POLARISATION FLUOROIMMUNOASSAY FOR QUININE IN SERUM AND URINE

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INTRODUCTION

Quinine is one of the oldest drugs in the pharmacopoeia and is still widely used both for the prevention of night cramps and the treatment of chloroquine-resistant falciparum malaria (Black *et al.*, 1981). Because of its narrow therapeutic window, measurement of serum concentration is important when using this drug. Detection of quinine and/or its metabolites in urine may also be of value in assessing compliance, especially in the developing world where many fail to understand the need for regular and continued medication. In the United Kingdom, where it is mainly used for treatment of nocturnal cramps and occasionally as an illicit abortifacient or narcotics filler, quinine poisoning due to overdose is becoming an increasing problem (Bateman *et al.*, 1985). Therefore, a rapid, simple and reliable method for assaying quinine in biological fluids is required.

Various quantitative analytical methods are available including a benzene or toluene extraction fluorescence technique (White *et al.*, 1983), high-performance liquid chromatography (Edstein *et al.*, 1983), gas chromatography/mass spectrometry (Furner *et al.* 1981), and immunoassays (Morgan *et al.*, 1985). Recently we described a fluoroimmunoassay for quinine using fluorescein-labelled drug as tracer and antiserum covalently linked to magnetisable solid-phase particles to facilitate the separation step (Sidki *et al.*, 1987). Here we report our development and validation of a polarisation fluoroimmunoassay (PFIA) which does not require a separation step. The assay was further simplified by exploiting the relatively rapid dissociation kinetics of the hapten-antibody complex which enables the tracer and the antiserum to be employed as a single, premixed reagent (Colbert *et al.*, 1984).

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MATERIALS AND METHODS

Reagents: Quinine, quinidine, chloroquine, trizma hydrochloride and trizma base were obtained from Sigma, Poole, Dorset, U.K.; Triton X-100 surfactant, sodium dodecyl sulphate, sodium tetraborate, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium azide, and all organic solvents ("Analar" grade) were from BDH Chemicals, Poole, Dorset, U.K. Halofantrine and mefloquine were gifts from the Walter Reed Army Institute of Research, Walter Reed Army Medical Centre, Washington DC 20307.

Fluorescein-labelled quinine: Quinine was reacted with dichlorotriazenyl aminofluorescein, the reaction mixture was purified by TLC, and the concentration of the pure tracer was estimated spectrophotometrically as described previously (Sidki *et al.*, 1987).

Quinine antisera: Quinine-9-hemisuccinate was conjugated to keyhole limpet hemocyanin and the conjugate was used to immunise three ewes as described before (Sidki *et al.*, 1987). Antiserum from the eleventh monthly bleed of sheep 27 was used in the studies described below.

Assay standards: A stock solution of quinine (1 g/l) in methanol was diluted to 1, 2, 5, 10 and 20 mg/l in drug-free pooled human serum and the standards stored at –20°C. The working urine standards were prepared by adding quinine (10 mg/l) in distilled water to synthetic urine (Sidki *et al.*, 1987) or drug free urine to obtain the following quinine concentrations: 0.1, 0.2, 0.5, 1 and 2 mg/l.

Serum digesting reagent: Serum samples and serum-based standards were diluted 10-fold with pepsin (40 mg/l) in 0.1M HCl in order to digest serum proteins prior to assay.

Single dose pharmacokinetic study: A single oral dose of 900 mg of quinine sulphate (three tablets) was adminstered to a volunteer and urine samples collected at regular intervals over three days. The quinine levels were measured by the present PFIA and by the benzene extraction fluorescence method (White *et al.*, 1983).

Assay diluent buffer used was Tris buffer (100 mmol/l, pH 7.4) containing 1 g/l of gelatin and 1 g/l sodium azide. Assay tubes used were disposable 50 × 10 mm round glass cuvettes (no. 9518), from Abbott Diagnostic Division, Basingstoke, Hampshire, U.K.

Polarisation fluorimeter used was Model 4000 polarisation fluorimeter (SLM Instruments, Urbana, IL 61801), as described previously (Sidki *et al.*, 1982), except that the sample compartment was fitted with an adaptor to accept the cylindrical cuvettes.

Antibody Dilution Curves: To aliquots (1 ml) of doubling dilutions of anti-quinine serum or non-immunised sheep serum was added 0.5 ml of fluorescein-labelled quinine (30 nmol/l) or 0.5 ml of diluent buffer (for the determination of the serum background signal). After incubation for 30 min at room temperature, fluorescence polarisation was measured with background correction (Sidki *et al.*, 1982).

