A specific ELISA method for determining chloroquine in urine or dried blood spots*

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Reported is an enzyme-linked immunosorbent assay (ELISA) that has been optimized and validated for the determination of chloroquine in urine or dried blood spots. The assay employs antisera raised in sheep to a chloroquine derivative conjugated to keyhole limpet haemocyanin and chloroquine conjugated to porcine thyroglobulin adsorbed onto the wells of a microtiter plate. The competitive binding of the antiserum to the wells was monitored using an alkaline-phosphatase-conjugated second antibody and a specific substrate. The assay exhibits no cross-reactivity with known chloroquine metabolites, other antimalarials, and commonly used drugs. The method was used to determine chloroquine in dried blood spot extracts and urine from a patient who was receiving a prescribed prophylactic chloroquine regimen. The drug was detected in the urine for 17 weeks and in the dried blood spots for 4 weeks after termination of the therapy.

INTRODUCTION

Malaria is still the most important parasitic disease in the tropics. Worldwide, some 200 million people are believed to be chronically infected, and in Africa alone it is estimated that the disease is responsible for the deaths of 1 million infants and children each year. Chloroquine has been the drug of choice for the treatment and prevention of all forms of malaria; unfortunately, however, increasing numbers of Plasmodium falciparum strains have become resistant to it. Nevertheless, oral chloroquine remains the drug of choice for the treatment of malaria other than falciparum and for all malarious areas where a high level of resistance is not prevalent.

Specific and sensitive methods for the determination of chloroquine in body fluids permit the calculation of more accurate pharmacokinetic parameters, which are important in planning dose schedules to obtain optimal plasma levels, in minimizing toxicity, and in drug formulation studies. Walker et al. have indicated that such parameters are particularly important for chloroquine, since the drug is widely administered to children in whom large differences in body weight and metabolic rate occur, particularly if they are suffering from malaria (1). Also, Ofori-Adjei et al. have demonstrated the necessity of determining chloroquine levels in hospitalized children prior to their further treatment, in order to avoid giving dangerously high levels of the drug (2). Furthermore, the determination of the levels of chloroquine in biological fluids is important also because of the presence of parasites that are resistant to the drug. Factors such as insufficient blood concentration of chloroquine due to malabsorption, differences in drug metabolism, or incorrect dose schedules may play a role in the spread of chloroquine resistance.

Chloroquine can be determined in biological fluids by a variety of analytical methods. Several workers have described assays based on fluorometry (3, 4), gas-liquid chromatography (5, 6), high-performance liquid chromatography (7, 8), and radioimmunoassay (9). However, even though the chromatographic methods offer the required sensitivity, selectivity, and reliability for analysis in biological fluids, they require expensive and complex equipment as well as highly trained staff. Their use is thus restricted to well-equipped laboratories, which may not be available in areas where malaria is endemic.

There is accordingly a need for a specific assay for chloroquine that requires only simple equipment and that can be used by relatively untrained staff, especially in the peripheral health services where...
decisions on the use of antimalarials have to be made. We have therefore developed a simple enzyme-linked immunosorbent assay (ELISA) for chloroquine that fulfils these requirements and describe here the method and some results we obtained using it.

MATERIALS AND METHODS

Reagents

Chloroquine, quinine, primaquine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, N-hydroxysuccinimide, fluorescein isothiocyanate, ethylenediamine hydrochloride, porcine thyroglobulin (PTG), sodium amide, Triton X-100, sodium azide, and triethylamine were obtained commercially, as were salts for buffers, all organic solvents ("Analal" grade), and keyhole limpet haemocyanin. N-Desethylchloroquine and N,N-didesethylchloroquine were synthesized according to the method described by Surrey & Hammer (10), while nitrophenyl phosphate disodium salt was obtained commercially. Halofantrine and mefloquine were gifts from the Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC, USA.

Assay standards

A 0.001-mol/l stock solution of chloroquine phosphate in distilled water was diluted in the appropriate buffer or urine to give standard solutions corresponding to 5 × 10⁻⁴, 10⁻⁴, 5 × 10⁻⁴, 10⁻⁴, 5 × 10⁻⁵, and 5 × 10⁻⁷ mol/l chloroquine.

Urine and blood specimens

A male patient aged 43 years was given two tablets (each containing 155 mg chloroquine base) on Mondays and Fridays for 4 weeks during exposure to risk in a malarious area for 6 weeks after leaving the area. For a week prior to the completion of this regimen and immediately following its completion, urine samples were taken from the patient together with fingerprick blood samples, and the latter were spotted onto a Whatman No. 1 qualitative filter-paper according to the method described by Rowell & Rowell (11). Blood and urine samples were taken daily for 1 week and periodically thereafter for a total of 17 weeks. The urine samples were stored at -20 °C and the dried blood-spot samples in plastic bags at +4 °C until assayed.

