Journal of Chromatography, 493 (1989) 196–201
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4797

Note

Contribution to the determination of chloroquine in biological fluids

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(First received November 11th, 1988; revised manuscript received March 23rd, 1989)

Of all the common protozoal diseases, malaria is the major cause of morbidity and mortality. It is estimated that 20% of the world population contracts this disease. Chloroquine (CQ) has been for many years the drug of choice for the treatment of acute attacks of malaria. It remains a useful drug for therapy and prophylaxis of malaria, except in cases of parasite resistance or in severe and complicated cases [1,2]. Although CQ has been used for more than 40 years, its pharmacokinetic behaviour is still not clear.

The analysis of CQ requires strict standardization of several factors. Good extractive and analytical methods are therefore of the utmost importance. Consequently, for the determination of CQ and its metabolites in biological fluids a number of different extraction and analytical methods have been proposed [3–17].

This paper describes a fast and efficient extraction method with a high CQ recovery from serum, whole blood and urine. Gas chromatographic (GC) analysis was performed using a nitrogen-selective detector [5].

EXPERIMENTAL

Chloroquine diphosphate and papaverine hydrochloride, used as internal standard (I.S.), were supplied by National Industry of Pharmaceutical Products, Cuba. All chemicals were of analytical or equivalent grade and used without further purification; methanol was Aldrich HPLC grade; ethyl acetate and

0378-4347/89/$03.50

trifluoroacetic anhydride (TFA) were from Merck (Darmstadt, F.R.G.); double-distilled chloroform was from Reactivul (Bucarest, Roumania).

Glassware and centrifuge tubes were pre-cleaned with 0.1 *M* hydrochloric acid in an ultrasonic bath, rinsed with double-distilled water and silanized with 2% dimethyldichlorosilane in 1,1,1-trichloroethane (BDH, Poole, U.K.).

The standard solutions of CQ base (0.025–10.0 $\mu g\ ml^{-1}$) and of papaverine base (40 $\mu g\ ml^{-1}$) were prepared in methanol. The CQ diphosphate standard solutions, calculated as CQ base (0.025–10.0 $\mu g\ ml^{-1}$), were prepared in double-distilled water. Sequential dilutions were prepared from the 1 $mg\ ml^{-1}$ and 100 $\mu g\ ml^{-1}$ solutions.

A Pye Unicam Model 204 gas chromatograph equipped with a nitrogen-specific detector and a CDP-1 computing integrator was used. The glass column (1.5 m×2 mm I.D.) was packed with 3% OV-1–OV-17 (1:3) on Chromosorb G (100–120 mesh, AW/DMCS) [5]. The column temperature was 285°C, and the injection port and detector temperatures were 300°C. The carrier gas (argon) flow-rate was 40 $ml\ min^{-1}$.

The serum samples and whole blood, free from CQ, used for spiked extractions were supplied by the Ciudad de la Habana Blood Bank.

Urine specimens were collected from two normal volunteers, who had never taken CQ before. Patients' whole blood samples were taken with potassium oxalate as anti-coagulant and frozen for 24 h at −20°C.

The serum, urine and whole blood blanks were made with 3.0 ml of each and analysed by GC.

For spiked extraction, 2.0 ml of serum, whole blood or urine were combined with 2.0 ml of each CQ diphosphate standard solution, 0.1 ml of I.S. (40 $\mu g\ ml^{-1}$) and 5 ml of 0.1 *M* sulphuric acid in a 20-ml screw-cap centrifuge tube. The mixture was extracted with 6 ml of chloroform for 5 min with gentle mixing on a platform shaker. After centrifugation for 10 min, the aqueous layer was transferred to another centrifuge tube, and 0.5 ml of 60% potassium hydroxide solution and 6 ml of chloroform were added. After shaking for 5 min and centrifugation, the aqueous phase was discarded and the chloroform layer was evaporated to dryness at 45°C under a stream of nitrogen. The residue was dissolved in 200 μl of methanol, then transferred to a micro-tube and evaporated to dryness again. This residue was dissolved in 10 μl of methanol, and 2–3 μl were injected into the gas chromatograph.

Patients' whole blood and urine samples were derivatized with TFA according to Bergqvist and Eckerbom [8].

The statistical and regression studies were employed to fit plots of peak-height ratio (CQ/I.S.) versus CQ concentration. A Microstat program for the NEC PC-9801-F computer was used.

RESULTS AND DISCUSSION

Papaverine was chosen as the I.S. because this quinoline alkaloid, in addi-

acteristics [5]. Moreover, papaverine is not often associated with chloroquine in therapeutic applications. Evaluation of the assay was carried out using nine standard calibration points in the concentration range 0.025–10.0 $\mu g\ ml^{-1}$ in three biological media.

