A Radioimmunoassay for the Antimalarial Drug Chloroquine

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We describe a 3H-based RIA for the antimalarial drug chloroquine (CLQ), the most commonly used antimalarial drug. In the assay a monoclonal antibody is used that is directed against 4-amino-7-chloroquinoline conjugated to keyhole limpet hemocyanin by the glutaraldehyde method. Besides CLQ, this antibody also recognizes with good affinity the 4-aminoquinoline homologs, hydroxychloroquine and amodiaquine. No extraction step or sample preparation is required, and the method can detect as little as 10 μg/L, the lower concentration in plasma of humans who are taking CLQ as a preventive measure. The between-assay CV is <10%, the within-assay CV <3%. Results correlate with those by liquid chromatography (r2 = 0.96). The speed and simplicity of the RIA method make it useful in evaluating the CLQ concentrations in acutely toxic patients.

Additional Keyphrases: malaria · intestinal parasites · monoclonal antibodies · liquid chromatography · monitoring therapy · pharmacokinetics · toxicology

Chloroquine [CLQ; 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline], a 4-aminoquinoline derivative (Figure 1), is used worldwide as an antimalarial drug (1) and to treat other tropical affections such as lambliasis and chronic rheumatoid disease (2). Prolonged administration can produce severe effects, including cerebral, dermatologic, myopathic, neuropathic, and psychiatric disorders, irreversible retinopathy, and maculopathy (3–5). Severe overdoses of this drug can be life threatening (6, 7). Acute toxicity may lead to cardiac and (or) respiratory arrest (8, 9). Although doses exceeding 2 g are considered potentially dangerous for adults (7), patients have been rescued after ingestions of up to 10 g (10). Nevertheless, the prognosis is dose-dependent and assay of the drug’s concentration in blood is helpful for monitoring patients. Rapid assays are especially important, the most critical phase after ingestion being the first several hours (7); moreover, there is a significant difference between the toxicokinetic and the pharmacokinetic parameters (11).

Although CLQ has been used since the late 1940s, no simple, rapid, sensitive, and specific analytical method for it has been developed (12). Generally, CLQ is routinely determined, after an extraction step, by its absorbance at 343 nm (13); using a fluorimetric detection procedure improves the sensitivity of the method (14). Chromatographic procedures with either spectrophotometric or fluorimetric CLQ detection have been developed (15–18) to increase the specificity for and the quantification of different CLQ metabolites. However, these methods still involve an extraction step, which keeps the assay somewhat complicated and is an easy source of error. To develop a rapid immunoassay for CLQ, we have thus produced monoclonal antibodies (Mab) directed against CLQ, as described elsewhere (19), based on the recognition of the 7-chloro-4-aminoquinoline (ACQ) moiety plus the bridge used to link hapten and carrier in the immunogen. This approach is briefly summarized below.

Materials and Methods

Materials: CLQ, ACQ, and glutaraldehyde solution (250 mL/L) were supplied by Rhône-Poulec, Saint Fons, France; hydroxychloroquine (HCLQ) by Winthrop Laboratories, Longvic, France; amodiaquine (ADQ) by Roussel-Uclaf, Paris, France; mefloquine by Roche, Neuilly, France; [3H]CLQ (specific activity, 82.2 kCi/mol) and Biofluor scintillation fluid by New England Nuclear, Boston, MA; the CMECDI by Fluka, Buchs, Switzerland; bovine serum albumin (BSA) and quinine by Merck, Darmstadt, F.R.G.; succinic anhydride, keyhole limpet hemocyanin (KLH), o-phenylenediamine, dextran, and activated charcoal by Sigma Chemical Co., Saint Louis, MO; gelatin by Vahiné, Aubignan, France; and triethylamine by J. T. Baker Chemicals, Deventer, The Netherlands. All other reagents were of the best grade available.

