Journal of Chromatography, 221 (1980) 119—127
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 660

SENSITIVE METHOD FOR THE DETERMINATION OF CHLOROQUINE AND ITS METABOLITE DESETHYL-CHLOROQUINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

YNGVE BERGQVIST

Clinical Chemistry Department, Falun Central Hospital, S-792 82 Falun (Sweden)

and

MARIANNE FRISK-HOLMBERG*

Section of Clinical Pharmacology, Department of Pharmacology, Box 573, BMC, Uppsala University, S-751 23 Uppsala (Sweden)

(First received March 18th, 1980; revised manuscript received June 20th, 1980)

SUMMARY

A method has been developed for the rapid quantitative analysis of chloroquine and its metabolite desethyl-chloroquine in plasma, blood and urine using high-performance liquid chromatography. An ethylene dichloride extract of the alkalinized biological samples was extracted with dilute acid and chromatographed on a reversed-phase column. Phosphate buffer in acetonitrile was used as the mobile phase with perchlorate as the counter-ion. Ultraviolet absorption at 254 or 340 nm or fluorescence detection was used. The fluorescence spectra and the fluorescence quantum yield of the substances were determined.

Chloroquine and desethyl-chloroquine concentrations in the range of 10 nmol/l (UV-detection) and of 0.5 nmol/l (fluorescence detection) could be accurately measured with a relative standard deviation of 12%. The method should be adequate for therapeutic and pharmacokinetic studies.

INTRODUCTION

Chloroquine (CQ) is a potent antirheumatic drug [1, 2] and is used frequently as an antimalarial drug. Although the drug has been used for more than 40 years the pharmacokinetic behaviour of this drug has not yet been clarified. This is most probably due to the lack of highly sensitive and selective analytical methods allowing measurements of chloroquine in biological samples after administration in therapeutic doses. The methods used so far have been liquid extraction with fluorescence detection [3, 4]. These methods are not sensitive enough and are unselective since the major metabolite desethyl-chloroquine (CQM) is determined simultaneously [4].

The aim of the present work was to develop a selective and sensitive method for the determination of CQ, an amine, in human plasma and urine. The method includes a separation by ion-pair high-performance liquid chromatography (HPLC), of the protonated forms of CQ and CQM using the perchlorate ion as a counter-ion. Ion-pair partition chromatography has been used for the separation of various synthetic amines [5] and biogenic amines [6]. Recently a method for the separation and determination of divalent amines as perchlorate ion-pairs has been described [7]. The choice of the conditions for the extraction and chromatographic procedure is discussed in more detail elsewhere [8]. The method provides complete separation of CQ and the main metabolite CQM. Furthermore, it is sensitive enough to determine the low concentrations anticipated for pharmacokinetic studies and is comparatively rapid, which permits its use in the routine monitoring of therapeutic plasma concentrations [9]. These have been reported in the range of 0.60—2.5 μmol/l serum during chronic treatment of rheumatoid patients (0.25 g/day) using liquid extraction with fluorescence detection [9].

EXPERIMENTAL

Instruments

A Laboratory Data Control pump, Model 712-74, and a Rheodyne Model 7120 high-pressure injection valve provided with a 150-μl loop were used. The detectors were a Schoeffel Model FS 970 fluorescence detector or a Waters Model 440 UV detector. The excitation wavelength of the fluorescence detector was set at 335 nm with a 370 nm emission cut-off filter. The UV detector had a measuring wavelength of 254 or 340 nm. The detector output was connected to a Vitatron 2001 recorder. The pH was measured with a Radiometer PHM 64.

Chemicals and reagents

The molecular structures of CQ, CQM and the internal standard 6,8-dichloro-4-(1-methyl-4-diethylamino-butylamino)-quinoline (D) are presented in Fig. 1. They were kindly supplied by Sterling-Winthrop (Skärholmen, Sweden). Quinine bisulphate, pharmacopoeial grade (Apoteksbolaget, Solna, Sweden) was recrystallized from ehtanol—water mixtures. Acetonitrile (chromatographic purity, Merck, Darmstadt, G.F.R.) and ethylene dichloride (Merck) were used. All aqueous solutions were prepared using high-purity water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical quality. Glassware and centrifuge tubes were cleaned by standing overnight in 5 *M* nitric acid, and were then ultrasonically cleaned and rinsed with Milli-Q water.

