

ELISA tests for dapsone and pyrimethamine and their application in a malaria chemoprophylaxis programme

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Enzyme-linked immunosorbent assays (ELISAs) are described for determining levels of dapsone and pyrimethamine in urine. Both assays have a sensitivity of about 20 µg/l and are reproducible, but each produces some false positives. The problem of false positive reactions was partially obviated by requiring positive results in both assays. In a pilot study involving 50 children aged 3 months to 4 years who were given a single dose of Maloprim (pyrimethamine + dapsone), 75% were positive for dapsone 7 days after administration of the drug, while 25% were still positive 15 days after its administration. The corresponding proportions for pyrimethamine were 73% and 30%, respectively. Comparison of the results obtained in a larger chemoprophylaxis trial with those from the pilot study indicated that the assays described could be used to investigate whether antimalarials had been taken.

Use of chemoprophylaxis by the population of areas where malaria is highly endemic is controversial. In general, mass chemoprophylaxis is inadvisable; however, it can be argued that malaria chemoprophylaxis should be given to pregnant women, especially primigravidae, and young children. If chemoprophylaxis is employed it is important that as high a degree of coverage as possible be achieved, since otherwise drug resistance will probably be encouraged.

Analysis of urine samples is the most reliable method of assessing coverage in chemoprophylaxis programmes; unfortunately, however, no simple and reliable assays are available for the standard antimalarials in urine. Simple colorimetric assays for chloroquine in urine have been reported (1, 2), but gave a high rate of false positives in a study of young Nigerian children^a as well as a high rate of false

negatives in children with high blood levels of chloroquine (3). No simple colorimetric assays for pyrimethamine or proguanil are available and their assay requires the use of complex biochemical tests or chromatography. In 1979 Huikeshoven et al. (4) described an enzyme-linked immunosorbent assay (ELISA) for dapsone and subsequently showed that it could be used to monitor treatment of leprosy patients (5). Here, we describe a modification of this assay, which together with a new ELISA test for pyrimethamine, was used to monitor patient compliance in a trial of malaria chemoprophylaxis using Maloprim (pyrimethamine + dapsone).^b

MATERIALS AND METHODS

The study was carried out in the village of Sarakunda, 20 km east of Farafenni, situated on the north bank of the River Gambia. In October 1981 a survey of the inhabitants of the village showed that its population was 1161, of whom 152 were below 5 years of age. Tribally, 53% of the population were Wollof and 43% Mandinka, while 4% belonged to other smaller tribal groups. A register of births and deaths has been maintained in the village since this initial census.

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Pilot survey

In April 1982 a pilot study was undertaken in Sarakunda in which Maloprim was administered to 50 children under 5 years of age (10 in each 1-year age cohort). One quarter of a tablet of the drug (containing 6.25 mg pyrimethamine + 25 mg of dapsone) was given to children aged 3–11 months and half a tablet (containing 12.5 mg pyrimethamine + 50 mg dapsone) to children aged 1–4 years. Administration of the drug was supervised by one of the investigators. Children were kept under observation for 30 minutes after administration, and samples of urine and saliva were collected 24 hours after the drug had been given as well as on alternate days for the following 3 weeks. Mothers collected the urine samples early on the morning of the designated day and samples were taken to Farafenni on the same day and stored at -20°C until assayed. At each sample collection, mothers were questioned about any side-effects of the drug, and children were examined for signs of methaemoglobinemia.

Main survey

In March 1983 a chemoprophylaxis trial was begun in Sarakunda. Compounds in the village were randomly assigned to one of the following treatments: (1) Maloprim + folic acid; (2) Maloprim + placebo; (3) chlorproguanil + folic acid; (4) chlorproguanil + placebo; (5) placebo + folic acid; and (6) placebo + placebo. Maloprim was given in the dose described above as specially formulated quarter-strength tablets, while the dosage of chlorproguanil was 20 mg and that of folic acid 5 mg. All children under 5 years of age and all pregnant women were included in the trial. Prophylaxis was given at fortnightly sessions held in the village community centre by Medical Research Council field staff. Records of drug administration were kept using a system designed for use by illiterate village health workers (6).

