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Determination of chloroquine and its metabolites in urine: a field method based on ion-pair extraction*

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A new straightforward photometric method for the assay of the antimalarial drug chloroquine and its metabolites in urine is described. The method involves an ion-pair extraction procedure with dichloromethane using the acid-base indicator bromthymol blue as counter-ion. The ion pair formed with chloroquine in the organic phase is yellow, and absorbance is measured at λ = 410 nm using a filter photometer. The absorbance is a linear function of concentration up to 400 μmol/l (120 mg/l) chloroquine. The method is suitable for the determination of chloroquine and its metabolites in urine down to a limiting concentration of about 10 μmol/l (3 mg/l). Additionally, the method is suitable for semi-quantitative visual estimation of the concentration of chloroquine in urine. A single dose of 5 mg/kg chloroquine base could be determined in urine from two volunteers for at least 8 days after administration of the drug. The results obtained for the analysis of chloroquine and its metabolites with the colorimetric method described here correlate well with those obtained using high performance liquid chromatography.

Selective and sensitive chromatographic methods for the determination of chloroquine and its metabolites have recently been developed in our laboratory (*1*, *2*), but are unsuitable for carrying out measurements in the field since they require sophisticated instrumentation. Under field conditions, qualitative tests for detecting chloroquine in urine are used to monitor compliance with medication (*3*). However, current simple methods for the analysis of urine, such as the Dill-Glazko test (*4*), Haskins's test (*5*), and the Wilson-Edeson test (*6*), have low specificity, since they are subject to interference from other drugs.

We report here a simple method suitable for the quantitative and semiquantitative determination of chloroquine and its metabolites in urine. The method, which has good sensitivity, involves extraction of chloroquine as an ion pair with bromthymol blue into dichloromethane and its subsequent colorimetric determination. The results obtained correlate with those obtained by high performance liquid chromatography.

MATERIALS AND METHODS

Ion-pair extraction of amino compounds with the anion of sulfonic acid dyes, such as bromthymol blue, was investigated (*7*) in the early 1960s for the quantitative photometric determinations of drugs. The technique involves extraction of ionized compounds (anionic or cationic), e.g., chloroquine and metabolites, from an aqueous into an organic phase with the aid of a counter-ion, e.g., bromthymol blue, as shown in the following equation:

$$HA^+_{aqueous} + X^-_{organic} \rightleftharpoons HAX_{organic}$$

(HA^+ = chloroquine; X^- = bromthymol blue)

In the case of chloroquine and its metabolites, with dichloromethane as extracting solvent, the ion pair (HAX) in the organic phase with bromthymol blue is yellow.

Reagents

Samples of chloroquine and deethylchloroquine were kindly donated,[a] while bromthymol blue sodium salt[b] and dichloromethane[c] were obtained

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[a] Sterling-Winthrop, Skarholmen, Sweden.

[b] Sigma Chemical Company, St. Louis, MO, USA.

[c] Merck, Darmstadt, Federal Republic of Germany.

commercially. All other chemicals and drugs were of analytical quality and commercially available.

Carbonate buffer (pH 9.5 ± 0.1) was prepared by mixing solutions of $KHCO_3$ (1.0 mol/l) and K_2CO_3 (1.0 mol/l) in the ratio of 4:1 by volume.

Bromthymol blue solution (0.65 mmol/l) was prepared by adding the carbonate buffer to a 0.01 mol/l stock solution of bromthymol blue sodium salt in distilled water. The solution is stable for at least 4 months at 35 °C.

Apparatus

For the quantitative determinations, a spectrophotometer (Shimadzu 210 A) and a digital pH-meter (Radiometer PHM 64) fitted with a combined glass electrode (GK 2401 C) were used. The liquid chromatography method has already been described (*1*).

Procedure

The following procedure is used to determine the concentration of chloroquine in urine.

A mixture of 1 ml of urine containing chloroquine + 2 ml of bromthymol blue solution + 3 ml of dichloromethane is shaken for approximately 30 seconds in a glass test tube. The organic and aqueous phases are separated either by being allowed to stand for 15–30 min or by centrifugation for 5 min. The aqueous phase is discarded, and 1.0 ml of water and 2.0 ml of the bromthymol blue solution, prepared as described above, are added. After being shaken for about 30 seconds, the phases are allowed to separate and the aqueous phase again discarded. The absorbance of the organic phase is then measured using a filter spectrophotometer at $\lambda = 410$ nm against a water blank sample that has been subjected to the same extraction procedure. The concentrations of chloroquine and its metabolites in the urine are obtained by comparison with a calibration curve obtained with urine samples to which had been added known amounts of chloroquine and then extracted as described above.

