detected in urine by HPTLC (except for the sample from patient 39, see below) are listed in Table I. Haskins MMII results for Cq plus DECq in urine and HPLC results for Cq and DECq in finger-stick blood are also listed (Table I).

The HPTLC method is a sensitive and selective indicator of the presence of Cq and DECq and thus of a history of Cq use. The HPTLC results, however, provide only an estimate of the Cq and DECq concentrations based on visual comparison with standards run concurrently. The Haskins MMII method is less sensitive (detection limit of 1.0 μg/ml compared to 0.25 μg/ml for the HPTLC method) and provides a determination of Cq plus metabolite which represents an intermediate value between Cq and the sum Cq plus DECq [6]. The most useful concentration parameter in characterizing the contribution of Cq dosing to antimalarial effect is that of Cq and DECq concentration in blood, listed in the fourth column of Table I. The HPTLC method is effective as a screening tool, indicating which blood samples should be analyzed in the laboratory. The Haskins MMII method complements the HPTLC method by providing a more precise estimate of Cq plus metabolite on urine samples in the field.

The HPTLC and Haskins MMII results compare reasonably well. The urine sample for patient 39 was insufficient for a 5-ml HPTLC test. In samples from patients 45 and 67, the amount of Cq and DECq present was barely detectable by HPTLC, while the values from the colorimetric tests were 2.03 and 1.75 μg/ml, respectively. This is probably due to a contribution to the less specific colorimetric result by compounds seen at higher R_F by HPTLC. These may well be ion-pair-extractable with methyl orange and thus contribute to the apparent Cq measured [6]. The fact that the weak spots seen by HPTLC are real and attributable to Cq and DECq is confirmed by the blood analysis results. No evidence of Cq or DECq is seen by the HPTLC urine test or the blood analysis for three samples (from patients 21, 47, and 53; not shown in the table), although the Haskins MMII result is between 1 and 2 μg/ml for each. In each case, high R_F spots are seen for the sample by HPTLC, indicating the likelihood of low-level, false-positive results for these samples from non-specificity of the Haskins MMII test for Cq.

A representation of an HPTLC plate used in the field study in Ecuador is shown (Fig. 2). The figure is a black-and-white photograph taken under ultraviolet (254 nm) illumination of a plate which had been spotted with standard and sample extracts and eluted as described in the Experimental section. The plate itself, when viewed under ultraviolet light, displays dark spots on a fluorescent green background. Direct viewing of the plate under ultraviolet light permits slightly more sensitive detection than is apparent from the photograph. For example, the spots at the R_F values corresponding to Cq and DECq are distinctly visible by direct viewing under ultraviolet light for the samples at positions 7 and 11, whereas these spots are not readily apparent in the photograph. The standards (positions 2, 3, and 22) and the samples in positions 13, 17, and 21 on the plate exhibit spots which are easily seen on the photograph as well as under direct observation of the original plate under ultraviolet light. The correspondence between plate position and sample number is listed in the legend to Fig. 2. The sample extract spotted in position 13 was that of a Peace Corps Volunteer (PCV) following a chemoprophylactic regimen; the corresponding Haskins MMII result was 2.25 μg/ml.

Urine samples from patients 8 and 57 were found to contain marginally greater than 12.2 μg/ml Cq plus metabolite by the Haskins MMII test. A more precise

assay of these samples by this method was not run. The urine sample from patient 75, a 5-year-old boy, was found by the initial Haskins MMII test to contain much greater than 12.2 μg/ml. Subsequent determinations using a dilution of the urine demonstrated that the concentration, expressed as Cq, in the urine of patient 75 was 226 μg/ml. A sample of this urine was returned to the laboratory, analyzed by HPLC [3], and found to contain 195 μg/ml Cq and 47 μg/ml DECq. The Haskins MMII assay routinely yields concentration values between those for Cq and the sum of Cq and DECq as measured by HPLC [6]. Of the ten persons from whom no urine samples were collected (all children 3 years of age or less), HPLC assay of the blood detected Cq in two of the ten with the concentration of Cq less than 40 ng/ml in both cases.

CONCLUSIONS AND RECOMMENDATIONS

The detection limit of 0.25 μg/ml for the HPTLC method is ideal for screening urine samples to detect recent Cq use. Cq concentrations in urine of less than 1.0 μg/ml are indicative of Cq concentrations in corresponding blood of less than 100 ng/ml [9]. If one subscribes to the often-cited plasma Cq concentration value of 90 nmol/l (about 30 ng/ml) as the therapeutic threshold concentration for Cq-sensitive strains of *P. falciparum* [10,11] and uses the value of 3.5 as a representative ratio between corresponding whole-blood and plasma Cq concentrations [12], a therapeutic threshold whole-blood concentration of about 100 ng/ml Cq results. Thus a negative result by the HPTLC assay, which sets an upper limit of 0.25 μg/ml on Cq concentration in urine, indicates the presence of appreciably less than the estimated therapeutic threshold concentration of Cq in blood.

The HPTLC method may be used in concert with earlier published field-adapted [6] and field-interfaced [3] methods to provide analytical support of chemoprophylactic and chemotherapeutic studies in malarious areas. As mentioned above, the HPTLC method serves as a convenient and sensitive means to screen for the presence and to estimate concentrations of a number of antimalarial drugs and metabolites in urine in the field. The Haskins MMII test [6] may be used to quantify Cq plus metabolite concentrations in HPTLC-positive urines in the field. Finger-stick blood samples may be preserved on filter paper for each patient and then selected for analysis in the laboratory based on the HPTLC results.

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