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Determination of pyronaridine in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies

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Abstract

A method is described for the determination of pyronaridine in plasma using high-performance liquid chromatography with fluorescence detection. The method involves liquid–liquid extraction with phosphate buffer (pH 6.0, 0.05 *M*) and diethyl ether–hexane (70:30%, v/v) and chromatographic separation on a C_{18} column (Nucleosil, 250×4.6 mm I.D., 5 μm particle size) with acetonitrile–0.05 *M* phosphate buffer pH 6.0 (60:40%, v/v) as the mobile phase (1 ml/min) and detection by fluorescence (λ_{ex}=267 nm, λ_{em}=443 nm). The detector response is linear up to 1000 ng and the overall recoveries of pyronaridine and quinine were 90.0 and 60.3%, respectively. The assay procedure was adequately sensitive to measure 10 ng/ml pyronaridine in plasma samples with acceptable precision (<15% C.V.). The method was found to be suitable for use in clinical pharmacological studies.

Keywords: Pyronaridine

1. Introduction

Pyronaridine (2-methoxy-7-chloro-10-[3′,5′-bis-(pyrolidinyl-1-methyl)-4′-hydroxy anilino]benzo-[*b*]-1,5-naphthyridine tetraphosphate) is an antimalarial drug synthesized in China during the mid-1970s. It is less toxic than chloroquine and is highly active against chloroquine-sensitive and resistant parasites both in rodent malarias in vivo and *P. falciparum* in vitro. Pyronaridine affects haemoglobin degradation [1] possibly through an effect on parasite topoisomerase II [2].

Clinical trials in China have shown pyronaridine to be a well tolerated drug, effective against *P. falciparum* and *P. vivax*, with only mild side effects [3]. Recently, trials carried out in Cameroon and Thailand proved the effectiveness of this drug against parasites unresponsive to chloroquine and in areas of multidrug resistance [4,5].

A high-performance liquid chromatographic (HPLC) method using ultraviolet detection for the determination of pyronaridine in human plasma has been reported [6]. However, the method is not adequately sensitive (detection limit is 70 ng/ml) for analysis of pyronaridine in human plasma samples obtained from clinical pharmacokinetic studies. The method described here is rapid, sensitive and selective for the chromatographic determination of pyronaridine in blood plasma. It involves the isolation of pyronaridine from plasma samples by liquid–

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liquid extraction followed by HPLC separation and determination by fluorescence detection.

2. Experimental

2.1. Materials and reagents

Pyronaridine tetraphosphate was obtained from the Institute of Parasitic Diseases, Academy of Preventive Medicine, China. The internal standard, (quinine hydrochloride) was obtained from Sigma (St. Louis, MO, USA). All chemicals and solvents were of analytical grade. Acetonitrile and hexane were obtained from Merck (Darmstadt, Germany). Dipotassium hydrogen phosphate and diethyl ether were purchased from Ajax (Auburn, Australia) and orthophosphoric acid 85% from Riedel-de-Haen (Germany).

2.2. Apparatus

A constant flow high-performance liquid chromatograph, consisting of Model 1050 pumping system (Hewlett-Packard, USA), Model 1050 automatic injector (Hewlett-Packard) and a Model 470 fluorescence detector (Waters, USA), was connected to an electronic integrator SP4600 Data Jet (Thermo Separations, UK).

2.3. Preparation of standards

Aliquots of pyronaridine standard solution in methanol with concentrations of 0.05, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 ng/μl were prepared by serial dilutions of a stock solution (100 ng/μl in methanol). The stock solution was protected from light and was found to be stable for at least 3 months at −20°C. The internal standard solution (quinine; 10 ng/μl) was also prepared in methanol and stored under similar conditions. Fig. 1 shows the structures of pyronaridine and quinine.

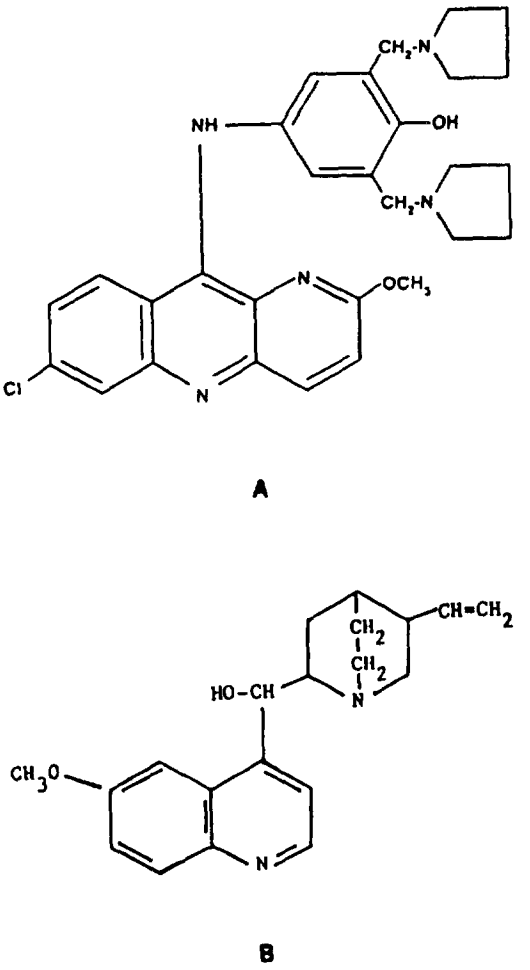

Fig. 1. Structures of (A) pyronaridine and (B) quinine.

