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Short communication

Alternative method for determination of pyrimethamine in plasma by high-performance liquid chromatography

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Abstract

A rapid, selective, sensitive and reproducible reversed-phase high-performance liquid chromatography (HPLC) procedure for the quantitative determination of pyrimethamine (PYR) in plasma is described. The procedure involved the two-step extraction of PYR and the internal standard, quinine (QN) with acetonitrile and dichloromethane at basic pH. Chromatographic separation consisted of the mobile phase (methanol–water containing 0.005 *M* octanesulfonic acid, 50:50, v/v) running through the column (Techopak-10 C_{18}) at a flow-rate of 1.6 ml/min. Detection was at UV wavelength of 240 nm. The mean recoveries of PYR and QN at a concentration range of 50 and 500 ng/ml were 98.9 and 89%, and 94.7 and 96% for PYR and QN. The within-day coefficients of variation were 2.1–5.1% for PYR and 5.9% for QN. The day-to-day coefficients of variation were 2.1–4.1% for PYR and 5% for QN. The minimum detectable concentrations for PYR and QN in plasma were 3 and 10 ng/ml. The method was found to be suitable for use in clinical pharmacokinetic study.

Keywords: Pyrimethamine

1. Introduction

Pyrimethamine (PYR) is a dihydrofolate reductase inhibitor which has been used for therapy or prophylaxis of parasitic diseases including chloroquine-resistant falciparum malaria and toxoplasmosis. In malaria chemotherapy, PYR is usually used in combination with other drugs, e.g., sulfadoxine (SP: Fansidar®), sulfadoxine and mefloquine (MSP: Fansimef®) and dapsone (Maloprim®). Furthermore, as the multidrug resistance of *P. falciparum* is increasing, the use of PYR with artemisinin compounds, e.g., artemether, has been shown to be a promising antimalarial combination [1]. Pharmacodynamic (antiparastic effect) and/or pharmacokinetic (plasma active drug concentrations) synergistic effects are expected from this combination therapy. With the occurrence of multidrug resistant *P. falciparum* malaria, as well as epidemic AIDs-associated toxoplasmosis, monitoring of PYR levels during drug therapy, as well as pharmacokinetic studies, are necessary. This requires a rapid, sensitive and selective method of drug assay in biological fluids. Several HPLC methods have been described for PYR measurement in plasma, serum and CSF. However, these methods suffer form one or more of the following shortcomings, i.e. relatively large sample volume, low sensitivity, lack of or requirement of

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specific synthetic internal standard, long or sophisticated extraction/chromatographic procedures [2–9].

In the present report, we describe an alternative reversed-phase HPLC method for quantitation of PYR in biological fluids.

2. Experimental

2.1. Chemicals

Pyrimethamine base (Fig. 1), 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine, was a gift from Dr. G. Edwards, University of Liverpool, UK. Quinine base (Fig. 1), the internal standard, was supplied by Sigma (St. Louis, MO, USA). PYR and QN were prepared as 0.5 μg/μl stock solutions in absolute methanol. Working standard solutions were prepared prior to use by diluting the stock standard solution with methanol to a concentration of 25 ng/μl. All standards were stored at 4°C in vials.

Absolute methanol, dichloromethane and acetonitrile were of HPLC grade (Fisons, Loughborough, UK). Octanesulfonic acid was obtained from Fisons. All other reagents and solvents were analytical grade quality which were supplied by BDH (Poole, UK).

2.2. Chromatography

The method was developed on a liquid chromatographic system consisting of a Model CM 4000 solvent delivery system (LDC Analytical, USA), a Waters 745 data module (Waters, Milford, MA, USA) equipped with a Rheodyne 7125 injector with a 20-μl loop (Rheodyne, Berkeley, CA, USA) and a SM 4000 variable-wavelength UV detector (LDC Analytical, USA). The wavelength was set at 24O nm and sensitivity at 0.005 AUFS. The separation was carried out on a Techopak-10 C_{18} reversed-phase column (15 cm×4.6 mm I.D., 10 μm particle size; HPLC-Technology Ltd., serial no. pp 45739). The mobile phase consisted of absolute methanol–water (50:50, v/v) containing octanesulfonic acid (0.005 *M*) as an ion-pair reagent, buffered to pH 2.2 with orthophosphoric acid. It was delivered at a flow-rate of 1.6 ml/min. The chromatographic analysis was operated at ambient temperature.

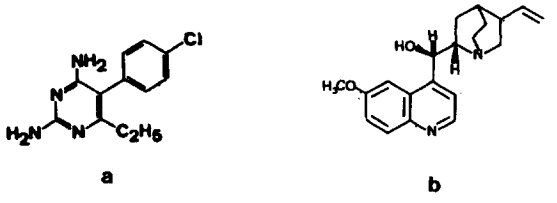

Fig. 1. Chemical structures of (a) pyrimethamine and (b) quinine (internal standard).