Extraction and chromatography conditions

It has been found that divalent 4-amino-quinoline derivatives give quantitative extractions > 99% from pure aqueous solutions at pH 10.5—11.0 in ethylene dichloride, methylene chloride and chloroform using equal phase volumes. When extracting biological samples from the solvents mentioned ethylene dichloride gave the lowest blank disturbance of the chromatogram

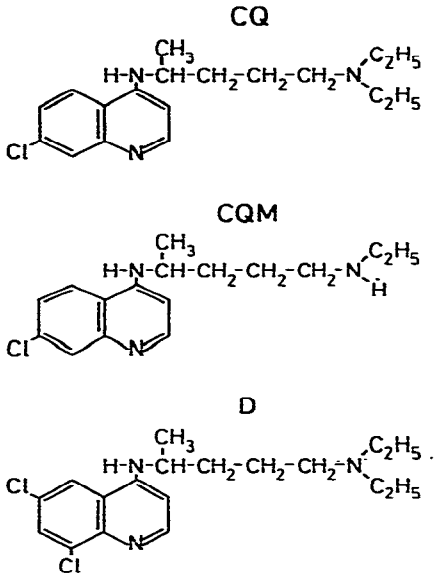


Fig. 1. Molecular structures of chloroquine (CQ) desethyl-chloroquine (CQM) and internal standard (D).

when using fluorescence detection. Acetonitrile (40%, v/v) has been found a suitable organic modifier for the separation of the 4-amino quinolines when perchlorate (75 mmol/l) is used as a counter-ion [8]. A mobile phase without perchlorate gave no separation of CQ and CQM [8]. An optimal chromatographic efficiency was obtained at a low pH (pH < 4.5) of the mobile phase. Above this pH (> 4.5) a decrease in chromatographic efficiency was seen.

In the present study the analytical column (200 × 4.0 mm I.D.) was packed by the upward-slurry packing method with pure methylene chloride as described by Bristow [10] using modified Nucleosil C_{18} 5 or 10 μm (Macherey, Nagel & Co., Düren, G.F.R.). When method II (direct injection) was used the analytical column was protected by a guard column (30 × 1.4 mm I.D.) packed with LiChrosorb RP-8, 10 μm (Brownlee Labs, Santa Clara, CA, U.S.A.). The flow-rate of the mobile phase [acetonitrile—phosphate buffer (40:60), ionic strength 0.1, pH 3, and perchlorate 75 mmol/l] was 0.8—1.0 ml/min. The mobile phase was degassed ultrasonically immediately before use and the experiments were performed at room temperature.

When using fluorescence detection a T-union was used to combine the column eluate with 0.2 *M* borate buffer, pH 11.5. The fluorescence signal was maximal at a flow-rate of approximately 0.30 ml/min, which gave a pH in the detector of 9.3—9.6. To eliminate noise in the detector a short stainless-steel coil (150 × 0.2 mm I.D.) was inserted after the T-union.

Analytical procedure

Method I

Injection after extraction (plasma, whole blood and urine). A 0.5--2.0-ml sample was made alkaline with 2.0 ml of 1 *M* sodium hydroxide and extracted for 15 min with 7.0 ml of ethylene dichloride containing the internal standard (D) at 100—200 nmol/l. The aqueous phase was discarded after centri-

fugation (step 1). Then 4.0 ml 0.05 *M* borate buffer, pH 11.0, was added. After shaking for 10 min and centrifuging the aqueous phase was removed by aspiration (Step 2). Next, 300 μl 0.1 *M* hydrochloric acid was added. After shaking for 10 min and centrifuging, 150 μl of the aqueous phase were injected. Whole blood was hemolyzed by diluting with distilled water and freezing at −70° C. Step 2 in the extraction was repeated.

Method II

Injection without extraction (plasma, urine). If high concentrations were anticipated:

Plasma. A 500-μl sample of plasma was deproteinized by adding it dropwise to a solution (2.0 ml) of trichloroacetic acid 5% (w/v) in methanol containing the internal standard (D) in concentrations of 100—200 nmol/l, during vigorous vortex mixing for 30 sec. The sample was left standing at room temperature for 10 min and after centrifugating for 20 min, 150 μl of the supernatant were used for chromatographic determination. Microprecipitates were easily removed by a 0.45-μm filter inserted before the injection loop.

Urine. A 200-μl sample of urine was diluted to 5.0 ml with the mobile phase containing the internal standard (D) at 1 μmol/l. The mixture was vortex mixed for 30 sec, then 150 μl were injected.

