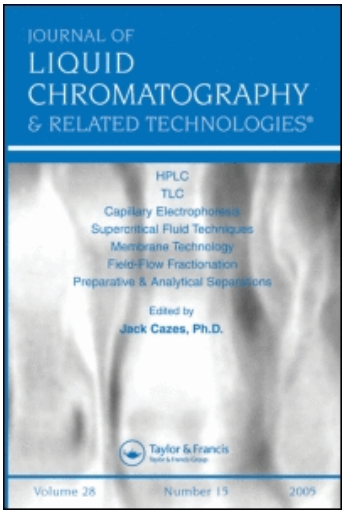

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SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF QUININE IN HUMAN PLASMA WITHOUT EXTRACTION

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ABSTRACT

A simple, rapid and sensitive assay, capable of quantitating quinine (Q) in human plasma samples is reported. The assay uses a reversed-phase C18 HPLC column packed with 5 μm ODS Hypersil. The chromatographic separation was accomplished with an isocratic mobile phase comprising acetonitrile-aqueous phosphate buffer pH 2 (50:50, v/v) containing 25 mM sodium dodecyl sulfate and 3 mM tetrabutylammonium bromide at a flow rate of 0.5 ml/min. The eluant was monitored by a fluorescence detector (excitation wavelength at 350 nm and emission wavelength at 450 nm). The assay was based on a simple plasma protein precipitation technique. To 200 μl of plasma sample, 400 μl of internal standard (cinchocaine 30 μg/ml in methanol) was added. After brief vortexing and centrifugation, the clear supernatant was injected onto the HPLC column. The inter- and intra-assay coefficients of variation were found to be less than 10%. The lowest limit of detection for Q in plasma was 18 ng/ml.

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INTRODUCTION

Quinine is considered one of the drugs of choice for the treatment of acute attacks of malaria in areas such as Southeast Asia, South America, East Africa and Western Pacific islands where *Plasmodium falciparum* is resistant to chloroquine (1 - 3). Despite the fact that quinine is one of the oldest drugs in the pharmacopeia and the most widely used antimalarial drug, there is relatively limited information on its pharmacokinetics and drug interactions (3,4). In humans approximately 20% of an oral dose is excreted unchanged in the urine (1 - 3). Its elimination half-life in healthy subjects and patients with falciparum malaria is highly variable ranging from 3.0 to 47.2 hours (2). A mean plasma concentration of 2 to 5 μg/ml is probably necessary to reduce parasitaemia in acute malaria and plasma concentrations above 10 μg/ml are considered toxic (1).

A much clearer elucidation of the pharmacokinetics and metabolism of quinine is required. This can be achieved with a specific assay for the determination of plasma and urine concentrations of both parent drug and its metabolites. In contrast to quinidine, only limited number of assays to quantitate quinine in plasma are reported. Non-specific assays including single and double extraction-fluorescence methods (5-8) are available for quinidine and quinine. However, they all suffer from the fluorescent contributions from either metabolites or unknown constituents of the plasma which often lead to spurious estimates of quinine concentrations. Thin-layer chromatographic methods (9-11) are specific assays, but they are tedious and time-consuming and, hence, inconvenient for the routine processing of large numbers of samples.

The published high-performance liquid chromatographic (HPLC) methods have offered rapidity, specificity and sensitivity for analysis of quinidine and quinine in plasma samples. These methods included the use of a normal-phase column with alkaline extraction and either UV detection (12) or post-column acidification and fluorescence detection (7). Reversed-phase HPLC methods with direct injection of plasma (13) or urine (14) are also reported as are injection of a supernatant obtained after plasma protein precipitation (15,16) or extraction of the plasma samples (17-20). Most of these methods are tedious. Some lack sensitivity and do not use internal standards.

The present report describes a rapid, simple, sensitive and specific HPLC method for the determination of quinine in human plasma. This method involves a simple plasma protein precipitation procedure, requires no extraction steps and employs an internal standard.