2.3. Sample extraction procedure

In order to minimize possible drug adsorption, extraction was carried out in 15-ml screw-cap glass test tubes precoated with dimethyldichlorosilane in toluene (5%, v/v). To a 1-ml plasma sample was added an internal standard QN (1000 μg), followed by vortex-mixing for 30 s. Acetonitrile (2 ml) was added, the mixture was then vortex-mixed for 30 s and centrifuged at 1200 *g* for 15 min. The acetronitrile layer was transferred to a new test tube, mixed with 2 ml of glycine buffer (pH 9.2) and vortex-mixed for 30 s. The resultant mixture was extracted with 6 ml of dichloromethane by mechanical tumbling for 30 min. After centrifugation at 1200 *g* for 10 min, the clear organic layer was transferred to a clean tube using a pasteur pipette. Evaporation to dryness was by a stream of air at 37°C. The residue was dissolved in 100 μl of mobile phase and 20 μl was injected onto the column.

2.4. Calibration curves

A solution of PYR in absolute methanol, concentration ranging from 50 to 1000 ng/ml, was injected into the HPLC system in order to assess detector linearity. Peak height was plotted against the quantity of PYR injected. PYR was linear ($r>0.999$) over the concentration range observed.

Calibration curves were prepared by triplicate analysis of 1-ml plasma samples spiked with concentrations of PYR in the range of 50–1000 ng/ml, with a fixed concentration of internal standard (1000 ng). Samples were analyzed as described above and the peak-height ratios of PYR to internal standard were plotted against the corresponding drug concentrations. Peak-height ratios of the samples were

determined and the concentrations calculated from the standard curves.

2.5. Method recovery, precision, accuracy and selectivity

The analytical recoveries of the extraction procedure for both PYR and QN were estimated by comparing the peak heights obtained from an extracted sample with those measured with equivalent amounts of each compound in methanol. The concentration range used was 50 and 1000 ng/ml.

The precision of the method, based on within-day repeatability, was determined by replicate analysis of six samples spiked with three different concentrations of PYR (50, 600 and 1000 ng/ml). The reproducibility (day-to-day variation) of the method was established using the same concentration range as above, but only a single determination of each concentration was made on three different days. Coefficients of variation (C.V.) were calculated from the ratio of standard deviation (S.D.) to the mean.

Accuracy was determined by replicate analysis of three different levels and comparing the difference between spiked value and that actually found.

The selectivity of the method was verified by checking for interference by some antimalarials (sulfadoxine, dapsone, mefloquine, primaquine, chloroquine) after subjecting them to the extraction procedure.

2.6. Application of the method to biological samples

In order to validate the assay method, the method was applied to investigate the pharmacokinetics of PYR in a male Thai volunteer (aged 24, weighing 52 kg) following the administration of a single oral dose of 100 mg PYR (Wellcome, UK). The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University. The subject was fasted overnight prior to drug administration. Venous blood samples (5 ml each) were collected into sodium heparinized plastic tubes at 0 (pre-dose), 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 18 and 24 h and on days 2, 3, 4, 5, 6, 7, 10 and 14 after dosage. Plasma was separated by centrifugation at 1500 *g* for 10 min, immediately after collection and frozen at −20°C until analysis.

Pharmacokinetic analysis was done by a model-independent method [10]. The maximum plasma concentration (C_{max}) and the time to maximum concentration (t_{max}) were observed values. The terminal phase elimination rate constant (λ_z) was determined by least squares regression analysis of the post absorption and distribution plasma concentration–time data and the terminal phase elimination half-life ($t_{1/2z}$) from the ratio $0.693/\lambda_z$. The area under the plasma concentration–time curve (AUC) was calculated by trapezoidal rule. Oral clearance (CL/F) was calculated from dose/AUC. The apparent volume of distribution (V_z/F) was calculated from CL/F divided by λ_z.