Standard curves for the drug in each spiked biological fluid exhibited excellent linearity over the concentration range 0.025–10.0 $\mu g\ ml^{-1}$, with correlation coefficients (r^2) that were consistently 0.997 at least, and the standard error was 0.01–0.03. The intercepts did not differ significantly from zero.

The limits of determination from spiked serum and whole blood samples are presented in Table I, together with the coefficient of variation (C.V.). These limits of determination in both media using 2.0 ml of the sample were found to be ca. 25 $ng\ ml^{-1}$ with a C.V. of less than 14%, and are below the expected drug concentration in biological specimens from patients [18].

Adding known concentrations (0.1, 0.05 and 0.025 $\mu g\ ml^{-1}$) of CQ to whole blood, serum and urine gave an average recovery (mean of seven determina-

TABLE I

PRECISION OF THE METHOD USING WHOLE BLOOD AND SERUM

Sample	Added ($\mu g\ ml^{-1}$)	Coefficient of variation[a] (%)
Whole blood	0.5	2.1
	0.1	1.5
	0.05	4.3
	0.025	14.0
	0.020	33.0
Serum	0.5	1.8
	0.1	1.3
	0.05	4.8
	0.025	13.0
	0.020	25.0

[a]Within-day precision of spiked samples; $n = 7$.

TABLE II

CQ CONCENTRATIONS IN WHOLE BLOOD EXTRACTS FROM TWO PATIENTS AT VARIOUS TIMES AFTER A DOSE OF CQ

Patient	Dose (as base)		CQ concentration ($nmol\ l^{-1}$)			
	mg	$mg\ kg^{-1}$	3 h	4 h	5 h	6 h
10954	600	11.11	4496.9	5817.6	5062.9	2987.4
10874	600	6.52	2558.5	2673.0	1603.8	1289.3

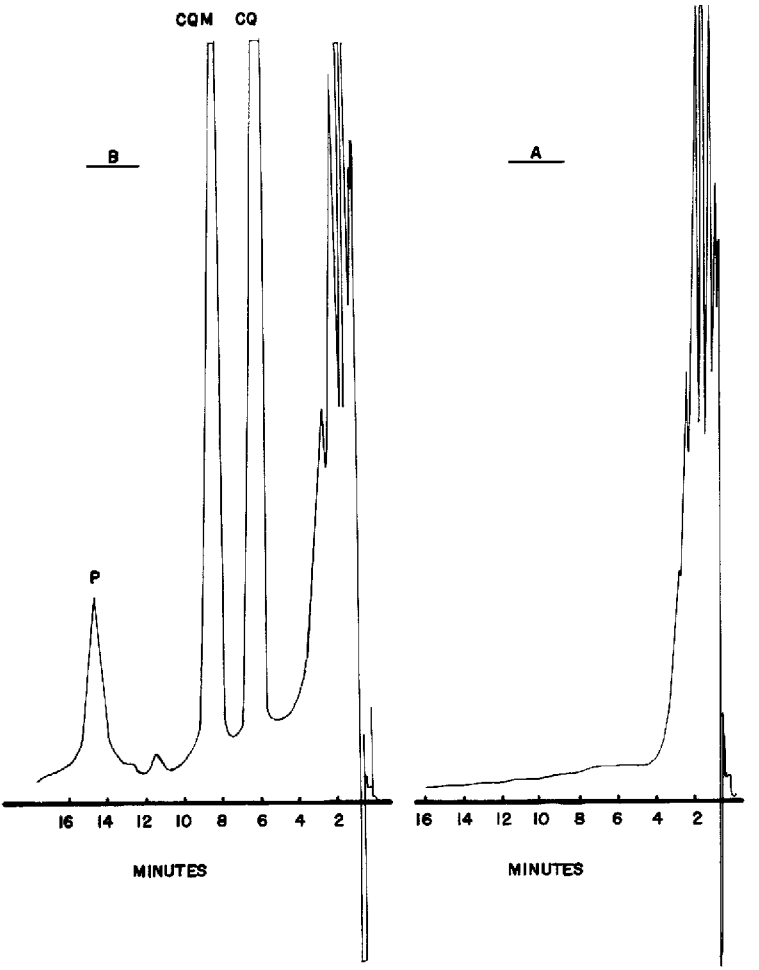

Fig. 1. Gas chromatograms from (A) a urine blank and (B) a urine extract 12 h after a CQ dose, obtained by the proposed extraction method. Peaks: CQ = chloroquine (1.99 ± 0.03 mg); CQM = desethylchloroquine (0.70 ± 0.05 mg); P = papaverine (I.S.; 0.1 ml of 40 $\mu g\ ml^{-1}$ solution).

tions for each biological medium) of 86 ± 7, 89 ± 12 and 83 ± 9%, respectively, by the above extraction procedure.

