Journal of Chromatography, 306 (1984) 147—153
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1958

AN IMPROVED GAS CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CHLOROQUINE AND TWO METABOLITES USING CAPILLARY COLUMNS

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(First received June 30th, 1983; revised manuscript received October 11th, 1983)

SUMMARY

A gas chromatographic method for the simultaneous determination of therapeutic levels of chloroquine, and the metabolites deethyl chloroquine and bideethyl chloroquine in human whole blood, plasma and urine has been developed by use of the capillary column technique. The analytes were extracted as bases with *n*-hexane—1-pentanol (90:10) and re-extracted into an acidic aqueous phase. After a further extraction to a small volume of chloroform, the substances were injected by the falling needle technique onto a fused-silica capillary column followed by nitrogen-selective detection. Limits of determination using 2 ml of the sample were found to be 10 nmol/l (3 ng/ml) for chloroquine and deethyl chloroquine and 30—40 nmol/l (10 ng/ml) for bideethyl chloroquine with a coefficient of variation of < 15%. The precision of the method at the 100 nmol/l level was about 4% (n = 8) for chloroquine.

INTRODUCTION

Chloroquine (CQ) is an antimalarial drug which is also used in the treatment of rheumatoid arthritis. Methods based on liquid chromatography (LC) for determination of CQ and its main metabolite deethyl chloroquine (CQM) in human plasma, whole blood and urine were recently reported [1, 2]. An alternative method with gas chromatography (GC) for the simultaneous determination of CQ and CQM, with acylation of CQM to obtain a separation between CQ and CQM on a packed column with OV-17 has been developed in our laboratories [3]. With this method CQ and CQM could be determined down to 100—200 nmol/l.

In this paper we describe a modification of the GC method which uses the capillary column technique. CQ and CQM as well as the bideethyl metabolite (CQMM) could be separated without derivatization. With split or splitless

0378-4347/84/$03.00

injection techniques problems such as "ghosting peak" and adsorption of the substances occur. The falling needle technique [4] seems to be the superior injection technique for CQ and its main metabolites. The evaporation step in the extraction procedure could be omitted, and the injection technique is simple and easy to handle. By isothermal chromatography 4—5 min give a sufficient separation of CQ, the two metabolites and the internal standard.

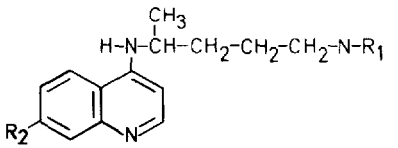
EXPERIMENTAL

Gas chromatography

A Varian VISTA 6000 gas chromatograph with a standard injector for split or splitless injection, equipped with a nitrogen-selective thermoionic detector was used. Fused-silica capillary columns, type J & W DB-5 (12 m × 0.32 μm, 0.25 μm film) and SGE BP-5 (7 m × 0.32—0.50 μm, 0.50 μm film) were used. The sample was injected by an all-glass solid injector with a falling needle from Chrompack and chromatographed isothermally at 255° C. The carrier gas (nitrogen) flow-rate was approximately 1.0 ml/min. The hydrogen and air flow-rates were 4.5 ml/min and 175 ml/min, respectively. In order to obtain optimized detector performance, auxiliary carrier gas was added at the end of the column at 30 ml/min. The nitrogen was purified from oxygen by OXY-TRAP (Alltech, U.S.A.).

Chemicals and reagents

Chloroquine, deethyl chloroquine and 7-bromo-4-(1-methyl-1-diethylamino-butylamino)quinoline, used as an internal standard, were kindly supplied by Sterling-Winthrop (Skärholmen, Sweden). Bideethyl chloroquine (CQMM) was synthesized at the Department of Organic Chemistry, Uppsala University, Uppsala, Sweden. The molecular structures are shown in Fig. 1. All the reagents were of analytical quality from Merck (Darmstadt, F.R.G.). Polypropylene



Compound	R_1	R_2
Chloroquine, CQ	$-N(C_2H_5)_2$	-Cl
Deethyl chloroquine, CQM	$-NH(C_2H_5)$	-Cl
Bideethyl chloroquine, CQMM	$-NH_2$	-Cl
Internal standard, IS	$-N(C_2H_5)_2$	-Br

Fig. 1. Molecular structures of the compounds of interest.

tubes from Sarstedt (Malmö, Sweden) and glass tubes used in the extraction procedure were cleaned by washing in a non-phosphate detergent Decon-90R (Decon Laboratories, Hove, U.K.), followed by cleaning in nitric acid (5 mol/l) in an ultrasonic bath, and by a rinse with deionized Milli Q filtered water (Millipore, Bedford, MA, U.S.A.).