Samples of urine for assays were obtained from all children who attended a monthly clinic held in the village by a project physician, and further samples were obtained in November 1984 during a cross-sectional survey of all cohort children. The nature and date of the last drug administration were recorded. Samples of urine from controls were obtained in November 1984 from children in neighbouring hamlets where no drugs had been administered.

Preparation of samples

Samples of urine and saliva were stored at -20°C until tested. Before being assayed, each sample was tested with phenol red indicator and adjusted to approximately pH 7.0 by addition of either a 10%

solution of ammonium hydroxide or 0.1 mol/l hydrochloric acid. A 1:10 dilution of samples of urine or saliva in phosphate-buffered saline (PBS)/Tween (0.05% Tween-20 in PBS (v/v), pH 7.3) produced the optimum discrimination between positive and negative samples.

Standard solutions

Standard solutions of dapsone and pyrimethamine (pH 7.0) covering the concentration range 0.5–1000 $\mu\text{g/l}$ were prepared in aliquots of urine pooled from individuals who had never taken either drug. Part of the urine pool was also used to provide a blank. The standard solutions were preserved by addition of thiomersal to a concentration of 0.02% (w/v) and were diluted 1:10 in PBS/Tween before use.

Preparation of antisera to dapsone and pyrimethamine and enzyme conjugates

Dapsone was conjugated to horseshoe crab haemocyanin, and antiserum to the conjugate was raised by immunizing rabbits with conjugate plus Freund's adjuvant (4). Antiserum to a pyrimethamine/haemocyanin conjugate was prepared similarly, and both antisera were conjugated with peroxidase (4).

ELISA test for dapsone

Nunc Immuno 1 microtitre trays were used for the ELISA test. Initially, 100 μl of a solution of dapsone antiserum in carbonate buffer (pH 9.6) was added to each well and the trays were incubated at 37°C for 12 hours (Fig. 1). The trays were then washed ($\times 6$) with PBS/Tween, and 75 μl of either standard or test sample diluted 1:10 in PBS/Tween was added followed by 25 μl of enzyme-dapsone conjugate in PBS/Tween supplemented with 10% horse serum. After incubation for 2 hours at 37°C , the trays were washed ($\times 6$) and 100 μl solution of ABTS (2,2'-azino-di-(3-ethyl)benzothiazolinesulfonic acid) in hydrogen peroxide was added to each well. This solution of 5 mg ABTS in 10 ml of 0.1 mol/l phosphate-citrate buffer (pH 4.0) + 18 μl of 1% hydrogen peroxide was prepared immediately before use. After a further 2 hours' incubation at room temperature, the contents of each well were analysed colorimetrically at $\lambda = 405\text{ nm}$ using a Titertek Multiskan instrument.^c Samples were analysed in duplicate and the mean absorbance determined. The absorbance of the test samples was compared with that of blanks from the urine pool and the proportional reduction in absorbance determined. An inhibition of the colour

^c From Flow Laboratories, Irvine, Scotland.

reaction of about 20% could be detected with the naked eye, and this level was therefore taken as a convenient cut-off for negative and positive samples. For dapsone antiserum the optimum concentration of IgG for coating was 6.25 mg in 100 μ l coating buffer, while for the enzyme-dapsone conjugate the optimum dilution was 1:500. These concentrations were used in all subsequent assays.

