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Note

Estimation of plasma hydroxychloroquine by high-performance liquid chromatography with ultraviolet detection

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The use of antimalarial aminoquinoline drugs is well established in treatment of rheumatoid arthritis. Toxicity with prolonged use is often associated with binding to melanin-rich tissue, e.g. the retinal epithelium [1—3]. However, the relationship between blood levels and likely therapeutic efficacy or toxicity is not resolved. Laaksonen et al. [4] provided evidence of such a relationship for hydroxychloroquine (HCQ) and chloroquine (CQ), suggesting a maximum safe serum concentration of HCQ of around 1.5 μmol/l. Wollheim et al. [5], however, failed to observe any relationship between plasma CQ levels and therapeutic response.

Many of these early studies relied on relatively non-specific, poorly standardised fluorimetric methods which would not distinguish parent drug from oxidation products or any other component fluorescing at the chosen wavelength. More recently more selective chromatographic methods have been developed to determine CQ and its de-ethylated metabolites in plasma [6—8].

The present paper reports a selective reversed-phase high-performance liquid chromatographic (HPLC) method which requires a single solvent extraction in sample preparation, and can detect the parent drug (HCQ) in plasma at least fourteen days after a single oral 400-mg dose. The results of five such single-dose studies illustrate the application of the method in rheumatoid patients.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade and aqueous solutions prepared in glass-

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distilled water. Aqueous solutions of HCQ sulphate (Sterling Pharmaceuticals, Australia) and CQ diphosphate (20 μmol/l, Sigma) were stored at 4°C and prepared fresh monthly. The extracting solvent, chloroform (Mallinckrodt, Australia), was used without further distillation. A solution of sodium tetraborate (2.28 g) was prepared in 100 ml of 1 mol/l sodium hydroxide.

Standards

Stock solutions of HCQ (6 and 60 μmol base per l) were used to prepare calibration standards. Known amounts were added to plasma, previously shown to contain no compounds which interfered with the chromatography, to give a concentration range of 0.03—15 μmol/l. The internal standard, CQ, was employed to allow for inter-sample variability in recovery through the extraction procedure.

Chromatography

The analysis was performed using a Spectra-Physics (SP8000B) high-performance liquid chromatograph coupled with an SP8400 UV—VIS detector set at 340 nm (range of 0.002—0.08 a.u.f.s.). The separation was performed at 40°C on a 10-μm 30 cm × 3.9 mm μBondapak™ Phenyl column (Waters Assoc., Australia, Part No. 27198). The mobile phase was acetonitrile (Burdick & Jackson Labs., HPLC grade)—0.001 *M* disodium orthophosphate buffer (pH = 3.5) (9:1). These solutions were filtered under vacuum through a 0.45-μm filter (Millipore) before use and degassed continuously with helium. The flow-rate was 2 ml/min and injection volume was 100 μl.

Metabolite analysis

The oxidiation metabolites, N-desethyl chloroquine, N,N-didesethyl chloroquine and chloroquine-diol (Winthrop Labs.) at concentrations of 0.03, 0.06 and 0.13 μmol/l, respectively, were extracted using the present method and subjected to the same chromatographic separation to assess potential interference and quantitation of these compounds.

Sample preparation

Aliquots (2 ml) of heparinised plasma were added to glass extraction tubes. The internal standard (100 μl) and sodium hydroxide—borate mix (250 μl) were added and briefly vortexed before adding 5 ml of chloroform. The tubes were then capped and shaken horizontallly for 10 min at 100 oscillations per min. The two phases were separated by centrifugation at 1000 *g* for 10 min. The upper aqueous layer was aspirated to waste.

In order to avoid traces of aqueous contamination, the chloroform phase was tipped firstly into one glass tube (75 × 12.5 mm) and then to a second similar tube. This latter fraction was evaporated to dryness under a gentle stream of nitrogen in a heating block at 45°C. As soon as the chloroform had evaporated, each tube was removed from the heating block and reconstituted in either 125 μl or 250 μl of the same mobile phase used for the chromatographic separation.

The performance of the above technique was assessed by assaying in a single assay run five replicates of plasma samples spiked with HCQ to give final concentrations 0.03, 1.5 and 15 μmol/l. The variability between assay runs was

assessed by monitoring the standard curve stability in five successive assays. The recoveries of HCQ and CQ were estimated by comparing the peak heights obtained in six extracted samples (1.5 and 1.0 μmol/l, respectively), with the peak heights of six replicate injections of unextracted aqueous solutions of HCQ and CQ. These extracts were subsequently reinjected after sitting at room temperature (20° C) for 24 h to assess potential sample deterioration over the time course of a long assay run.

