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Note

Assay method for the simultaneous determination of proguanil, chloroquine and their major metabolites in biological fluids

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Both chloroquine and proguanil have been extensively used for malaria chemoprophylaxis as single agents for more than thirty years. However, with the widespread appearance of chloroquine-resistant falciparum malaria, it has been necessary to combine the preparations to provide adequate protection against falciparum malaria. The combination of chloroquine and proguanil is now the mainstay of malaria chemoprophylaxis [1] and is advised for all areas where chloroquine-resistant malaria exists. Since this combination chemoprophylaxis was launched, an increase in the frequency of mild side-effects has been reported [2,3] and evidence from a recent study [3] looking at the use of chloroquine and proguanil separately and in combination reports that 34% of a population using chloroquine and proguanil had adverse reactions, which was two-fold higher than comparable groups using either drug alone. Altered drug

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handling when administered in combination may be one mechanism to explain the increased incidence of the mainly gastrointestinal side-effects. Information on the kinetics and metabolism of these drugs when combined is therefore of increasing importance and techniques to quantify both drugs and their respective metabolites simultaneously are required.

Proguanil was originally determined by non specific colorimetric assay [4]. Other methods based on high-performance liquid chromatography (HPLC) now exist for this drug and one or both of its main metabolites [5–7]. Chloroquine, likewise, was originally assayed by spectrofluorimetry [8–10]. This technique was non-specific due to the interference of the desethyl metabolite. HPLC methods now also exist for chloroquine which are adequately sensitive and specific to allow determination of the parent drug and the main desethylchloroquine metabolite in biological fluids [11,12].

It is the purpose of the present work to report the results obtained in the simultaneous assay of these compounds by a reversed-phase ion-pairing HPLC assay method based on the previous publication for proguanil and its metabolites [7].

EXPERIMENTAL

Materials and equipment

A Waters Assoc. liquid chromatograph was used consisting of an M6000A pump and fixed-wavelength (254 nm) ultraviolet detector, Model 441, fitted with a nominal 18-μl flow cell. Injection was by a Rheodyne 7125 valve incorporating a 20-μl loop. The column was 100 mm$\times$2 mm I.D., slurry packed in the laboratory with 3-μm ODS Hypersil (HETP, Macclesfield, U.K.). Solid-phase extraction pretreatment of sample was by Bond-Elut (Analytichem, Harbor City, CA, U.S.A.) C_{18} cartridge in conjunction with a Vac-Elut (Jones Chromatography, Llanbradach, U.K.) ten-cartridge manifold.

Proguanil, cycloguanil, 4-chlorophenylbiguanide (CPB) and chlorproguanil were supplied by ICI Pharmaceuticals as the hydrochlorides. Chloroquine sulphate was supplied by May & Baker (Dagenham, U.K.). Desethylchloroquine hydrochloride was donated by the World Health Organisation. Acetonitrile and methanol were obtained from Rathburn (Walkerburn, U.K.), sodium lauryl sulphate (SLS) from FSA (Loughborough, U.K.) and tetraethylammonium bromide (TEA) from Aldrich (Gillingham, U.K.). Water was purified by a Millipore Milli-Q system (Harrow, U.K.) and all other reagents were of AnalaR or equivalent grade.

Chromatographic separation

The chromatographic solvent used was acetonitrile–10 m*M* phosphate buffer pH 2.0 (50:50) containing 230 m*M* SLS and 10 m*M* TEA. A flow-rate of 0.4 ml min^{-1} was used. The rationale of this mobile phase is described below.

Sample pretreatment

Pretreatment of plasma and urine was similar to that described previously for proguanil and metabolites [7]. Plasma samples were extracted using C_{18} Bond-Elut cartridges which had previously been wetted with 2 ml of methanol and conditioned with 2 ml of water. A 1-ml sample was added to the cartridge followed by the appropriate amount of chlorproguanil solution as internal standard. The cartridge was washed with 1 ml water and 1 ml methanol. The compounds were eluted with 1 ml methanol containing 0.1% perchloric acid. The collected extract was evaporated and reconstituted in 50 μl of water immediately before chromatography. Urine samples were treated similarly except that after application of sample and internal standard the cartridge was washed with water only and the final solution was made up to 1 ml in water. These procedures were applied to both spiked plasma and urine standards and to samples.