ELISA test for pyrimethamine

For the pyrimethamine assay, in contrast to the dapsone assay, antigen was bound to the microtitre tray (Fig. 1). Optimum results were obtained with Dynatech M129 A microtitre trays. Initially, 100 μ l of pyrimethamine-haemocyanin conjugate in carbonate buffer (pH 9.6) was added to each well of the trays, which were then incubated for 1 hour at 30 $^{\circ}$ C. After washing (\times 6) in PBS/Tween, 75 μ l of test sample (or standard) diluted 1:10 in PBS/Tween was added to each well followed by 25 μ l of anti-pyrimethamine immunoglobulin conjugated with peroxidase. After incubation for 2 hours at 37 $^{\circ}$ C, trays were washed (\times 6) and 100 μ l of ABTS/hydrogen peroxide solution added to each well. After incubation for a further 2 hours at room temperature, the absorbance of the solutions was determined colorimetrically at $\lambda=405$ nm. Positive and negative responses were defined as described for the dapsone assay. The optimum concentration of pyrimethamine-haemocyanin conjugate was 2.5 mg/l buffer, and that of the enzyme conjugated anti-pyrimethamine antiserum 1:200 in PBS/Tween supplemented with 10% horse serum. These concentrations were used in all subsequent assays.

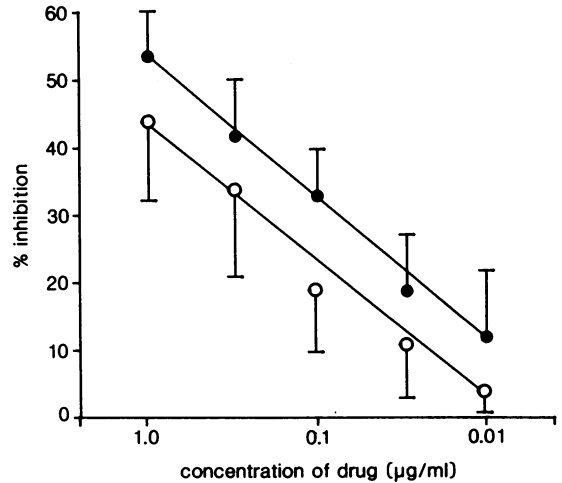


Fig. 2. Inhibition of colour reaction in dapsone (○) and pyrimethamine (●) ELISA tests by various concentrations of dapsone and pyrimethamine in normal urine. Plots show the mean + standard deviation of 35 observations.

RESULTS

Sensitivity, specificity, and reproducibility

The lower limit of detection for dapsone and pyrimethamine in urine was approximately 20 μ g/l and the reproducibility of both assays for standard solutions was good (Fig. 2). In order to determine the reproducibility of the assays for test solutions, the following samples of urine were assayed five times each: 10 samples that were definitely positive ($>40\%$ inhibition), 10 that were weakly positive (20–40% inhibition), and 10 that were negative ($<20\%$ inhibition). All the samples that produced $>40\%$ inhibition were positive in each of the five dapsone assays. Nine were positive in each of the pyrimethamine assays, while the remaining sample was negative in only one of the five pyrimethamine assays. Similar results were obtained with the negative samples: nine were negative in each assay, while one was positive on one occasion in each assay. More variable results were obtained among the weakly positive urines, and nine and eight samples, respectively, were negative on at least one occasion in the pyrimethamine and dapsone assays.

Samples of urine from 45 children were obtained approximately 24 hours after administration of a quarter or half tablet of Maloprim. A positive dapsone assay ($\geq 20\%$ inhibition) was exhibited by 44 samples (98%), while 42 samples (93%) were positive

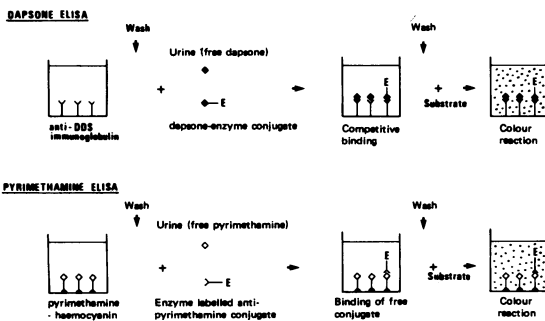


Fig. 1. Schematic representation of the ELISA tests for dapsone and pyrimethamine (anti-DDS = dapsone antiserum).