Diluent buffer. Phosphate-buffered saline containing 0.05% Tween 20 (PBST) (pH 7.4) was prepared from 36.0 g sodium chloride, 2.15 g potassium dihydrogen phosphate, 7.4 g disodium hydrogen phosphate, 2.5 ml Tween 20, and 0.5 g sodium azide and made up to 5 litres with distilled water.

Coating buffer. Carbonate-bicarbonate buffer was prepared using 5.6 g of sodium carbonate and adjusted to pH 9 with sodium bicarbonate before being made up to 1 litre with distilled water.

Microtiter plates. Ninety-six-well plates were used.

Fluorimetry. A Baird atomic spectrofluorimeter was used to determine chloroquine extracted from spiked urine standards and urine samples using a method reported by Brodie et al. (12).

Preparation of the immunogens

7-(2-Aminethylamino)-4-(4-diethylamino-1-methylbutylamino)quinoline (compound A). To a solution of chloroquine (0.48 g, 1.5 mmol) in 10 ml dry ethylenediamine under nitrogen at room temperature was added sodium hydride (200 mg, 8.33 mmol) and the reaction mixture was stirred overnight. The solvent was then removed, the residue taken up in chloroform, washed with water, dried over anhydrous magnesium sulfate, evaporated down, and subsequently dried under vacuum to yield a red oil, which was used without further purification.

Chloroquine-keyhole limpet haemocyanin immunogen. To a solution of compound A (100 mg, 0.29 mmol) and keyhole limpet haemocyanin (100 mg) in 8 ml water and 4 ml pyridine was added dropwise, over 40 minutes, a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (92 mg, 0.52 mmol) and N-hydroxysuccinimide (37 mg, 0.32 mmol) in 50% water/pyridine (4 ml). The reaction mixture was stirred at room temperature overnight, dialysed for 3 days, and then lyophilized to yield the immunogen conjugate (90 mg).

Chloroquine-fluorescein tracer. A solution of compound A (10 mg, 0.03 mmol), fluorescein isothiocyanate (12 mg, 0.03 mmol), and triethylamine (50 μl) in 1 ml methanol was stirred at room temperature for 1 hour. After acidification of the mixture by addition of 0.300 μl of 1 mol/l hydrochloric acid, the solvent was removed. The solid product was purified by preparative thin-layer chromatography (eluant: methanol

a Sigma Chemical Co. Ltd., Poole, Dorset, England.
b BDH Chemicals, Poole, Dorset, England.
c Calbiochem-Behring, San Diego, CA 92112, USA.
d Aldrich, Gillingham, Dorset, England.
e Avloclor®.
0.88 ammonium hydroxide (95:3)) to yield a major band (Rf 0.18).

Preparation of coating conjugate and coating of microtiteration plates

A solution of compound A (5 mg, $1.5 \times 10^{-5}$ mol) in 250 µl dry dioxane was added over 15 minutes in 50-µl portions to a solution of 3 mg cyanuric chloride in 500 µl dry dioxane under nitrogen. The mixture was heated to 60 °C for 15 minutes, cooled, and then added in 50-µl portions over 30 minutes to an ice-cold solution of 10 mg PTG in 2 ml of 100 mmol/l sodium phosphate buffer (pH 7.4). The solution was stirred at room temperature for 3 hours and then applied to a Sephadex G25 column (16 mm × 15 cm) that had been equilibrated with phosphate-buffered saline. The purified PTG-chloroquine conjugate was lyophilized and stored at -20 °C.

The wells of microtiteration plates were coated by passive adsorption using 100 µl of a 5 mg/l solution of the PTG-chloroquine conjugate in coating buffer. After being allowed to stand overnight at 4 °C, the plates were washed five times with tap water, dried at 45 °C, and stored at room temperature. The plates were soaked with 250 µl per well of PBST for 5 minutes, emptied, and shaken dry just before being used.

Anti-chloroquine antisera. Three ewes were immunized with chloroquine-keyhole limpet haemocyanin using a protocol described by Sidki et al. (13). Samples of blood were taken from the sheep 2 weeks after the second booster dose and subsequently every 4 weeks for 6 months. The sera were assessed for the presence of antichloroquine antibodies by fluorescence polarization immunoassay using the method described by Sidki et al. (14).

