Journal of Chromatography, 229 (1982) 241—247
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1167

Note

Determination of chloroquine and its desethyl metabolite in plasma, red blood cells and urine by liquid chromatography

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(Received October 20th, 1981)

Chloroquine (Fig. 1) is used for prophylaxis and treatment of malaria and also in rheumatoid arthritis and systemic lupus. Determination of chloroquine in biological material is generally accomplished with the aid of spectrophotofluorometry. Several methods using this technique have been published [1—3]. These methods lack sensitivity and also specificity due to interference of metabolites such as desethylchloroquine (Fig. 1). Moreover, the extraction procedures used in these methods do not completely separate chloroquine from its main metabolite.

Recently a liquid chromatographic method using fluorimetric detection was published [4]. This utilizes a reversed-phase column and an aqueous eluent

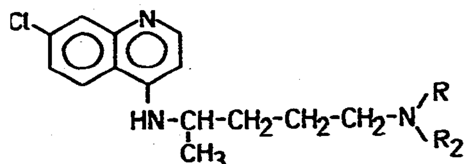

Fig. 1. R = R_2 = ethyl: chloroquine. R = H, R_2 = ethyl: desethylchloroquine. R = R_2 = methyl: internal standard.

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which was mixed with base after separation on the column, thereby generating fluorescence of the eluting chloroquine and its metabolite.

The purpose of the present method was to enable the determination of chloroquine and its desethyl metabolite, retaining the specificity and sensitivity of the latter method but using a simpler system. This was achieved by using a silica column that was eluted with a non-aqueous solvent mixture containing diethylamine, and sufficient fluorescence of chloroquine and its metabolite was generated to obtain a high sensitivity.

Several substances with a structure similar to chloroquine were synthezised and tested for use as internal standard. 7-Chloro-4-(1-dimethylamino-4-pentylamino)quinoline had the best properties of these substances.

The method was used to assay samples from healthy volunteers who had been given chloroquine equal to 300 mg of the base orally and by infusion.

MATERIALS AND METHODS

Instrumental

The liquid chromatographic system consisted of an M45 pump, a U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a 970 fluorescence detector (Spectra-Physics, Santa Clara, CA, U.S.A.)

The excitation wavelength was 335 nm and a 370-nm filter was used. The column (0.15 m × 4.6 mm I.D.) was slurry-packed with LiChrosorb Si 60, 5 μm (Merck, Darmstadt, G.F.R.) and eluted with acetonitrile—methanol—diethylamine (80:19.5:0.5). The flow-rate of the eluent was 1 ml/min. A Model 204 spectrofluorometer (Perkin-Elmer, Norwalk, CT, U.S.A.) was used to obtain the fluorescence spectra.

Standards and solvents

Several 4-substituted 7-chloroquinolines were synthezised by reacting 1-dimethyl-amino-3-aminopropane, 1-diethyl-amino-3-aminopropane, 1-dipropyl-amino-3-aminopropane [5], 1-dibutyl-amino-3-aminopropane and 1-dimethyl-amino-4-aminopentane with 4,7-dichloroquinoline [6]. The products were purified on preparative silica plates developed with acetonitrile—methanol diethylamine (60:39.5:0.5).

1-Dimethyl-4-aminopentanone was prepared in a way similar to the diethyl analogue [7] by heating 1-chloro-4-pentanone (2 g) with dimethylamine (1.5 g) in a screw-capped tube for 4 h at 70°C. After distillation under vacuum, the oxime was prepared and reduced with sodium in ethanol [8]. After addition of water and extraction with diethyl ether, the organic phase was dried (sodium sulphate) and the solvent evaporated, after which the product could be used directly.

The diethyl ether used for extraction was of analytical grade and was distilled to remove stabilizers which interfered with the chromatogram. Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade; the water content of the acetonitrile was less than 0.01%.