MATERIALS AND METHODS

Reagents and Chemicals

All chemicals were of analytical grade. Quinine (Q) bisulfate was kindly supplied by Kimia Pharma, Indonesia. Cinchocaine hydrochloride was obtained from Orgamol Evionnaz, Switzerland. HPLC-grade methanol and sodium dodecyl sulfate were purchased from BDH Limited (Poole, England). Tetrabutylammonium bromide was obtained from Sigma Chemicals Co (St Louis, MO, USA). Water was double glass distilled and MilliQ® filtered. All glassware was cleaned and silanized with 0.5% Aquasil ® (Pierce, Rockford, IL, USA) before use.

A stock solution containing 1 mg/ml of quinine free base was prepared in 50% (v/v) methanol-water. The internal standard stock solution of cinchocaine (1 mg/ml) was prepared in pure methanol. This solution was then stored at -20°C until required and renewed every week. Plasma standard solutions containing known concentrations of Q were prepared by appropriate dilution of the stock Q solutions with drug-free plasma. The internal standard solution of cinchocaine (30 μg/ml) was prepared by dilution of the stock solution with methanol and prepared freshly at each day of analysis. All quinine plasma standards were freshly prepared each day of analysis.

Analysis Procedure

A protein precipitation technique was used in the preparation of plasma standards and samples. To 200 μl of plasma sample or standard in a 1.5 ml plastic Eppendorf ® microcentrifuge tube, 400 μl of the internal standard solution (cinchocaine 30 μg/ml in methanol) was added. The mixture was vortexed for 10 seconds and then centrifuged at 2000 g for 3 minutes. 20 μl of the clear supernatant was injected onto the HPLC column. The concentrations of Q in samples were determined from calibration plots of the chromatographic peak height ratios (Q/cinchocaine) versus concentration.

Chromatographic Conditions

The HPLC system consisted of an LKB 2150 pump (LKB, Stockholm, Sweden) connected to a Waters 712 autoinjector (Milford, MA, USA). The fluorescence detector used was a Shimadzu RF 540 (Shimadzu, Kyoto, Japan) equipped with a 12 µl HPLC flow cell. The excitation wavelength was set at 350 nm (slit width 10 nm) and the emission wavelength at 450 nm (slit width 20 nm). The chromatographic response was recorded by a Shimadzu R3A integrator. The HPLC column was 100 x 2 mm I.D. packed with a reversed phase C18 material, 5 µm ODS Hypersil (Shandon, Southern London, UK). Analysis of the samples of Q was performed using a mobile phase consisting of an acetonitrile-aqueous phosphate buffer (10 mM) mixture (50:50, v/v) containing 25 mM sodium dodecyl sulfate (SDS) and 3 mM tetrabutylammonium bromide (TBA) and adjusted to pH 2 with orthophosphoric acid. The flow rate of the mobile phase was 0.5 ml/min (back pressure approximately 110 bars). Chromatographic separations were performed at room temperature.

RESULTS AND DISCUSSION

The C18 bonded stationary phase is the most popular used in reversed-phase liquid chromatography. ODS-Hypersil, being a typical modern capped octadecyl stationary phase material, was thus selected as the stationary support. As the experiments were conducted in a constant temperature environment, the remaining important factors that govern the retention and separation of basic drugs such as Q in the reversed-phase HPLC system were the organic modifier concentration and the pH of the mobile phase. Q has two pKas (4.1 and 8.4) and is ionized in the acidic and neutral solvents commonly used in reversed-phase HPLC. In this study, retention of Q was achieved by the addition of the anionic hydrophobic pairing ion sodium dodecyl sulfate (SDS), to the mobile phase at low pH. The organic modifier concentration, the pH and ionic strength of the mobile phase were empirically optimized as previously described (21,22). The variation of the capacity factor (k') of Q and the internal standard cinchocaine as a function of the mobile phase SDS concentration is shown in Figure 1A. Both cationic solutes go through the predicted maxima. Such chromatographic behavior can be adequately

