Journal of Chromatography, 344 (1985) 241—248
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2722

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR HYDROXYCHLOROQUINE AND METABOLITES IN BLOOD AND PLASMA, USING A STATIONARY PHASE OF POLY(STYRENE DIVINYLBENZENE) AND A MOBILE PHASE AT pH 11, WITH FLUORIMETRIC DETECTION

S.E. TETT, D.J. CUTLER* and K.F. BROWN

Department of Pharmacy, University of Sydney, Sydney 2006 (Australia)

(First received March 26th, 1985; revised manuscript received May 17th, 1985)

SUMMARY

A sensitive rapid high-performance liquid chromatographic assay for hydroxychloroquine and three major metabolites, desethylhydroxychloroquine, desethylchloroquine and bisdesethylchloroquine, has been developed. An ion-suppression separation technique is used with a macroporous polymer, poly(styrene divinylbenzene), stationary phase and a mobile phase of methanol—water with triethylamine, pH 11. A single ether extraction from alkalinized plasma or whole blood, with chloroquine added as an internal standard, is used. Using fluorescence detection, with excitation at 337 nm, the limit of sensitivity is 1 ng/ml. Some anti-inflammatory drugs which may be used concurrently for rheumatoid disorders are shown not to interfere with the assay. Samples of whole blood and plasma obtained from rheumatoid patients contained hydroxychloroquine and all three metabolites.

INTRODUCTION

Hydroxychloroquine (HCQ) (Fig. 1), a structural analogue of chloroquine (CQ), was initially synthesized for use as an antimalarial drug [1]. Clinical trials have since shown that both CQ and HCQ are effective in the treatment of rheumatoid diseases [2, 3]. HCQ has been reported to exhibit lower toxicity [4].

In early research with HCQ a fluorescence assay, with solvent extraction, was used [5]. More recently a high-performance liquid chromatographic (HPLC) assay has been described [6]. Both of these assays are non-selective, neither separating parent drug and metabolites nor quantifying metabolites.

0378-4347/85/$03.30

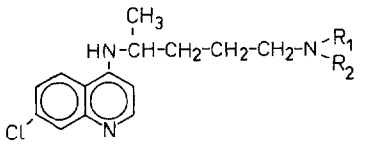

Fig. 1. Structures of chloroquine, hydroxychloroquine and metabolites.

	$-R_1$	$-R_2$
Hydroxychloroquine (HCQ)	$-CH_2CH_3$	$-CH_2CH_2OH$
Chloroquine (CQ)	$-CH_2CH_3$	$-CH_2CH_3$
Desethylchloroquine (CQM)	$-CH_2CH_3$	$-H$
Desethylhydroxychloroquine (HCQM)	$-H$	$-CH_2CH_2OH$
Bisdesethylchloroquine (CQMM)	$-H$	$-H$

Three assays developed for the determination of CQ reported separation of HCQ from CQ and desethylchloroquine [7–9]. None of these reported resolution between HCQ and all three of its major metabolites, desethylhydroxychloroquine (HCQM), desethylchloroquine (CQM) and bisdesethylchloroquine (CQMM) (Fig. 1). Using the reversed-phase HPLC assay developed by Bergqvist and Frisk-Holmberg [9], resolution between HCQM and CQMM was not achieved and in our experience resolution between HCQ and CQM was lost within one month.

HCQ and its metabolites are basic compounds. The assay described here uses a reversed-phase ion-suppression HPLC separation technique, with a mobile phase of pH 11. CQ, HCQ and its metabolites all fluoresce at this high pH, allowing sensitive and selective detection. A porous polymer packing material, poly(styrene divinylbenzene), is used in the analytical column. This material is reported to be stable when used with mobile phases of pH range 1–13 [10]. The more commonly used reversed-phase packing material of silica particles with chemically bonded alkyl chains are unstable at extremes of pH.

EXPERIMENTAL

Instruments

A Gilson Model 302 HPLC pump with a Gilson Model 802 manometric module and a Rheodyne injection port Model 7125, fitted with a 20-μl sample loop, were used. A Rheodyne Model 7302 column inlet filter (2-μm filter) was fitted pre-column. The analytical column (150 × 4.1 mm) was obtained pre-packed with poly(styrene divinylbenzene) (PRP-1, Hamilton, Reno, NV, U.S.A.); 95% of the particles lies within the size range 3–6 μm.