Plasma assay

In a 10-ml screw-capped tube, 1 ml of plasma, 1 ml of 1 *M* sodium

hydroxide, 100 μl of internal standard solution and 6 ml of diethyl ether were shaken for 15 min on a shake-board. The concentration of the internal standard solution was adjusted so that the detector signal corresponded to 0.5, 1 and 6 μg/ml chloroquine for the concentration ranges 2—25, 25—200 and 200—1000 ng/ml, respectively. The extracted sample was centrifuged for 5 min at 500 *g* (Wifug X1) and the ethereal phase was transferred to a new tube and dried (0.2—0.3 g sodium sulphate). The ether was removed after centrifugation and evaporated in a new tube by a stream of nitrogen (30°C). The residue was dissolved in 200 μl of the eluent and, after passage through a pasteur pipette closed with fine glass-wool, 100 μl were injected in the chromatograph. When plasma levels of chloroquine were below 25 ng, 2 ml of plasma were used. To the sample were then added 2 ml of 1 *M* sodium hydroxide, whereupon the whole mixture was extracted with 7 ml of diethyl ether in a 15-ml screw-capped tube. Apart from that, the procedure was the same as described for a 1-ml sample.

Red blood cell assay

Frozen and thawed red blood cells (2 ml) were diluted three times with distilled water, and 1 ml of the mixture was treated as described above.

Urine assay

Urine samples (100 μl) were diluted to 1 ml with water and then extracted as described above for the plasma samples.

Clinical samples

Blood samples were collected in heparinized Venoject^{R} tubes, cooled and centrifuged. The plasma and red blood cells were frozen separately and stored at —20°C until analysis.

RESULTS AND DISCUSSION

Sample handling

Since red blood cells of a chloroquine-treated subject have a 3—4 times higher concentration of the drug than the surrounding plasma [9], it is important that no hemolysis of erythrocytes occurs to increase the original plasma level. This is especially important with malaria plasmodium infected cells since there is an even greater proportion of chloroquine inside the red blood cells [10].

Duplicates of plasma samples that were analysed within a two-months interval did not show any variation in the measured concentrations of chloroquine that could be detected with the method of determination used. Between determinations the plasma was stored in a freezer at —20°C.

Extraction

The absolute recovery of chloroquine (and desethylchoroquine) from plasma, red blood cells and urine was 88%, 80% and 88%, respectively, with the methods described here. Drying of the ether extract with anhydrous sodium sulphate prior to evaporation was performed in order to decrease the humidity,

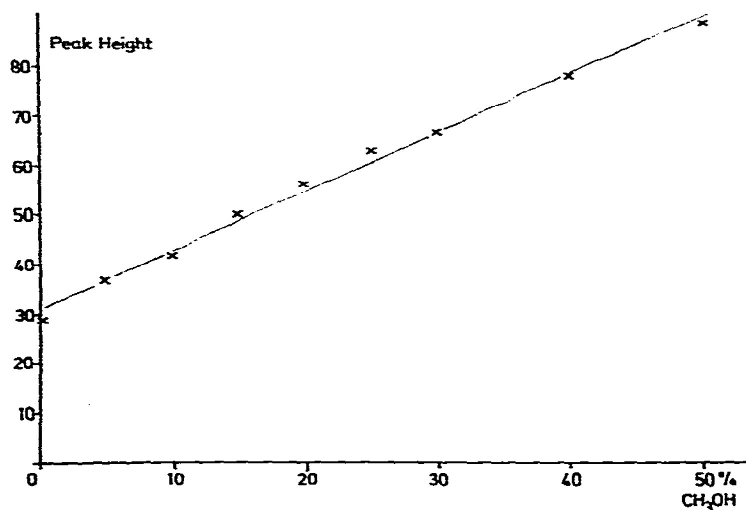
which would be detrimental to the column in the presence of diethylamine. After injection of about 100 undried samples, the back-pressure of the column had increased to an unacceptable level. No such increase could be registered after injection of several hundred dried samples.

Evaporation of the ether phase had to be performed by venting the tubes with a stream of nitrogen gas, since it was found that the use of vacuum evaporation, even at moderate temperatures (20—25°C), caused a substantial decrease in the recovery of chloroquine.

Liquid chromatography and internal standard

The water content of the eluent was found to be critical for the resolution of internal standard and desethylchloroquine. It could also cause the back-pressure on the column to increase. However, most batches of commercially available acetonitrile and methanol (HPLC grade) could be used without extra drying. If the performance of the column deteriorated it was conditioned with hexane—acetic acid—2,2-dimethoxypropane (180:20:5) [11]. The eluent was composed to give good separation of the compounds of interest and low absorption at the excitation wavelength used. However, a higher content of methanol increased the fluorescence of chloroquine (Fig. 2). With the eluent composition described here, chloroquine had 43% of the fluorescence resulting from measurement in pH 9 borate buffer [4]. The desethyl metabolite of chloroquine, however, produced the same fluorescence as the mother compound in borate buffer at equimolar concentrations in both solvent systems. Diethylamine in the eluent increased the fluorescence of chloroquine twice compared to the same amount of a 35% ammonia solution.