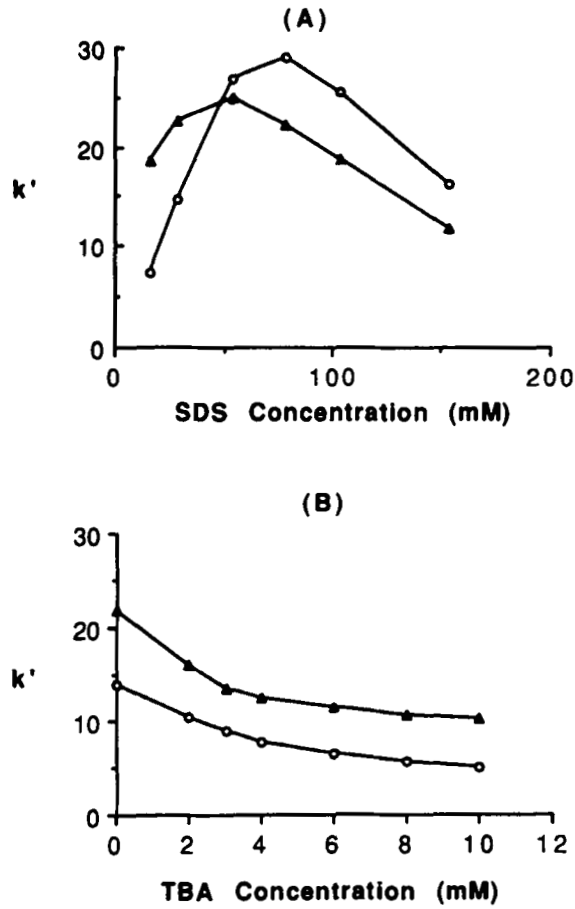

FIGURE 1

Variation in capacity factor (k') of quinine (O) and the internal standard cinchocaine (▲) as a function of sodium dodecyl sulfate, SDS concentration (**A**), and as a function of tetrabutylammonium bromide, TBA concentration (**B**). The mobile phase for Figure 1(A) was 50% (v/v) acetonitrile and 10 mM phosphate buffer (pH2); the flow rate was 0.5 ml/min. The mobile phase used in Figure 1(B) was 50% (v/v) acetonitrile and 10 mM phosphate buffer (pH2) containing 25 mM SDS; the flow rate was 0.5 ml/min.

explained by the ion-exchange desolvation mechanism (23). A mobile phase of an acetonitrile-aqueous phosphate buffer (10 mM, pH2) mixture (50:50, v/v) containing 25 mM SDS was chosen as it provides a good resolution between Q and the internal standard. It also permits the internal standard to be eluted after Q. With these chromatographic conditions the retention times for Q and the internal standard were 7.6 and 11.7 min respectively. The addition of tetrabutylammonium bromide (TBA) was necessary to reduce the broadening effect that was observed using the mobile phase outlined above. The effect of TBA on the k' of Q and the internal standard cinchocaine is presented in Figure 1B. Addition of TBA up to 4 mM caused a marked decrease in the k' of both solutes of interest, i.e. it shortened the retention times. The existing mobile phase with further addition of 3 mM TBA was chosen as the mobile phase for the analysis of Q in plasma as it provided acceptable retention times for Q and the internal standard.

Figure 2 shows chromatograms of blank plasma, plasma spiked with 0.018 μg/ml of Q and a typical subject's plasma chromatogram 12 hours post dose. Under these chromatographic conditions, no endogenous sources of interference were observed and the resolution between Q and the internal standard (cinchocaine) was satisfactory. Blank plasma samples from more than 20 subjects were analysed and no plasma endogenous peaks co-eluting with Q and the internal standard were detected. The elution sequence and retention time were: Q, 4.5 min and cinchocaine (internal standard), 6.8 min.