For use in field studies, a semiquantitative estimation of the concentration of chloroquine and its metabolites in urine can be made by visual comparison of the solutions with urine samples containing known amounts of chloroquine by holding the sample tube against a white surface.

RESULTS

Calibration plot

The ion pair formed between bromthymol blue and chloroquine in the organic phase shows an absorption maximum at $\lambda = 410$ nm, and the calibration curve at this wavelength for urine samples to which had been added 25–400 μmol/l of chloroquine is linear over this range (Fig. 1); the small positive intercept with the absorbance axis indicates that the blank urine and the aqueous samples contain a low concentration of bromthymol blue.

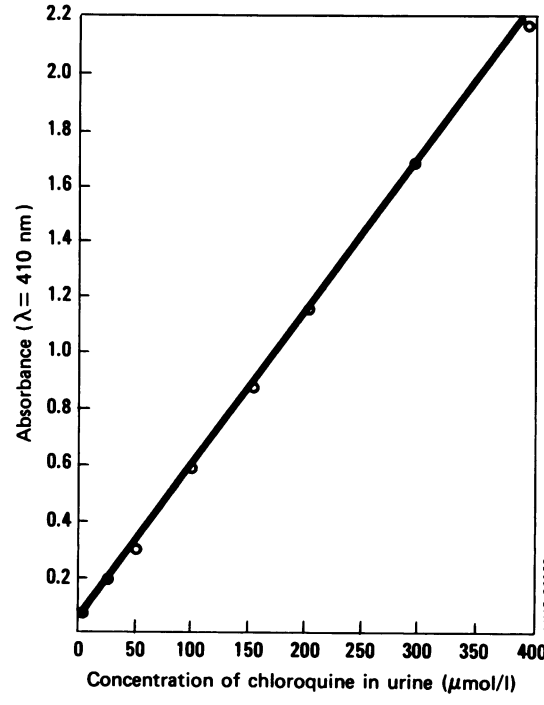

Fig. 1. Calibration curve for the quantitative photometric determination of chloroquine in urine.

Effect of pH on the extraction

The pH value of the bromthymol blue solution is important, since in the extraction of chloroquine the specificity and the urine blank value are strongly pH-dependent (*7*). The optimum pH for extraction of chloroquine and deethylchloroquine in the presence of related 4-aminoquinoline drugs was found to be 9.5 (*8*).

Lower limit of the determination

The lower limit of determination for the method is a function of the magnitude of the blank value. To determine the precision of the method for urine samples obtained from the same or different individuals, chloroquine was added to 10 different

urine samples from healthy volunteers to give chloroquine concentrations of 0, 5, 10, 15, 25, 50, and 100 μmol/l. All samples were analysed in duplicate by the bromthymol blue method. For urine samples from the same individual the relative standard deviation of the method is less than 2% at chloroquine concentrations above 25 μmol/l; for samples from different individuals the respective figure is 15% at a chloroquine concentration of 10 μmol/l. The practical lower limit of determination of the method was therefore taken to be 10 μmol/l, at which concentration the relative standard deviation is acceptable.

Comparison with a reference method

Correlation studies were performed using urine samples from volunteers given a single oral dose of 5–10 mg/kg chloroquine base. Urine samples were taken 1–15 days after administration of the dose and were analysed by high performance liquid chromatography (*1*) or by the bromthymol blue method. An acceptable level of agreement between the two methods was found at the 95% confidence limit over the concentration range 10–200 μmol/l of chloroquine and its metabolites in urine.

Evaluation of visual determination

Five individuals were asked to place 49 randomly assigned samples, obtained from urine treated as described above, into four concentration ranges (0–25, 26–50, 51–100, >100 μmol/l) by visual comparison with four standard solutions containing known concentrations of chloroquine + metabolites (0, 25, 50, 100 μmol/l). Excellent agreement was found between visual assignment of the yellow colour of the samples into the above ranges and the total concentration of chloroquine and its metabolites as determined by chromatography.