Calibration procedure

Five standard concentrations of each compound were prepared in plasma to cover what was considered from the literature information to be appropriate ranges. These were proguanil (0–200 ng ml^{-1}), cycloguanil (0–80 ng ml^{-1}), CPB (0–20 ng ml^{-1}), chloroquine (0–90 ng ml^{-1}) and desethylchloroquine (0–20 ng ml^{-1}), respectively. Corresponding standards were prepared using urine as matrix. Concentration ranges used were proguanil (0–5 μg ml^{-1}), cycloguanil (0–2 μg ml^{-1}), CPB (0–1 μg ml^{-1}), chloroquine (0–4 μg ml^{-1}) and desethylchloroquine (0–1 μg ml^{-1}), respectively. Each standard was subjected to the pretreatment procedure described above. For plasma standards 50 μl of 1 μg ml^{-1} internal standard solution were added and for urine standards 50 μl of 50 μg ml^{-1} internal standard solution were used. Peak heights were measured at appropriate absorbance ranges, usually at 0.005–0.02 a.u.f.s. for plasma and 0.02–0.05 a.u.f.s. for urine. Calibration equations were obtained by linear regression of peak-height ratio (compound/internal standard) on concentration. The recoveries of chloroquine and its major metabolite at 100 ng ml^{-1} and at 5 μg ml^{-1} in plasma and urine, respectively, were determined by comparing peak heights obtained after extraction with those obtained by direct injection of the appropriate concentration of aqueous standards of each of the compounds.

Detection limits were estimated in plasma by injecting successively lower concentrations until a signal-to-noise ratio of approximately 3 was obtained.

The within-day accuracy and precision of the method was determined by assaying a single spiked plasma or urine sample ten times using a single calibration procedure. The day-to-day precision was taken as the variation in the slope of the calibration lines obtained on different days over a period of five days.

RESULTS AND DISCUSSION

The chromatographic solvent used was developed from that found suitable for assay of proguanil alone [7]. In order to obtain satisfactory resolution of both chloroquine and desethylchloroquine from the proguanil it was necessary