Among the substances synthesized for use as internal standard, 7-chloro-4-(1-dimethylamino-4-pentylamino)quinoline was selected because of its great similarity to chloroquine and suitable retention in the chromatographic system.



Fir. 2. Fluorescence response of chloroquine in mixtures of acetonitrile and methanol with 0.75% diethylamine added. The excitation and emission wavelengths were 355 nm and 390 nm, respectively.

7-Chloro-4-(1-diethylamino-3-propylamino)quinoline was an alternative, but was considered to elute too close in front of the chloroquine peak. The three other substances tested for use as internal standard eluted either too close to the solvent front or fused with the chloroquine peak.

Capability of the method

The limit of detection of the method described was 1 ng/ml of plasma for chloroquine and 0.5 ng/ml for desethylchloroquine. The precision of the method was 3.5% (n = 10) at 50 ng/ml plasma (or urine) for chloroquine and 5% (n = 10) at the 25 ng/ml for desethylchloroquine. The corresponding values when analysing red blood cells were 4% and 17% for chloroquine and desethylchloroquine, respectively.

Typical chromatograms resulting from the analysis of plasma samples are shown in Fig. 3. No interfering peaks could be detected in the chromatogram. Chromatograms obtained on analysis of red blood cells and urine samples had a similar appearance.

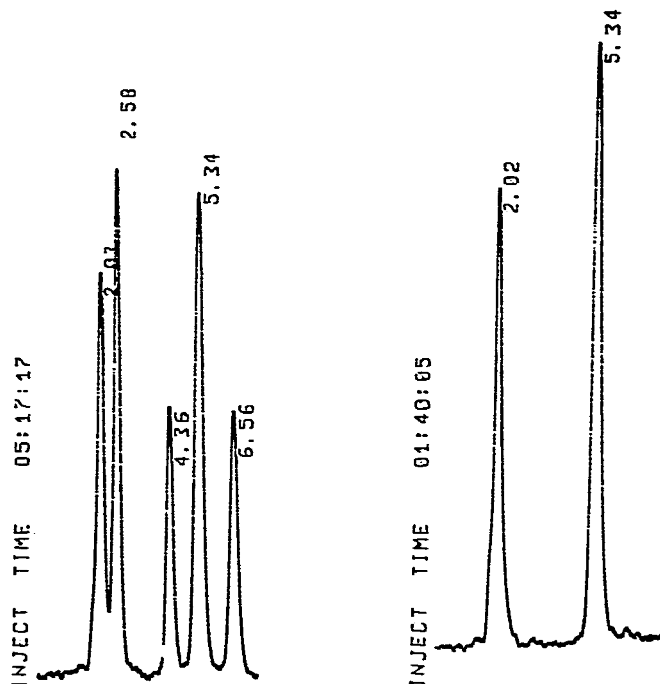


Fig. 3. Chromatograms of plasma samples. Left: sample containing 45 ng/ml chloroquine (t = 4.36), internal standard (t = 5.34) and 20 ng/ml desethylchloroquine (t = 6.56). Right: blank sample with internal standard.

The calibration curves were constructed by analysing samples spiked with chloroquine and metabolite and plotting the resulting peak height ratios (chloroquine or metabolite/internal standard) against concentration. The curves were linear and had the same slope regardless of whether plasma or urine was used. The corresponding curve for red blood cell concentrations of chloroquine had a slightly different slope.

Clinical application

Fig. 4 shows plasma concentration curves of chloroquine obtained by analysing samples from two healthy subjects according to the described method. The dose was equal to 300 mg of the base given orally and by infusion during 20 min.

An accurate chloroquine assay is much needed to elucidate the complex pharmacokinetics of this drug. There is also a great need for such an assay in field conditions where supposed resistance of the plasmodia to chloroquine has to be assessed by measuring the drug in samples from patients. Lack of compliance with the dosage prescription is a common cause for failure of malaria prophylaxis. Monitoring of drug concentrations in the treatment of malaria and rheumatic diseases is also a possibility after appropriate studies of concentration—effect relationships have been made.