2.4. Sample preparation and extraction

All glassware was silanized with 5% dimethylchlorosilane in toluene to minimise adsorption of the drugs onto glass surfaces. An aliquot of the internal standard (quinine; 100 μl; 1000 ng) in methanol was added into a 15 ml glass test-tube with a teflon-lined screw cap. Plasma (250 μl) was transferred into the test tube and the mixture was vortex-mixed (30 s). This was followed by the addition of 0.05 *M* phosphate buffer (pH 6.0; 500 μl) and vortex stirring for 30 s. Diethyl ether–hexane (5 ml; 70:30, v/v) was then added and the resultant mixture thoroughly vortex-mixed for 90 s, followed by centrifugation (2500 rpm; 10 min). The upper organic layer was aspirated into a new test tube and the mixture evaporated to dryness in the Reactitherm module at 40°C under a gentle stream of nitrogen. When

evaporation was completed, acetonitrile (100 μl) was added to the dried residue, the tubes were vortex-mixed for 60 s and a 50-μl aliquot analysed by HPLC.

2.5. Chromatographic conditions

The mobile phase used was acetonitrile–0.05 *M* phosphate buffer of pH 6.0 (60:40, v/v) adjusted to pH 6.0 with orthophosphoric acid (85%), filtered and degassed with a flow-rate of 1.0 ml/min. Samples (50 μl) were applied with an automatic injection system onto an analytical column (5 μm, 250×4.6 mm I.D.) (Nucleosil). No guard column was employed in this assay. The fluorescence detector was set at λ_{ex} 267 nm and λ_{em} 443 nm. Chromatography was performed at room temperature. Analyte peak areas were digitized and integrated using an electronic integrator.

2.6. Linearity

The linearity of the analytical procedure was tested at 0.05, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 ng/μl in triplicate at each concentration. Aliquots (20 μl) of each of these solutions were injected into the HPLC to evaluate the detector response.

2.7. Calibration

Calibration graphs were prepared by spiking drug-free plasma samples with standard solution of pyronaridine to give a concentration range of 10–400 ng/ml in plasma. The samples were extracted using the procedure described in Section 2.4 and the peak heights of the drug were plotted against their corresponding concentrations. Linear regressions of peak-height ratio versus drug concentrations were performed in order to calculate the slope, intercept (peak-height ratio for zero concentration) and correlation coefficient for each calibration graph. Unknown concentrations were calculated using each observed samples/internal standard peak-height ratio and the calibration graph parameters.

2.8. Recovery and within-day and day-to-day precision

The absolute recoveries of the extraction procedures for pyronaridine and quinine were determined by comparing the peak heights obtained from extracted plasma samples containing known amounts of the substance with those obtained from equivalent amounts of the compounds in methanol by direct injection. Within-day precision was determined by conducting repeated analysis of spiked plasma samples at six different concentrations. The day-to-day precision was assessed by analysing spiked plasma samples at three different concentrations over a period of five days.

2.9. Drug disposition study

In order to test the application of the method to in vivo pharmacokinetic studies, pyronaridine was orally administered (6 mg/kg; 400 mg; capsule) to a healthy male subject (68 kg). Blood samples were withdrawn at 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 72, 96, 120, 144, 252 and 336 h. The blood samples were centrifuged (1000 *g*; 20 min) immediately after collection and the plasma supernatant was stored at −70°C until analysis by HPLC.

2.10. Preliminary pharmacokinetic analysis

Elimination half-life, area under the plasma concentration–time curve (AUC), clearance and volume of distribution were calculated using the non-linear least square regression programme, TOPFIT [7]. Maximum concentration (C_{max}) and time to achieve the maximum concentration (t_{max}) were the observed values.

3. Results and discussion

A typical chromatogram for pyronaridine standard (250 ng, on column) and quinine (500 ng, on column) is shown in Fig. 2A. Under the described conditions, the retention times of pyronaridine and quinine were around 4.6 and 6.3 min, respectively. Fig. 2B shows a chromatogram of a drug-free plasma sample. Fig. 2C shows a chromatogram obtained

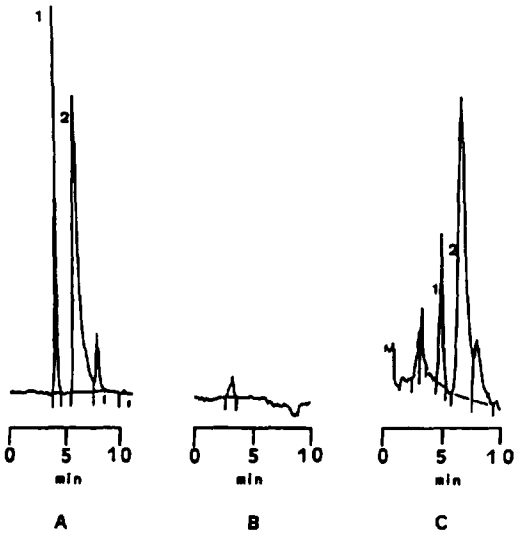

Fig. 2. Typical chromatograms (A) standard of pyronaridine (1) (250 ng, on column) and quinine (2) (500 ng, on column); (B) blank plasma and (C) plasma sample spiked with pyronaridine (1) (100 ng, on column) and quinine (2) (500 ng, on column). Detection sensitivity high, integrator attenuation 8.

from a plasma sample spiked with pyronaridine (100 ng, on column) and quinine (500 ng, on column). The method provides good resolution of pyronaridine and quinine from normal plasma constituents.