A Perkin-Elmer fluorescence spectrophotometer Model 204 fitted with a continuous flow cell was used as the detector. The excitation wavelength was set at 337 nm, and an emission filter with cut off at 370 nm was used. Chromatographic tracings were recorded on a Sekonic SS250F chart recorder. The pH was measured using a Townson expansion pH meter.

Chemicals and reagents

Hydroxychloroquine and two of its metabolites, desethylhydroxychloroquine and desethylchloroquine, were donated by Sterling Pharmaceuticals

(Ermington, Australia). Another metabolite, bisdesethylchloroquine, was kindly supplied by the Army Malaria Research Unit (Ingleburn, Australia). Chloroquine, the internal standard, was donated by May and Baker Australia (West Footscray, Australia).

Water was freshly distilled. Methanol was purified by first refluxing with sodium and potassium hydroxide, followed by redistillation. Triethylamine (laboratory grade) and ammonia solution 30% (analytical grade) were obtained from Ajax Chemicals (Sydney, Australia). Diethyl ether was freshly distilled before use. Glassware coming into contact with the compounds was silanized prior to each use with Aquasil silanizing liquid (Pierce, Rockford, IL, U.S.A.).

Mobile phase selection

Mobile phases of varying methanol—water composition, adjusted to pH 11 with 1 *M* sodium hydroxide solution, were investigated initially to find a binary mixture which gave desirable retention times when an aqueous solution of HCQ and metabolites was injected. Methanol—water (80:20) was the most suitable, but the peaks were broad and tailing. Triethylamine was added to improve peak shape.

Ionic strength was varied from 0.04 *M* to 0.5 *M* by adding sodium chloride to a mobile phase of methanol—water (80:20) with 100 m*M* triethylamine, pH 11.

Mobile phase pH was varied between 8.5 and 12 using borate buffer—methanol (20:80) with 100 m*M* triethylamine. Ionic strength was 0.2 *M*.

Triethylamine concentrations were varied between 0 and 300 m*M*, with mobile phase pH held constant at 10.9 using a glycine buffer (ionic strength 0.1 *M*)—methanol (20:80).

The mobile phase finally chosen was methanol—water (80:20), with 100 m*M* triethylamine, adjusted to pH 11 with 1 *M* sodium hydroxide. The mobile phase was filtered through a 10-μm filter system (Millipore) and degassed prior to use. Flow-rate was 1 ml/min.

Analytical procedure

Hydroxychloroquine and metabolites were extracted from 1 ml of diluted blood (1:1 with distilled water) or plasma, to which 50 μl of an aqueous CQ solution (0.6, 12 or 120 μg/ml) had been added as internal standard. A 300-μl volume of ammonia solution, 30%, was added, raising the pH to 13. The sample was vortexed with 3 ml of freshly distilled diethyl ether for 1 min. After centrifugation for 5 min at 1200 *g* the aqueous phase was frozen by immersion of the tube in a slurry of dry ice and acetone. The ether phase was decanted and evaporated under a stream of nitrogen at room temperature. The residue was reconstituted in 50 μl of 0.1 *M* sulphuric acid and 50 μl of acetonitrile. A 20-μl aliquot was injected onto the column.

Calibration curves

A 1-ml aliquot of drug-free plasma or diluted blood was spiked with 20—100 μl of aqueous solutions containing known amounts of HCQ and all three metabolites before proceeding with the analytical method described above.

The aqueous solutions were made by dilution from a standard solution containing 50 μg/ml of each compound. The amount of internal standard added to each spiked sample, 30 ng, 600 ng or 6 μg, was chosen to give peak-height ratios between 0.2 and 5 to minimize measurement errors. Peak-height ratios were used to construct calibration curves.

Recovery

Recoveries were determined by adding known quantities of each compound to drug-free plasma or whole blood and extracting as described. The mean peak heights resulting from three injections of the reconstituted extracts were compared with the mean peak heights resulting from three injections of aqueous solutions containing the same amount of compound.

Blood collection

Silanized Vacutainers (Mallinckrodt, Sydney, Australia), heparinised by the addition of 100 I.U. lithium heparin, were used to collect blood samples. Plasma samples were obtained by centrifuging whole blood at 1200 *g* for 15 min within 1 h of collection. Plasma was removed using the Vacutainer plunger and frozen at −22°C in plastic tubes (Disposable Products, Sydney, Australia).

