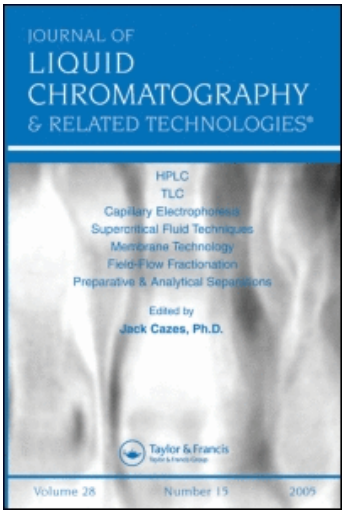

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Original Article

HPLC METHOD FOR THE DETERMINATION OF CHLOROQUINE AND ITS MAIN METABOLITE IN BIOLOGICAL SAMPLES

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ABSTRACT

An ion-pair reversed phase HPLC-method for the determination of chloroquine and desethylchloroquine in plasma, blood and filter-paper-absorbed blood spots is described.
Using 75 µl samples, concentrations ranging from 2 to 1000 ng/ml can be measured.
Intraday precision as well as interday precision are lower than 6 % (RSD).
The filter-paper method could be used for the determination of chloroquine and desethylchloroquine in samples originating from areas with a lack of laboratory equipment.
The procedure is sensitive enough to be used in monitoring of drug levels as well as for pharmacokinetic studies.

INTRODUCTION

Chloroquine is since 40 years one of the most important drugs for the prophylaxis and the treatment of malaria. It is also used for the treatment of rheumatoid arthritis.

Chloroquine resistant strains of Plasmodium falciparum are causing a serious problem. One should be aware that various therapeutic incidents such as malabsorption, subtherapeutic doses and a lack of compliance could lead to false conclusions about resistance. Therefore, reliable methods for the determination of chloroquine in biological material are needed.

Total blood concentrations of 3.5 μg/ml (1) could be lethal.So, chloroquine levels should also be followed to avoid toxic levels. There is indeed a poor relationship between dosage regimens and blood levels.

Various methods for the determination of chloroquine are described : spectrofotometry (2), fluorimetry (3), gaschromatography (4,5,6), HPLC (7-13) and EMIT (14). Nowadays, HPLC is the method of choice.

For the separation by HPLC, normal phase (8,11) and reversed phase (7,9,10,12,13) chromatography has been used.

Fluorimetric detection (8,9,11) is preferable to UV detection (7,9,10,12,13) because of greater sensitivity. We describe an ion-pair reversed phase HPLC method with fluorimetric detection.

Because of the lack of technical possibilities in areas where chloroquine levels should be determined, a method was developed based on filter-paper dried blood (11,15). These samples allow easy transport to suitable laboratories.

MATERIALS AND METHODS

Reagents

Methanol, dichloromethane and acetonitrile were obtained from Sigma; potassium dihydrogenphosphate,

disodiumtetraborate and sodiumhydroxide from UCB and $HClO_4$ 60 % from Merck.
Silicon oil was purchased from Dow Chemicals.
Chloroquine diphosphate was obtained from Sigma.
Hydroquinidine, used as the internal standard, was obtained from Aldrich Chemie.
Desethylchloroquine was kindly supplied by Sterling-Winthrop Research Institute and bisdesethylchloroquine by Rhône-Poulenc. All reagents were used as recieved.
The water we used was purified by a Milli-Q system (Millipore).

Apparatus

HPLC analyses were carried out with a Waters Assoc. Model 6000 A pump, connected to a U6K injector (Waters Assoc.) and a spectrofluorimetric detector (Kontron). Chromatograms were recorded with a Chromatopac C-R1B integrator (Shimadzu). A second Model 6000 A pump was used to adjust the pH of the column effluent.

Glassware

All glassware was cleaned in a solution of potassium dichromate in concentrated sulfuric acid (100 g/l) for several days. It was rinsed with purified water and dried at a temperature of 150 °C.
To prevent the adsorption of chloroquine on glass (16,17), all glassware was siliconized by treating it with an emulsion of silicon oil in water. It was then dried at a temperature of 180 °C.

Chromatographic conditions

Separations were carried out on a Novapak C-18 column (Waters Assoc.) under radial compression. The solvent system consisted of KH_2PO_4 buffer (0,06 M) - Methanol - Acetonitrile - perchloric acid 60 % (600:220:130:6 v/v), degassed in an ultrasonic bath. The separations were done at room temperature. The flow-rate was 1.2 ml/min. After separation the column effluent was mixed with a borax-buffer (pH = 11, 0.25 M) generating optimal fluorescence of the eluting components at pH = 9.5 (excitation wavelength = 325 nm; emission wavelength = 380 nm). The flowrate of the borax-buffer was 0.1 ml/min.