Apparatus: Radioactivity was counted with a liquid scintillation counter (Model LS 7000; Beckman Instruments, Irvine, CA). The ELISA plates (Nunc, Roskilde, Denmark) were read in a Titertek Multilkan MC (Etabl, Helsinki, Finland). Protein was determined by using a protein assay from Bio-Rad Laboratories, München, F.R.G. Liquid-chromatographic assays were performed with use of a Beckman microflow-114 pump equipped with a sample valve (no.
7125) from Rheodyne, Cotati, CA. The 250 × 6.4 mm normal-phase column was packed with Microporasil (7 µm) from Waters Associates, Bedford, MA. The absorbance from the effluent was measured with a spectrofluorimeter (JY-3; Jobin-Yvon, Paris, France).

**Plasma samples:** Heparinized blood was centrifuged (2500 × g, 15 min) within 1 h of collection, and the plasma was transferred to polystyrene tubes and stored frozen at −22 °C. Standards were prepared by adding exact quantities of the drugs to pooled fresh plasma that we obtained from a blood bank and had previously tested for the absence of CLQ and its derivatives.

**Anti-CLQ Mabs:** The description of how the antibodies were obtained has been previously reported elsewhere (19). Briefly, the immunogen consists in a conjugate between ACQ and KLH, obtained via the glutaraldehyde method. Another conjugate, prepared by coupling HCLQ-hemisuccinate with BSA via the CMEDCI method, was used for the screening of antibodies by ELISA. After a six-week immunization schedule performed on high-responder Biozzi mice, two Mabs, termed ACQ-01 and ACQ-02 were chosen on the basis of their high affinity constants. ACQ-01, showing the highest affinity constant (3.1 × 10^8 L/mol vs 5.6 × 10^7 L/mol for ACQ-02), was selected for use in the clinical RIA.

**RIA:** Pipet into 7.5 × 1.2 cm polypropylene tubes 100 µL of a solution containing 0.25 pg (25000 counts/min) of [3H]CLQ in phosphate buffer (10 mmol/L, pH 7.4). Add 300 µL of samples or standards (20, 40, 80, 160, 320, and 480 µg/L of CLQ, calculated as CLQ base). Mix and add 100 µL of the ACQ-01 preparation (diluted 500-fold). Mix and incubate for 1 h at room temperature. Cool to 4 °C and add 300 µL of cold dextran-coated charcoal: 12 g of charcoal and 1.2 g of dextran and gelatin, each in phosphate-buffered saline (phosphate 10 mmol/L, NaCl 150 mmol/L, pH 7.4). Mix immediately and centrifuge (5000 × g) for 15 min at 4 °C. Transfer 600 µL of the supernate to a vial that contains 7 mL of scintillation cocktail, and count the radioactivity. Assay all standards and samples in triplicate.

**Liquid chromatography:** To 1 mL of plasma in a capped tube add 1 mL of 1 mol/L sodium hydroxide and 30 µL of an aqueous 1 g/L solution of quinine, used as an internal standard. Add 10 mL of hexane to this alkaline aqueous phase, and vortex-mix for 30 s. Once the phases have separated, decant the hexane layer and evaporate it, under a gentle stream of nitrogen, on a 40 °C water bath. Rinse the tube twice with 1 mL of hexane and, after a second evaporation step, dissolve the residue in 100 µL of the mobile phase (acetonitrile/methanol/diethylamine, 80/19.5/0.5 by vol). Inject 50 µL of the dissolved residue onto the normal-phase column (flow rate 3 mL/min; pressure 7 MPa). Set the excitation and emission wavelengths for fluorimetric detection to 335 and 390 nm, respectively.

**Results**

**Cross-reactivity studies:** To determine the structure recognized by the two Mabs, we examined the inhibition of the [3H]CLQ binding by different homologs or metabolites of the drug (Figure 2). There was complete cross-reactivity between CLQ and its hydroxy derivative, HCLQ. The major metabolite of both drugs, DCLQ, competes similarly for the antibodies. Another metabolite, ACQ, which was used for immunization, showed a 1000-fold lower affinity than CLQ. ADQ, which contains an aromatic cycle in its side chain, possessed the highest apparent affinity constant. Two other quinoline-like antimalarial drugs did not react with either Mab, indicating that the antibodies are specific for the 4-amino-7-chloroquinoline structure.