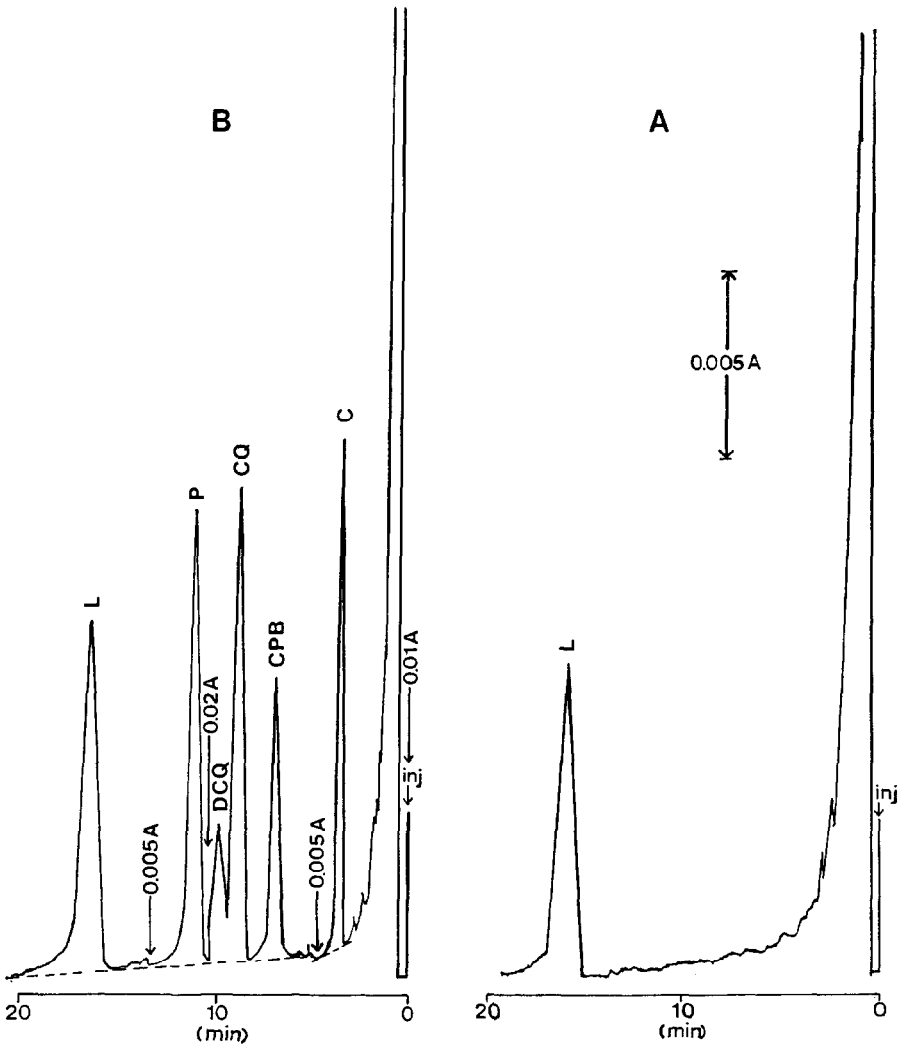

Fig. 1. Representative chromatograms obtained after solid-phase extraction of (A) blank plasma and (B) plasma spiked with 59.5 ng ml^{-1} cycloguanil (C), 14.5 ng ml^{-1} 4-chlorophenylbiguanide (CPB), 64.8 ng ml^{-1} chloroquine (CQ), 16.3 ng ml^{-1} desethylchloroquine (DCQ) and 151.8 ng ml^{-1} proguanil (P). Chromatographic conditions: column, 100 mm × 2 mm I.D., 3 μm ODS-Hypersil; mobile phase, acetonitrile–10 m*M* phosphate buffer (pH 2) containing 230 m*M* SLS and 10 m*M* TEA; flow-rate, 0.4 ml min^{-1}.