In a further evaluation of the bromthymol blue method, drug-free urine samples from 10 volunteers were treated with chloroquine (5, 10, 15, 25, 50, 100 μmol/l) and extracted as described above. Twelve individuals then visually examined the yellow solutions and classified them as positive or negative relative to drug-free urine. At concentrations greater than 15 μmol/l chloroquine all solutions were classified as positive.

To test the clinical applicability of the bromthymol blue method, we analysed the urine on different days from two volunteers given an oral dose of 5 mg/kg chloroquine base. Table 1 shows that chloroquine and its metabolites could be detected in urine up to 8 days after administration of the drug.

Table 1. Mean results for quantitative and semiquantitative determination of chloroquine and its metabolites in urine by the bromthymol blue method[a]

	Day							
Determination	0	1	2	3	6	8	10	14
Quantitative (μmol/l)	0	52	31	28	30	24	17	18
Semiquantitative[b]	0	2	1	1	1	1	0	0

[a] Subjects administered 5 mg/kg chloroquine base.

[b] Semiquantitative ratings: 1 = 0–25 μmol/l; 2 = 26–50 μmol/l; 2 = 26–50 μmol/l; 3 = 51–100 μmol/l.

Analytical specificity

We have compared the specificity of the bromthymol blue method with other qualitative tests for chloroquine in urine: Dill-Glazko test (*4*), Haskins's test (*5*), and Wilson-Edeson test (*6*). For this purpose, different concentrations of various drugs were added to drug-free urine and these samples analysed quantitatively in the spectrophotometer. The interference from most drugs listed in Table 2 was found to be comparable for the bromthymol blue method, Haskins's test, and the Dill-Glazko test.

DISCUSSION

Patient compliance to antimalarial treatment is always a problem. The patient must be encouraged to complete the full course of treatment in order to prevent recurrence of the disease. By use of the colorimetric method described here for quantitative determination of chloroquine in urine, compliance could be monitored and suspect resistance of malaria parasites to the drug could be screened without use of high-technology instrumentation.

Samples of urine taken from five adult volunteers at different times after administration of a single oral dose of 2.2–8.8 mg/kg chloroquine base were analysed and the relation between the sum of concentrations of chloroquine and its metabolites in urine, whole blood, and plasma determined (Fig. 2A and B). All the samples were assayed by high performance liquid chromatography (*1*).

A dose of 10 mg/kg chloroquine base given orally for treatment of malaria produces a mean plasma chloroquine concentration of 222 ± 116 nmol/l after 24 hours (*9*). In contrast, the concentration in whole blood 7 days after six children had been given a total of 25 mg/kg chloroquine in divided doses (10 mg/kg on days 1 and 2 and 5 mg/kg on day 3) ranged from

Table 2. Comparison of the concentrations of various drugs in urine that produce a colour equivalent to 20 μmol/l chloroquine.

	Drug concentration (μmol/l) by:			
Drug	Bromthymol blue[a]	Haskins	Wilson-Edeson	Dill-Glazko
Amodiaquine	700	500	100	–
Bideethylchloroquine	100	–	–	–
Deethylchloroquine	20	10	20	500
Hydroxychloroquine	50	20	10	100
Quinine	20	10	20	10
Proguanil	30	30	> 1000	> 1000
Primaquine	500	> 1000	200	500
Pyrimethamine	> 1000	200	> 1000	300
Salicylic acid	> 1000	> 1000	> 1000	> 1000
Acetaminophene	> 1000	> 1000	> 1000	> 1000
Levomepromazine	> 1000	300	75	200
Promethazine	400	200	10	100
Metoprolol	300	50	> 1000	500
Ephedrine	300	100	> 1000	> 1000
Sulfadoxine	> 1000	> 1000	> 1000	> 1000
Oxazepam	> 1000	> 1000	> 1000	> 1000
Nortriptyline	200	200	20	200
Tetracycline	> 1000	> 1000	10	> 1000
Nicotine	> 1000	> 1000	> 1000	> 1000

[a] The bromthymol blue method is free of interference from haemoglobin concentrations up to 200 mg/l and albumin up to 1000 mg/l.