Patient studies

Venous blood samples (10 ml) were drawn into lithium heparin (125 I.U.) tubes at 0, 0.5, 1.0, 1.5, 3, 5, 9, 12 h and 1, 2, 3 and 4 days following a single oral 400-mg dose of Plaquenil™ from five patients with rheumatoid arthritis and who were both clinically assessed to be candidates for HCQ therapy and had not previously received HCQ therapy. These blood samples were centrifuged for 15 min (1000 *g*) and the plasma fraction was stored at −20° C until assayed. These patients were hospitalised for the first 24 h to facilitate sample collection. Subsequent samples were drawn in an outpatient clinic. Patients participating in the study were invited in advance to complete an informed consent form before being admitted to the study.

RESULTS

Fig. 1 shows sample chromatograms obtained using the method described above. Fig. 1a shows the result of injecting an aqueous mixture of HCQ and CQ (peaks 1 and 2, respectively) which had not been subjected to the extraction procedure described. Fig. 1b, shows the result of an extracted plasma sample spiked with HCQ (0.15 μmol/l) and CQ (1.0 μmol/l). Fig. 1c and d show extracts of patient plasma samples (spiked with the internal standard, CQ) before and 1.5 h after an oral 400-mg dose of Plaquenil, respectively. Fig. 1e, shows a mixture of the oxidation products N,N-didesethylchloroquine (peak 5), chloroquine-diol (peak 4), N-desethylchloroquine (peak 3), HCQ (peak 1), and CQ (peak 2).

Using the ratio of the height of peaks (HCQ:CQ) obtained from spiked plasma standards, a calibration curve was constructed in each assay run and showed a linear relationship between the peak height ratios and HCQ concentration over the range 0.03—15 μmol/l (r^2 = 0.998, slope = 2.137). The detection limit of the method was 0.01 μmol/l.

The performance and reproducibility of the method, assessed by comparing five replicate estimations obtained using three concentrations within a single assay and five concentrations between assay runs are presented in Table I. This shows a within-run coefficient of variation ranging from 9.1% (at 0.03 μmol/l) to 6.7% (at 15 μmol/l). The corresponding between-run values were 7.4% and 6.6%, respectively. The mean recoveries of HCQ (1.5 μmol/l) and CQ (1.0 μmol/l) through the extraction procedure were 91.5 ± 3.9% (n = 6) and 84.3 ± 4.4% (n = 6), respectively. Samples were found to be stable at room temperature in mobile phase for at least 24 h, as no deterioration or alteration in chromatography could be detected when samples were injected a second time on the day following the initial assay.

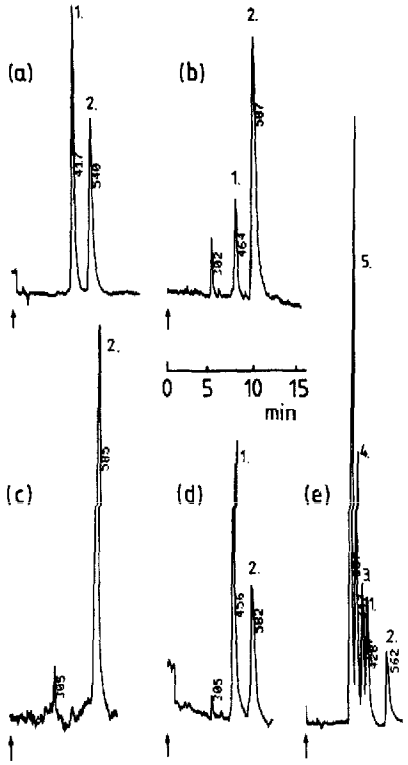

Fig. 1. Sample chromatographic traces obtained using the method described. (a) An unextracted aqueous mixture of HCQ and CQ; (b) a plasma extract spiked with 0.15 μmol/l HCQ and the internal standard, CQ (1.0 μmol/l); (c) a pre-HCQ dose plasma sample blank spiked with internal standard; (d) a patient plasma sample 1.5 h after a 400-mg dose of HCQ (Plaquenil); (e) a mixture of HCQ, CQ, N,N-didesethylchloroquine, chloroquine-diol and N-desethylchloroquine. Peaks: 1 = HCQ; 2 = CQ; 3 = N-desethylchloroquine; 4 = chloroquine-diol; and 5 = N,N-didesethylchloroquine.

TABLE I

PRECISION OF THE METHOD (n = 5)

HCQ concentration (μmol/l)	Coefficient of variation (%)	
	Between-assay	Within-assay
0.03	7.4	9.1
0.3	7.2	—
1.5	5.1	5.5
7.5	7.5	—
15	6.6	6.7

Patient studies

The results of the single oral Plaquenil (400-mg) dose studies (Fig. 2) illustrate the application of the method to patient samples. Fig. 2 shows means and standard errors of plasma HCQ levels (in nmol/l) estimated using the above assay technique in five rheumatoid patients. It can be seen that mean time to