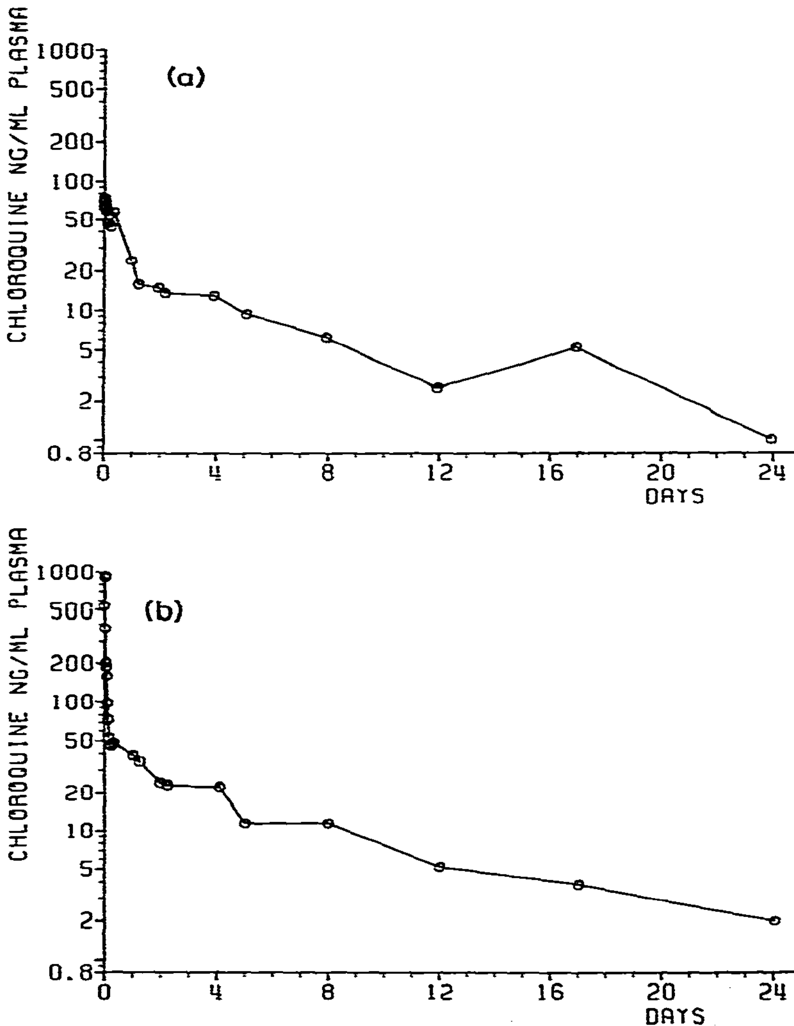


Fig. 4. Plasma concentration curves obtained from two subjects who had been given chloroquine orally (a) or intravenously (b) in doses equal to 300 mg of the base.

ACKNOWLEDGEMENT

This work was supported by a grant from the Swedish Agency for Research Co-operation with Developing Countries (79/145:2).

REFERENCES

1 B.B. Brodie, S. Udenfriend, W. Dill and T. Chenkin, J. Biol. Chem., 168 (1974) 319.
2 E.W. McChesney, H.S. Wyzan and J.P. McAuliff, J. Amer. Pharm. Assoc., 45 (1956) 640.
3 S.A. Adelusi and L.A. Salako, J. Pharm. Pharmacol., 32 (1980) 711.
4 Y. Bergqvist and M. Frisk-Holmberg, J. Chromatogr., 221 (1980) 119.
5 F.C. Whitmore, H.S. Mosker, R.R. Adams, R.B. Taylor, E.C. Chapin, C. Weisel and W. Yanko, J. Amer. Chem. Soc., 66 (1944) 725.
6 H. Andersag, S. Breitner and H. Jung, U.S. Pat., 2,233,970 (1941).
7 S.E. Forman, Brit. Pat., 604884 (1948).
8 I.L. Knunjanz, G.W. Tschelinzew, S.W. Benewolenskaja, E.D. Ossetrowa and A.I. Kurssanowa, Bull. Acad. Sci. U.S.S.R., 7 (1934) 165.
9 M. Frisk-Holmberg, Y. Bergkvist, B. Domeij-Nyberg, L. Hellström and F. Jansson, Clin. Pharmacol. Ther., 25 (1979) 345.
10 P.B. Macomber, R.L. O'Brien and F.E. Hahn, Science, 152 (1966) 1374.
11 R.A. Bredeweg, L.D. Rothman and G.D. Pfeiffer, Anal. Chem., 51 (1979) 2061.