Intra-assay precision was determined at three concentrations (0.1, 0.05 and 0.025 $\mu g\ ml^{-1}$) for seven analyses. Inter-assay precision was determined at the same three concentrations in four replicates (samples were frozen for four weeks, and the determinations were carried out each week). The within-day C.V.s for CQ in the three media were 6.4% (serum), 6.6% (whole blood) and 7.8% (urine) and the day-to-day C.V.s were 8.45% for whole blood, 8.1% for serum and 9.27% for urine samples.

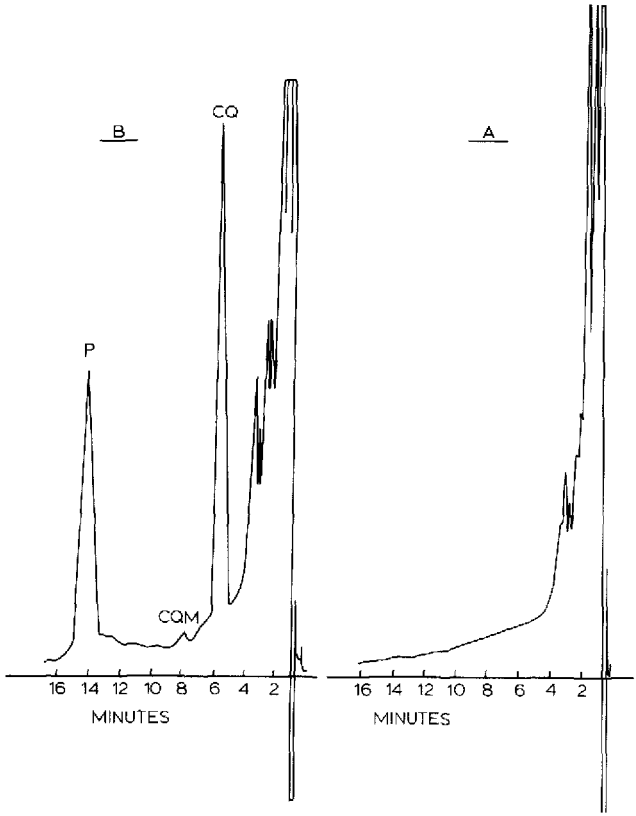

Fig. 2. Gas chromatograms from (A) a whole blood blank and (B) a whole blood extract 3 h after a CQ dose, obtained by the proposed extraction method. Peaks: CQ = chloroquine (2558.5 ± 0.10 nmol l^{-1}); CQM = desethylchloroquine (441.2 ± 0.7 nmol l^{-1}); P = papaverine (I.S.; 0.1 ml of 40 μg ml^{-1} solution).

The method was applied to patients' urine samples and whole blood, which were previously frozen to liberate the CQ from the red blood cells by the rupture of the cell membrane. Two *Plasmodium vivax* patients received 1000 mg of CQ diphosphate as tablets (600 mg of CQ base). The CQ maximum concentration (C_{max}) was reached ca. 4 h later. Table II shows the CQ concentration in whole blood samples 3, 4, 5 and 6 h after the initial dose. The values represent the mean of the sample and its replicate ($n = 2$). These results are in agreement with the Bergqvist values [18].

Early methods [5,8] for extracting CQ and its metabolites involved treatment with sodium or potassium hydroxide to destroy the protein binding of CQ, followed by re-extraction with acid solutions, and finally back-extraction with alkali solution. Bonini et al. [7] used only one extraction step, with a phosphate buffer solution of pH 13.0, then evaporation, followed by injection into the gas chromatograph.

The present study shows an alternative extraction method, which involves the extraction of CQ base from the biological fluids with sulphuric acid solution, and then back-extraction of the uncharged form with potassium hydroxide. In both steps chloroform was used, because in the first step the aqueous phase is then the upper phase and thus transfer to a new test-tube is facilitated. This two-step method gave blood, serum and urine extracts that were free of interfering endogenous substances in a shorter period of time. Figs. 1 and 2 are representative chromatograms of blood and urine samples obtained from patients receiving an oral dose of CQ.

The present extraction method can thus be applied to pharmacokinetic studies and also to therapeutic control of patients receiving the drug.

ACKNOWLEDGEMENTS

We thank Mrs. Lucía Iturralde for typing of the manuscript. The study was supported by grants from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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