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Note

Determination of chloroquine and its de-ethylated metabolites in human plasma by ion-pair high-performance liquid chromatography

NESBITT D. BROWN*

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20012 (U.S.A.)

BING T. POON

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20012 (U.S.A.)

and

JEFFREY D. CHULAY

U.S. Army Medical Research Unit-Kenya, Box 401, Army Post Office, NY 09675 (U.S.A.)

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During the past 35 years, a prodigious amount of research has been conducted on the compound chloroquine [1—6] in an attempt to better understand the pharmacokinetic actions of this drug as a therapeutic modality in treating malaria in human subjects. Although much of this work has been performed on the modes of action of chloroquine in various biological systems, many questions remain unanswered.

The introduction of high-performance liquid chromatography (HPLC) has played a great part in enhancing specificity. Bergqvist and Frisk-Holmberg [7] and Staiger et al. [8] demonstrated the practicability of HPLC in assaying chloroquine and some of its metabolites in physiological fluids.

In this report, we describe a simple, specific, and sensitive method, using an ion-pair reversed-phase HPLC procedure for separating and quantifying chloroquine, desethylchloroquine, and bidesethylchloroquine in plasma samples (Fig. 1). A pretreated organic extract of plasma is required before performing the assay. Amounts as low as 5 ng on column of each compound can be quantified, and analysis time is only 12 min per sample. The use of an isocratic system minimizes analysis time by not having to equilibrate the column after each assay.

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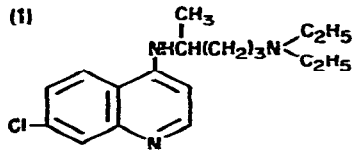
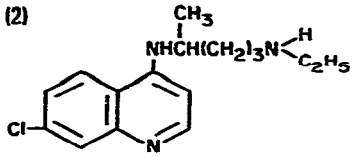
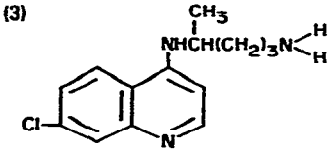


CHLOROQUINE



DESETHYLCHLOROQUINE



BIDESETHYLCHLOROQUINE

Fig. 1. Free base structural formulae of (1) chloroquine, (2) desethylchloroquine, and (3) bidesethylchloroquine.

The employment of this new method is applicable in both the research and clinical laboratories.

EXPERIMENTAL*

Apparatus

The method was developed using a Waters Model ALC/GPC-204 liquid chromatograph. The total system consisted of two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a Model 440 absorbance detector, set at 340 nm, a Houston Instrument Omni-Scribe A5000 dual-pen recorder, and a Columbia Scientific Industries Supergrator-3 integrator.

Reagents

Spectroquality acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) mixed with PIC B-7 reagent (1-heptanesulfonic acid, Waters Assoc., Milford, MA, U.S.A.) was used as the mobile phase. Chloroquine diphosphate (Sigma, St. Louis, MO, U.S.A.), desethylchloroquine sesquioxalate and bidesethylchloroquine hydrobromide (Walter Reed Army Institute of Research's Inventory, Washington, DC, U.S.A.) were used to prepare all working standards. Stock solutions containing 100 ng/μl of each compound were prepared

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in glass-distilled water. Working standards were prepared from the stock standards.

Procedure

A 300 mm × 3.9 mm I.D., 10 μm μBondapak C_{18} column (Waters Assoc.) was used to chromatograph chloroquine and its metabolites in standard solutions and experimental plasma specimens. The mobile phase consisted of 0.02 *M* 1-heptanesulfonic acid and acetonitrile. PIC B-7 reagent was prepared by dissolving 40 ml of the pre-packaged reagent into 460 ml of glass-distilled water. The pH of the solution was 3.4. A pumping ratio of 66:34 of PIC B-7 to acetonitrile was used in an isocratic mode to separate each compound. Flow-rate was 1.0 ml/min. Column pressures ranged between 62 and 76 bar. A 10-μl volume of a methanolic phosphoric acid extract of plasma was introduced onto the column through a continuous flow loop injector. Peak areas and heights were measured and computed with an on-line integrator.

Samples

Plasma specimens were collected from two normal subjects. Each volunteer received a single 300-mg dose of chloroquine diphosphate. Plasma samples were taken before dosing (control) and 12 and 24 h after dosage. A 1-ml sample of plasma and an equal volume of 1 *N* sodium hydroxide were pipetted into a 50-ml screw-capped polypropylene tube. *n*-Heptane (30 ml) was added to the mixture and the tubes were shaken for 30 min. After the extraction, 25 ml of the organic phase, containing chloroquine and its metabolites were placed into a similar type tube and the solution was blown to dryness with streams of nitrogen gas in a 30°C water bath. The residue remaining within the tubes was redissolved into 1 ml of methanol—0.1 *M* phosphoric acid (1:1). The dissolved samples were then transferred to a 3-ml polypropylene screw-capped tube for further concentration. The dried specimens were finally reconstituted with the methanol—phosphoric acid solution to a volume of 100 μl. The samples were analyzed using the previously described HPLC method. An 85% recovery of chloroquine and its de-ethylated metabolites was obtained from spiked specimens. The extraction method used for our sample preparation is a modification of the procedure described by Vogel and Konigk [9].