Polarisation Fluoroimmunoassay: Assays were performed at room temperature in duplicate. All serum specimens or serum-based standards were initially treated with the digesting reagent. For the conventional assay procedure, 10 µl of urine or 10 µl of treated erum was mixed with 0.5 ml of labelled quinine (30 nmol/l) followed by 1 ml of antiserum (2,000-fold diluted). After incubation for 30 min, fluorescence polarisation was measured. For the single-reagent procedure, labelled quinine (10 nmol/l) and antiserum (3,000-fold diluted) were premixed indiluent buffer and the bulk mixture stored in the dark at 4°C until used. To 1.5 ml of the single reagent was added 10 µl of urine or treated serum and after 2 h incubation, fluorescence polarisation was measured.

RESULTS

Assay optimisation

Choice of buffer and additive: The fluorescence polarisation of the quinine label was affected by the buffer constituents, the pH and the type of additive added to prevent adsorption of the label to the cuvette wall. We monitored the fluorescence intensity of quinine label in three different buffers, namely, sodium phosphate (100 mmol/l, pH 7.4), Tris (100 mmol/l, pH 7.4), and sodium borate (100 mmol/l, pH 9.0) over 4 h with various additives included. Bovine albumin (1 g/l) and the non-ionic detergent Triton X-100 (1 mL/l), were unsuitable because high polarisation values indicated non-specific binding of the labelled drug. With an equimolar mixture of Triton X-100 (220 mg/l) and SDS (100 mg/l) in Tris buffer, the fluorescence intensity of the quinine label was stable over 4 h and the polarisation signal was resonably low (80 mP). Using gelatin (1 g/l) in Tris buffer, similar results for the fluorescence intensity and polarisation signal (90 mP) were obtained.

Protein effect: Due to the presence of various proteins in serum samples, high polarisation readings were obtained following the addition of quinine tracer due to its non-specific binding. Pepsin present in the pretreatment reagent digested serum proteins and therefore prevented the spurious high polarisation readings. Urine specimens contain negligible amounts of proteins and no pretreatment was necessary when using up to 20 µl of urine.

Anti-quinine sera: All three sheep produced high titre antisera working at dilutions ranging from 1:3000 to 1:5000 in the PFIA system. They possessed slightly different cross-reactivity and affinity to quinine and its congeners. There was no significant non-specific binding of the fluorescein-labelled quinine as determined using normal sheep serum as control (Fig. 1.)

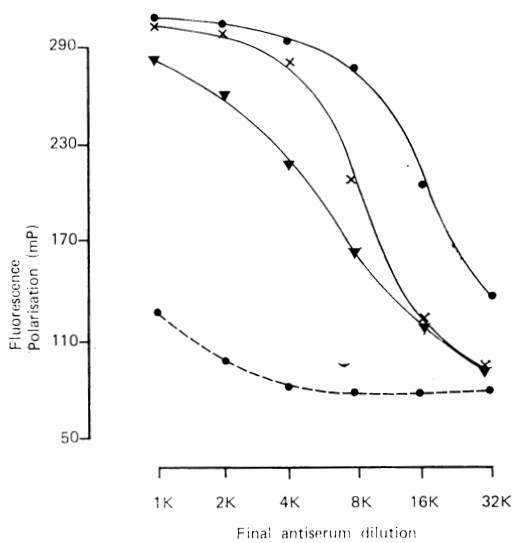

Fig. 1—Dilution curves of anti-quinine sera obtained from the fourth bleed of sheep 26 (●) and 28 (×) and the eleventh bleed of sheep 27 (▲) and with a normal sheep serum (●....●) $K = 10^3$

Standard curve: Fig. 2 shows the standard curves obtained with both the conventional and single-reagent methods using both urine

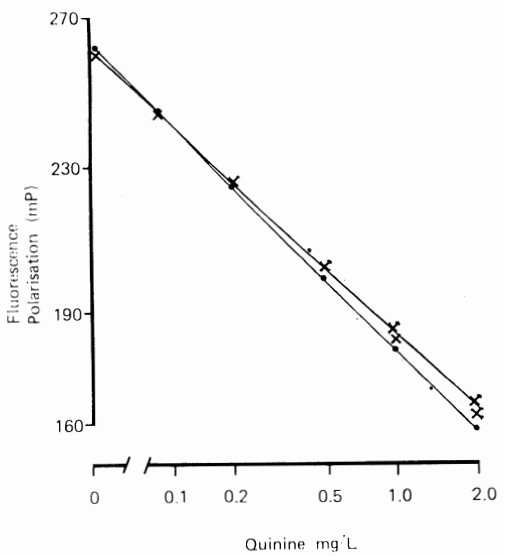

Fig. 2—Standard curves obtained using the conventional (●) and single-reagent (×) polarisation fluoroimmunoassay for quinine in serum or urine.

standards and 10-fold diluted serum standards The serum assay covered the sub-therapeutic, therapeutic, and toxic levels of quinine while the urine assay was sufficientlly sensitive to quantitate quinine in urine.

