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Simultaneous determination of chloroquine, proguanil and their metabolites in human biological fluids by high-performance liquid chromatography

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Abstract: A reversed-phase ion-pair high-performance liquid chromatographic method with ultraviolet detection is described for the simultaneous measurement of chloroquine, proguanil and their major metabolites in human plasma, erythrocytes and urine. After a liquid–solid extraction on a Bond Elut® C8 cartridge, the compounds are separated on a C8 Lichrospher 60 RP select B column by isocratic elution; the mobile phase is water–acetonitrile–methanol (78:28:4, v/v/v) with 0.5 M ammonium formate and 0.075 M perchloric acid. The eluent is monitored with an ultraviolet detector at 254 nm. The lower limits of quantification in plasma are near 6.0 ng ml^{-1} for chloroquine and near 9.0 ng ml^{-1} for proguanil. No chromatographic interference can be detected from endogenous compounds or from other antimalarial drugs. The method is accurate and precision is good with inter- and intra-assay relative standard deviations lower than 6.8% for plasma samples. *N*-(2-6 dichlorobenzylidene amino)guanidine is used as an internal standard. The chromatographic procedure takes 35 min and can be used for therapeutic drug monitoring and clinical studies.

Keywords: *Chloroquine; proguanil; reversed-phase ion-pair chromatography.*

Introduction

Malaria is one of the oldest and most pervasive diseases with half of the world's population still encountering *Plasmodium*. The increasing resistance of *Anopheles* vectors to insecticides and of malaria parasites to antimalarial compounds have contributed to a resurgence of malaria. It has been estimated that some 110 million cases occur and 1 million people die every year [1].

Proguanil, a biguanide antifolate drug has been used as an antimalarial prophylactic agent since 1946. Ineffective by itself, proguanil undergoes cyclic oxidation by hepatic microsomal oxidases of the cytochrome P 450 system to form its active metabolite, cycloguanil [2]. Chloroquine, a 4-aminoquinoline, has been the most prescribed compound for the chemoprophylaxis and treatment of malaria for more than 50 years. However, with the widespread appearance of chloroquine-resistant *Plasmodium falciparum* strains it has been necessary to co-administer the drugs to provide adequate protection against malaria [3]. The combination of chloroquine and proguanil is now being widely used as an alternative for malaria prophylaxis in endemic areas, particularly for short periods of time.

The determination of the drug level in blood is a predictive tool for assessing the patient's compliance with chemoprophylaxis, establishing whether a therapeutic concentration has been obtained or suggesting the presence of a resistant strain of the parasite. Information on the kinetics and metabolism of these compounds when combined is therefore of increasing interest and methods to quantify both drugs and their respective metabolites simultaneously are required. The analytical method of choice for measuring the antimalarial drugs administered either singly or together is high-performance liquid chromatography (HPLC). Several methods have been reported for the determination of chloroquine concentration; these methods include the use of normal- or reversed-phase columns after liquid–liquid extraction of the drugs, followed by either ultraviolet (UV) or fluorescence detection. In contrast to the analysis of chloroquine, few

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methods for the determination of proguanil and its metabolites using reversed-phase ion-pair chromatography with UV detection have been reported. Only one method has been described for the simultaneous determination of chloroquine, proguanil and their major metabolites by HPLC [3].

For measuring the nanogram levels found in biological fluids after prophylactic doses or in pharmacokinetics studies, a simple, reproducible, specific and sensitive method is required. The purpose of the present work is to report the results obtained in the simultaneous determination of chloroquine, proguanil and their metabolites in various biological fluids by a reversed-phase ion-pair HPLC method.