ELISA protocol

Urine samples. Frozen or fresh urine was diluted 1:100 and 1:1000 with PBST, and 50-µl samples were assayed, in triplicate, in microtiteration plate wells using 50 µl of an IgG fraction of the chloroquine-specific antiserum diluted 1:1000 with PBST. After a first incubation for 30 minutes at 37 °C, the plate was washed with cold tap water, blotted dry, 100 µl of anti-sheep IgG conjugated with alkaline phosphatase diluted 1:250 with PBST added, and the plate incubated for 30 minutes at 37 °C. Subsequently, the plate was washed three times with tap water, and 100 µl of the substrate 4-nitrophenyl phosphate (2 g/l in 1 mol/l diethanolamine buffer (pH 9.8) containing 0.5 mmol/l magnesium chloride) was added to the plate, which was re-incubated for 30 minutes at 37 °C until the colour developed. The colour intensity of the solutions was read using a Dynatech Minireader II or a Cambridge Life Sciences 961 portable plate reader.

Dried blood spots. By means of a paper punch, a 6-mm circle was removed from the centre of each dried blood spot to be assayed. The circles were placed in polystyrene test tubes (12 mm × 55 mm) and 250 µl PBST was added to each tube. The samples were then agitated on a multitube vortex mixer for 5 minutes until the colour had been extracted from the paper. And 50-µl samples were taken from the tubes and assayed, in triplicate, without further dilution following the same procedure described above for urine.

Recovery of chloroquine

Fluorimetric assay. Five chloroquine standards containing 1.1–5.5 mg/l of the free base were made up in synthetic urine; 1-ml aliquots of each standard were then treated with 3–4 drops of 0.5 mol/l sodium hydroxide and the chloroquine was extracted into 5 ml of n-hexane by stirring on a vortex mixer for 1–2 minutes. After centrifugation at 1500 g for 5 minutes, the hexane layer was pipetted off and retained. The residual urine was extracted again with hexane, as before, and the extract obtained added to the first. To the combined extracts was added 3 ml of 0.12 mol/l hydrochloric acid, and the chloroquine extracted into the acid layer by vortex mixing for 1–2 minutes. The hexane layer was pipetted off and re-extracted with hydrochloric acid, as before. Finally, the two aqueous extracts were combined, transferred to a 10-ml volumetric flask, and the solution was treated with 2 ml of a solution of 0.5 mol/l sodium hydroxide, and 1 ml of a 4g/l solution of cysteine in distilled water added. The resultant mixture was made up to 10 ml with borate buffer (pH 9.4; 0.01 mol/l), allowed to stand for 30 minutes, and the fluorescence of the solutions determined using a Baird atomic spectrofluorimeter (excitation, $\lambda = 305$ nm; emission, $\lambda = 381$ nm).

ELISA method. (1) From urine. Synthetic urine that had been diluted 1:1000 with PBST was used to prepare $2.5 \times 10^{-2}$, $5.0 \times 10^{-2}$, and $10^{-3}$ mol/l chloroquine standards. Ten sets of triplicates for each of these solutions were assayed against triplicate standards in urine on the same microtiteration plate.

(2) From dried blood spots. Citrated whole blood was used to prepare $5 \times 10^{-8}$, $10^{-7}$, and $5 \times 10^{-7}$ mol/l chloroquine standards, and these were used to prepare dried blood spots on filter-papers. Sets of triplicate dried blood spots for each standard concentration were assayed against solutions of the corresponding standards in PBST.

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1 See footnote f, p 212.
RESULTS

Anti-chloroquine sera

All three ewes produced high titres of anti-chloroquine antibodies. The third sample of blood from sheep G-5 was used to develop the assays described.

Optimization of assay conditions

An investigation of the effect of using various buffers in the assay indicated that PBST minimized the non-specific binding of the first and second antibodies, and this buffer was therefore used subsequently. The assay was most rapid at 37 °C, and the incubation times were optimized at this temperature.

In the fluorimetric assay of chloroquine, addition of cysteine in the final extraction was necessary to give stable readings.

Standard curve

A typical standard curve for the ELISA method, obtained using 0.5 μl of urine or 50 μl of dried blood spot extract (equivalent to 1 μl of capillary blood), is shown in Fig. 1. Urine samples were diluted 1:100 or 1:1000 in PBST prior to analysis.