for pyrimethamine. Forty-two samples (93%) were also positive in both assays. Urine samples were also assayed from 247 children who lived in 27 hamlets in the Farafenni area where no drug prophylaxis had taken place and where access to drugs was limited: 23 samples (9.5%) were positive for dapsone and 11 (4.5%) for pyrimethamine. Twelve of the samples that were positive for dapsone were from children who lived in a cluster of five adjacent hamlets. Only seven samples (2.8%) were positive in both assays. Urine from children who had not been prescribed anti-malarials remained positive in the assays after samples were treated with phosphate-citrate buffer (pH 2) or boiled for 2 minutes. The combined results of the dapsone and pyrimethamine assays were therefore more specific than the results of either assay alone. If it is assumed that all the samples of urine obtained 24 hours after administration of Maloprim contained dapsone and pyrimethamine (some children may have vomited after the observation period), for the combined assays the sensitivity and specificity were 93% and 97%, respectively.

Pilot study

Samples of urine were collected from 45 children aged less than 4 years over a period of 19 days following administration of Maloprim (quarter tablet for 9 children aged 3–11 months and a half tablet for 36 children aged 1–4 years). The mean inhibition of absorbance at $\lambda = 405$ nm of these samples compared to that of normal urine is plotted in Fig. 3 and 4 against the time elapsed after administration of the drug. The proportion of children whose urine produced $\geq 20\%$ inhibition in the dapsone and pyrimethamine assays (equivalent to about 50 $\mu\text{g/l}$ of dapsone or 20 $\mu\text{g/l}$ of pyrimethamine) as a function of the number of days after treatment is shown in Fig. 5. Urinary levels of dapsone and pyrimethamine were generally higher for children aged 1–4 years who had been given a half tablet of Maloprim than for children aged 3–11 months given a quarter tablet. Dapsone levels in urine fell more rapidly than pyrimethamine levels.

Relative to phosphate-buffered saline, samples of saliva collected from 49 children immediately before

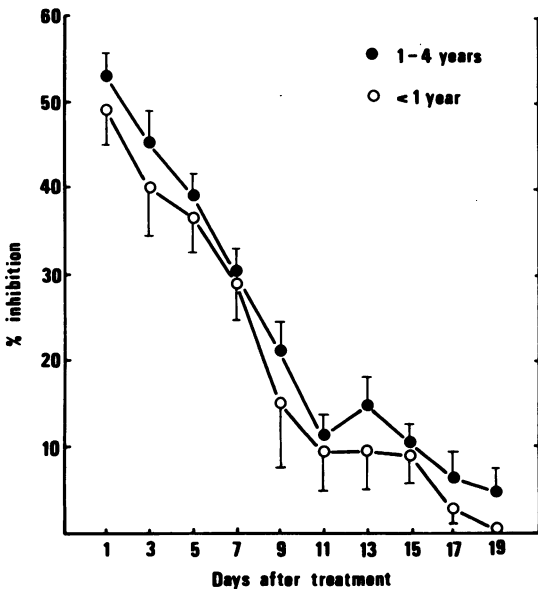


Fig. 3. Inhibition of colour reaction in the dapsone ELISA test by post-treatment samples of urine from 9 children aged <1 year (○) given a quarter tablet of Maloprim and from 36 children aged 1–4 years (●) given a half tablet of the drug.