Experimental

Chemicals and reagents

All chemicals were of analytical reagent grade unless indicated otherwise. Formic acid ammonium salt and *N*-(2,6 dichlorobenzylidene amino)guanidine as the internal standard were purchased from Aldrich (St Quentin Fallavier, France). HPLC grade methanol, hydrochloric acid, 65% (w/w) perchloric acid, 85% (w/w) phosphoric acid were from Merck (Darmstadt, Germany) and acetonitrile (R.S. for HPLC) was from Carlo Erba (Rueil-Malmaison, France). Bond Elut® C8 cartridges were obtained from Prolabo (Paris, France). Chloroquine sulphate (CQ), monodesethylchloroquine (MDCQ) and bidesethylchloroquine (BDCQ) as base were kindly supplied by Rhône Poulenc (Vitry, France). Proguanil hydrochloride (PG), cycloguanil (GG) and 4 chloro-phenylbiguanide (4CPB) as base were a generous gift from ICI-Pharma (Cergy, France). The chemical structures are shown in Fig. 1. Stock solutions containing 500 $\mu g\ ml^{-1}$ of each compound were prepared in methanol–water (50:50, v/v) and stored at −20°C. Working solutions at a concentration of 5 $\mu g\ ml^{-1}$ for drugs, metabolites and the internal standard were obtained by appropriate dilution from the stock standard solutions just before use; these solutions were used for drug-free plasma, erythrocytes, whole blood and urine spiking. All standards were freshly prepared each day. All aqueous solutions were prepared using high-purity water obtained from the water purification system UHQ® Elga (Villeurbanne, France).

Apparatus

The HPLC equipment comprised a 590 high-pressure pump connected to a refrigerated WISP 712 autoinjector Waters (Milford, MA, USA). The UV detector used was a 481 Waters detector equipped with a 14-µl flow cell and the wavelength was set at 254 nm. A Waters 990 photodiode array detector allowed spectral studies. The chromatographic response was recorded by Maxima 820 workstation software (Waters) running on a Power Mate SX Plus personal computer NEC (Boxborough, MA, USA). The Maxima 820 chromatography workstation also included a system interface module (Waters). Chromatograms shown in this report were obtained on a 740 data module (Waters).

Chromatographic separations were performed at ambient temperature on a 250 × 4 mm i.d. column packed with 5-µm C8 Lichrospher 60 RP Select B specially treated to separate basic compounds (Hewlett Packard, Evry, France).

A C8 Lichrospher guard column (10 × 4 mm i.d.) was placed between the injector and the analytical column. The solid-phase extraction pretreatment of the sample was carried out on a Bond Elut® C8 cartridge connected to a Vac Elut® SPS 25 manifold (Prolabo, Paris, France).

Chromatographic conditions

The chromatographic eluent was water–acetonitrile–methanol (72:28:4, v/v/v) with 0.5 M ammonium formate and 0.075 M perchloric acid as counter ions. The apparent pH was adjusted to 4 using 1.1% (v/v) phosphoric acid. The flow rate was set at 0.5 $ml\ min^{-1}$ and the back pressure was 1000 $lb\ in^{-2}$. Before analysis the mobile phase was filtered and degassed through a 0.5-µm FH filter (Millipore, Bedford, USA) with a Pyrex filter holder.

Sample collection

Blood samples were provided from subjects undergoing a chemoprophylactic regimen of CQ (100 mg daily) and PG (200 mg daily) over 4 months. Whole blood was collected by venepuncture into heparinized tubes and centrifuged immediately at 1500*g* for 15 min [4]. The upper two-thirds of the plasma and the lower two-thirds of the erythrocytes layer were separated, frozen as aliquots and stored at −20°C. The whole blood was kept at −20°C

