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Detection and estimation of antimalarial drugs in body fluids — I. Field test for amodiaquine in urine and saliva

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Abstract: Simple and specific tests for the detection of antimalarial drugs are required during clinical studies on the sensitivity of *Plasmodium falciparum* to these drugs. A specific colour test has been developed for amodiaquine which is sensitive enough to permit the visual detection of the drug, following the oral administration of a single 600 mg dose, for up to 10 days in saliva and up to seven weeks in urine. The test is based on the oxidation of amodiaquine to a yellow-coloured derivative using an aqueous solution of potassium persulphate and disodium hydrogen orthophosphate as oxidant. The colour has also been determined spectrophotometrically at 560 nm to achieve a lower detection limit of 0.5 μg amodiaquine in 10 ml biological fluid.

Keywords: *Amodiaquine; detection in urine and saliva.*

Introduction

The development of *P. falciparum* resistance to chloroquine, the most widely used antimalarial drug, has prompted a re-evaluation of available alternative drugs for the treatment of acute falciparum malaria. Amodiaquine (I) and chloroquinine are both 4-aminoquinoline derivatives, but amodiaquine has not been used or studied to the same extent as chloroquine. Recent studies have shown that although cross-resistance to amodiaquine occurs, chloroquine-resistant *P. falciparum* infections are frequently either cured by or are less-resistant to amodiaquine [1, 2]. For optimum exploitation of this therapeutic advantage of amodiaquine, more extensive clinical studies are required.

A prime requirement in the clinical assessment of drug sensitivity of plasmodia *in vivo* are simple and sensitive analytical methods for the detection and estimation of antimalarial drugs under field conditions.

Colour tests have been described for amodiaquine, one being based on the dye eosin and the other on bromophenol blue [3, 4]. Since the colour changes undergone by these dyes in the presence of amodiaquine can also be effected by other basic nitrogenous

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compounds, these tests are not specific for amodiaquine. Furthermore, quantitative estimation of the drug using these methods is not possible. Because of the differences in the activities of amodiaquine and chloroquine against chloroquine-resistant plasmodia, a field test which is specific for amodiaquine would be advantageous during clinical studies.

A procedure is described for the specific detection and spectrophotometric determination of amodiaquine in urine and saliva based on its oxidation with potassium persulphate to an intensely-coloured derivative.

Experimental

Reagents

Amodiaquine hydrochloride (Parke Davis & Co., UK), Camoquine® tablets (Parke Davis & Co.) were obtained from commercial sources.

Other reagents are: diethylether (BDH); disodium hydrogen orthophosphate (BDH); potassium persulphate (BDH) and sodium carbonate solution (50% m/v).

Oxidising solution: prepared by dissolving 10.8 g (0.04 mole) of potassium persulphate and 15.4 g (0.08 mole) of disodium hydrogen orthophosphate in 100 ml of water.

Instrumentation

Absorbance measurements were recorded on a SP 26-200 Pye–Unicam Single beam spectrophotometer, using a glass cell with a pathlength of 1 cm.

Oxidation of amodiaquine

Two tablets of Camoquine® equivalent to about 400 mg of amodiaquine base were crushed to a fine powder in a mortar and 20 ml of 50% sodium carbonate solution added to form a slurry. The slurry was transferred to a beaker and the mortar rinsed with a further 20 ml of the sodium carbonate solution. The addition of 20 ml oxidising solution to the slurry immediately produced an intense orange colour. The mixture was allowed to stand for 30 min at ambient temperature with intermittent stirring, after which it was transferred to a separating funnel and extracted twice with 50 ml portions of diethyl ether. The combined ether extracts were dried over anhydrous sodium sulphate.