3. Results and discussion

Chromatograms of extracts of drug-free plasma, spiked plasma and plasma obtained from the volunteer 2 h after a single oral dose of PYR (100 mg) are shown in Fig. 2. PYR and the internal standard, QN, were resolved with retention times of 4.9 and 6.8 min, respectively. Endogenous peaks from extracted drug-free plasma did not interfere with drug analysis. The method was free from chromatographic

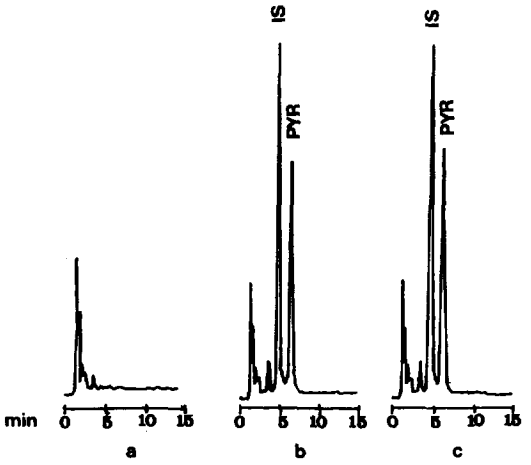

Fig. 2. Chromatograms of extracts of (a) drug-free plasma (b) spiked plasma (800 ng PYR, 1000 ng QN) and (c) plasma obtained from healthy male Thais at 2 h after a single oral dose of 100 mg PYR base.

interference from endogenous compounds and the commonly used antimalarials. Retention times of mefloquine, chloroquine, primaquine, sulfadoxine, dapsone and 3-*N*-oxide metabolite of PYR were 8.2, 8.3, 10.2, 3, 3.1 and 2.1 min, respectively. The combination partner–artemisinin compounds did not interfere with the detection as they lack a UV chromophore. QN, the internal standard, showed reproducible extraction, suitable retention and was well resolved from the PYR peak. The minimum detectable concentrations, defined as a peak three times the base-line noise at 0.005 AUFS in a 1-ml plasma sample were 3 and 10 ng/ml for PYR and QN, respectively. Analytical recoveries were 98.9 and 89%, and 94.7 and 96%, for PYR and QN, at concentrations of 50 and 200 ng/ml, respectively.

Calibration curves for PYR were linear over the range 50–1000 ng/ml, with a correlation coefficient of 0.9999 or better. There was little variation in PYR assays; coefficients of variation in all cases were below 10%. The intra-assay (within-day), inter-assay (day-to-day) variations and accuracy for the assay are given in Table 1.

To validate for the clinical applicability of the method, the pharmacokinetics of PYR was investigated in healthy Thai males following an oral administration of 100 mg pyrimethamine base. The plasma concentration–time profile is shown in Fig. 3. The profile was in agreement with those reported in previous studies, showing a bi-exponential decline of the concentration [11]. PYR was rapidly absorbed from the gastrointestinal tract following a single oral dose of 100 mg. C_{max} (757 ng/ml) was reached at 1.5 h (t_{max}). Plasma concentrations of PYR were measurable up to 14 days of the investigation period (the time when the level of PYR was undetectable). AUC, $t_{1/2z}$, V_z/F and CL/F were 62.9 μg h/ml, 58.6 h, 1.91 l/kg and 0.379 ml/min/kg, respectively.

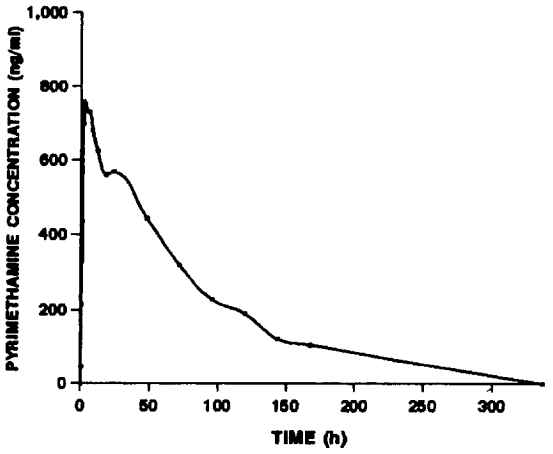

Fig. 3. Plasma concentration–time profile of PYR in healthy male Thai following a single oral dose of 100 mg PYR base.

The analytical method for the determination of PYR presented in this paper meets the criteria for application to routine clinical drug level monitoring or pharmacokinetic study. The advantage of the method over that previously reported are, basically, its simplicity and the use of an easily available internal standard. More importantly, its sensitivity allows the detection of a concentration as low as 3 ng/ml, which is lower than the reported concen-

Table 1
Precision, accuracy for PYR assay in plasma

Compound	Concentration added (ng/ml)	Concentration measured (mean±S.D.) (ng/ml)	%C.V.	Difference between measured and added concentrations (%)
Within-day variation				
PYR	50	49.7±2.1	4.2	−0.66
	600	604.7±12.9	2.1	+0.78
	1000	996.3±50.5	5.1	−0.37
QN	1000	1007±59.6	5.9	+0.7
Day-to-day variation				
PYR	50	50.3±2.1	4.1	+0.66
	600	609.3±13.0	2.1	+1.56
	1000	985.3±33.1	3.4	−1.47
QN	1000	996.3±50.9	5.0	−0.37

tration range required for sensitive *P. falciparum* [12].

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