Assay validation

Sensitivity. The sensitivity of the ELISA method was determined from the mean and standard deviation \((n=20)\) of the assay response at zero dose using the method described by Rodbard (15). Standards and samples were determined in triplicate. The lowest concentration of chloroquine detectable with 95% confidence in 1-μl samples of urine was 0.3 μg/l and 0.6 μg/l in 1-μl dried blood spot extracts.

Precision. The within-assay variation (coefficient of variation) of the ELISA method determined using the results of 10 sets of triplicate analyses for urine samples was 6.2%, 5.8%, and 7.1% for the low, medium, and high concentrations, respectively. Similar results were obtained for dried blood spot extracts. Between-assay coefficients of variation were 7.3%, 8.4%, and 9.1% for both methods. A linear standard curve \((r=1.000, n=4)\) was obtained for the fluorimetric assay over the concentration range 200–800 μg/l. Within-assay coefficients of variation were 0.6%, 1.1%, and 1.8% for urine replicates \((n=6)\) at chloroquine concentrations of 800 μg/l, 400 μg/l, and 200 μg/l, respectively.

Analytical recovery. Analytical recovery for the ELISA method averaged 96.4%, 98.7%, and 98.1% for the low, medium, and high urine standards (see above), respectively. The recovery from dried blood spot extracts was also within this range. In contrast, the average recovery for the fluorimetric assay for urine samples was 66.0% with urine standards containing 200 μg/l and 600 μg/l chloroquine.

Stability of the reagents. The coated microtitration plates were stable for over 6 weeks at room temperature. However, the chloroquine-fluorescein tracer, which was used in the assessment of the antisera by polarization fluorimunoassay, was unstable in solution and decomposed within 24 hours at 4 °C.

Interferences. Interferences from the sample matrices were assessed by using increased volumes of urine or dried blood spot extracts in the ELISA assays. The results indicate that for curves for different standards in PBST and those for samples to be superimposable the volume of the sample matrix should not exceed 1 μl.

Specificity. The cross-reactivity in the ELISA method of chloroquine metabolites, the antimalarial drugs, quinidine, mefloquine, halofantrine, and pirimetaine, and of other commonly used drugs was determined by measuring the amount of each compound required to give a 50% reduction in the absorbance signal of the zero standard. No significant cross-reactivity was observed at concentrations of these substances below \(10^{-4}\) mol/l.
phyllactic therapy are shown in Fig. 2. Steady-state capillary blood levels were 150–160 µg/l and the elimination half-life was 7.2 days. Significant levels of chloroquine were detected in dried blood spots for 28 days after termination of therapy. The corresponding profile for urine samples is shown in Fig. 3. High levels of chloroquine were detected in 0.1-µl samples of urine for 61 days after termination of therapy and this increased to 115 days for 1-µl samples.

The cross-reactivity of the fluorimetric assay was determined for the major chloroquine metabolite, N-desethylchloroquine. With the extraction procedure described, only 14% of the metabolite in urine was recovered.

Correlation of the ELISA and fluorimetric methods. Eight urine samples were assayed in triplicate by each method. The correlation of the results was good ($r = 0.963; y_{fluorimetric} = 0.839x_{(ELISA)} + 2.02$).

Urine and dried blood spot levels

The levels of chloroquine in dried blood spot extracts from a volunteer during and following pro-

Fig. 2. Logarithmic plot of chloroquine concentration in dried blood spot extracts from a patient following prophylactic medication with the drug. The plot represents the best straight line fit obtained by linear regression analysis of the data after day 3 ($r = -0.944, n = 9$). The broken line is an extrapolation of the line of best fit.

Fig. 3. Logarithmic plot of chloroquine concentration in urine during and after ending prophylactic medication with the drug. The plot represents the best straight line fit obtained by linear regression analysis of the data after day 6 ($r = -0.824, n = 14$).
DISCUSSION

Chloroquine that had been derivatized to form compound A and coupled to keyhole limpet haemocyanin gave a good response in the ELISA method. This same derivative was also used to produce a PTG-chloroquine conjugate that was coated onto the microtitration plate wells. Competition for a limited number of chloroquine antibodies between free chloroquine in a sample or standard added to the well and chloroquine bound on the well's surface served as the basis for the immunoassay. The amount of bound chloroquine-specific antibody per well after the competitive reaction was determined using alkaline-phosphatase-conjugated anti-sheep IgG and the specific substrate, \( p \)-nitrophenyl phosphate.