Assay kinetics

Association kinetics: The association kinetics of the fluorescein-labelled quinine with the quinine antibody was investigated by obtaining serial polarisation readings after adding 0.5 ml of tracer (30 nmol/l) to 1 ml of the antiserum (diluted 2,000-fold). The polarisation readings increased rapidly and reached a plateau within 1 min.

Dissociation kinetics: The rate of dissociation was also investigated by premixing the antiserum (1 ml of a 2,000-fold dilution) with 0.5 ml of the fluorescein-labelled quinine (30 nmol/l) for at least 2 h at room temperature and then adding excess quinine (50 µl of 1 g/l solution) and taking a series of polarisation readings. These were plotted against time on

semi-logarithmic paper and a straight line corresponding to the antibody population with the slowest dissociation rate (polarisation half-life about 2 h) was drawn. The difference in polarisation units between this line and the points of the early part of the displacement was then plotted (Fig. 3) and from the straight line obtained, the polarisation half-life for the fast-dissociating antibody population was calculated as 5 min.

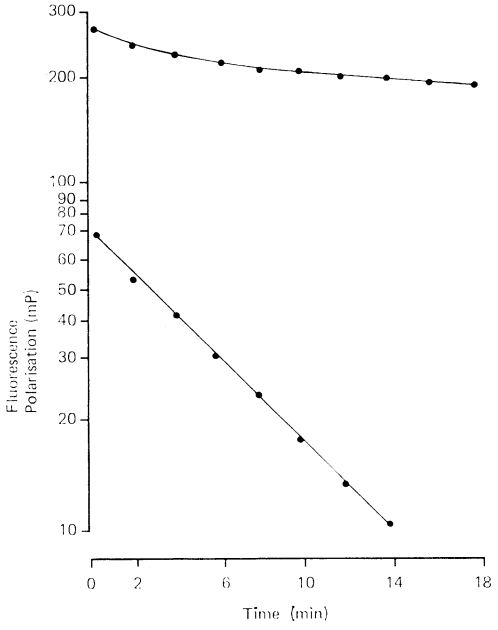

Fig. 3—Tracer dissociation kinetics (upper line) and the kinetics of the fast-dissociating antibody population of the anti-quinine serum (lower line) monitored by fluorescence polarisation.

Incubation time: With serial conventional PFIA, it was found that equilibrium was reached within 5 min and the standard curve remained stable for over 1 h. Addition of the standards to premixed reagent required a longer incubation time (2 h) to reach equilibrium.

Quinine excreted in urine: Fig. 4 shows the amount of quinine in mg excreted per mmol creatinine in each sample. Quinine and its metabolites appeared in urine within 2 h with the peak level at 4 h. The curve then shows an exponential decrease but quinine was still detectable after three days. The urinary clearance half-life of quinine and its metabolites as detected by the present assay and calculated from Fig. 4 was 8.2 h. The amount of total quinine recovered in the urine during the three days period represents 49% of the administered does.

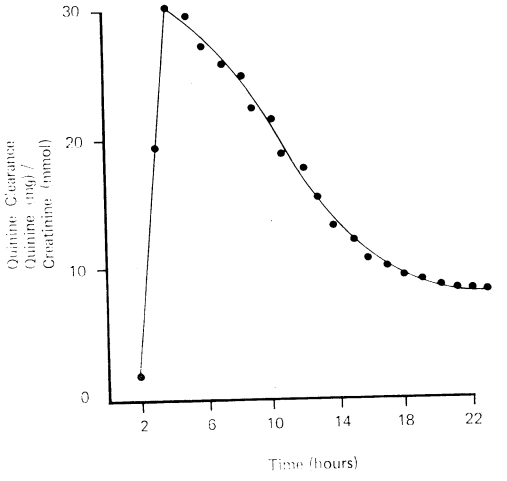

Fig. 4—Renal clearance of quinine shows first order kinetic in normal subject (male, 26 year old) given an oral single dose of 900 mg quinine sulphate.

Assay validation

Sensitivity: We calculated the standard deviation (SD) of the polarisation readings from 20 replicates of a zero standard. The minimum detectable concentration of quinine at the 95% confidence level (Rodbard, 1978) was 0.05 mg/l.

