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Development and validation of a liquid chromatography–mass spectrometry assay for the determination of pyronaridine in human blood for application to clinical pharmacokinetic studies

Himanshu Naik, Paul Imming, Mark S. Schmidt, Daryl J. Murry, Lawrence Fleckenstein *

College of Pharmacy, University of Iowa, Iowa City, IA, United States

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Abstract

A reliable and sensitive method for the determination of pyronaridine in human blood was developed and validated. A 0.3 ml aliquot of whole blood was extracted using liquid–liquid extraction after addition of amodiaquine as an internal standard. Analysis was performed on a Shimadzu LCMS-2010A in single ion monitoring positive mode using atmospheric pressure chemical ionization (APCI) as an interface. The extracted ion for pyronaridine was *m/z* 518.20 and for amodiaquine was *m/z* 356.10. Separation was achieved on a Gemini 5 μm C_{18} 3.0 × 150 mm column using a mobile phase composed of 2 mM perflurooctanoic acid–acetonitrile mixture delivered at a flow rate of 0.5 mL/min. The mobile phase was delivered in gradient mode. The retention times of pyronaridine and amodiaquine were 9.2 and 8.2 min, respectively, with a total run time of 14 min. The estimated calibration range of the method was 5.7–855 ng/mL. The analysis of quality control samples for pyronaridine at 11.4, 285, and 760 ng/mL demonstrated excellent precision with relative standard deviation of 11.1, 4.8 and 2.2%, respectively ($n = 5$). Recoveries at concentrations of 11.4, 285 and 760 ng/mL were all greater than 75%. No interference peaks or matrix effects were observed. This LC-MS method for the determination of pyronaridine in human blood has excellent specifications for sensitivity, reproducibility and accuracy. This LC–MS technique was found to improve the quantitation of pyronaridine in whole blood allowing its use in pharmacokinetic studies with clinically relevant doses.

Keywords: Pyronaridine; Antimalarial; Liquid chromatography–mass spectroscopy

1. Introduction

Development of resistance is an increasing problem for antimalarial chemotherapy because resistance against most available drugs has developed in the majority of world-wide parasite populations. There is an urgent need for development of new antimalarial drugs, particularly in less developed tropical countries. From the pharmaceutical side, identification of new targets and compounds are needed and fixed combination therapies are currently being pursued. Pyronaridine, in combination with artesunate is being developed as a 3-day treatment for acute uncomplicated malaria caused by *Plasmodium falciparum* or *P. vivax*.

Pyronaridine is a promising antimalarial drug under development for the treatment of acute falciparum malaria. Pyronaridine(7-chloro-2-methoxy-10-[3′,5′-bis (pyrolidinyl-1-methyl)-4′-hydroxyanilino]benzo[*b*]-1,5-naphthyridine tetraphosphate), is an acridine type schizonticidal drug synthesized in China by Zeng et al. in the early seventies and active against erythrocytic stages of malaria parasites. Pyronaridine is less toxic than chloroquine and highly active against chloroquine-sensitive and resistant parasites both *in vivo* and *in vitro* [1].

Pyronaridine has both acidic and basic functional groups and cannot be obtained in an uncharged form. [2,3]. The structures of pyronaridine and amodiaquine are shown in Fig. 1A and B, respectively. Pyronaridine is known to concentrate in red blood cells. Studies have reported a blood to plasma ratio ranging from 4.9 to 17.8 [4]. Thus, it is important to have a sensitive analytical method for the quantitation of pyronaridine in whole blood that is suitable for pharmacokinetic studies.

* Corresponding author. Tel.: +1 319 335 8804; fax: +1 319 353 5646.
E-mail address: l-fleckenstein@uiowa.edu (L. Fleckenstein).