Extraction procedure

Filter-paper-absorbed blood spots (75 µl) were cut into pieces. Chloroquine and desethylchloroquine were desorbed from the paper by sonication and stirring in 1.5 ml HCl 0.2 N.
Blood and plasma samples (75 µl) were diluted in 1.5 ml HCl 0.2 N.
After alkalinization with 0.5 ml NaOH 2 N and addition of 100 µl of a solution of the internal standard (4 µg/ml), the drugs were extracted in 6.0 ml dichloromethane by a rotating shaker during 15 min. Following centrifugation (4000 rpm, 10 min), the aqueous layer was removed by vacuum aspiration and the organic layer was transferred to a dry test tube and evaporated to dryness at 40 °C under a stream of air. The residue was dissolved in 100 µl or 300 µl of mobile phase and an aliquot was injected into the chromatographic system.

Stock solutions and calculations

Concentrations of chloroquine and its main metabolite desethylchloroquine were determined using a calibration curve in which peak height of the drug / internal standard ratio was plotted against concentration.
Stock solutions of chloroquine and desethylchloroquine contained 1 mg base/ml in HCl 0.1 N.
Chloroquine concentrations ranging from 15.6 - 1000 ng/ml and desethylchloroquine concentrations ranging from 7.8 - 500 ng/ml were prepared by dilution of a standard solution of 10 µg/ml chloroquine and 5 µg/ml desethylchloroquine in plasma or in blood.

RESULTS AND DISCUSSION

The fluorescent emission of chloroquine, its main metabolites as well as the internal standard is strongly pH dependent, which necessitated post-column alkalinization of the eluting components. The influence of pH on the emission of chloroquine is illustrated in fig. 1 showing optimal fluorescence at pH 9.5.
Desethylchloroquine, bisdesethylchloroquine and the internal standard show similar curves.
Fig. 2 shows typical chromatograms of a test solution, a blanco blood sample, a spiked blood sample and a patients blood sample.
The extraction recovery from plasma, blood and filter-paper-dried blood spot, determined at a concentration of 100 ng/ml, by comparing the results after extraction with the results of a standard solution in the mobile phase ranged from 86 to 92 % for chloroquine and

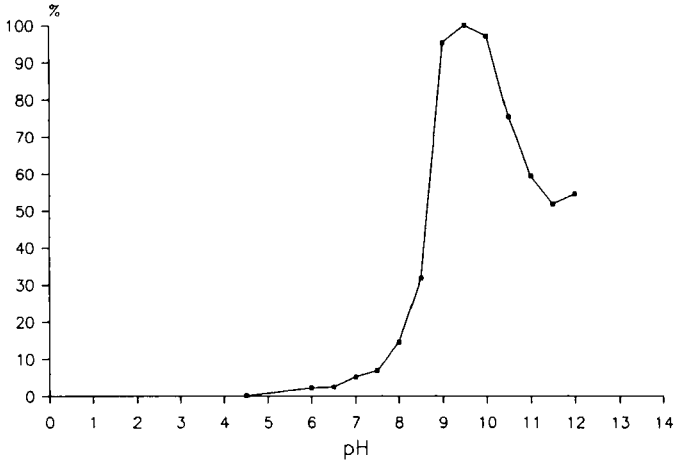


Figure 1 : Influence of pH on the fluorescence emission of chloroquine (expressed as % of the maximum respons) at 380 nm after excitation at 325 nm.

desethylchloroquine and from 89 to 99 % for hydroquinidine.

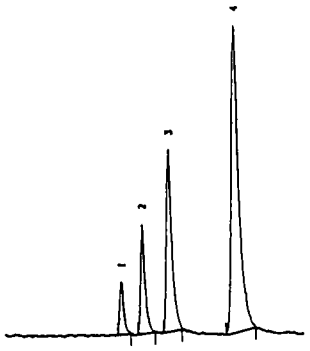
The intraday and interday (n=6) variability of the assay, determined at concentrations ranging from 7.8 to 500 ng/ml for desethylchloroquine and from 15.6 - 1000 ng/ml for chloroquine, expressed as the relative standard deviation, was lower than 6 % .

Standard curves for chloroquine (15.6 - 1000 ng/ml) and desethylchloroquine (7.8 - 500 ng/ml) showed good linearity and passed near the origin ($r = 0.998$).

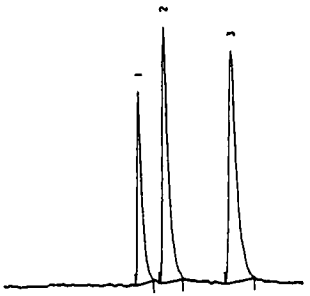
Typical standard curves ranging from 15.6 - 250 ng/ml for desethylchloroquine and from 31.3 - 500 ng/ml for chloroquine, using human blood are shown in fig. 3.