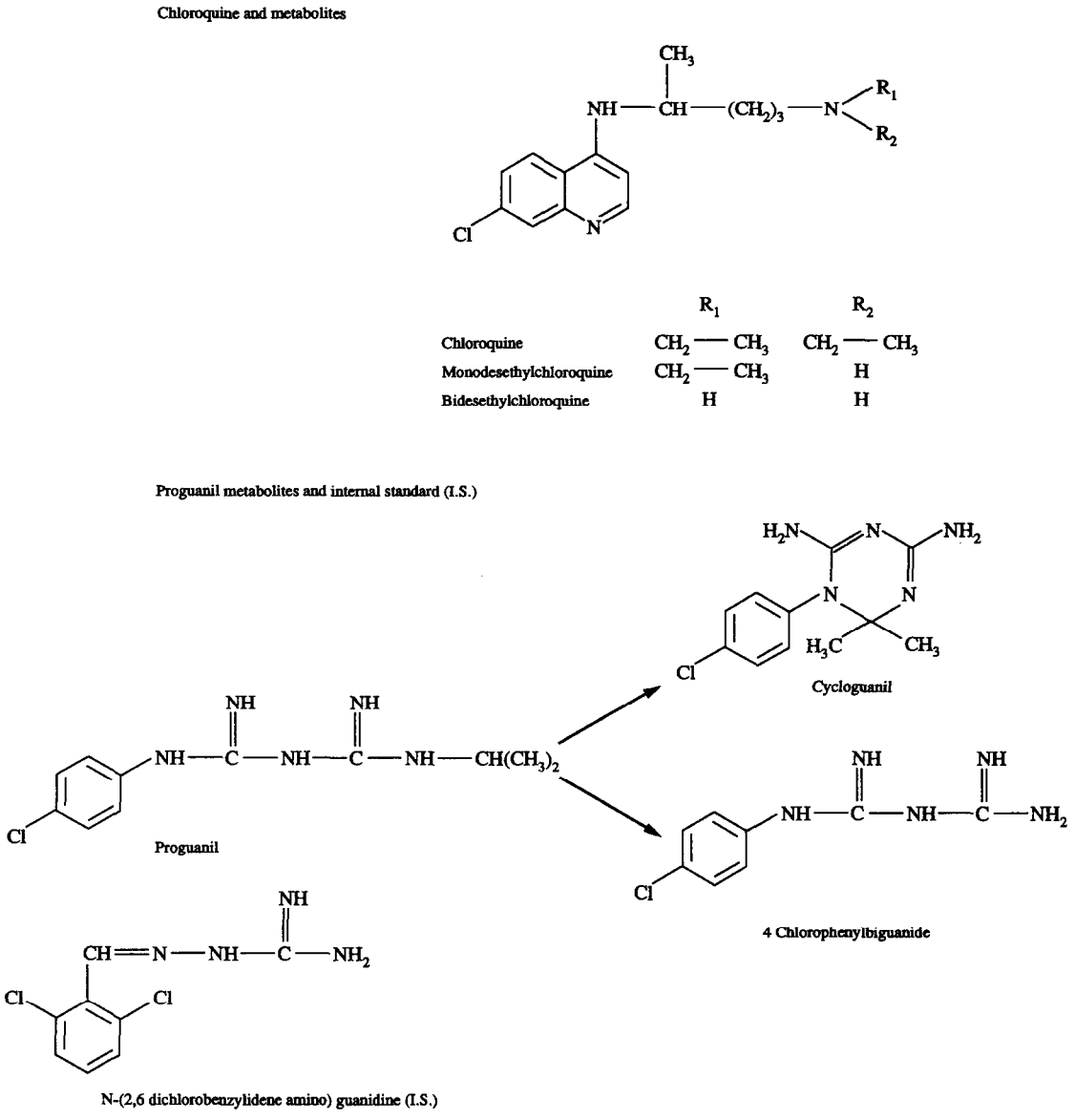

Figure 1
Molecular structures of drugs, metabolites and internal standard.

before analysis. Drug-free plasma, erythrocytes and urine were frozen at −20°C; calibration, recovery and precision measurements were subsequently made using these frozen specimens.

Before use, whole blood and erythrocytes diluted with distilled water (1:2, v/v) were completely lysed by ultrasonication, deproteinized with 1 ml of acetonitrile by vortex-mixing and centrifuged. Urine was diluted with distilled water (1:99, v/v).

Extraction procedure

Drugs were extracted from plasma, whole blood, erythrocytes or urine using a 3-ml Bond Elut® C8 cartridge pretreated successively with methanol (2 × 2 ml) and distilled water (2 × 2 ml). To 1 ml of plasma, diluted urine or deproteinized whole blood sample, 0.05 ml of internal standard (5 μg ml^{-1}) was added and the mixture was passed on to the cartridge. After adsorption of the components, the cartridge was successively washed with water (2 × 2 ml) and acetonitrile (2 × 2 ml).

Elution was carried out with 2 ml methanol containing 0.1% (v/v) hydrochloric acid. The eluate was evaporated to dryness at 30°C under a stream of nitrogen in a borosilicate tube. The

residue was reconstituted by vortex mixing with 200 µl of water, and 50 µl was injected into the column.

