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A new and simple solid-phase extraction method for LC determination of pyronaridine in human plasma

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Abstract

A new approach using a simple solid-phase extraction technique has been developed for the determination of pyronaridine (PND), an antimalarial drug, in human plasma. After extraction with C_{18} solid-phase sorbent, PND was analyzed using a reverse phase chromatographic method with fluorescence detection (at $\lambda_{ex} = 267$ nm and $\lambda_{em} = 443$ nm). The mean extraction recovery for PND was 95.2%. The coefficient of variation for intra-assay precision, inter-assay precision and accuracy was less than 10%. The quantification limit with fluorescence detection was 0.010 μg/mL plasma. The method described herein has several advantages over other published methods since it is easy to perform and rapid. It also permits reducing both, solvent use and sample preparation time. The method has been used successfully to assay plasma samples from clinical pharmacokinetic studies.
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Keywords: Pyronaridine; Plasma; LC; Malaria

1. Introduction

The constraints of antimalarial chemotherapy underscore the need for novel compounds to be developed. Relatively few new antimalarial drugs are undergoing clinical trials. Pyronaridine (PND) is one of them from the limited armamentarium of candidate antimalarial drugs [1]. PND (Fig. 1) is a Mannich base which was synthesized at the Institute of Parasitic Diseases, Academy of Preventive Medicine, Shanghai, China in 1970 [2]. Though currently available data do not support registration by international regulatory standards, it has proven to be well tolerated and effective against chloroquine-resistant falciparum malaria [3]. Currently available assay procedures for the determination of PND in biological fluids currently are laborious and involve conventional sample extraction techniques [4–8]. An automated solid-phase extraction (SPE) technique is available for the determination of PND in whole blood but not in human plasma [9]. However, several investigators reported limited storage stability of PND in whole blood [4,8,9]. Considering this limitation we have developed and validated a simple technique for the determination of PND in human plasma utilizing solid-phase extraction and its application in clinical studies.

2. Experimental

2.1. Chemicals and reagents

The solid-phase extraction (SPE) cartridges used were Supelclean LC-18 (Supelco Park, Bellefonte, PA, USA) with 1 mL capacity and containing 100 mg sorbent. Mass

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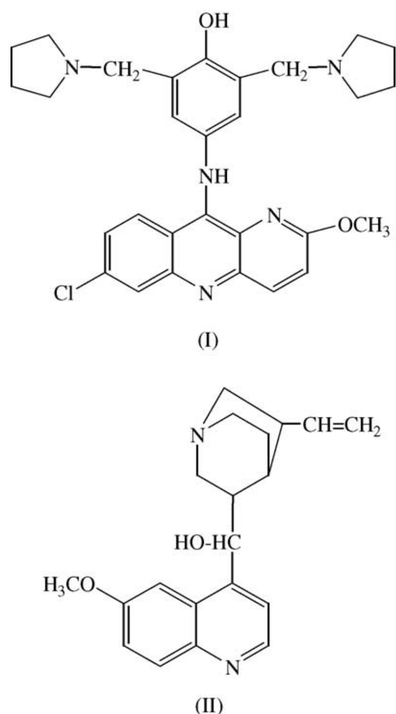

Fig. 1. Structure of pyronaridine(I) and quinidine(II).

spectrometry (Model TSQ 700, Finnigen Mat, USA) and NMR (Bruker ARX-300, Germany) has confirmed the identity of the compound. Quinidine sulphate (QN, internal standard) was obtained from Sigma (St. Louis, MO, USA). All chemicals and solvents used in the assay procedure were of analytical/chromatography grade. Methanol, acetonitrile, glacial acetic acid and hydrochloric acid were purchased from Merck (Darmstadt, Germany). The pyronaridine tetraphosphate raw material was supplied by Institute of Parasitic Diseases, Academy of Preventive Medicine, Shanghai, China, and formulated into capsules at School of Pharmaceutical Science, Universiti Sains Malaysia. In order to obtain pure standard of pyronaridine for analytical purposes pyronaridine tetraphosphate raw material was further purified in our laboratory.