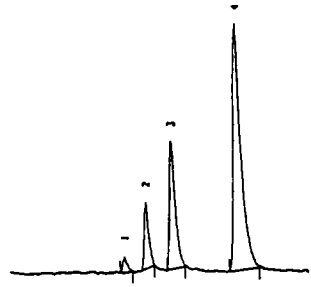
Dried on filter paper, chloroquine and desethylchloroquine were stable for at least 6 weeks.

The method described was used to investigate the pharmacokinetics of chloroquine after an oral dose of 300



(a) 1 bisdesethylchlor. 6.23 min (b)
2 desethylchloroq. 7.40 min
3 chloroquine 8.80 min
4 internal stand. 12.46 min





(c) 1 desethylchloroq. 7.38 min (d) 1 bisdesethylchl. 6.24
2 chloroquine 8.81 min 2 desethylchloroq. 7.38
3 internal stand. 12.42 min 3 chloroquine 8.78
4 internal stand. 12.45

Figure 2 : Chromatograms of HPLC assay using fluorimetric detection : (a) test solution, (b) blanco blood samples, (c) blood spiked with 125 ng/ml chloroquine and 62.5 ng/ml desethylchloroquine and (d) human sample after an oral dose.

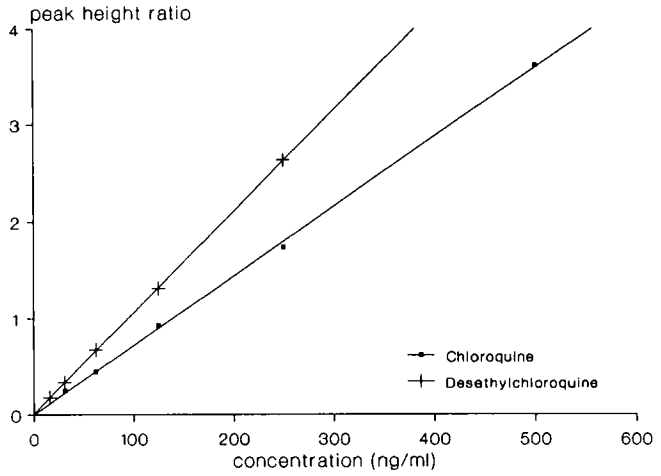


Figure 3 : Calibration curves for desethylchloroquine and chloroquine in blood.

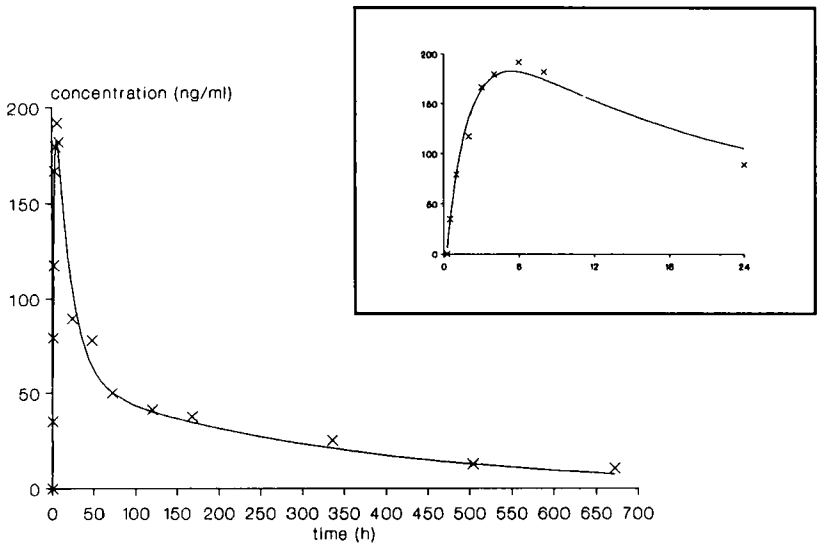


Figure 4 : A typical concentration - time curve for a human subject after an oral dose of 300 mg chloroquine. The insert represents the initial concentration on a modified time scale.

mg chloroquine. A typical concentration - time curve of one subject is shown in fig 4.
The sensitivity limit of the assay was 1 ng/ml for desethylchloroquine and 2 ng/ml for chloroquine.

CONCLUSION

A specific method for the determination of chloroquine and desethylchloroquine has been developed and validated using as little as 75 µl plasma or blood. Blood can be used as such or dried on filter paper. The low sample volume allows sample collection by the finger-prick method. This non-invasive method facilitates sample collection for pharmacokinetic studies and studies investigating drug-resistance. The method is also attractive for neonatal drug investigation.

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