Calibration procedure

The calibration procedure based on peak-height ratio (CQ/IS, MDCQ/IS, BDCQ/IS, PG/IS, CG/IS and 4CPB/IS) was performed using spiked drug-free plasma, deproteinized erythrocytes and diluted urine, which were carried through the whole analytical procedure.

Calibration points were obtained by spiking biological samples with working standard solutions to achieve concentrations of 25, 100, 200 and 600 ng ml^{-1} of CQ, PG and their metabolites. Five samples were run at each concentration. The peak-height ratio of drugs or metabolites to the internal standard was plotted against the corresponding concentration. A linear regression was performed in order to estimate the linearity, slope, intercept and correlation coefficient of each calibration line.

Analytical recovery and assay precision

The absolute recovery was determined by comparing the peak-heights obtained from plasma, haemolysed erythrocytes and diluted urine samples spiked at three levels of each compound (25, 100 and 200 ng ml^{-1}) with those obtained by direct injection of the appropriate concentrations of standards.

Estimates of inter-and intra-assay precision were obtained by replicate assays of samples from pools of spiked plasma, erythrocytes and urine at four concentrations (25, 100, 400 and 600 ng ml^{-1}). The intra-assay precision was assessed from 30 plasma and 10 urine or erythrocytes samples. The inter-assay precision was determined over a period of 2 weeks with 10 analyses of the same spiked biological fluids. The relative standard deviations (RSD) of the estimated concentrations were determined and used for the assessment of precision.

Limits of Quantification

The lower limit of quantification of the method for all compounds was calculated from the analysis of drug-free plasma (n = 30) urine (n = 10) and erythrocytes (n = 10) samples as the mean (M blank) and standard deviation (SD blank). These lower limits are functions of the magnitude of the blank value and were

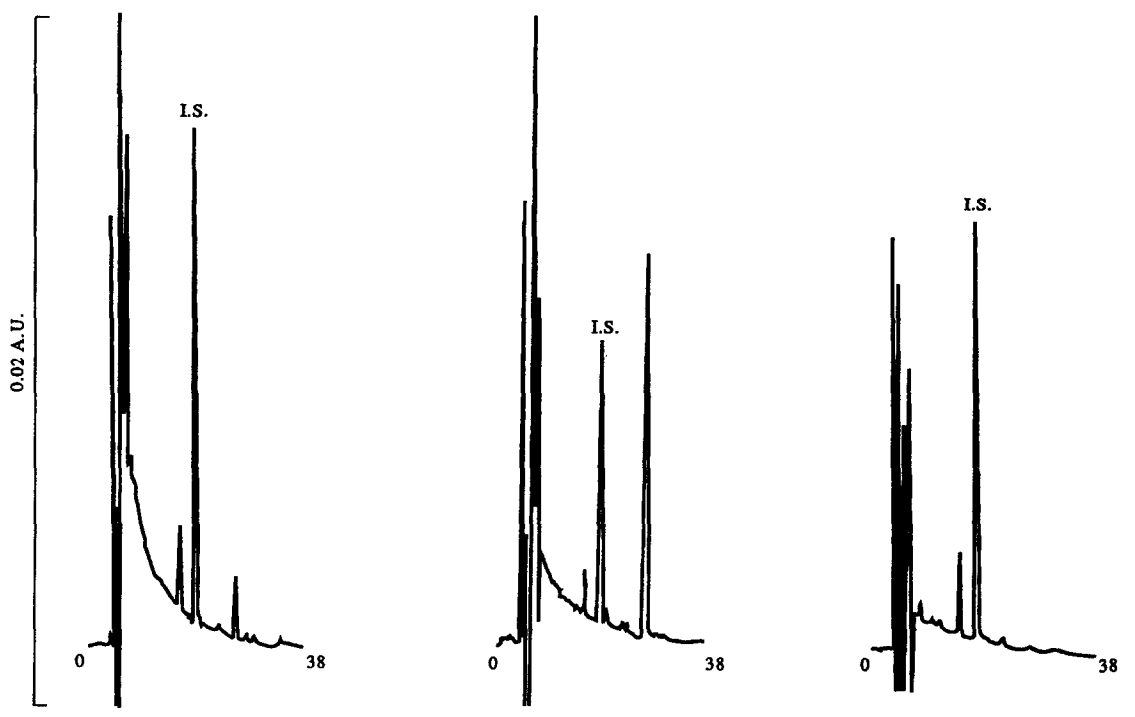

Figure 2
Chromatograms of extracted blank plasma (a), erythrocytes (b) and urine (c) spiked with internal standard.