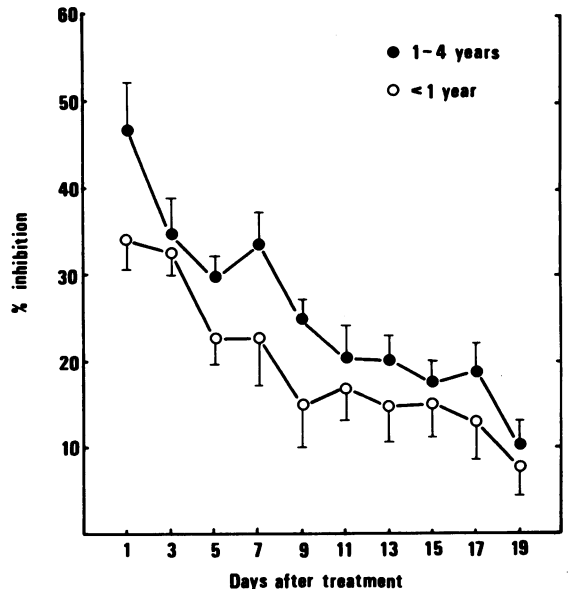


Fig. 4. Inhibition of colour reaction in the pyrimethamine ELISA test by post-treatment samples of urine from 9 children aged <1 year (○) given a quarter tablet of Maloprim and from 36 children aged 1–4 years (●) given a half tablet of the drug.

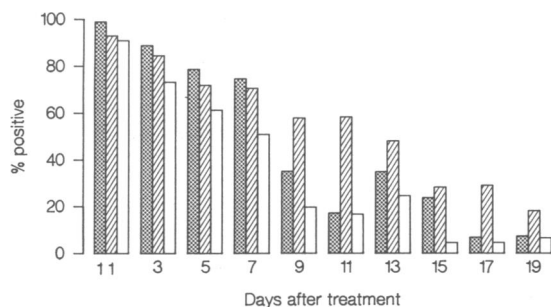


Fig. 5. Proportion of urine samples from 9 children aged < 1 year given a quarter tablet of Maloprim and from 36 children aged 1–4 years given a half tablet that were positive for dapsone (▨), pyrimethamine (▩), or dapsone + pyrimethamine (□) at various times after treatment.

and again 24 hours after Maloprim had been administered were strongly inhibitory in the dapsone ELISA test (mean inhibition before drug administration, -50.7% ; after administration, -59.2%). This degree of inhibition was not observed in the ELISA test for pyrimethamine, where a mean value of $+1.9\%$ was obtained before administration of Maloprim and -4.6% was observed 24 hours after the drug had been given. An increase in inhibition after drug administration occurred with both assays, but the difference was statistically significant only for pyrimethamine ($P < 0.01$). For both assays a wide degree of overlap occurred between pre- and post-drug administration values.

Main study

Samples of urine from children who attended a monthly clinic over a 2-year period and from children examined in a cross-sectional survey were assayed for dapsone and pyrimethamine. No significant differences were found between clinic and survey samples and results were therefore pooled. Table 1 shows the proportion of positive assays related to the period elapsed from the last drug administration recorded on the child's health card together with the corresponding data from the pilot study. The results of both studies are comparable for 2 weeks after the last drug administration. However, in the third week significantly more children examined during routine surveillance were positive for dapsone, pyrimethamine, and both assays combined than were children covered in the pilot study ($P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively). Analysis of these data indicated that samples from children in the main study had been collected 2 days earlier, on average, in the third week than those in the pilot survey. If the data are compared at 2-day, rather than weekly intervals, there is no significant difference between the results of the main and pilot studies (Mantel-Haenszel test; $P > 0.1$).

Table 2 shows the results of the assays of urine from Sarakunda children who received either chlorproguanil or placebo and those from children living in hamlets where no known chemoprophylaxis occurred. A slightly higher fraction of samples were positive in both dapsone and pyrimethamine assays for the Sarakunda children than for the controls, but the difference was only statistically significant ($P < 0.01$) if the results of the pyrimethamine assays of Sarakunda children who received placebo were compared with controls. There were no significant

Table 1. Results of ELISA tests for dapsone and pyrimethamine in urine according to the period elapsed after administration of Maloprim in the main study and pilot surveys

Mean number of days after administration		Proportion positive in ELISA test					
		Dapsone		Pyrimethamine		Dapsone + pyrimethamine	
		Pilot survey	Main study	Pilot survey	Main study	Pilot survey	Main study
3.0 ± 1.7	3.1 ± 1.6	119/135 (88) ^a	59/69 (86)	114/129 (88)	47/60 (78)	99/129 (77)	41/60 (68)
10.0 ± 2.2	8.6 ± 1.5	71/179 (40)	20/27 (74)	101/174 (58)	8/15 (53)	49/174 (28)	5/15 (33)
16.9 ± 1.6	14.9 ± 1.6	17/127 (13)	42/101 (42)	34/128 (27)	36/83 (43)	8/127 (6)	13/83 (16)

^a Figures in parentheses are percentages.