RESULTS AND DISCUSSION

Separation of HCQ and all three metabolites was achieved. Elution times were 2.7 min for HCQM, 3.2 min for CQMM, 4.4 min for HCQ, and 6 min for CQM. The internal standard, CQ, eluted after 12.2 min.

Changes of triethylamine concentration in the mobile phase altered the retention times of CQ, HCQ and metabolites (Fig. 2); 100 m*M* triethylamine was chosen as a satisfactory compromise between speed and resolution.

Retention times were also altered by changing the pH of the mobile phase (Fig. 3). At pH 11 resolution was achieved between HCQ and metabolites and sensitivity of retention times to pH variations was minimized.

Changes in ionic strength of the mobile phase had no effect on the retention times of the compounds.

Recovery from plasma and whole blood was approximately 85% for HCQ and 80% for CQM. Recovery from plasma was 65—70% for HCQM and CQMM, and approximately 45% from whole blood. Small daily fluctuations in recoveries (up to 5%) were apparent. Changed recovery of one compound was paralleled by changed recoveries of the others.

Repeated extractions with diethyl ether increased recovery of all compounds to above 90%. Extraction with chloroform also increased recovery, but problems with emulsification, separation from aqueous phase and evaporation render this solvent less convenient than diethyl ether for routine use.

As this assay was developed for rapid quantification of a large number of samples, the quicker and easier single ether extraction step was chosen, accepting lower recoveries.

Calibration curves for HCQ and metabolites extracted from plasma demonstrate linearity over a wide concentration range. To construct calibration curves the peak-height ratios were expressed relative to 600 ng of added internal

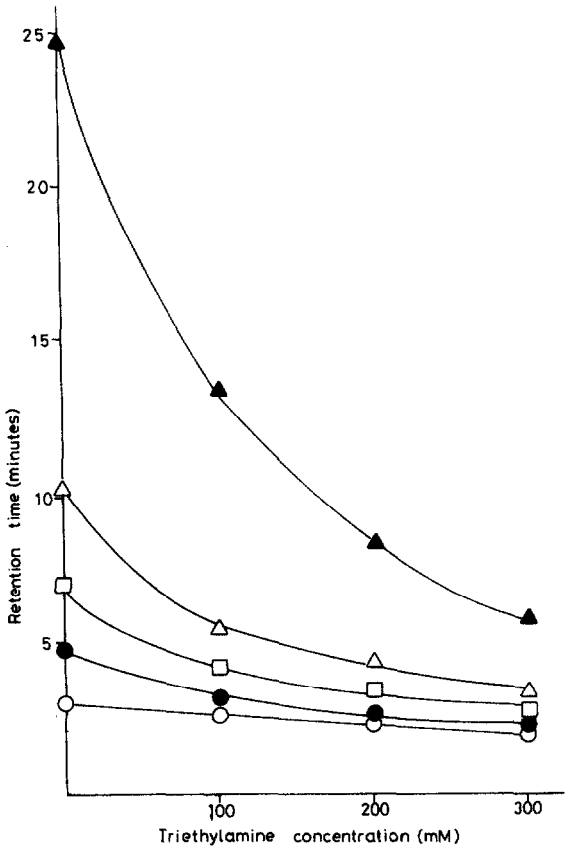

Fig. 2. Effect of triethylamine concentration on retention times of CQ, HCQ and its metabolites. Mobile phase: methanol—glycine buffer (ionic strength 0.1 *M*) (80:20), pH 10.9. ▲, CQ; △, CQM; □, HCQ; ●, CQMM; ○, HCQM.

TABLE I

CALIBRATION CURVE PARAMETERS FOR THE ANALYSIS OF PLASMA CONCENTRATION RANGE 1 ng/ml TO 4 μg/ml

y is the peak height ratio (expressed relative to 600 ng of internal standard); *x* is the plasma concentration (μg/ml).