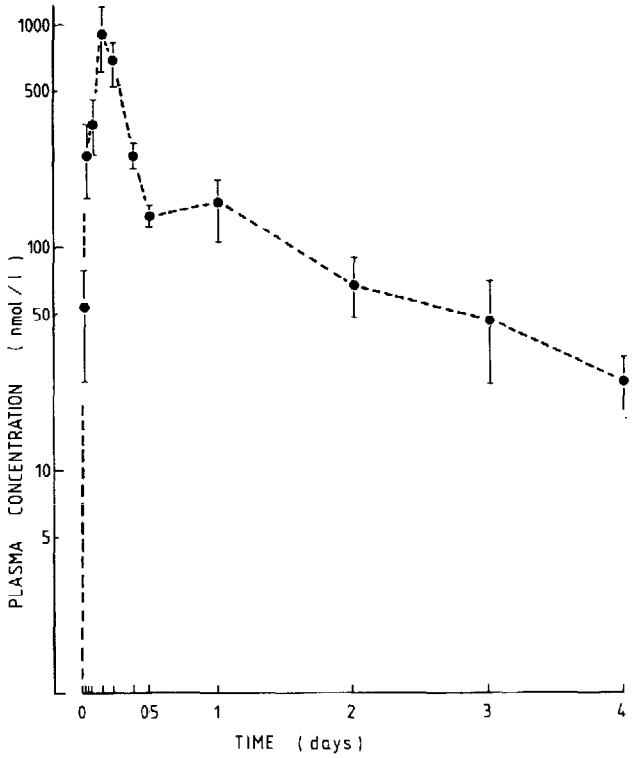

Fig. 2. Shows the estimated mean (± S.E.M.) plasma HCQ concentrations (nmol/l) up to four days following single oral 400-mg doses of Plaquenil in five rheumatoid patients, not previously medicated with HCQ, using the assay methodology described.

peak was between 3.0 and 5.0 h after the dose. The terminal phase is indicative of at least two compartments over the time course indicated. Three of the five patients had further samples drawn at seven and fourteen days following the dose. Plasma levels of HCQ could still be detected in each of these patients at fourteen days and in each case the level was less than or equal to the lowest standard (30 nmol/l) and approached the limit of detection of the present method (10 nmol/l).

DISCUSSION

The HPLC method presented allows a convenient and sensitive estimation of HCQ levels in heparinised plasma samples. The recovery and reproducibility of the extraction procedure are acceptable. No interfering compounds have yet been found either in samples assayed so far from patients receiving concominant therapy (including naproxen, aspirin, indomethacin, methyl-clothiazide, cimetidine and diclofenac) or as demonstrated with the three oxidation products presented.

Most previous methods for measuring HCQ have relied on non-specific fluorimetric methods and so could be criticised for the potential interference from metabolites and/or other fluorescing compounds. In the present HPLC method, fluorescence detection was avoided as it was found that these com-

pounds only fluoresce significantly at a pH greater than 8, which is inconsistent with HPLC column packing stability. An alternative approach could be to invoke a post-column pH adjustment to facilitate fluorescence. However, this approach obviously requires the dedication of a second pumping system which may not only be unavailable or inconvenient in many laboratories, but also introduces peak broadening and hence potential loss of sensitivity.

The single-dose studies in five patients presented suggests at least two compartments in the terminal phase, one with a half-life in excess of one day. As seen in the standard error bars in Fig. 2, the inter-patient variability in this latter compartment appears to be considerably greater than that of the first compartment. This could suggest that patients with the slower terminal half-life may achieve higher trough plasma levels at steady-state and, speculatively, be more likely to experience toxicity. These hypotheses will be tested in the next phase of the study where these and further patients, having been studied for two weeks following a single oral dose, progress to chronic HCQ therapy. Steady-state trough levels will be estimated for each patient to test whether these correlate with a slower terminal half-life in the single-dose study and the possible presentation of side-effects, especially retinal toxicity.

Further, it is hoped that the method, possibly in a modified form, may be employed to measure tissue levels of HCQ (e.g. leucocytes) as these may have a more direct relationship with disease suppression or the expression of toxicity than do plasma levels.

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REFERENCES

1 H.E. Hobbs, A. Sorby and A. Freedman, Lancet, ii (1959) 478.
2 H.N. Bernstein, Surv. Opthalmol., 12 (1967) 415.
3 A. Elman, R. Gullberg, R. Nilsson, I. Rendah and L. Wachtmeister, Scand. J. Rheumatol., 5 (1976) 161.
4 A.-L. Laaksonen, V. Koskiahde and K. Juva, Scand. J. Rheumatol., 3 (1974) 103.
5 F.A. Wollheim, A. Hanson and C.-B. Laurell, Scan. J. Rheumatol., 7 (1978) 171.
6 N.D. Brown, B.T. Poon and J.D. Chulay, J. Chromatogr., 229 (1982) 248.
7 L.C. Patchen, D.L. Mount, I.K. Schwartz and F.C. Churchill, J. Chromatogr., 278 (1983) 81.
8 G. Alvan, L. Ekman and B. Lindström, J. Chromatogr., 229 (1982) 241.