2.2. Chromatography

The analytical instrument used was a single LC pump (Waters 501, Milford, USA) equipped with a syringe-loading sample injector with a 50-μl sample loop coupled to a fluorescence detector (Model HP1046A, Hewlett Packard, USA) operating at $\lambda_{ex} = 267$ nm and $\lambda_{em} = 443$ nm. The chromatograms were recorded using an electronic integrator (Hewlett Packard 3392A, USA). Chromatographic separations were performed on a C_{18} reversed-phase Partisil 10 ODS (3) stainless-steel column 250×4.6 mm i.d.; (Phenomenex®, Torrance, CA, USA) maintained at room temperature (27 °C). The mobile phase was methanol–0.05 M ammonium acetate buffer (adjusted to pH 4.0 with glacial acetic acid (50/50, v/v). The flow rate was 1.2 mL/min.

2.3. Isolation and purification of the PND tetraphosphate

Two grams of pyronaridine raw material was dissolved in 75 ml of distilled water. The solution was adjusted to pH 9 using 0.1 M NaOH. The resulting solution was refluxed for 3 h and subsequently was extracted with diethyl ether. The extract was then concentrated in vacuo before subjecting to separation through wet column chromatography. Isolation was carried out using diethyl ether–chloroform and methanol–chloroform with increasing polarity as the eluting solvents. NMR and mass spectrometry analysis were determined for structural confirmation of the isolated and purified substance and results were compared with the literature values for PND. LC purity of PND were carried out by injecting in triplicates the known concentration of this substance in methanol into a LC system. The similar chromatographic conditions as described in Section 2.2 was used for purity testing except for detection was carried out with UV detector operating at λ 275. The purity was estimated as the ratio of peak area of substance of interest to total sum peak area after a chromatographic run.

2.4. Stability

The stability of standard solutions of PND with concentrations 5 and 20 μg/mL has been analysed immediately after preparation and after 1 week and 4 weeks of storage at −20 °C. The stability of PND in plasma during storage at −70 °C has been determined at two spiked concentrations, namely 0.040 and 0.500 μg/mL. Spiked plasma samples in replicates of 10 at each concentration were analysed immediately after preparation and 3 months after storage at −70 °C. A pool of spiked samples at high and lower concentrations has been prepared and stored at −70 °C on the day the clinical samples were received. Aliquots from this pool of spiked plasma served as quality control samples when the clinical samples were analysed. Aliquots from this pool of spiked QC samples were also used to determine the in process stability of PND and the impact of freezing and thawing. The dry extracts of samples after extraction were stored at −20 °C for not more than 2 days before analysis. Freeze/thaw effects were determined for not more than three cycles. The percentage ratios of concentrations determined to known added concentrations were calculated at each concentration level.

2.5. Selectivity

To assess method selectivity, six blank plasma samples, six spiked plasma samples and six plasma samples from patients who were given PND therapeutically were analysed. Several common antimalarial drugs were injected into LC in order to observe eventual interference with PND and IS.

2.6. Drug disposition study

The method was then used to determine PND in plasma samples from a separate pharmacokinetic study involving five Thai patients with uncomplicated falciparum malaria who received a 3-day course of 12 mg/kg of PND. This study was approved by the Ethics Committee of the Mahidol University, Bangkok, Thailand. Blood samples were drawn prior to drug administration and at 0.5, 1, 2, 4, 8, 12 and 24 h after the first drug administration. After the second dose, blood samples were drawn at 12 and 24 h later. Subsequent blood samplings were carried out at 4, 8, 12 h after the third and last dose, and at day 1, 2, 3, 4, 5, 8, 12 and 19 after the last drug treatment. Blood samples were centrifuged (1000 × *g*: 20 min) immediately after collection in order to separate the blood plasma from cellular elements, and the plasma supernatant was stored at −70 °C until analysis by LC.

2.7. Extraction procedure

In order to minimize potential drug adsorption, all glassware has been silanized with dichlorodimethylsilane in toluene (5%, v/v) before use. SPE extraction was carried out using a vacuum manifold (Supelco Park, Bellefonte, PA, USA). Plasma (0.25 mL) and internal standard QN (10 μl, 100 μg/mL), diluted to 1 mL with distilled water, was loaded onto a SPE cartridge already conditioned with methanol and distilled water (each 1 mL). Each cartridge was then washed with 1 mL of methanol–water (50:50, v/v). The elution was carried out with three aliquots of 0.5 mL of methanol–0.1 M HCl solution (99:1, v/v). The eluent was collected into a test tube and dried under a gentle stream of nitrogen at 35 °C. The residue was reconstituted in 100 μl methanol and 50 μl were injected into the LC column.