Compound	Regression equation*	r^2
Hydroxychloroquine	$y = 4.0x$	0.9884
Desethylhydroxychloroquine	$y = 14.2x$	0.9656
Bisdesethylchloroquine	$y = 6.2x$	0.9721
Desethylchloroquine	$y = 6.5x$	0.9948

*Quadruplicates at each of nine data points. Weighting = reciprocal of variance estimated from replicates.

standard. Table I gives the parameters of the regression lines of these standard curves. Calibration curves prepared from whole blood (concentration range 0.8—4 μg/ml) gave similar correlation coefficients, r^2 values were greater than

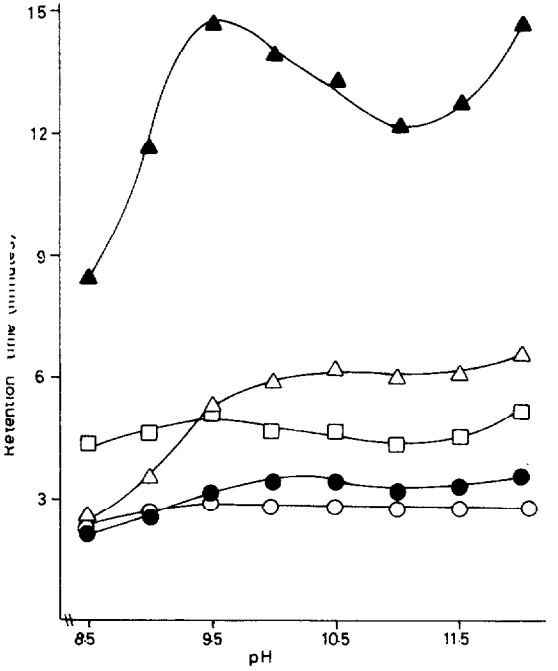

Fig. 3. Effect of mobile phase pH on retention times of CQ, HCQ and metabolites. Mobile phase: methanol—borate buffer (80:20) with 100 m*M* triethylamine, ionic strength 0.2 *M*. ▲, CQ; △, CQM; □, HCQ; ●, CQMM; ○, HCQM.

TABLE II

VARIATION IN ASSAY PRECISION (PERCENT RELATIVE STANDARD DEVIATION) WITH CONCENTRATION FOR HYDROXYCHLOROQUINE AND METABOLITES

Concentration (ng/ml)	Relative standard deviation (%)			
	HCQ	HCQM	CQMM	CQM
Plasma (n = 4)				
1	12	14	11	9
50	4	3	7	7
500	3	4	7	2
2 μg/ml	1	3	3	2
Whole blood (n = 6)				
2	9	17	12	6
100	5	3	9	4
2 μg/ml	3	6	7	4

0.99 for all of the compounds. The precision of the assay, expressed as relative standard deviations (coefficients of variation) of replicates at various concentrations in plasma and whole blood is shown in Table II.

The lower limit of detection of HCQ and its metabolites is 1 ng/ml in plasma and whole blood.

A number of non-steroidal anti-inflammatory drugs may be administered concurrently with hydroxychloroquine in the treatment of rheumatoid disorders. Ibuprofen, indomethacin, phenylbutazone, aspirin, fenoprofen, flurbiprofen, cicloprofen, carprofen and indoprofen were found not to interfere with the assay. With the single ether extraction used, a small peak eluted after 3.2 min from a plasma sample containing 125 μg/ml naproxen. This potentially interfering peak can be removed by making the sample acidic initially, vortexing for 1 min with 3 ml diethyl ether, then discarding the ether phase. The analytical procedure described above may then be performed, adding 0.5 ml instead of 0.3 ml of strong ammonia solution.

It has been reported that CQ binds to glass and to some plastics [11], and that, when using Vacutainers to collect blood, whole-blood-to-plasma ratios are altered for some basic drugs [12]. The silanized Vacutainers were found not to take up HCQ or metabolites, nor did they alter blood-to-plasma ratios. The plastic tubes used to store frozen samples did not take up the compounds.

Increased plasma levels have been reported for CQ and CQM if whole blood is not centrifuged at $> 1000\ g$ for 15 min, within 1—2 h of collection [13]. This procedure was therefore followed to isolate plasma containing HCQ and metabolites.

Chromatograms are shown for plasma (Fig. 4B) and whole blood (Fig. 4C) extracts obtained from a rheumatoid patient receiving hydroxychloroquine sulphate (Plaquenil, Winthrop Labs., Ermington, Australia) 200 mg orally (equivalent to 155 mg of HCQ base) daily for six months.