Upon evaporation of the solvent, a bright orange-coloured oil was obtained. A dark brown solid was obtained on keeping the oil in a deep freeze overnight. The solid was redissolved in ether and the ether allowed to evaporate at room temperature over a few hours. The crystals obtained had a melting point of 178–179°C (with decomposition). The visible absorption spectrum of a methanol solution of the solid showed a maximum at 560 nm and the nuclear magnetic resonance spectrum shows the following characteristics: 6-proton triplet at $\delta = 1.1$; 2-proton singlet at $\delta = 2.15$; 4-proton quadruplet at $\delta = 2.8$; doublets and unresolved multiplets in the region $\delta = 6.5$ to $\delta = 8.5$

Visual detection of amodiaquine in standard aqueous, saliva and urine solutions

A stock solution containing the equivalent of 200 μg/ml of amodiaquine base was prepared by dissolving 65.0 mg of amodiaquine hydrochloride in 250 ml of water. A 25 ml aliquot of the stock solution was diluted to 50 ml to give a standard solution of 100 μg/ml of amodiaquine base. To 0.01–1.0 ml aliquots of the standard solution were added 1.0 ml of water to give standard aqueous solutions containing 1.0–100 μg of amodiaquine base which were then treated with 3.0 ml of the oxidising solution.

Using 50 ml stoppered test tubes, standard urine (or saliva) solutions containing 1.0–100 μg of amodiaquine base were prepared as described for aqueous solutions but using 2.0 ml of drug-free urine (or saliva) in place of water. To these solutions were added 5.0 ml of sodium carbonate solution and 10.0 ml of diethyl ether. After shaking for 2 min the aqueous layers were removed with a Pasteur pipette, the ether extracts shaken briefly with a few milligrams of anhydrous sodium sulphate and transferred to a clean 10 × 2 cm test tube. The ether extracts were evaporated with the aid of a hand pump and the residues treated with 2.0 ml of the oxidising solution. The reaction mixtures were kept at ambient temperature and observed for the appearance of a yellow colour. Blank urine and saliva were similarly treated for comparison.

Visual detection of amodiaquine in urine and saliva from dosed volunteers

A single oral therapeutic dose of Camoquine®, equivalent to 600 mg of amodiaquine base was administered to each of five volunteers from whom pre-dose urine and saliva samples had been obtained. All urine samples were collected separately within the following 12–18 h. Thereafter, sample collection continued on a daily basis (once daily) for the next two weeks and then once a week for the next two months for one of the volunteers. About 10–15 ml of unstimulated saliva samples were also obtained at 24 h intervals for two weeks.

For the detection of amodiaquine, 10 ml of urine (or saliva) samples were placed in 50 ml stoppered test tubes, made alkaline with 5.0 ml of sodium carbonate solution and extracted with 10.0 ml of ether as described above for standard urine (and saliva) solutions. Subsequent drying of the ether extract with anhydrous sodium sulphate, evaporation and treatment of the residue with 2.0 ml of the oxidising solution were as described above.

Spectrophotometric determination of amodiaquine in biological fluids — calibration in water, plasma, saliva and urine

A standard solution containing 100 μg/ml of amodiaquine base was prepared as described above. To 0.05–0.2 ml aliquots of this solution were added 2.0 ml of water (or drug-free plasma, or saliva or urine) to give standard solutions containing 5.0–20.0 μg of the drug in these media. To prepare standards containing 0.5 μg and 1.0 μg of the drug respectively, an aliquot of the standard solution was diluted ten times to give a drug concentration of 10.0 μg/ml and two aliquots (0.05 and 0.1 ml) of the diluted solution were placed in test tubes followed by the addition of 2.0 ml of water (or drug free plasma, or saliva or urine).

The standard solutions were made alkaline with 5 ml of sodium carbonate solution and extracted with 10 ml of ether as described above. Emulsion formation was observed during extraction of the saliva standards but the emulsion was usually cleared by the addition of a further 2.0 ml of ether when shaking had stopped.

After removing the aqueous layers, the ether layer was treated as described for the visual detection of standard solutions above. After evaporating the ether, the residues were treated with 3.0 ml of oxidising solution and the mixture allowed to stand at ambient temperature for 15 min. The absorbances of the reaction mixtures were measured at 560 nm using drug-free water (or plasma or saliva or urine) which had been similarly treated as references, respectively.