2.8. Calibration procedure

Stock solutions of PND (100 μg/mL) and internal standard (100 μg/mL) were prepared by dissolving each substance in methanol. The vials were wrapped in aluminium foil and stored at −20 °C. Calibration curves were prepared by spiking drug-free plasma with standard solutions of PND and internal standard (I.S.) (100 μg/mL, 10 μl) to give a final plasma concentration range of 0.010–0.800 μg/mL of PND and 4 μg/mL for the internal standard. The samples were taken through the extraction procedure and the peak-height ratio of the drug was plotted against the corresponding concentration of drug. Linear regression of the peak-height ratio versus the drug concentration ranging from 0.010 to 0.800 μg/mL was performed in order to estimate slope, intercept and coefficient of correlation for each standard curve.

2.9. Analytical recovery

The analytical recovery of the extraction procedure for PND was determined by comparing the peak height ratio obtained from extracted plasma samples containing known amounts of the substance with those obtained from equivalent amounts of the compound in methanol by direct injection. The within-day and day-to-day precision has been determined at four different concentrations with replicate assays of samples. The day-to-day assay variation was assessed over a period of 5 days. The internal standard recovery was determined in the presence of high and low concentrations of PND.

2.10. Pharmacokinetic analysis

Data are presented as mean ± SD values. The elimination half-life was calculated by regression analysis of the log linear portion of the plasma concentration versus time curve. The total area under the plasma concentration–time curve ($AUC_{0-\infty}$) was a summation of AUC_{0-t} (the area from time zero to the last sampling time) and $AUC_{t-\infty}$ (the area from time *t* to infinity). AUC_{0-t} was calculated using the linear trapezoidal formula and $AUC_{t-\infty}$ was determined by dividing the last measurable plasma drug concentration by the elimination rate constant. Maximum concentration (C_{max}) and time to reach maximum concentration (T_{max}) were the observed values.

3. Results

The reported sample preparation, which was clean, rapid and simple, allowed the compounds of interest to be extracted from plasma under the chromatographic conditions. Fig. 2 shows the chromatograms obtained from: (a) drug-free

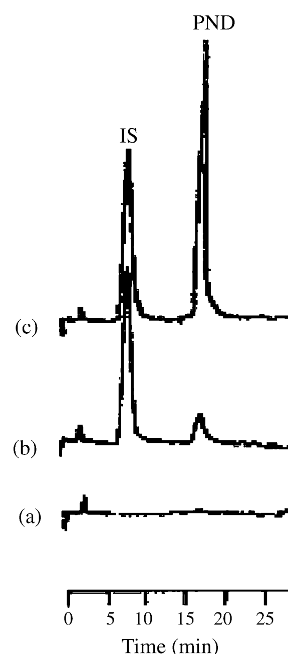

Fig. 2. Typical chromatograms obtained from: (a) drug-free plasma (b) extracted spiked samples (0.010 μg/mL) and (c) extracted patient sample (0.093 μg/mL).

Table 1
Intraday and interday precision and accuracy data for assay of pyronaridine in plasma ($n = 5$)

Spiked concentration (μg/ml)	Precision		% Deviation (found vs. spiked)
	Mean	CV %	
Within day			
0.040	0.039	3.4	2.5
0.080	0.079	3.3	1.2
0.300	0.319	2.7	6.3
0.600	0.562	3.4	6.3
0.010 (LLOQ)[a]	0.011	18.7	10.0
Day-to-day			
0.040	0.038	7.7	5.0
0.080	0.079	7.4	1.2
0.300	0.307	6.8	2.3
0.600	0.578	7.7	4.2
0.010 (LLOQ)[a]	0.009	18.9	10.0

[a] Indicates analysed in replicates of 10.