The ELISA method is highly specific for chloroquine and exhibits no significant cross-reactivity with its major metabolites and other commonly administered drugs. It can therefore be used to assay biological fluids for chloroquine. Urine and reconstituted capillary blood from dried blood spots obtained from fingerprick samples soaked onto filter-paper were used as the physiological matrices. Such samples did not interfere in the assay at volumes less than 1 \( \mu l \). The method is sufficiently sensitive to determine chloroquine in these matrices and has acceptable precision and recovery levels. The results correlated well with those obtained with a conventional fluorimetric assay for urine samples from a patient who was receiving prophylactic doses of chloroquine.

The ELISA method was used to determine chloroquine levels in dried blood spots and in urine samples from the patient both during and after termination of the therapy. For reconstituted dried blood spots, the steady-state chloroquine levels were 150–160 \( \mu g/l \) and the elimination half-life was 7.2 days. These values are in agreement with those reported previously for plasma samples (16) and filter-paper-absorbed fingerstick blood (17). Lindstrom et al. have shown that the level of chloroquine determined in blood samples collected by fingerprick onto filter-paper correlates well with the corresponding level in venous whole blood, although the concentrations are significantly higher in the latter (8). Dried blood spot samples were also demonstrated by these workers to be stable for at least 7 weeks at 20 °C.

The ELISA method is sufficiently sensitive to detect chloroquine in dried blood spot extracts for up to 28 days and in urine for up to 115 days after administration of the last dose of the drug using 1-\( \mu l \) samples in the assay. The method therefore appears to be sensitive, simple, and specific enough for use in pharmacokinetic studies on dried blood samples or fresh fingerprick samples in developing countries or in screening studies of the drug sensitivity of malaria parasites.

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RÉSUMÉ

DOSAGE DE LA CHLOROQUINE DANS LES URINES ET DANS LE SANG SéCHÉ PAR UNE MÉTHODE ELISA

On a utilisé comme immunogène de la chloroquine couplée à de l'hémocyanine de fissurelle (keyhole limpet) par l'intermédiaire d'une unité structurale d'aminoquinoléine, pour susciter la production d'anticorps anti-chloroquine chez trois moutons. Les immunéséums obtenus étaient caractérisés par une augmentation de la polarisation de la fluorescence après liaison d'un dérivé chloroquine-fluorescénique à l'immunésérum spécifique de la chloroquine. Tous les moutons ont produit des titres élevés d'immunéséums, et des prélevements de sang de l'un d'entre eux ont été utilisés pour mettre au point un dosage immunoenzymatique (ELISA) de la chloroquine. Les plaques de microtitrage ont été sensibilisées avec un conjugué thyroglobuline de porc-chloroquine. On a procédé à l'ELISA en prenant de l'immunésérum anti-mouton marqué à la phosphatase alcaline et un substrat constitué de phosphate de nitro-4 phénylé, de manière à quantifier la liaison de l'anticorps anti-chloroquine aux plaques au cours de l'étape de liaison compétitive.

Ce dosage nécessite au plus 1 \( \mu l \) de solution témoin ou d'échantillon (gouttes de sang séché provenant de piqûres au doigt, absorbées sur du papier filtré et reconstituées, ou urine), et 50 \( \mu l \) d'une fraction IgG de l'immunésérum chloroquine-spécifique dilué à 1/1000 par godet. Au bout de 30 minutes d'incubation à 37 °C, on lave les plaques à l'eau du robinet et on procède à une seconde
incubation pendant 30 minutes à 37 °C avec 100 µl d'IgG anti-mouton conjuguée avec de la phosphatase alcaline diluée à 1/250. Après un second lavage à l'eau de robinet, on incube pendant 30 minutes à 37 °C avec 100 µl d'une solution de phosphate de nitro-4 phénylé (substrat). L'intensité de la couleur dans le godet est ensuite déterminée à l'aide d'un lecteur de plaques de microtitrage.

La méthode ELISA décrite ici est spécifique de la chloroquine et ne montre aucune réaction croisée avec ses métabolites ni avec les autres médicaments d'usage courant. Ce dosage a une précision et un rendement acceptables et se corrèle bien avec le dosage fluorimétrique classique de la chloroquine. On a déterminé à l'aide de cette méthode les concentrations de chloroquine dans les urines et dans le sang séché d'un malade soumis à une médication prophylactique. Le médicament a été décéléré pendant 17 semaines dans les urines et pendant 4 semaines dans le sang séché, après la fin du traitement. Cette méthode ne nécessite qu'un matériel simple pouvant être utilisé par un personnel relativement peu qualifié, et pourrait trouver une application intéressante dans les services de santé périphériques des pays en développement où se décide l'emploi des antipaludiques.

REFERENCES

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