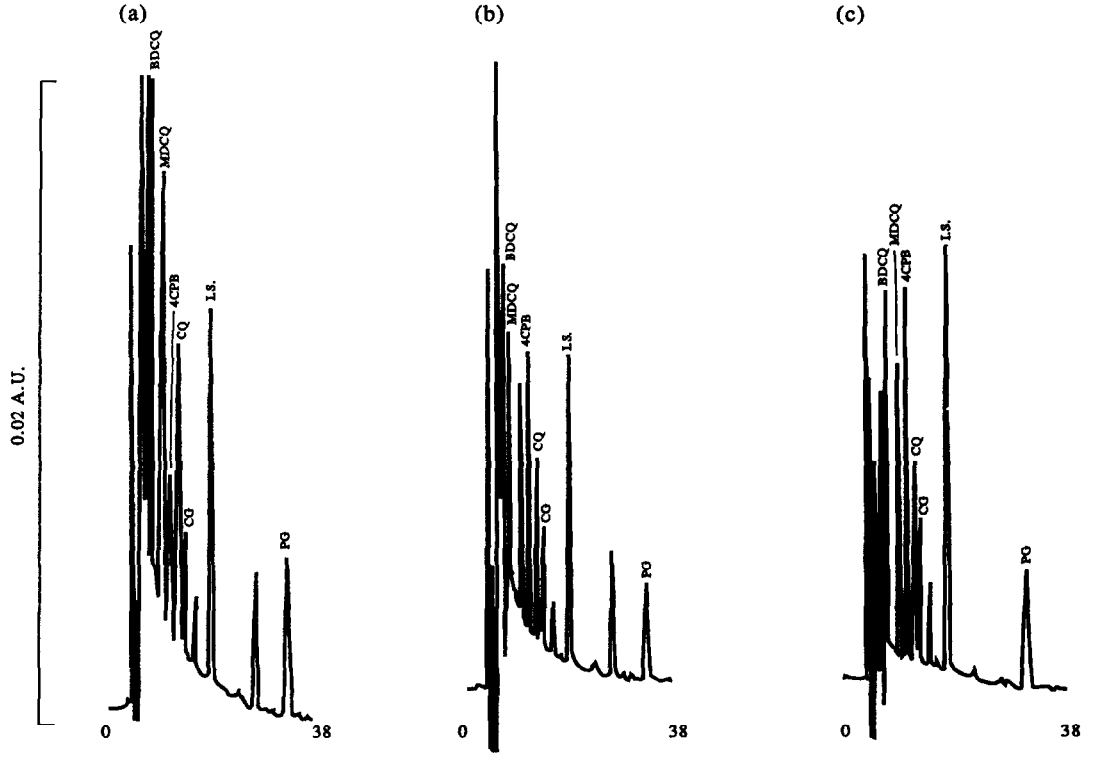

Figure 3
Chromatograms of spiked human plasma (a), erythrocytes (b) and urine (c) containing 100 ng ml^{-1} of each drug and metabolite.