Absolute recovery was calculated by comparing the peaks of Q and the internal standard with those obtained by direct injection of the pure drug standards of Q and cinchocaine of equivalent quantities. The mean recovery for Q (n = 3) from plasma sample was 89 ± 8% (S.D.) at 0.09 μg/ml, 100 ± 7% at 0.91 μg/ml and 105 ± 8% at 9.1 μg/ml. The absolute recovery for the internal standard was 95.9 ± 1.7% (n = 4). The calibration curve for Q was linear over the concentration range of 0.09 to 10 μg/ml with the square of the correlation coefficient (r^2) greater than 0.99 (peak height ratio = 1.3111 x plasma Q concentration). The day-to-day coefficient of variation (C.V.) of the slope of the calibration curves of Q were 5.0% (n = 4). The precision and accuracy of the assay was evaluated for three concentrations of Q (0.018, 0.45 and 9.1 μg/ml) by analysing each one four times on the same day. The intra-assay C.V. for Q were 7.7% at 0.018 μg/ml (18 ng/ml), 5.9% at 0.45 μg/ml and 4.3% at 9.1 μg/ml. Between day variation of the assay was assessed by

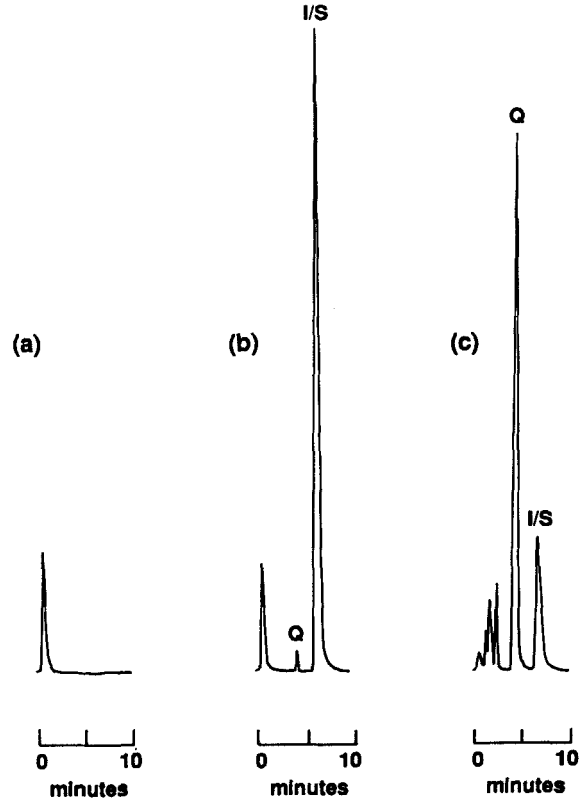

FIGURE 2

Typical chromatograms of human plasma: (a) blank plasma; (b) blank plasma spiked with 18 ng/ml quinine (Q) and the internal standard (I/S), cinchocaine; and (c) plasma with 2.2 μg/ml Q taken from a healthy volunteer 12 hours after an oral dose of 600 mg quinine sulfate.

TABLE 1

Possible Interferences Under Assay Conditions[1]

(a) Drugs which are not fluorescent and so do not cause an interference under the assay conditions

Acetaminophen	Diltiazem	Naproxen
Antipyrine	Ephedrine	Phenacetin
Aspirin	Flurbiprofen	Phenylbutazone
Butobarbital	Frusemide	Phenytoin
Caffeine	Ibuprofen	Pindolol
Cefoxitin	Indapamide	Primaquine
Cephalothin	Indomethacin	Proguanil
Chloroquine	Ketoprofen	Propranolol
Chlorproguanil	Lidocaine	Pyrimethamine
Cocaine	Mefloquine	Sulfanilamide
Codiene	Mepivacaine	Theophylline
Diclofenac	Methadone	Triazolam

(b) Drugs which are fluorescent under the assay conditions

Cetriaxone (1.3 min)[2]	Labetolol (2.5 min)	Quinidine (4.5 min)
Cephradine (1.0 min)	Piroxicam (1.2 min)	Salicylic acid (1.0 min)
Diazepam (1.5 min)		

[1] Under these chromatographic conditions, the retention times for quinine and cinchocaine (the internal standard) were 4.6 and 7.0 min, respectively.