**Precision of the RIA method:** The within-assay CV was calculated by comparing means of triplicates from five separate assays. In the range of concentrations usually found in plasma, the CV varied from 2.1 to 3.0% (Table 1). The between-assay CV and the analytical recovery were determined for CLQ and DCLQ, as summarized in Table 2.

**Comparison with a liquid-chromatographic procedure:** Twenty plasma samples obtained from patients who were acutely overdosed with CLQ were assayed by the CLQ-RIA and by the liquid-chromatographic method described above. If necessary, samples were diluted to give a CLQ concentration ranging between the lower and the higher limit of the method (i.e., 10 to 500 µg/L). Figure 3 shows the results of this comparison.
Discussion

CLQ concentrations in blood are generally determined by spectrophotometric or spectrofluorimetric methods, recently improved by combining them with chromatographic procedures (17). However, an extraction step is required, and hence, although improved, this methodology is too complex to be used in most routine laboratories.

The present method is based on an immunological approach. Mabs directed against CLQ were raised in mice, after immunization by a metabolite, ACQ, coupled to a carrier by a glutaraldehyde bridge. Owing to this procedure of obtaining the immunogen, the epitope of the Mabs includes specifically the quinoline nucleus and nonspecifically its side-chain substitutions. Binding results clearly indicate that 4-aminooquinoline antimalarial drugs (CLQ, HCLQ, and ADQ) are more reactive than ACQ. The complete cross-reactivity of DCLQ with CLQ and HCLQ demonstrates that the N-desalkylation of the side-chain, occurring during the metabolism of the drug, will not modify the recognition. In other words, the RIA determines both CLQ and its active metabolites, and results better correlate with the clinical toxicity than do results for the isolated CLQ fraction (7, 14). The specificity of the Mabs is demonstrated by the lack of cross-reactivity towards unrelated antimalarial compounds (quinine, mefloquine).

The precision of the RIA is comparable to that found in recent liquid-chromatographic methods (within-assay CV <3%). Analytical recovery is good for concentrations of CLQ and (or) DCLQ ranging from 10 to 500 μg/L. However, incomplete recovery is observed for CLQ concentrations above 500 μg/L, owing to the relative imprecision of the measure at these concentrations. Therefore, samples must be diluted to CLQ concentrations that are within the optimum range, 10–500 μg/L. The between-assay CV is always <10%, the highest values being encountered for concentrations <40 μg/L. Thus, the RIA may be helpful for evaluating plasma CLQ concentrations, with a lower limit of sensitivity of 10 μg/L, and covering the range of concentrations generally achieved in humans who are taking CLQ prophylactically (20, 21).

When comparing the results obtained by the CLQ-RIA with those by liquid chromatography with fluorimetric detection, it should be noted that the latter method separately determines the drug and its major metabolite, whereas the RIA detects the sum of drug and metabolites. Figure 3 indicates the good correlation observed (r² = 0.96), owing to the fact that CLQ and DCLQ together represent about 95% of the circulating forms of the drug (14). In the context of CLQ acute overdoses, it is useful to determine quickly the concentration of the toxic compounds (i.e., CLQ + DCLQ), with regard to the marked toxicity of the N-desethyl metabolite (22, 23). Thus, the RIA appears to be superior to the laborious and lengthy chromatographic techniques.

Assaying CLQ still remains a challenge because of the increasing number of CLQ acutely toxic patients (24) and to the rapid dissemination of CLQ-resistant plasmodium (25, 26). To demonstrate whether our immunological method can be applied to epidemiological studies, it is necessary to avoid expensive materials such as radionabeled compounds. Furthermore, the method must be applicable to whole-blood samples because CLQ accumulates in erythrocytes (2, 27). Studies are in progress to modify our ELISA, initially used in the antibody screening, to adapt it for this expanded use. Such a test could therefore meet the requests recently made by the World Health Organization.

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References