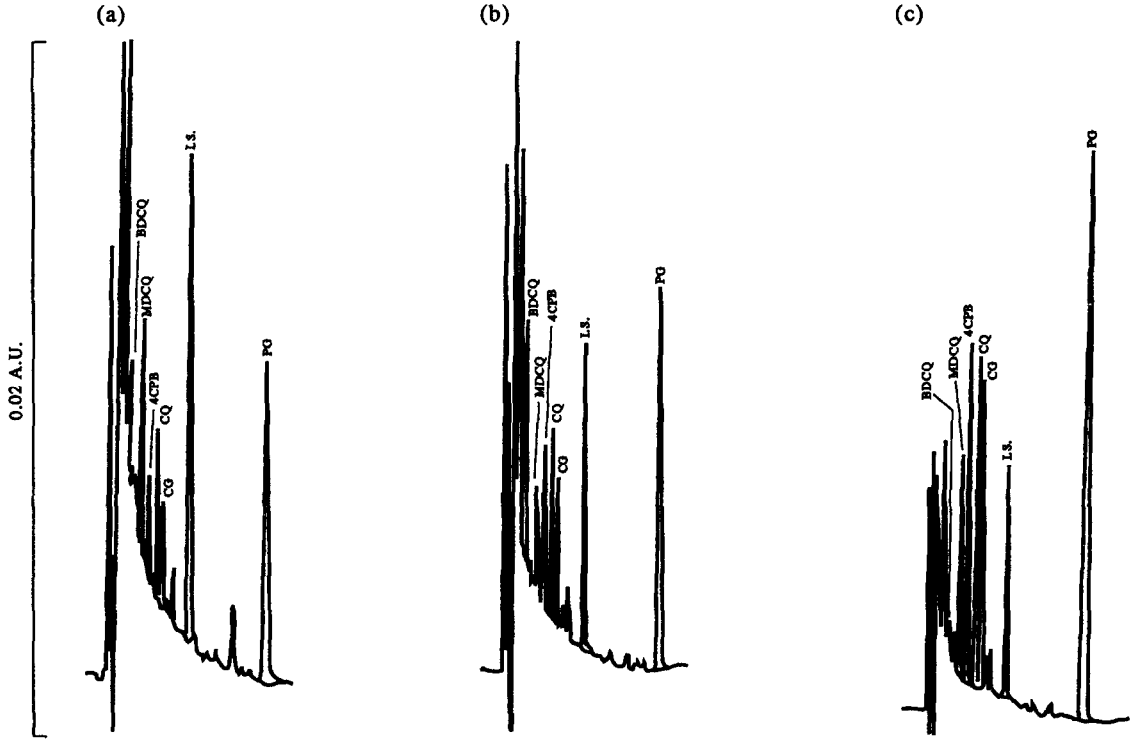

Figure 4
Chromatograms of extracted plasma (a), erythrocytes (b) and urine (c) samples from patient under chloroquine–proguanil chemoprophylaxis regimen.

obtained from the Massart *et al.* [5] formula: Lq = M(blank) + 10 SD(blank).

Results

Selectivity

Figure 2 shows HPLC profiles of an extracted blank plasma (a), erythrocytes (b) and urine (c) spiked with internal standard. Typical chromatograms obtained according to the described method from spiked plasma (a) erythrocytes (b) and urine (c) with 100 ng ml^{-1} of each compound are represented in Fig. 3. Chromatograms obtained from biological samples collected from a patient under the CQ/PG prophylaxis regimen are displayed in Fig. 4.

The elution sequence, retention times and capacity factors are shown in Table 1. No endogenous substances were found to interfere either with the analysis of plasma and erythrocytes or the analysis of urine. Only two minor peaks (retention times 16.1 and 25.2 min) can be observed from the blanks; these peaks are tentatively attributed to components of the Bond Elut® C8 cartridge.

The potential interference with other drugs was examined. The method was shown to be free of chromatographic interference from other antimalarial drugs including mefloquine (R_t = 28 min), mefloquine metabolite RO-21-5104 (R_t = 27.5 min), halofantrine (R_t = 27 min), pyrimethamine (R_t = 21 min), sulphadoxine (R_t: 26 min), and quinine (R_t = 14.6 min).

Calibration graphs for drugs and metabolites

The ratio between the peak-height of the drug analysed and that of the internal standard was calculated and plotted against the concentrations of the drug tested after analysis of blank plasma samples spiked with increasing concentrations of drugs (25–100–200–600 ng ml^{-1}) and a constant amount of internal standard. Within these concentration ranges linear plots were obtained for the six compounds from the three biological fluids. The correlation coefficients (r^2) were greater than 0.998.