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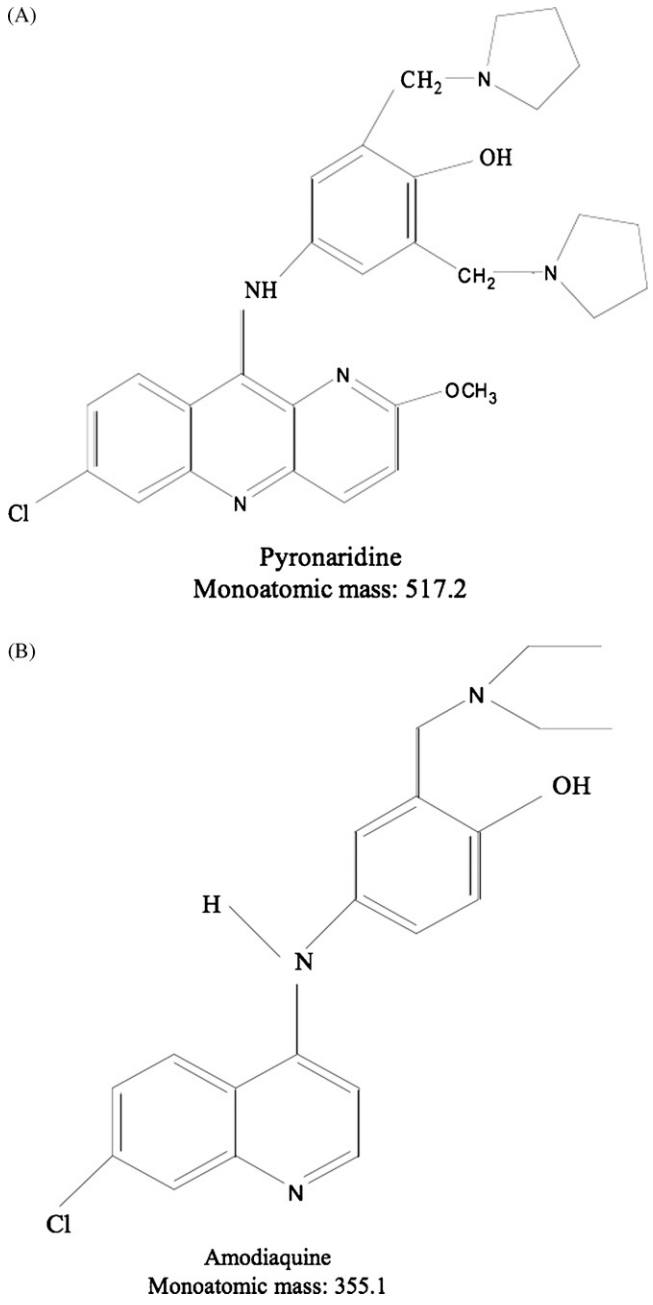

Fig. 1. Chemical structures of pyronaridine (A) and amodiaquine (B).

The pharmacokinetics of pyronaridine has been previously characterized in whole blood or plasma by high-performance liquid chromatography (HPLC) using spectrofluorimetric [1,5–7] or by electrochemical detection [3]. However, these methods are relatively non-specific, laborious and time-consuming and have long retention times. We previously reported an HPLC assay using UV detection for the analysis of pyronaridine in whole blood for application to clinical pharmacokinetic studies [4]. This method requires 0.5 mL of whole blood, and has a run time of 30 min and sensitivity of 28.5 ng/mL. The previously reported methods lacked sensitivity and had excessive sample volume requirements for application to clinical pharmacokinetic studies of pyronaridine in children.

The purpose of the present study was to develop and validate an accurate, reproducible and reliable method for the determination of pyronaridine in human blood. The target features of the method were to increase sample throughput by reducing sample run time (<15 min.), to increase assay sensitivity (LLOQ ≈ 5 ng/ml), and lower blood volume requirement (<0.5 ml). The analytical method established was to be applied to samples obtained from a clinical pharmacokinetic study of adults and children with malaria.

2. Experimental

2.1. Solvents and chemicals

Pyronaridine (Lot # PYROG-03006) was obtained from Shin Poong Pharmaceutical, Ltd (Seoul, Korea). The internal standard amodiaquine was obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile, methanol, ether, and *ortho*-phosphoric acid 85% were obtained from Fischer Scientific (Fair Lawn, NJ, USA). All solvents and chemicals were HPLC or analytical grade. Sodium phosphate tribasic dodeca-hydrate buffer and potassium phosphate (Monobasic) were obtained from Sigma-Aldrich (St Louis, MO, USA). Perfluorooctanoic acid was obtained from TCI America (Tokyo, Japan). Ultra-pure analytical grade Type 1 water for HPLC was produced by Milli-Q Plus water system (Millipore Corporation, Bedford, MA, USA) and used for the preparation of the sample and aqueous solutions.

2.2. Instrumentation

Chromatographic analysis was carried out on a Shimadzu Model 2010A liquid chromatograph and mass spectrometer (Shimadzu, Columbia, MD, USA) using a LC-10AD VP Solvent Delivery system (Pump: A, B). The injection was made with a Shimadzu SIL-10AD VP automatic injector and analysis uses Shimadzu model 2010A data analysis software Lab Solutions Version 3.30. The column was stored in a CTO-10AS VP column oven (Shimadzu, Columbia, MD, USA). Samples were stored in a ULT 2586-5-A14 freezer (Revco scientific, Asheville NC, USA) at −80 °C.