Preparation of standards

Equal amounts of CQ, CQM and CQMM were dissolved in hydrochloric acid (0.01 mol/l) to give a concentration of 100 μmol/l. Aliquots of this solution were diluted with drug-free plasma. The standards were handled and analysed simultaneously with the samples. An example of a calibration curve in the concentration range 100—1000 nmol/l, passing through the origin, is shown in Fig. 2.

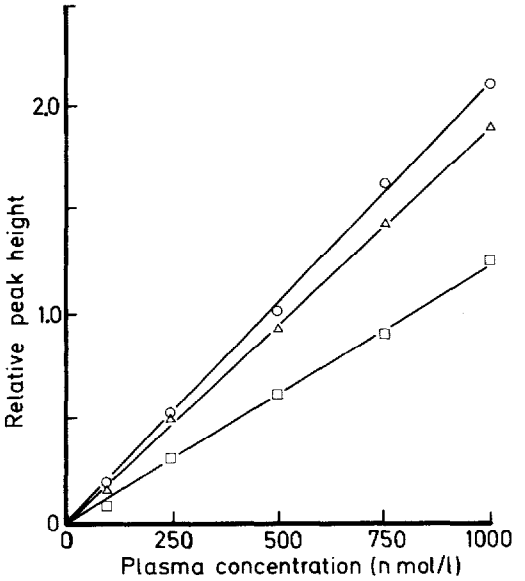


Fig. 2. Calibration curves for chloroquine (○), deethyl chloroquine (△) and bideethyl chloroquine (□).

Injection techniques

Different injection techniques were tested by means of repeated injections of the same extract of CQ, CQM, CQMM and internal standard at a concentration of 10 μmol/l with the same syringe (SGE SK-101) and on the same column. The tested injection techniques were as follows: split injection: split ratio 1:10, isothermal 255°C. Splitless injection: 170°C for 2 min, temperature-programmed 20°C/min up to 260°C. Falling needle injection: isothermal 255°C.

Injector and detector temperatures were 280°C. For split and splitless injection the solvent was ethyl acetate with and without *n*-decylamine (1000 μmol/l). For falling needle injection the solvent was chloroform with and without *n*-decylamine.

Evaporation experiment

One millilitre of an alkalinized aqueous solution of CQ, CQM and CQMM at a concentration of 500 nmol/l in polypropylene tubes was extracted with 3 ml of chloroform. For comparison of recovery, 2.5 ml of the chloroform extract were either re-extracted with 500 μl of 0.1 mol/l hydrochloric acid or evaporated to dryness at 50°C in a glass tube (evaporation interrupted just when completed) and the residue reconstituted in 500 μl of 0.1 mol/l hydrochloric acid. A 100 μl aliquot of each hydrochloric acid phase was injected into the LC system described in ref. 1 and peak heights for CQ, CQM and CQMM were compared.

Extraction procedure

A volume of 0.5—2.0 ml of sample and 100 μl of internal standard solution were made alkaline (pH > 11) with 1.0 ml of sodium hydroxide (1 mol/l) and extracted for 15 min with 4.0 ml of *n*-hexane—1-pentanol (90:10) in polypropylene tubes. Whole blood was hemolysed by diluting (1:2, v/v) with water before extraction. After centrifugation, 3.0 ml of the organic upper phase were transferred to a new polypropylene tube containing 3.0 ml of hydrochloric acid (0.2 mol/l). After extraction for 15 min and centrifugation, the aqueous phase was transferred to a conical glass test tube containing 200 μl of sodium hydroxide (5 mol/l), and 200 μl of chloroform were added. After mixing for 1 min on a Vortex mixer and centrifugation, 2 μl of the chloroform phase were injected into the gas chromatograph using the falling needle technique.

RESULTS AND DISCUSSION

Evaluation of injection techniques

Initial experiments testing the different injection techniques — split, splitless and falling needle — by repeated injection of the same extract of CQ, CQM, CQMM and internal standard, showed that the highest sensitivity and best precision were achieved with falling needle injection. An example of these experiments is shown in Table I. Addition of *n*-decylamine (1000 μmol/l)

TABLE I

REPRODUCIBILITY OF DIFFERENT INJECTION TECHNIQUES

Injection of 2 μl of the same extract (n = 10) of CQ, CQM and CQMM at a concentration of 10 μmol/l.