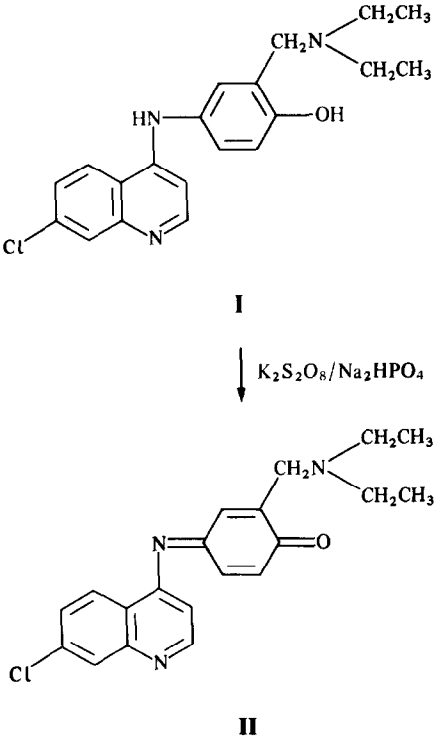
This procedure was repeated five times for statistical evaluation.

Results and Discussion

Oxidation of amodiaquine

Structurally, amodiaquine (**I**) differs from chloroquine only in the nature of the side chain attached to the 4-amino-7-chloroquinoline nucleus which is common to both compounds. A colour test which is specific to either of these compounds has, therefore, to be based on the different chemical behaviours of the side chains. Amodiaquine may be considered to be an *N*-substituted aminophenol. Aminophenols are known to be susceptible to oxidation by a variety of oxidising agents to give benzoquinones which are usually coloured compounds. Accordingly, the reaction of amodiaquine with a variety of oxidising agents was investigated as a basis for a simple field test for amodiaquine.

Although there has been no previous report on the oxidation of aminophenols with potassium persulphate, amodiaquine was found to be readily oxidised to the intensely coloured benzoquinone imine derivative 2-diethylaminomethyl-*N*-(7-chloro-4-quinolyl)-1,4-benzoquinone imine (**II**) as represented in Scheme 1. The compound was identified by comparison of its proton magnetic resonance with that of amodiaquine. The yield of the derivative was found to be 90.7% as determined by comparison of the absorbance of standard solutions of **II** with the absorbance of solutions obtained following oxidation of standard solutions of amodiaquine in methanol. Because of the high yield and the formation of a coloured product this oxidation reaction seems an ideal basis for a sensitive field test for amodiaquine.



Scheme 1

Visual detection of amodiaqine in standard aqueous, urine and saliva solutions

When the persulphate oxidation was applied to standard aqueous solutions of amodiaquine, visual detection of the drug was possible down to a level of about 5.0 μg. The colour reaction was observable almost instantaneously when large amounts of amodiaquine (>20 μg) were present, but 5–20 μg quantities required the reaction mixture to stand for about 15 min with intermittent shaking.

When the reaction was applied directly to urine, an intensification of the normal yellow colour of urine was observed for samples containing 20 μg or more of amodiaquine. For urine containing less than 20 μg of amodiaquine the colour change was not clear and extraction of **II** with ether was also not satisfactory. However, extraction of amodiaquine (**I**) from urine using ether followed by its oxidation with potassium persulphate permitted easy detection of amodiaquine in urine at the 5.0 μg level and its reproducible quantitation by spectrophotometry, if necessary.

Although saliva has rarely been used as a sampling fluid for the detection of antimalarial drugs, it has been suggested as a convenient fluid for the forensic detection of many basic compounds [5]. Amodiaquine may be detected in saliva by direct treatment with the oxidising solution and shaking the mixture with ether. However, the colour of the solution is usually attenuated by the cloudiness of the mixture due to the presence of oral debris. Prior extraction of the drug was therefore adopted as for urine.

No colour reaction was observed from chloroquine, aspirin, and paracetamol (and its metabolite, *p*-aminophenol), sulphadoxine and pyrimethamine. Promethazine gave a pink colour on reaction with the oxidising solution.

Visual detection of amodiaquine in dosed volunteers

Following the administration of a single oral dose of 600 mg of amodiaquine base to five volunteers, the drug could be detected in the saliva for a period of 10 days after drug administration.