Calibration curves based on peak ratios (CQ/D, CQM/D) were prepared daily by means of spiked drug-free plasma and urine samples which were carried through the whole analytical procedure. The choice of the internal standard D was justified by the distribution, extraction and fluorescence properties or UV characteristics similar to those of CQ and CQM [8]. The CQ and CQM peaks were identified on the basis of their retention times in relation to the internal standard.

D was eluted after CQ and CQM in the chromatography system and its peak was well separated from the other peaks. By selecting an internal standard which is eluted after CQ, interference with other polar metabolites can be avoided.

Fluorescence quantum yield measurements. These were performed using an Aminco-Bowman spectrofluorimeter equipped with Hanovia 150 W xenon lamp and IP 21 photomultiplier tube using a bandwidth of 12 nm. They were determined for CQ, CQM and D by the comparative method of Chen [11] using quinine bisulphate as a reference standard [12]. Spectra were corrected in quantum yields measurements.

RESULTS AND DISCUSSION

The fluorescence detection of CQ and CQM was found to be 20 times more sensitive than UV detection at 254 or 340 nm. The fluorescence of CQ and CQM was also found to be pH dependent with a maximum at pH 9.5. Since the chromatographic column is unstable at pH > 8, a post-column addition of borate buffer (pH 11.5) was made in order to obtain the maximal fluorescence in the detector. In Table I, the quantum yields of CQ and CQM are given and found to be 0.13 and 0.14, respectively, when the excitation wavelength was 330 nm. The internal standard (D) had a lower quantum yield.

Fig. 2 demonstrates calibration curves in plasma determined by method I and Fig. 3 shows a calibration curve in urine obtained with method II. Using method I it was possible to determine 0.5 nmol/l of CQ and CQM with a relative standard deviation of 12%. Method II had a considerably lower sensitivity, > 200 nmol/l, and was therefore used only when concentrations > 200 nmol/l were anticipated. The results from a comparative study of methods I and II when applied to plasma and urine samples are given in Table II. Method II is thus suitable for the rapid quantitation of CQ and CQM.

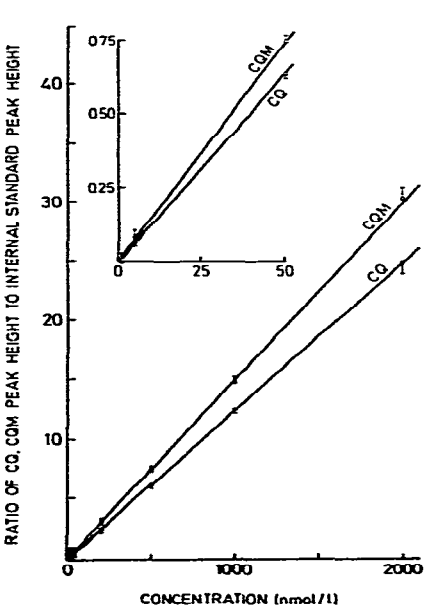
TABLE I

FLUORESCENCE QUANTUM YIELDS AND SPECTRAL PROPERTIES

Chloroquine, desethyl-chloroquine and the internal standard in borate buffer pH 9.5; quinine bisulphate 0.05 mmol/l in 0.1 mol/l H_2SO_4.

Substance	Excitation maximum (nm)	Emission maximum (nm)	Quantum yield = ϕ
Chloroquine	334	392	0.13
Desethyl-chloroquine	334	392	0.14
D (internal standard)	343	400	0.08
Quinine bisulphate	345	450	0.55*

*Ref. 12.



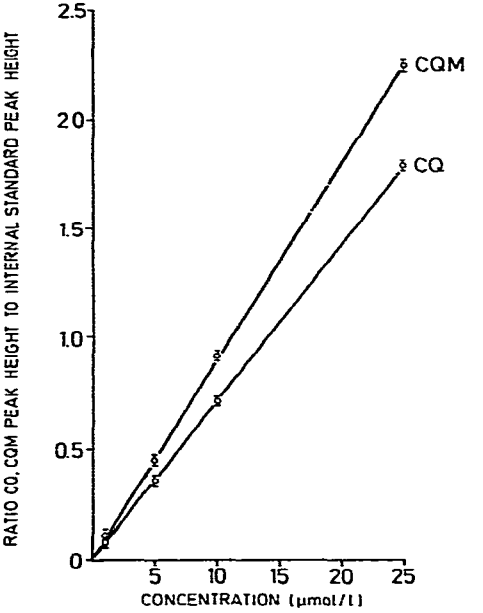


Fig. 2. Calibration standard curves of CQ and CQM determined by method I in plasma with fluorescence detection. The shaded area in the large figure is magnified in the upper part of the figure. Each point represents the mean value and S.D. of four determinations carried out on different days. The length of the bar corresponds to the estimated standard deviation.