plasma (b) extracted spiked samples and (c) extracted patient sample. The method yields clean chromatograms, with baseline resolution of PND and internal standard at the retention times of 16.7 and 7.8 min, respectively. Neither endogenous substance from plasma nor tested antimalarial compounds with fluorescence properties such as chloroquine, quinine, mefloquine, pyrimethamine were studied and found not to interfere with PND and IS retention time. The PND calibration curves were linear ($r > 0.999$) in the range of 0.010–0800 μg/mL. The equation of the calibration plots for PND ($n = 10$) was $y = 0.053$(SD: 0.007))$x+$ 0.019(SD: 0.017). y is the peak-height ratio of PND to the internal standard and x denotes various spiked plasma concentrations of PND (μg/mL). Mean analytical recovery of PND ($n = 5$) from human plasma was between 90 and 99% at low and high concentrations. The minimum detectable concentration of PND corresponding to a peak three times the baseline noise at PMT gain 13 and attenuation 1 was 0.005 μg/mL plasma. As for the studied concentration range (0.040–0.600 μg/mL) the within-day coefficient of variation (C.V.) ranged between 2.7 and 3.4% (Table 1); the corresponding C.V. for day-to-day ranged between 6.8 and 7.7% (Table 1). The PND standard solutions were remarkably stable as less than 2% change was found in the stored standard solutions (5 and 20 μg/mL) relative to freshly prepared standard solutions under the test conditions. The PND was stable in plasma at −70 °C over the study period since the percentage recovery was ±5% of the known added concentrations. We found that PND is stable in dry extract stored at −20 °C (<3 days) and for three freeze/thaw cycles as both studied PND concentrations demonstrated a percentage deviation of less than 15% from the nominal values. Quinidine, used as internal standard, showed reproducible extraction (mean recovery 82.3%) and baseline separation. The mean PND plasma concentration time profile over the period of 0–504 h in Thai patients with uncomplicated falciparum malaria, is shown in Fig. 3. After the first dose, PND first appeared 8 h later in plasma.

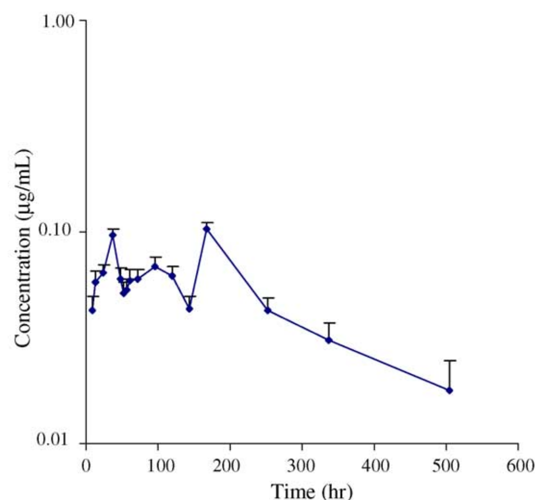

Fig. 3. The mean plasma concentration vs. time profile over the period of 0–504 h in Thai patients with uncomplicated falciparum malaria ($n = 5$) having received 12 mg/kg × 3 of PND orally.

The maximum plasma concentration (C_{max}), the time to reach C_{max} (T_{max}), and $AUC_{o-\infty}$, were 0.12 ± 0.03 μg/mL, 80.0 ± 79.9 h and 29.4 ± 13.1 μg h/mL, respectively. PND was eliminated from the plasma with a mean half-life of 194.8 ± 47.8 h.

4. Discussion

Though the volume of plasma (0.25 mL) for extraction was similar to values reported in the literature, this simple solid-phase extraction procedure has several advantages over other published methods [4–7]. The limit of detection for PND was 0.010 μg/mL standard solution. In this study the limit of quantification was 0.010 μg/mL plasma, which was better than that reported by Chen and Fleckenstein (about 0.028 μg/mL) who used whole blood and plasma. A recent automated solid-phase extraction method for whole blood also did not offer a better limit of quantification (about 0.025 μg/mL) [8]. In addition, the method requires blood haemolysis and sample buffering before loading onto the SPE column. Our sample clean-up procedure using solid-phase cartridges is easy, reduces solvent use and laboratory time. Neither buffer solutions nor pH adjustment were required during sample preparation, sample loading or sample reconstitution prior to injection which makes the method suitable for routine analysis of large numbers of clinical samples. The solid-phase extraction method described herein is both rapid and reliable, and offers a sample extraction procedure for PND in human plasma alternative to conventional liquid extraction techniques which are laborious.