The recovery of pyronaridine from blood plasma is shown in Table 2. According to Saleh and Loh [6], the use of diethyl ether resulted in a clean extract with good recovery (85%). We found that a higher recovery could be obtained by adding hexane to the diethyl ether (30:70; v/v, 5 ml). The extraction recoveries were 86.9 and 92.2% at pyronaridine concentrations of 40 and 320 ng/ml, respectively.

The linearity was determined by plotting the peak heights versus the amount of drug injected, both pyronaridine and quinine were linear ($r>0.999$) up to 1000 ng on column.

Linear regression analysis performed for calibration graphs ($n=5$) yielded correlation coefficient which was $r=0.992\pm0.003$. The equation of the calibration plots ($n=5$) for pyronaridine was $y=0.0024x\pm0.013$.

The results obtained for the within-day precision determined by conducting repeated analysis of spiked plasma samples at six different concentrations (10, 20, 40, 80, 160 and 320 ng/ml) are listed in Table 1. The mean percentage coefficient of variation was 7.98% within a range of 4.27% to 14.12% indicating that a good within-day precision was achieved. Table 2 shows the results obtained for the day-to-day precision. The mean percentage coefficient of variation was 4.06% within a range of 0.60 to 7.25% indicating good reproducibility. The minimum detectable concentration of pyronaridine corresponding to a peak three times baseline noise at the highest sensitivity setting is 10 ng/ml.

Table 1
Within-day precision of the HPLC method for the determination of pyronaridine in plasma ($n=4$)

Concentration added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	C.V. (%)
10	12.75±1.80	14.12
20	20.70±1.79	8.63
40	50.85±4.52	8.88
80	94.46±5.83	6.17
160	161.76±9.40	5.81
320	318.91±13.62	4.27

The validated method for blood plasma was used to study the pharmacokinetics of pyronaridine in a healthy volunteer after a single oral dose (400 mg). The corresponding pyronaridine plasma concentration over the period of 0–336 h is depicted in Fig. 3. The maximum blood concentration (C_{max}) of 495.8 ng/ml was reached at 0.5 h post dose (t_{max}). The half-life, AUC $_{(0-\infty)}$, clearance and volume of distribution are 251 h, 51 700 ng.h/ml, 1.90 ml/min/kg and 41.2 l/kg, respectively.

The assay method described in this study is highly specific, selective and sensitive. The extraction procedure is simple and the chromatographic time is

Table 2
Day-to-day precision of the method for the determination of pyronaridine in plasma ($n=5$)

Concentration added (ng/ml)	Concnetration found (mean±S.D.) (ng/ml)	Recovery (%)	C.V. (%)
20	21.15±1.53	90.5	7.25
80	83.34±3.61	92.0	4.33
320	316.73±1.89	92.2	0.60

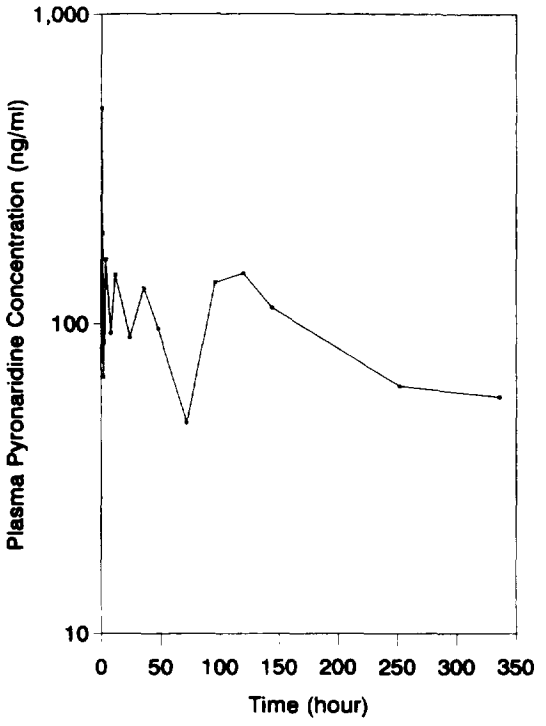

Fig. 3. Plasma concentration–time curve for a healthy subject given a 6 mg/kg oral dose (capsule) of pyronaridine.

rapid. The method is currently being used as a routine technique for the determination of pyronaridine in plasma samples obtained from a clinical pharmacokinetic study recently conducted in Thailand.

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