The chromatograms shown in Fig. 4 were obtained after the column had been in use for four months. Separation of HCQ and the two major metab-

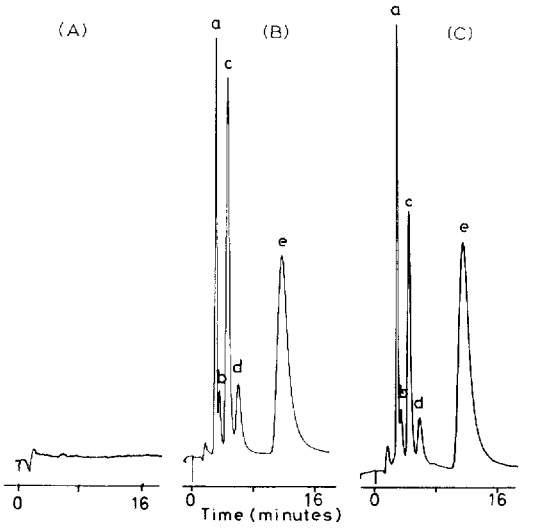

Fig. 4. Chromatograms of extracts of (A) blank plasma; (B) plasma from a rheumatoid patient receiving HCQ containing HCQM (101 ng/ml), CQMM (20 ng/ml), HCQ (207 ng/ml), CQM (23 ng/ml) and CQ 436 ng added; (C) whole blood from the same patient containing HCQM (219 ng/ml), CQMM (51 ng/ml), HCQ (704 ng/ml), CQM (77 ng/ml) and CQ (436 ng added per 0.5 ml). Peaks: a = HCQM; b = CQMM; c = HCQ; d = CQM; e = CQ.

olites, HCQM and CQM, remains excellent. Resolution between HCQM and the minor metabolite, CQMM, calculated as the difference in retention times between the two compounds divided by half the sum of their peak widths at the baseline, decreased in this time from 0.96 to 0.85. Since calibration curves were obtained simultaneously with spiked plasma samples containing all the compounds, interference between peaks should be evident as departures from linearity. This was not observed indicating that resolution between HCQM and CQMM remained satisfactory.

The stationary phase used in the analytical column, PRP-1, appears to be durable. Mobile phase, pH 11, is pumped through the column continuously. When losses of resolution were observed, the column was reversed and flushed overnight with water. Back-pressure has remained constant, at 15.7 MPa using a flow-rate of 1 ml/min throughout four months intensive use.

This assay is rapid and selective, separating HCQ and all three metabolites. The method is sensitive and reproducible, allowing accurate quantification of HCQ and metabolites in blood and plasma at concentrations as low as 1 ng/ml.

REFERENCES

1 A.R. Surrey and H.F. Hammer, J. Amer. Chem. Soc., 72 (1950) 1814.
2 E.M. Adams, D.E. Yocum and C.L. Bell, Amer. J. Med., 75 (1983) 321.
3 A.J. Popert, Rheum. Rehabil., 15 (1976) 235.
4 E.W. McChesney, Amer. J. Med., 75 (1983) 11.
5 E.W. McChesney, W.D. Conway, W.F. Banks, Jr., J.E. Rogers and J.M. Shekosky, J. Pharmacol. Exp. Ther., 151 (1966) 482.
6 R.L. Nation, L.P. Hackett, L.J. Dusci and K.F. Ilett, Brit. J. Clin. Pharmacol., 17 (1984) 368.
7 N.D. Brown, B.T. Poon and J.D. Chulay, J. Chromatogr., 229 (1982) 248.
8 Y. Bergqvist and A. Olin, Acta Pharm. Suec., 19 (1982) 161.
9 Y. Bergqvist and M. Frisk-Holmberg, J. Chromatogr., 221 (1980) 119.
10 D.P. Lee, J. Chromatogr. Sci., 20 (1982) 203.
11 T.G. Geary, M.A. Akood and J.B. Jensen, Amer. J. Trop. Med. Hyg., 32 (1983) 19.
12 R.H. Cotham and D. Shand, Clin. Pharm. Ther., 18 (1975) 535.
13 Y. Bergqvist and B. Domeij-Nyberg, J. Chromatogr., 272 (1983) 137.