RESULTS AND DISCUSSION

In recent years, HPLC has proven to be an effective technique for studying the pharmacokinetic actions of various drugs in both man and animal [10,11]. The results of these efforts are demonstrated in the ability of newly developed procedures to enhance knowledge of drug metabolism in various biological systems, based on the utilization of highly specific and sensitive methods. For certain drugs, where the biotransformations of the parent compounds are subtle, previous analytical methodologies were incapable of distinguishing the small differences among chemical structures.

Chloroquine diphosphate is an example of a drug in which its metabolism in man is not completely understood, due to the non-specificity of past method-

ologies. Because of this deficiency, we designed an experiment using a new analytical method in which the metabolic fate of chloroquine could be studied in human subjects. Chloroquine, desethylchloroquine and bidesethylchloroquine were separated without interferences from endogenous compounds present in plasma. The metabolite hydroxychloroquine, which is present in urine, can also be separated using this method.

The application of the method is demonstrated in a series of chromatograms taken from the separation of standard solutions and experimental samples. Fig. 2 represents the separation of a standard containing bidesethylchloroquine, desethylchloroquine, and chloroquine, detected at 0.005 a.u.f.s. Linearity was observed for all concentrations used in this study (5—200 ng). Correlation coefficients for bidesethylchloroquine, desethylchloroquine, and chloroquine were 0.992, 0.989, and 0.978, respectively.

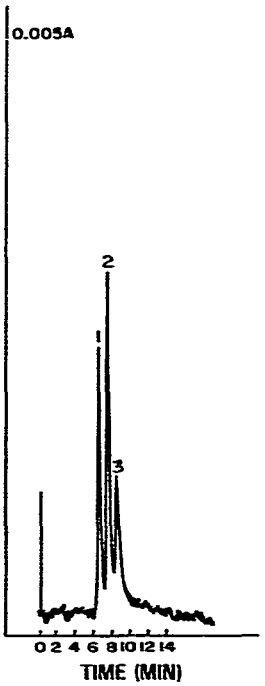


Fig. 2. Separation of a standard solution containing (1) 76 ng of bidesethylchloroquine, (2) 77 ng of desethylchloroquine, and (3) 60 ng of chloroquine. Column: 300 × 3.9 mm I.D. μBondapak C_{18}; mobile phase: 66% PIC B-7 reagent (0.02 *M*) and 34% acetonitrile; flow-rate: 1.0 ml/min; column temperature: ambient.

We analyzed and quantified a series of spiked plasma specimens containing chloroquine and its de-ethylated metabolites. Sample concentration of ten prepared specimens was 84 μg/ml for each compound. Data on within-run precision for chloroquine, desethylchloroquine, and bidesethylchloroquine were 83.2 ± 1.1 (C.V., 1.36%), 82.9 ± 1.3 (C.V., 1.55%), and 82.6 ± 1.3 (C.V., 1.62%) μg/ml, respectively. Day-to-day precision data for the ten spiked plasma specimens as evaluated during a 5-day period were 83.1 ± 1.4 (C.V., 1.42%), 82.7 ± 1.3 (C.V., 1.59%), and 82.2 ± 1.4 (C.V., 1.69%) μg/ml for

chloroquine and its mono- and di-de-ethylated metabolites, respectively. Based on the excellent resolution of the compounds present in the spiked plasma samples, experimental plasma samples collected from the two human subjects were chromatographed. Chromatograms shown in Fig. 3 and 4 depict two time frames of subject A and B. For subject A, three characteristic peaks were observed in the 12-h plasma sample (Fig. 3). Calculating their percentages, chloroquine was present in the sample at 63.1%, followed by desethylchloroquine at 28.3% and bidesethylchloroquine at 8.5%.