Table 2. Results of ELISA tests for dapsone and pyrimethamine in urine from children administered chlorproguanil or placebo and from controls

Group	Proportion positive in ELISA test		
	Dapsone	Pyrimethamine	Dapsone + pyrimethamine
Main study:			
Placebo	24/183 (13) ^a	21/175 (12)	9/175 (5)
Chlorproguanil	24/173 (14)	14/159 (9)	7/159 (4)
Controls	23/247 (9)	11/247 (5)	7/247 (3)

^a Figures in parentheses are percentages.

differences when the results of both the dapsone and pyrimethamine assays were combined.

DISCUSSION

The dapsone and pyrimethamine assays were sensitive, reproducible, and easy to perform. Consistent results were obtained for samples of urine that were strongly positive or negative. In contrast, less reproducible results were obtained with samples that were weakly positive (20–40% inhibition), and the specificity of the assay would have been increased had 40% instead of 20% inhibition been used as the positive cut-off level; however, a 40% inhibition level would have lowered the sensitivity of the assay. Fortunately, only about 15% of all positive reactions were weak, and hence any inconsistencies in urine assays should not have any marked overall effect on the results.

Use of the assays to monitor the administration of Maloprim was problematic since about 5–10% of urine samples from children who were not known to have received dapsone or pyrimethamine were positive in one or other of the assays for these substances. Many of these samples were strongly positive and remained so even after they had been diluted to a concentration of 1:100 or when a higher level of inhibition was used as the criterion for a positive test.

In the Gambia dapsone is occasionally given by unqualified medical practitioners for conditions other than leprosy, while pyrimethamine is sometimes available in pharmacies. However, it is unlikely that either these or related drugs were available in the isolated hamlets around Sarakunda, and investigations in hamlets where several positive dapsone assays were found failed to reveal any source of this drug. It is therefore likely that these reactions were

false positives caused perhaps by reaction between a dietary metabolic product and the antisera used in the ELISA tests; this possibility is being investigated further. Urine from children who had received Maloprim was usually positive in both the dapsone and pyrimethamine assays, while urine from children who had probably not received either of these drugs was normally positive in only one assay. Use as selection criterion of a positive result in both assays discriminated between true and false positives and gave a test with satisfactory sensitivity and specificity (93% and 97%, respectively).

Both dapsone and pyrimethamine are excreted in saliva (7), and since its collection could be directly supervised by the investigators both saliva and urine were assayed in the pilot study. Surprisingly, all samples of saliva collected prior to drug administration were strongly positive in the dapsone assay, which could therefore not be used to monitor Maloprim compliance. This may have been caused by a cross-reaction with a substance normally present in saliva. In the pyrimethamine ELISA test there was a small but statistically significant difference between the mean inhibition level in assays of saliva collected before and after administration of Maloprim, but the large spread of results precluded use of this assay.

In order to validate the results of the ELISA tests, samples of urine were collected from a group of children under 5 years of age who had been given a quarter or a half tablet of Maloprim under direct surveillance. As expected from their respective half-lives in plasma (8), dapsone disappeared from the urine more rapidly than pyrimethamine, but 40% of samples collected during the second week after administration of Maloprim were still positive in the dapsone ELISA test. Dapsone was therefore probably present in the plasma of these children in sufficient concentrations to produce a synergic effect with pyrimethamine for at least 1 week after drug administration.