Fig. 3. Calibration standard curves of CQ and CQM determined by method II in urine with UV detection. Each point represents the mean value and S.D. of four determinations carried out on different days. The length of the bar corresponds to the estimated standard deviation.

TABLE II

COMPARISON OF METHODS I AND II

Method I (x)	Method II (y)	Number of samples	Correlation coefficient	Regression equation
Plasma				
CQ	CQ	9	0.974	$y = 0.913x + 0.012$
CQM	CQM	9	0.994	$y = 0.987x - 0.014$
Urine				
CQ	CQ	15	0.996	$y = 0.964x - 1.29$
CQM	CQM	15	0.998	$y = 0.965x + 0.42$

TABLE III

PRECISION OF THE ANALYTICAL METHODS

R.S.D. = relative standard deviation.

	Concn. (nmol/l)	S.D.	R.S.D. (%)	n
Method I with fluorescence detection				
Within-day: plasma				
CQ	0.6	0.07	11.7	9
	4.9	0.15	3.1	9
	51.4	2.0	3.9	5
	821	32	3.9	5
CQM	0.5	0.05	10.0	9
	5.1	0.27	5.3	9
	49.7	2.1	4.2	5
	782	27	3.5	5
Day-to-day: plasma				
CQ	50.2	6.7	13.4	5
	775	40	5.2	5
CQM	51.0	4.6	9.1	5
	790	35	4.4	5
Method II with fluorescence detection				
Within-day: plasma				
CQ	213	15	7.0	7
	725	38	5.2	7
CQM	264	20	7.5	7
	770	27	3.5	7
Within-day: urine*	(μmol/l)			
CQ	5.0	0.19	3.8	5
	25.5	0.40	1.6	5
	49.4	0.36	0.7	5
CQM	5.0	0.20	4.0	5
	25.3	0.66	2.6	5
	49.7	0.32	0.6	5

*UV-detection.

Table III gives some results of tests of the within-day and the day-to-day reproducibilities for both methods.

The absolute recoveries of CQ and CQM from drug-free plasma, whole blood and urine, were also determined; the samples were analyzed by methods I and II. The absolute recovery was obtained by comparison of the peak heights from these samples with a calibration curve obtained by directly injecting known amounts of CQ and CQM into the chromatograph. The results are presented in Table IV.

None of the following drugs interfered with the determination of CQ and CQM in plasma: ibuprofen; phenylbutazon; naproxen; prednisolon; indometacin; salicylic acid; salicylazosulphapyridine. The drugs are commonly used in combination therapy for rheumatoid diseases.

Fig. 4 shows the plasma concentration—time data of CQ and CQM ob-

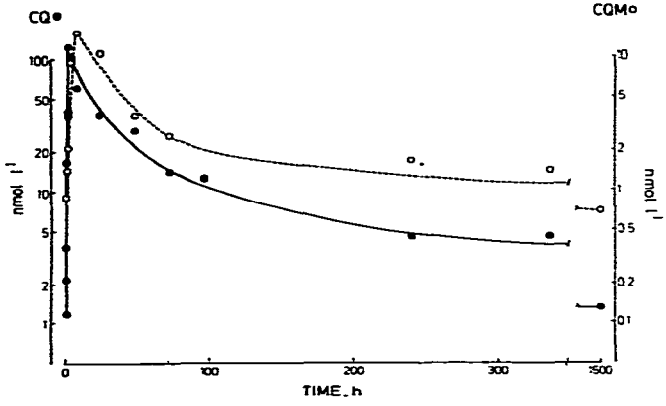


Fig. 4. Plasma concentration—time data of CQ and CQM obtained from a patient after a single oral dose of 0.25 g of chloroquine phosphate. Method I with fluorescence detection.