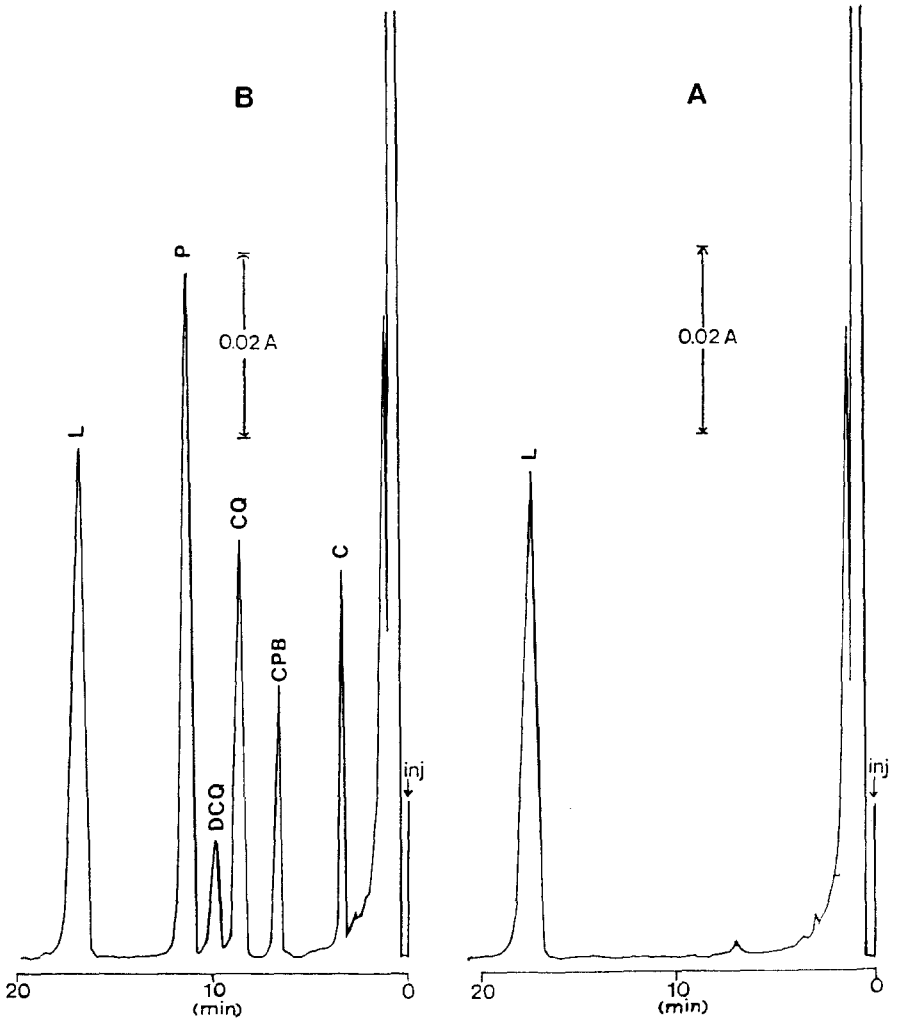

Fig. 2. Representative chromatograms obtained after solid-phase extraction of (A) blank urine and (B) urine spiked with 0.992 μg ml^{-1} cycloguanil (C), 0.518 μg ml^{-1} 4-chlorophenylbiguanide (CPB), 2.16 μg ml^{-1} chloroquine (CQ), 0.544 μg ml^{-1} desethylchloroquine (DCQ) and 2.53 μg ml^{-1} proguanil (P). Chromatographic conditions as in Fig. 1.

to increase the pairing-ion concentration. Separation was decided subjectively from the k' versus pairing-ion concentration curves for the various compounds in the range of pairing-ion concentration 200–300 m*M*. To achieve adequate resolution between the chloroquine and its desethyl metabolite it was found necessary to add TEA as organic counter ion [13]. This, as expected, decreased retention of all compounds as well as increasing the resolution between chloroquine and metabolite. The separation achieved in plasma and urine is shown in Figs. 1 and 2. The critical resolution is that between chloroquine and des-

TABLE I

ANALYTICAL CHARACTERISTICS OF THE METHOD IN PLASMA

Compound	Recovery at 100 ng ml^{-1} (n=9) (%)	Concentration spiked (ng ml^{-1})	Mean concentration found (n=10) (ng ml^{-1})	R.S.D. (n=10) (%)	Calibration slope (mean ± S.D., n=6) ($\times 10^3$)	Detection limit (ng ml^{-1})
CQ	77	42.3	41.4	±4.9	21.5 ± 1.5	1.4
DCQ	91	10.88	11.30	±4.6	26.4 ± 1.5	1.0
C	75[a]	39.68	38.81	±4.3	50.4 ± 1.6	0.5[a]
CPB	74[a]	9.66	9.70	±6.2	58.1 ± 2.2	0.5[a]
P	99[a]	101.2	103.33	±3.9	35.2 ± 1.1	1.0[a]
L	96	–	–	–	–	–

[a]Results from ref. 7.

TABLE II

ANALYTICAL CHARACTERISTICS OF THE METHOD IN URINE

Compound	Recovery at 5 μg ml^{-1} (n=5) (%)	Concentration spiked (μg ml^{-1})	Mean concentration found (n=10) (μg ml^{-1})	R.S.D. (n=10) (%)	Calibration slope (mean ± S.D., n=4)
CQ	100	2.16	2.17	±1.6	0.374 ± 0.008
DCQ	100	0.544	0.546	±1.8	0.410 ± 0.009
C	91[a]	0.992	0.992	±3.4	0.749 ± 0.026
CPB	89[a]	0.5175	0.514	±2.9	0.938 ± 0.034
P	89[a]	2.53	2.56	±1.7	0.521 ± 0.012

[a]Results from ref. 7.

ethylchloroquine but resolution in both matrices is sufficient to allow quantitation of all components. Tables I and II summarise the quantitative aspects of the method. Table I refers to plasma and Table II to results obtained in urine.