The ELISA tests were also used to monitor compliance in a larger chemoprophylaxis study involving six drug regimens. Comparison of the results of urine assays from children who should have received chlorproguanil or placebo with those from control children confirmed that the former children had not received Maloprim. Also, the results of assays of urine from children designated to receive Maloprim were closely similar, for the first two weeks after administration, to those from children in the pilot study who received Maloprim under surveillance. Samples obtained from study children during the third week after drug administration exhibited a

higher proportion of positives than comparable samples from children in the pilot study; however, this variation largely arose because of differences in the collection times for the two groups, almost all samples from children in the test group having been obtained during the early part of the third week, while those from the pilot study were collected more evenly throughout this week. Nevertheless, the study shows that the ELISA tests for dapsone and pyrimethamine, despite the problem posed by false positive reactions, can provide useful information about the efficacy of a malaria chemoprophylaxis programme.

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RÉSUMÉ

LES ÉPREUVES ELISA POUR LA RECHERCHE ET LE DOSAGE DE LA DAPSONE ET DE LA PYRIMÉTHAMINE, ET LEUR UTILISATION DANS LES PROGRAMMES DE CHIMIOPROPHYLAXIE DU PALUDISME

Dans le présent article sont décrites des méthodes de titrage immunoenzymatique (ELISA) pour la dapsone et la pyriméthamine, et leur application à l'étude de l'observance du traitement par un antipaludéen, le Maloprim (pyriméthamine + dapsone). Les 2 titrages reposent sur l'inhibition de la réaction colorée provoquée par l'action de la peroxydase sur une solution d'acide azino-2,2' bis(éthyl-3) benzothiazoline sulfonique dans l'eau oxygénée. Une inhibition de 20% de la réaction colorée est décelable à l'oeil nu et c'est ce seuil qui a été utilisé pour distinguer les échantillons positifs et négatifs. La limite de détection est de 20 µg/l de dapsone ou de pyriméthamine dans l'urine normale, et avec des échantillons négatifs ou fortement positifs on obtient une bonne reproductibilité; les résultats varient davantage avec les échantillons de teneur moyenne.

Dans une étude pilote, des échantillons d'urine ont été prélevés chez 45 enfants âgés de 3 mois à 4 ans, 24 heures environ après l'administration d'un quart ou d'un demi-comprimé de Maloprim. En ELISA, 44 échantillons (98%) se sont révélés positifs pour la dapsone, 42 (93%) pour la pyriméthamine et 42 (93%) pour les deux médicaments. Des titrages ont également été pratiqués sur des échantillons d'urine prélevés chez 247 enfants habitant des hameaux où aucune chimioprophylaxie n'avait été administrée: 23

(9,5%) se sont montrés positifs pour la dapsone et 11 (4,5%) pour la pyriméthamine. Sept échantillons seulement (soit 2,8%) étaient positifs dans les deux épreuves. La sensibilité et la spécificité des deux épreuves associées sont par conséquent de 93% et 97% respectivement. Les "faux positifs" n'ont pas été supprimés en chauffant ou en acidifiant l'urine et ils font l'objet d'une investigation plus approfondie. Des échantillons d'urine ont été recueillis un jour sur deux pendant trois semaines chez 45 enfants âgés de 3 mois à 4 ans après administration d'un quart ou d'un demi-comprimé de Maloprim. Les échantillons se sont montrés positifs pour la dapsone chez 75% des enfants 7 jours après la prise du médicament, et au bout de 15 jours 25% étaient encore positifs. En ce qui concerne la pyriméthamine, les chiffres correspondants étaient de 73% et 30%.

Les résultats fournis par cette enquête pilote ont été employés pour surveiller l'observance dans un essai chimioprophylactique comportant six schémas thérapeutiques. Les échantillons d'urine ont été recueillis chez les enfants conduits dans les dispensaires pour les visites systématiques ainsi qu'au cours d'une enquête transversale. En comparant les résultats obtenus avec ceux de l'enquête pilote, il ressort que les médicaments ont été administrés conformément à ce que les mères avaient indiqué.

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