Injection technique	Solvent	Mean peak height (mm) (attenuation 128 · 10^{-12})			Peak height relative to internal standard (R.S.D.*, %)		
		CQ	CQM	CQMM	CQ	CQM	CQMM
Split	Ethyl acetate	13	11	—	1.35(9.5)	1.35(7.1)	—
	Ethyl acetate + DA**	14	13	5	1.37(5.4)	1.33(7.6)	0.46(8.6)
Splitless	Ethyl acetate	123	98	10	1.29(15.2)	1.04(15.3)	0.09(11.0)
	Ethyl acetate + DA	178	145	52	1.33(9.5)	1.07(7.9)	0.38(10.6)
Falling needle	Chloroform	402	385	228	1.39(3.5)	1.35(3.8)	0.79(8.9)
	Chloroform + DA	430	410	232	1.36(3.5)	1.35(2.4)	0.81(5.8)

*R.S.D. = relative standard deviation.
**DA = *n*-decylamine, 1000 μmol/l.

to the solvent as an adsorption suppressor improved the sensitivity, especially for the primary amine CQMM with split and splitless injection.

With falling needle injection no significant improvement of sensitivity by addition of *n*-decylamine could be observed. However, when column performance had been degraded due to injection of a large number of samples, non-linear standard curves were obtained for CQMM in the concentration range 25—1000 nmol/l unless *n*-decylamine was added to the solvent before injection.

With falling needle injection no "ghosting peak" (< 5%) was observed, which was a problem reported by Churchill et al. [5] in their method using on-column injection and which we also observed with split and splitless injection.

Evaporation

Adsorptive losses of CQ and metabolites must be considered whenever the drug is in contact with glass surfaces [6], especially in the low (ng/ml) concentration range. The results in Fig. 3 indicate that 18% of CQ, 42% of CQM and 43% of CQMM were lost during the chloroform evaporation in comparison with re-extraction to an aqueous phase. The precision was also decreased with evaporation.

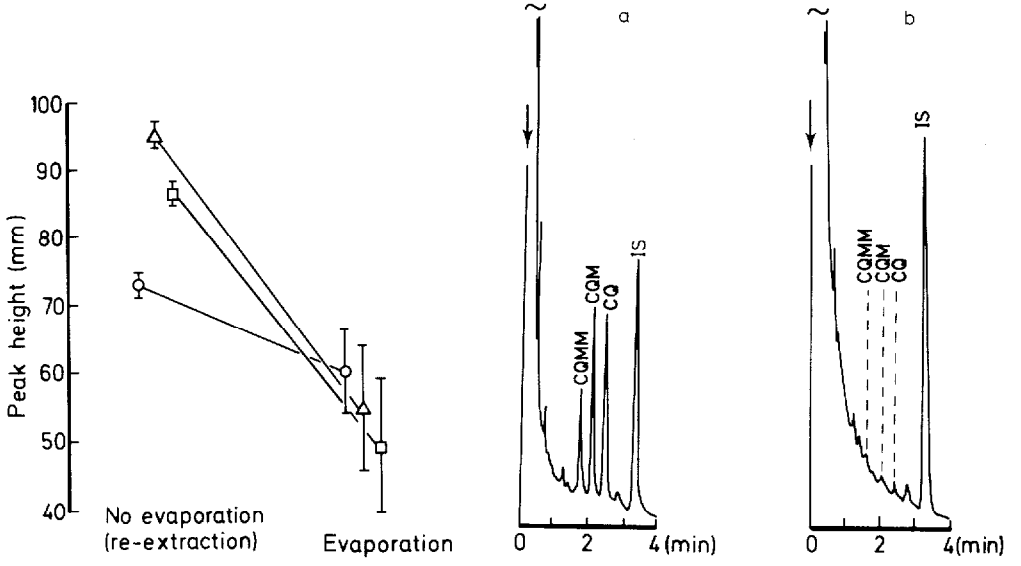


Fig. 3. Comparison of re-extraction and evaporation as a concentration step of 500 nmol/l chloroquine (○), deethyl chloroquine (△) and bideethyl chloroquine (□). The length of the bar corresponds to the standard deviation (n = 5).

Fig. 4. Capillary gas chromatograms from the analysis of (a) chloroquine in plasma with "spiked plasma sample" at a concentration of 100 nmol/l of each substance, and (b) drug-free plasma sample, with internal standard (IS). Column: 7 m × 0.32 μm I.D., BP-5. Injection: 2 μl by falling needle. Column temperature: 255°C. Detector temperature: 280°C.