The recovery of all compounds using the solid-phase extraction method described is greater than 74% (the values quoted for proguanil and metabolites are those obtained previously [7]). With the exception of proguanil the recoveries are higher from urine. This is probably due to the need to wash plasma samples with methanol to obtain clear extracts at the levels encountered. Tables I and II also show the results obtained by repeated analysis of spiked samples using the purposed procedure. It can be seen that in all cases the accuracy described as a percentage of spiked concentration found is between 97.8 and 103.9% for the difficult matrix, i.e. plasma. The precision is expressed as the relative standard deviation (R.S.D.) for each compound and is 6.1% in the worst case of the minor proguanil metabolite. The day-to-day precision is shown as the constancy of the slope of the calibration line and the maximum relative

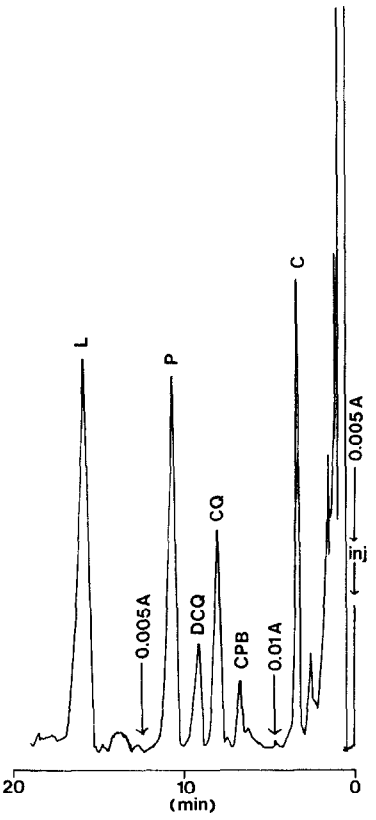

Fig. 3. Representative chromatogram obtained after solid-phase extraction of plasma sample from a subject receiving oral doses of paludrine (200 mg daily) and chloroquine (300 mg base weekly). Sample taken 5 h after ingestion. Peaks (concentrations in parentheses): C = cycloguanil (45.0 ng ml^{-1}; CPB = 4-chlorophenylbiguanide (11.6 ng ml^{-1}); CQ = chloroquine (64.1 ng ml^{-1}); DCQ = desethylchloroquine (36.5 ng ml^{-1}); P = proguanil (108.1 ng ml^{-1}). Chromatographic conditions as in Fig. 1.

standard deviation was 7% which shows the long-term stability of the chromatographic system. In all cases the correlation coefficient of the calibration line produced values of r^2 of 0.995 or greater.

The detection limit for each compound is shown for plasma. Values for the biguanide series are taken from the previous work [7]. Those for chloroquine and desethylchloroquine are comparable but slightly higher presumably due to their lower molar absorptivities.

Fig. 3 shows a representative chromatogram of a plasma sample from a subject following oral combined dosing with both drugs. It can be seen that no substantial differences exist between the subject plasma chromatogram and that shown for spiked human plasma. The minor peak preceding the 4-chlor-

phenylbiguanide proguanil metabolite in the subject sample is tentatively attributed to an additional metabolite of chloroquine since it is present in all subjects studied to date. Its magnitude, however, is such that it does not affect the quantitation of the 4-chlorophenylbiguanide. The concentrations found as indicated in the legend to Fig. 3 show that the detection limits quoted are adequate to quantitate the various compounds at the realistic levels present.

In practice the method developed has proved reliable. The major factor causing deterioration of performance appears to be associated with the column. About 500 injections are possible before the column needs replacement. In term of cost this is not a major factor since the column size is 100 mm × 2 mm I.D. and requires only 0.5 g of packing material.

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