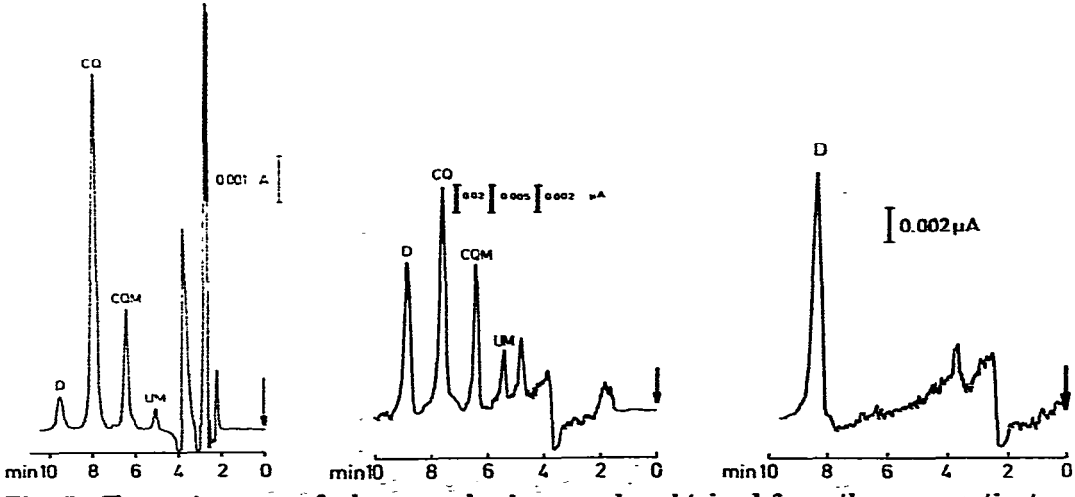


Fig. 5. Chromatograms of plasma and urine samples obtained from the same patient as in Fig. 4. UM is an unidentified metabolite. 1, Plasma, CQ, 82 nmol/l; CQM, 16 nmol/l; D, internal standard; sample was processed by method I with fluorescence detection. 2, Urine, CQ, 8.5 µmol/l; CQM, 3.1 µmol/l; D, internal standard; sample was processed by method II with UV detection at 340 nm. 3, Drug-free plasma with internal standard D was processed by method I with fluorescence detection.

TABLE IV

RECOVERY OF THE ANALYTICAL METHODS

$n = 4$.

	Recovery (% ± S.D.)									
	Concentration (nmol/l)									
	0.5	5	10	25	50	200	500	800	1000	1500
Method I: plasma										
CQ	100 ± 11.4	97 ± 5.3			97 ± 3.2	86 ± 2.0	87 ± 1.5		88 ± 2.0	
CQM	104 ± 9.4	92 ± 6.9			94 ± 3.0	90 ± 4.0	85 ± 1.4		90 ± 1.2	
Method I: whole blood										
CQ			87 ± 2.5			72 ± 3.4		75 ± 3.5		
CQM			89 ± 1.1			75 ± 5.0		70 ± 2.1		
Method II: plasma										
CQ						102 ± 5.4		98 ± 6.1		103 ± 3.4
CQM						94 ± 3.0		100 ± 2.8		100 ± 3.7
Method II: urine	Concentration (μmol/l)									
CQ	100 ± 1.5		99 ± 0.7	98 ± 1.7	100 ± 1.0					
CQM	97 ± 1.4		97 ± 1.1	97 ± 3.2	99 ± 1.3					

tained after the first single dose of 0.25 g chloroquine phosphate to a rheumatoid patient. Venous samples were taken and analyzed by method I (fluorescence detection) at regular intervals. The plasma concentration of CQ is approximately 10 times higher than that of CQM during a dosage interval. It is obvious that the disposition patterns of CQ and CQM are similar and seem to follow a multi-exponential curve. The chromatogram of this patient's plasma and urine (see Fig. 5) indicates that another yet unidentified metabolite is present.

CONCLUSIONS

The analytical method described exhibits good reproducibility and permits the simultaneous determination of CQ and its metabolite CQM at concentrations of 0.5 nmol/l in plasma and urine. If higher concentrations (> 200 nmol/l) are anticipated, the samples (plasma, urine) can be injected directly into the liquid chromatograph after dilution. The chromatographic system has a stable column life for up to 10 months and allows analysis of 20—30 samples daily. The method could be a valuable tool for the further elucidation of CQ disposition and pharmacokinetics in man and an aid in the monitoring of chronic CQ therapy which has to be followed closely to avoid the undesirable side effects [9].

ACKNOWLEDGEMENTS

Christer Hed is thanked for excellent technical assistance. Åke Olin, Ph.D, Institute of Chemistry, Uppsala University and Karl Gustav Wahlund, Ph.D, Department of Analytical Pharmaceutical Chemistry, Biomedical Center, Uppsala are thanked for their constructive criticism of the methods.

This work was kindly supported by the Swedish National Association against Rheumatoid Diseases.

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