1315 to 1815 nmol/l (*10*). All the assays were performed using chromatography.

The following concentrations of chloroquine and its metabolites in morning urine samples from patients on a continuous daily dosage regimen of 150 mg chloroquine base were found: chloroquine (144 ± 60 μmol/l), deethylchloroquine (54 ± 30 μmol/l), and bideethylchloroquine (12 ± 9 μmol/l).[d] Any method for the determination of chloroquine and its metabolites in urine must therefore be capable of covering the clinically relevant concentration range, 50–300 μmol/l.

Chloroquine and its metabolites are diamines with $pK_{H_2A} = 8.1$ and pK_{HA} in the range 10.8–11.5 (*8*). Bromthymol blue is a sulfonic acid derivative with a $pK_a = 7.12$ (*11*). At pH 9.5, both chloroquine and bromthymol blue exist predominantly in ionic form. In contrast, at this pH value basic drugs tend to be present predominantly in the nonionic form and, hence, are not extracted as ion pairs with bromthymol blue. The lower concentration limit of this method is determined by the background extraction of the urine blank, which decreases with increasing pH value. To reduce the degree of coextraction of endogenous compounds in the urine, the organic phase obtained after the first extraction is re-extracted with bromthymol blue. The absorbance of the extracted solutions obtained from urine blanks from healthy volunteers is approximately the same as that of an aqueous blank. The extent of background extraction was reasonably constant at pH 9.5, and variations between individuals was low. A buffer of high ionic strength (1.0 mol/l) must be used to obtain a constant pH value. In other semi-quantitative tests for determination of chloroquine in urine, no buffer is used.

The method described here is suitable for use in laboratories lacking sophisticated equipment, since it requires only a filter photometer of reasonable

[d] BERGQVIST, Y. Ph.D. thesis, University of Uppsala, Sweden, 1983 (Abstracts of Uppsala Dissertations on Science, No. 683, 1983).

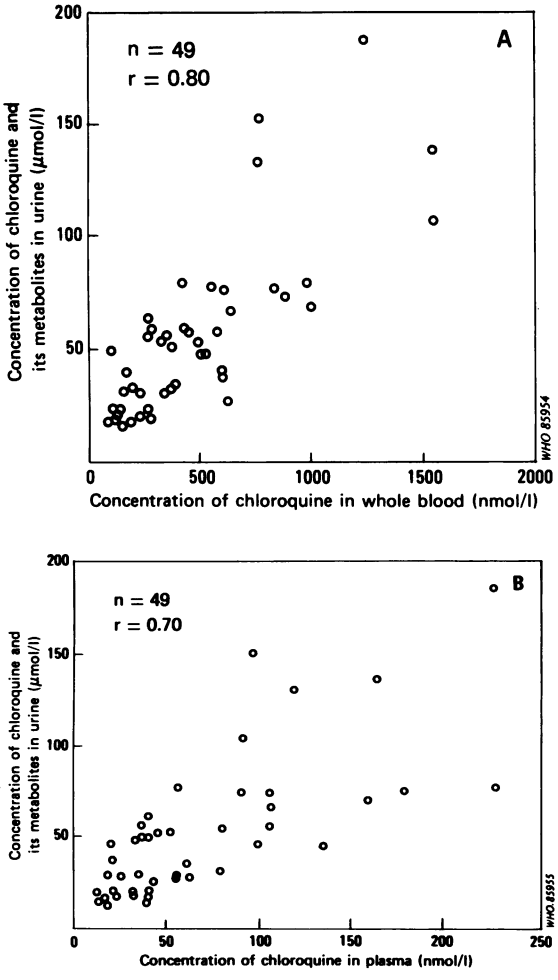

Fig. 2. Relation between the concentrations of chloroquine in whole blood (A) and plasma (B) and the sum of the concentrations of chloroquine and its metabolites in urine.

quality for quantitative determinations down to a limiting concentration of 10 μmol/l chloroquine. Good agreement was found between the bromthymol blue method and high performance liquid chromatography for quantitative ($r = 0.95$) and semi-quantitative determinations (Fig. 3).