Precision: Quinine in methanol (1g/l) was added to pooled normal human serum or urine to obtain low, medium, and high concentrations. Each was assayed 10 times in duplicate in one assay and on 10 different days. Within-assay coefficients of variation (CVs) were 6.0, 5.4 and 2.9% for serum

samples and 3.2, 3.1 and 7.6% for urine samples at mean quinine levels of 0.3, 0.7 and 1.5 mg/l, respectively. Between-assay CVs were 8.0, 6.5 and 5.0% for serum samples and 4.2, 9.3, and 9.4% for urine samples at the same levels.

Recovery: Quinine was added at concentrations of 0.3, 0.7 and 1.5 mg/l to pooled normal human sera. Analytical recovery by PFIA was 102.9, 101.6 and 98.4%, respectively. Corresponding recoveries for quinine added to pooled human urine were 99.0, 102.0 and 104.0%.

Specificity: No cross-reaction was observed with other major anti-malarial drugs including primaquine, chloroquine, halofantrine, and mefloquine or their main metabolites at levels up to 50 mg/l. Quinidine, the optical isomer of quinine, did not show any interference in the present assay at a concentration of 50 mg/l. The cinchona alkaloid cinchonidine (demethoxy quinine) cross-reacted 4.0% with antisera obtained from sheep 26 and 27. Sheep 28 showed 87% cross reaction with this alkaloid indicating that most of its antibody populations are not directed against the quinoline ring of the molecule.

The present assay was used to determine quinine levels ranging from 6 to 115 mg/l in 26 urine specimens in which quinine concentrations were also determined by the benzene extraction fluorescence method (White *et al.*, 1983). The regression equation relating PFIA (y) and fluorescence method (x) results was $y=1.2x-0.2$ mg/l, with $r=0.96$, calculated on the assumption that the two methods have equal precision characteristics (Cornbleet and Gochman, 1979).

DISCUSSION

In developing assays for antimalarial drugs one should note their application in third world countries as well as in the developed world. Chromatographic methods such as gas-liquid and high-performance liquid chromatography, although reliable and accurate, require sophisticated instruments and skilled personnel to operate and maintain them. By contrast, once developed, an immunoassay can be easily manipulated to suit its intended application.

Development of an immunoassay for a hapten involves lengthy and complicated procedures starting with the preparation of a suitable derivative followed by conjugation to a carrier protein to make the immunogen. An appropriate species is then immunised according to a selected protocol and serum collected for assessing the presence of antibodies. Such assessment normally requires the production of labelled hapten with a close resemblance to the unlabelled molecule which can be quantitated easily (Chard, 1982). The use of tritiated hapten labels fulfill these criteria. However, because of the complexity and costs involved in making and keeping radioactive tracers and the cost and time required to count tritium, we have adopted an alternative approach for several years. This involves making fluorescein-labelled hapten and using polarisation of fluorescence for end-point detection (Sidki and Landon, 1985). These labels are easy to prepare and purify and are very stable with a shelf-life of many years. The assay only requires the mixing of standard or sample with the assay reagent (tracer and antibody) followed by a a brief incubation and polarisation measurement. The only obstacle, until recently, was the need for a simple and reliable instrument to perform the polarisation measurements.

With the availability of fully automated polarisation instrument from Abbott (Diagnostic Division, Basingstoke, Hampshire, U.K.) and more recently the PF1-20 system from Perkin-Elmer (Beaconsfield, Bucks., U.K.), polarisation fluoroimmunoassay is becoming popular for therapeutic drug

monitoring and the detection of drugs in general. Although we used a complex scientific instrument (Model 4000 polarisation instrument from SLM) for developing the present assay, it is easily adopted to any polarisation fluorometer once the antibody and tracer are available.

The present PFIA is simple to perform on serum samples from patients on quinine in order to maintain the drug concentration within therapeutic levels. When applied to urine samples collected from a volunteer following a single oral dose, results showed that the drug and its metabolites were still detectable after three days (3.7 mg/l). Values were expressed per mmol of creatinine to overcome urine clearance fluctuations. Urinary assays could also be used to determine compliance.

SUMMARY

The development and validation of a polarisation fluoroimmunoassay for the antimalarial drug quinine is described. The assay is performed either by sequential addition of the reagents or by a single-reagent technique whereby the tracer and antibodies are premixed. Serum samples require pepsin digestion prior to assay while urine specimens are assayed directly. The reliability criteria of the assay are satisfactory and no cross-reaction is detected with quinidine (the optical isomer of quinine) or with common antimalarial drugs. The assay was applied to the measurement of quinine in urine specimens obtained from a single-dose pharmacokinetic study and the results correlated with those of the benzene extraction fluorescence method for quinine measurement.

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