The various calibration equations relating y (peak-height ratio) to x (concentration ng ml^{-1}) were:

CQ: $y = 0.00616x - 0.00879$ ($r^2 = 0.998$)
MDCQ: $y = 0.00861x + 0.01961$ ($r^2 = 0.999$)
BDCQ: $y = 0.01298x + 0.0901$ ($r^2 = 0.999$)
PG: $y = 0.00295x + 0.00936$ ($r^2 = 0.999$)
CG: $y = 0.00328x + 0.00101$ ($r^2 = 0.999$)
4CPB: $y = 0.00982x + 0.00776$ ($r^2 = 0.999$)

Linearity was tested by triplicate analysis of 12 standard samples; an excellent linearity was exhibited over the concentration range 12.5–2000 ng ml^{-1} for all compounds.

Analytical precision and accuracy

The intra-assay precision for all compounds was typically 1.8–6.8% in plasma, 1.0–5.6% in erythrocytes and 2.1–8.8% in urine. The inter-assay precision was 0.8–4.4% in plasma, 1.4–8.5% in erythrocytes and 1.5–9.1% in urine. These values demonstrate that the precision of the method is good over the range of concentrations studied for all biological fluids.

Accuracy was calculated as the relative difference (%) between the amount of drug added to drug-free plasma and the amount of drug measured. For CQ, MDCQ and BDCQ the values were respectively 1.5, 1.4 and 1.7% (100 ng ml^{-1}; n = 5), and for PG, CG and 4CPB were of 1.4, 1.3 and 1.4% (100 ng ml^{-1}; n = 5).

Analytical recovery

The absolute recoveries of drugs, metabolites and the internal standard were estimated by comparison of the peak-height obtained after extraction from plasma, erythrocytes and urine samples containing a known amount of the substance with the peak-height obtained after direct injection of a simple solution [methanol–water (50:50, v/v)] containing the same amount of each compound. Five analyses were performed at each level. The mean recoveries of the drugs from biological samples are shown in Table 2.

The mean recovery of the internal standard

Table 1
Retention times and capacity factors

Drug	Retention time (min)	Capacity factor
BDCQ	7.9	4.3
MDCQ	10	5.7
4CPB	11.5	6.7
CQ	12.9	7.6
CG	13.8	8.2
SI	18.8	11.5
PG	32.9	20.9

Table 2
Recovery of drugs by the analytical method

Concentration (ng ml^{-1})	Sample (n = 5)	% Recovery					
		CQ	MDCQ	BDCQ	PG	CG	4CPB
25	Plasma	86	85	104	73	74	67
	Erythrocytes	75	70	66	66	67	57
	Urine	72	69	82	64	71	61
100	Plasma	86	74	101	64	67	65
	Erythrocytes	77	60	52	61	63	55
	Urine	77	70	66	57	67	58
200	Plasma	77	68	88	60	65	64
	Erythrocytes	71	59	52	61	64	57
	Urine	71	68	65	55	65	57

Table 3
Limits of quantification of drugs

Drug	Limits of quantification (ng ml^{-1})		
	Plasma (n = 3)	Erythrocytes (n = 10)	Urine (n = 10)
CQ	6.1	2.4	3.0
MDCQ	2.5	2.5	4.4
BDCQ	2.1	2.9	1.5
PG	5.6	6.6	10
CG	6.8	5.2	2.9
4CPB	2.2	3.1	2.6

was 93% (n = 20) at a concentration of 250 ng ml^{-1}.

Limits of quantification

The lower quantification limit for each compound is shown for plasma, erythrocytes and urine. Table 3 summarizes the results for all biological fluids.

Discussion and Conclusions

The HPLC method described in this report for the simultaneous determination of CQ and PG in human biological fluids is accurate, sensitive and selective. The precision is good with RSDs always lower than 9.5% and the linearity is acceptable over the whole range of therapeutic concentrations. The time required for the chromatographic procedure is about 35 min, which is longer than that of the procedure reported by Taylor *et al.* (20 min) [3].

Distinct advantages of the present assay compared with other HPLC methods include the simultaneous determination of the parent drugs and their two respective major metabolites in all biological fluids with no interference from other antimalarial or endogenous compounds, the use of a rapid solid-phase extraction on a Bond Elut® C8 cartridge and the choice of an appropriate internal standard which is not an antimalarial compound. In conclusion, the reversed-phase ion-pair HPLC method described here for the measurement of CQ, PG and metabolites in biological fluids is suitable for routine analysis in drug monitoring or clinical studies.

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