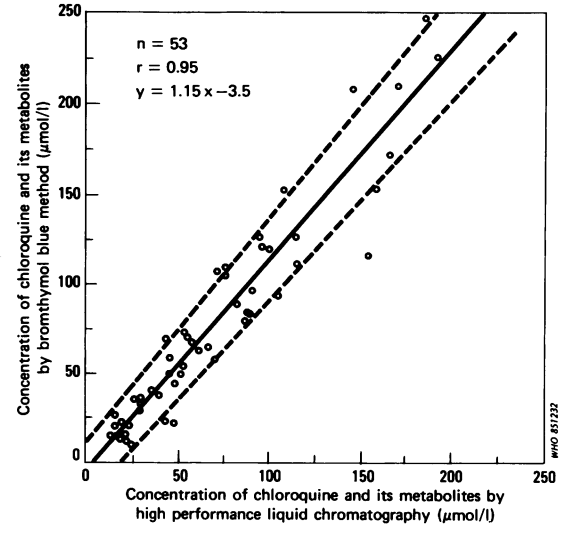

Fig. 3. Comparison of the bromthymol blue and high performance liquid chromatography methods. Patients were administered a single dose of 5–10 mg/kg chloroquine dihydrogen phosphate, and samples of urine were collected 1–15 days afterwards. The broken lines indicate the 95% confidence limits of the regression line.

For fieldwork, the bromthymol blue method permits visual estimation of the concentration of chloroquine and its metabolites down to 10–15 μmol/l by comparison of the intensity of the yellow coloration with that of standard solutions. The limiting concentrations for other qualitative tests are: 7 μmol/l (Wilson–Edeson), 13 μmol/l (Haskins) and about 240 μmol/l (Dill-Glazko).[e] However, visual estimation of the concentration of chloroquine is more difficult in these cases, since the colour development is not a linear function of concentration, for concentrations of chloroquine that exceed 75–100 μmol/l.

[e] ROMBO, L. ET AL. Evaluation of three qualitative tests for detection of chloroquine in urine, and their correlation with concentrations determined by liquid chromatography. *Annals of tropical medicine and parasitology* (in press).

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RÉSUMÉ

RECHERCHE ET DOSAGE DE LA CHLOROQUINE ET DE SES MÉTABOLITES DANS L'URINE: MÉTHODE DE TERRAIN REPOSANT SUR L'EXTRACTION PAR FORMATION DE PAIRES D'IONS

On trouvera décrite dans cet article une méthode simple d'extraction par paires d'ions en vue de l'analyse quantitative ou semi-quantitative de la chloroquine et de ses métabolites dans l'urine. Leur extraction se fait au moyen du dichlorométhane et on utilise un indicateur coloré, le bleu de bromothymol, comme ion de signe contraire. La paire d'ions présente dans la phase organique donne une couleur jaune dont l'intensité est proportionnelle à la concentration de chloroquine dans l'échantillon d'urine. L'absorbance est mesurée à 410 nm et elle est comparée à celle d'étalons connus. Si l'on recherche un résultat semi-quantitatif, on peut se contenter de comparer à l'œil nu la couleur jaune de la phase organique à celle de solutions étalons. Pendant au moins 8 jours après l'administration d'une dose unique de 5 mg/kg de chloroquine base à deux volontaires, on a pu déceler la présence de chloroquine dans les échantillons d'urine. La méthode est utilisable pour la recherche et le dosage de la chloroquine dans l'urine à des concentrations descendant jusqu'à environ 10 μmol/1 (3 μg/ml) et elle est linéaire de 25 à 400 μmol/1. Les résultats obtenus concordent parfaitement ($r = 0,95$) avec ceux que donne la chromatographie en phase liquide à haute performance (HPLC).

L'écart-type relatif interindividuel dépasse 15% à une concentration de l'ordre de 10 μmol/1. L'écart-type relatif intra-individuel est inférieur à 2% pour une teneur en chloroquine supérieure à 25 μmol/1.

Cette méthode peut s'employer pour doser les métabolites principaux de la chloroquine ainsi que d'autres antipaludéens, la quinine et le proguanil. On a pu constater que la présence de divers produits basiques ne faussait guère les résultats.

La présente méthode devrait être utilisable dans les laboratoires dépourvus de matériel sophistiqué vu que seul un spectrophotomètre à filtres est nécessaire. Sur le terrain, la concentration peut s'estimer par simple examen visuel pour les valeurs supérieures à 10–15 μmol/1.

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