It is interesting to note that three chromatographic peaks were resolved after injection of PND tetraphosphate solution prior to purification using the earlier described current chromatographic method (Fig. 4). Furthermore a capsule

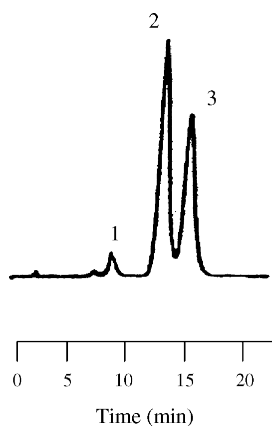

Fig. 4. Typical chromatograms of pyronaridine tetraphosphate capsule.

formulation of PND which was administered to Thai patients in clinical studies also yielded a chromatogram with three peaks similar to Fig. 4. These unknown peaks were labeled as 1–3. Compound 3 was isolated and purified in our laboratory using column chromatography and subsequent mass spectrometry and NMR analysis confirmed substance 3 as PND. The NMR and mass spectrometry results for substance 3 conform with published values [2]. The LC purity of the purified PND substance is found to be 99.6%. However, compound 1 and 2 could not be isolated and purified. The structural identification of the two impurities is in course and has been given high priority. The chromatographic method described by Jayaraman et al. [6] was unable to resolve peaks 1–3. The PND tetraphosphate used in our study was obtained from the same source that used by Jayaraman et al. The lack of resolution of compounds 1–3 by the assay method of Jayaraman et al. might explain the erratic plasma levels of PND obtained in their pharmacokinetic study. The presence of hitherto unknown, undeclared, yet important impurities in both, the pyronaridine reference standard and the capsule formulation, raises quality control issues. Based on the experiences in this study, the quality of the reference standard should be ensured and the drug formulations submitted to rigid analysis prior to their clearance for clinical studies.

Chen and Fleckenstein, using a rabbit model, showed high concentrations of PND accumulated in blood cells and hence concluded that whole blood is a suitable matrix for clinical sample analysis of PND [8]. However, Blessborn et al. demonstrated that PND in whole blood appears to have limited stability when held at refrigerator temperature (6 °C) [9]. However, in real clinical situations it is widely accepted that the effect of a drug is related to the exposure of a patient to the unbound concentration of drug in plasma rather than the total concentration in blood [10]. It is also important to note that storage stability of PND in whole blood at both refrigerator and frozen temperatures (−20 °C) exhibited significant loss of drug with time [4]. Wages et al. suggested that PND loss is due to irreversible binding to blood constituents. In addition, antimalarial compounds such as artemisinin and its derivatives have been shown to be covalently bound to parasitized red blood cells [11,12]. Edlund et al. experienced difficulties with the determination of artemisinin derivatives in whole blood; they observed that artesunate and dihydroartemisinin bound irreversibly to haemoglobin to form a complex from which the compounds could not be recovered [13]. Even instant freezing after blood sampling could not overcome this problem. In consequence, Edlund et al. developed an assay for the measurement of these compounds in blood plasma [13]. Since the inactivation of pyronaridine is apparently due to irreversible binding which is a dynamic, time-dependent process, it is reasonable to separate cellular blood elements and blood plasma immediately after blood sampling, analogous to the procedures used for artemisinin derivatives (4, 5, 9, 13). Therefore, in our laboratory, plasma was selected as the preferred matrix for the analysis of PND in clinical samples. Hence in our clinical study, blood samples were centrifuged immediately after collection to separate plasma and store it frozen at −70 °C until analysis by LC.

5. Conclusion

In conclusion, the above-described analytical method fulfills all criteria required for a PND assay to be suitable for clinical pharmacokinetic studies. The advantage over liquid–liquid extraction and its speed and simplicity make it useful for routine drug analysis in human plasma. The reported method could also have the potential for SPE automation, thereby significantly economizing analysis time in the laboratory.

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