The drug was readily detected in urine for the two weeks during which sampling lasted in four of the volunteers. For the fifth volunteer from whom urine samples were obtained for three months, the drug could be detected for up to 7 weeks. The last urine sample in which the drug could be visually detected was found by spectrophotometry to contain 4.1 μg per 10 ml of urine, a result which is consistent with the earlier finding that the lower limit of visual detection was about 5 μg of amodiaquine.

Spectrophotometric determination of amodiaquine in aqueous, saliva, urine and plasma solutions

Calibration graphs of absorbance of the oxidation product (**II**) against the amount of amodiaquine in saliva, urine and plasma showed good linearity and reproducibility over the range 0.5–20 μg (Table 1).

When the oxidation procedure was applied directly to 0.5–20 μg of the drug in methanol, the calibration graph obtained was described by the equation $y = 0.0147\ (\pm 0.00035)x + 0.0098\ (\pm 0.0035)$ where x μg is the amount of drug oxidised and y is the absorbance of the oxidation product. Comparison of this equation with those for water, saliva, urine and plasma standards, shows the recovery of the drug to be 97.1, 89.14, 94.35 and 63.54%, respectively. The simple extraction procedure described above may be considered adequate for urine and saliva samples. Furthermore, comparison of the regression equations for the aqueous, saliva and urine standards shows there is no significant difference between these equations. Thus, aqueous standards may be

Table 1
Calibration data for amodiaquine in water, saliva, urine and plasma

Amount of amodiaquine oxidised (μg)	Absorbance*			
	Water†	Saliva‡	Urine§	Plasma¶
0.5	0.012 (19.00)	0.010 (8.00)	0.011 (25.32)	0.009 (39.38)
1.0	0.022 (17.39)	0.021 (10.43)	0.021 (17.66)	0.011 (24.30)
5.0	0.075 (11.57)	0.089 (9.68)	0.076 (8.92)	0.054 (8.92)
10.0	0.147 (4.34)	0.132 (4.67)	0.148 (3.13)	0.102 (10.04)
20.0	0.291 (1.31)	0.274 (1.41)	0.282 (1.36)	0.194 (6.15)

*Each value is the mean of five determinations. The relative standard deviation are in parentheses.
Regression equations: where y is the absorbance of the oxidation product obtained from oxidation of x μg of amodiaquine.
†$y = 0.0143\ (\pm 0.0003)x + 0.0051\ (\pm 0.0029)$; $r = 0.9985$.
‡$y = 0.0132\ (\pm 0.0005)x + 0.0085\ (\pm 0.0048)$; $r = 0.9952$.
§$y = 0.0139\ (\pm 0.0004)x + 0.0066\ (\pm 0.0025)$; $r = 0.9988$.
¶$y = 0.0094\ (\pm 0.0004)x + 0.0070\ (\pm 0.0041)$; $r = 0.9930$.

conveniently used for the determination of unknown urine and saliva samples of the drug. Plasma standards will, however, be needed for determination of unknown plasma samples. For many drugs, plasma levels may be predicted from the saliva levels [5]. Thus, one significance of the results reported here is that it may be possible, under field conditions, to predict plasma levels of amodiaquine by measurement of the levels in saliva by this simple spectrophotometric method, once the effect of the factors which influence the partitioning of drugs into saliva has been established for amodiaquine.

Summary

For the specific detection of amodiaquine in urine and saliva, 10 ml of the sample is made alkaline with 5 ml of 50% sodium carbonate solution and then extracted by shaking with 10 ml of ether for 2 min by hand. The aqueous layer is removed with a Pasteur pipette and the ether layer shaken with a little anhydrous sodium sulphate to dry it. After transferring to another test tube, the ether is evaporated with the aid of a hand pump. Addition of 2 ml of a solution prepared from dissolving potassium persulphate (0.04 mole) and disodium hydrogen orthophosphate (0.08 mole) in 100 ml of water results in the formation of an intense yellow colour in the presence of amodiaquine.

The lower limit of visual detection is 5 μg of amodiaquine. Following the ingestion of a single therapeutic dose, amodiaquine can be detected in saliva for up to 10 days and in urine for up to 7 weeks after dosing.

Spectrophotometric measurement of the yellow colour at 560 nm permits the determination of amodiaquine in biological fluids to a lower limit of 0.5 μg/10 ml.

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