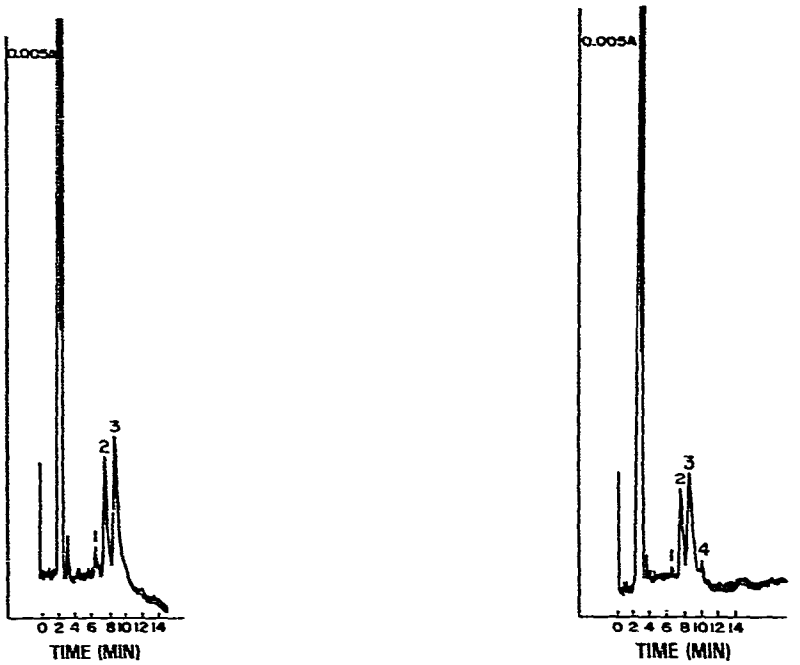


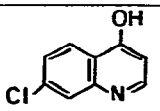
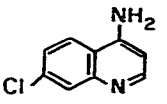
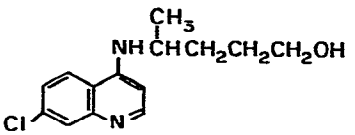
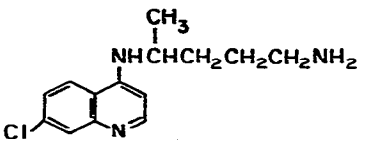
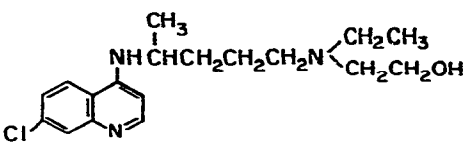
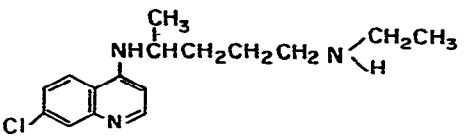
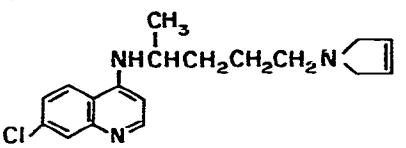
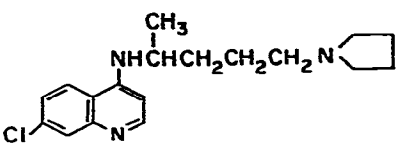
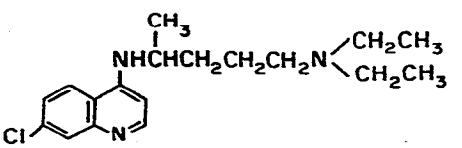
Fig. 3. Chromatogram of a 12-h plasma extract from human subject A. Peaks: (1) bidesethylchloroquine, (2) desethyl chloroquine, and (3) chloroquine. Sample volume: 10 μl; detection wavelength: 340 nm.

Fig. 4. Chromatogram of a 24-h plasma extract from human subject B. Peaks: (1) bidesethylchloroquine, (2) desethyl chloroquine, (3) chloroquine, and (4) unknown. Sample volume: 10 μl.

The chromatogram shown in Fig. 4, representing subject B, displayed a somewhat different profile. The total peak area for the same assayed volume of subject B sample was 1/3 less than that of subject A. Whereas three peaks were observed in the 12-h specimen, the 24-h specimen showed four peaks. Chloroquine again was the major compound present in the sample. The percentages for the assayed sample were 52.3% for chloroquine, 29.8% for desethylchloroquine, 2.9% for bidesethylchloroquine and 14.9% for an unknown compound. The retention time of this unknown metabolite did not match any of the retention times obtained for the 7-chloro-4-substituted derivatives shown in Table I. Additional information, using alternative analytical techniques is presently being obtained to characterize and identify the unknown metabolite.

TABLE I

7-CHLORO-4-SUBSTITUTED DERIVATIVES

Retention time (min)	Structure	Compound
4.93		7-Chloro-4-hydroxyquinoline
5.59		4-Amino-7-chloroquinoline
6.93		4-(4′-Hydroxy-1′-methylbutylamino 7-chloroquinoline
7.17		Bidesethylchloroquine
7.94		Hydroxychloroquine
8.39		Desethylchloroquine
9.06		7-Chloro-4[4′-(3-pyrrolidino)-1-meth butylamino]quinoline
9.47		4-(4′-Pyrrolidyl-1-methylbutylamino 7-chloroquinoline
9.62		Chloroquine

From this study, we demonstrated the applicability of a new analytical methodology for observing the metabolic fate of chloroquine in humans. Because of the advantages of this technique, further comprehensive studies are planned using various mammalian species in order to determine similarities and differences in the metabolism of chloroquine in mammals.

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