[2] Retention time.

re-analysing randomly selected subject samples (n = 54). The results indicate that the accuracy of the assay for Q was greater than 90% (mean ± S.D. = 100 ± 9) with C.V. of 9.0%

The predicted value of five 0.018 μg/ml (i.e. 18 ng/ml) plasma Q standards gave values of 0.0183, 0.0163, 0.0183, 0.0203 and 0.0183 μg/ml. This data gives a mean and S.D. of 0.0183 ± 0.0014 μg/ml. The C.V. of the assay at this concentration was 7.7%, which is much lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). A typical chromatogram of 0.018 μg/ml plasma Q standard is presented in Figure 2(b). Thus the MQC or the detection limit of sensitivity for this assay was assigned 18 ng/ml.

Plasma samples stored at -70°C for up to three months showed no signs of decomposition and practically the same concentration values were obtained (n = 6). This suggests that Q is stable under these storage conditions for at least three months.

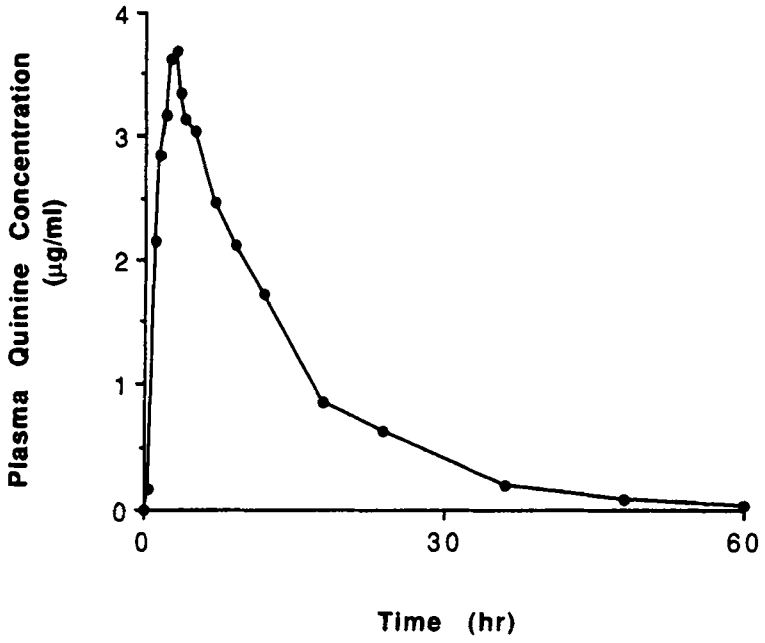

FIGURE 3

Plasma quinine concentration-time profile obtained from a representative subject who had taken an oral dose of 600 mg quinine sulfate.

Possible interference by other drugs was examined (Table 1). The assay method was shown to be free of chromatographic interference from the other antimalarial drugs including chloroquine, chlorproguanil, mefloquine, primaquine, pyrimethamine and proguanil. A number of basic and acidic drugs were also tested for possible interference. Most of them are not fluorescent at these wavelengths and do not interfere with the assay for Q. Table 1 shows that the diastereoisomer quinidine which is fluorescent under the assay conditions, has a similar retention time as Q. Thus, this assay cannot be used to quantitate Q in the presence of quinidine.

The assay method was applied to the measurement of Q concentrations in the plasma samples obtained from 18 Caucasian subjects participating in a bioavailability study. Figure 3 shows a representative plasma concentration-time

profile of Q in one subject after the oral administration of 600 mg Q sulfate. In the group of 18 subjects, plasma Q concentrations declined exponentially with elimination half-life values ranging between 8.0 and 16.1 hours with a mean of 10.5 hours (± 2.4, S.D.). None of the samples in this bioavailability study had a concentration of Q below the detection limit of 18 ng/ml.

In summary, the proposed assay has been shown to be simple, rapid, sufficiently sensitive and specific for the determination of quinine in human plasma samples. The procedure is suitable for the routine analysis in clinical and pharmacokinetic studies. This assay is being employed for the measurement of quinine in plasma samples from the study of effects of oral contraceptives on the pharmacokinetics , protein binding and *in vitro* placental transfer of quinine.

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