Extraction conditions

The earlier work [7] had shown that hexane—1-pentanol (90:10) and chloroform could quantitatively extract CQ and CQM from an alkalinized sample. When this extraction was used with biological samples, there were no interfering endogenous compounds with the same retention time as CQ, CQM and CQMM left after the double-extraction procedure. Fig. 4 shows a chromatogram from a plasma spiked with 100 nmol/l of each substance. This figure also shows a chromatogram from a drug-free plasma sample. It can be seen that the plasma does not contain endogenous substances that interfere with CQ and its metabolites. The substances were adequately separated within 4 min.

Recovery

Adding known quantities (100, 500 and 1000 nmol/l) of CQ, CQM and CQMM to plasma gave 100 ± 5% recovery for CQ, 97 ± 8% for CQM and 85 ± 7% for CQMM, by the above extraction procedure.

The limit of determination

The limits of determination from spiked plasma samples are presented in Table II together with the relative standard deviation. These limits of determination are for CQ and CQM about 10 nmol/l and for CQMM 30—40 nmol/l, and are well below the expected drug concentration in biological specimens from patients given therapeutic doses of CQ both in treatment against rheumatoid arthritis as well as for prophylaxis against malaria [8].

TABLE II

PRECISION OF THE DETERMINATION OF CHLOROQUINE AND ITS METABOLITES BY CAPILLARY GAS CHROMATOGRAPHY

Within-day precision of spiked samples; $n = 8$.

	CQ	CQM	CQMM
Mean (nmol/l)	478	460	500
C.V. (%)	5.1	4.8	12
Mean (nmol/l)	113	107	75
C.V. (%)	3.7	1.8	11
Mean (nmol/l)	65	59	34
C.V. (%)	7.8	7.7	14
Mean (nmol/l)	20	17	16
C.V. (%)	4.3	8.7	24
Mean (nmol/l)	14	10	—*
C.V. (%)	10	11	

*Relative standard deviation > 30%.

Selectivity of the present method

A comparison of the present method and the LC method [1] for the assay of CQ, CQM and CQMM is shown in Fig. 5. Samples were taken from patients undergoing chloroquine dihydrogen phosphate treatment (250 mg per day). The results indicate that the two methods are equivalent.

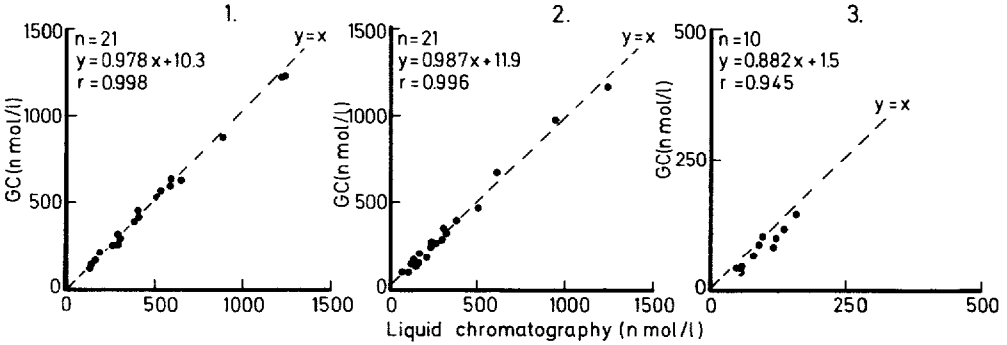


Fig. 5. Comparison of results for chloroquine (1), deethyl chloroquine (2) and bideethyl chloroquine (3) in plasma obtained by liquid chromatography (X) and gas chromatography (GC) with capillary column (Y).

With the present extraction procedure and the chromatographic system no interference with other commonly used drugs in combination therapy for rheumatoid diseases and malaria prophylaxis with CQ was seen. The tested drugs were primaquine, pyrimetamine, quinine, chlorproguanil, and phenylbutazone.

CONCLUSION

In the present work we have shown that sample preparation and injection technique are critical steps for analysis of amines like CQ and metabolites in the ng/ml range. By using the falling needle injection technique the method would be useful for simultaneous determination of CQ, CQM and CQMM down to 10 nmol/l (3 ng/ml) for both CQ, and CQM and for CQMM down to 30—40 nmol/l (10 ng/ml) with a precision of $< 15\%$. Recently other methods have been published usign GC [5, 9, 10] for the determination of CQ in biological specimens, but none of them dealt with the separation of the bideethyl metabolite (CQMM) of CQ.

ACKNOWLEDGEMENT

A grant from the I.F. Foundation for Pharmaceutical Research is gratefully acknowledged.

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