2.3. Standards

Standard stock solutions of pyronaridine and amodiaquine were prepared in methanol. Pyronaridine and amodiaquine were weighed on a Mettler Toledo AG 104 analytical balance (Mettler Toledo Inc., Hightstown, NJ, USA). Appropriate amounts of drugs were dissolved using methanol in volumetric flasks to make a 1 mg/mL stock solution of each. The stock solution was protected from light and stored at −20 °C. Calibration curves concentrations of 5.7, 28.5, 114, 285, 570, and 855 ng/mL were prepared by serial dilution of a stock solution (1 mg/mL in methanol) with methanol. A 10 μg/mL solution for internal standard (amodiaquine) was also prepared in methanol and kept in similar conditions.

2.4. Sample preparation

Extraction of pyronaridine and amodiaquine was carried out by liquid–liquid extraction [4]. The extraction procedure was validated by spiking human blood with known concentrations of pyronaridine and internal standard amodiaquine. Human blood obtained from the blood bank of University of Iowa Hospitals and Clinics was stored frozen in aliquots at −80 °C and used for control preparation. Blood aliquots (0.3 mL) were spiked with 10 μL of 10 μg/mL amodiaquine solution. For standard preparation accurate amounts of pyronaridine and internal standard were added to screw cap glass test tubes using working solution. 0.3 mL of blood sample was added to these tubes and solution was mixed on a vortex mixer for 15 min. Seven hundred fifty microliter of 0.5 M Sodium phosphate tribasic dodeca-hydrate buffer (pH adjusted to 10.3 with 85% *ortho*-phosphoric acid) was added to the tubes and tubes were vortex for 1 min. To this mixture 4.5 mL of ether was added and solution was vortexed for 15 min followed by centrifugation for 10 min. The organic layer was transferred in to a new glass tube and the eluent was evaporated by stream of nitrogen. The residue was reconstituted with 200 μL of solution containing acetonitrile and 0.02 M KH_2PO_4 (13:87, v/v). Test tubes were shaken for 1 min, sonicated for 10 min at 35 °C and again vortexed for 1 min before transferring the liquid layer to an autoinjector vial. A 15 μL aliquot of this solution was injected onto the chromatographic system. For lower QC (11.4 ng/mL) and calibration point (5.7 ng/mL) 75 μL of the solution was injected onto the column.

2.5. Chromatographic and mass spectrometer conditions

The mobile phase used for the analysis was a mixture of 2 mM perfluorooctanoic acid (solvent A) and acetonitrile (solvent B). Solvent A and B were combined in a gradient as follows: 35% B (3 min), 35–55% (7.5 min), 55–35% (0.5 min) and held at 35% until the end of the run. The mobile phase was filtered before being used to prevent entry of bubbles or impurities into the system. The solution was degassed and sonicated under vacuum for approximately 15 min before use. The mobile phase was delivered at a flow rate of 0.5 mL/min. The analysis was carried out using Gemini 5 μm C_{18} 3.0 × 150 mm column and a guard column (Phenomenex, USA) with C-18 Gemini cartridges. The total run time was 14 min. A divert valve was used to discard the LC effluent during the first 3 min and the last 3 min of each chromatographic run.

Curved desolvation line (CDL), heat block and APCI temperatures for the analysis were set at 200°, 200° and 350 °C, respectively. During method development all temperatures were varied but these values resulted in largest peak area. The nebulizer gas flow was set at 2.5 L/min; the detector voltage was set at 1.6 kV, and ion source polarity was set in positive mode. The autoinjector was kept at room temperature and daily temperature was recorded using a temperature recorder. The column was kept in a column oven maintained at 25 °C.

2.6. Validation

2.6.1. Selectivity

Six human blood samples with six individual donors receiving no medication were extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent response at the retention time of pyronaridine and amodiaquine was compared to the lower limit of quantification.

Experiments were carried out to investigate matrix effects to ensure that precision, selectivity and sensitivity was not compromised. Five different lots of blood were used and matrix effects were determined as referred by Matuszewski et al. [8]. The response of the analyte was compared with non-extracted external standard solution (in mobile phase) at the same nominal concentration. The difference from 100% is attributed to matrix effect as ion suppression.

2.6.2. Accuracy and precision

To validate the method for pyronaridine intraday accuracy and precision were evaluated by analysis at various concentration levels on the same day. Three different concentrations (11.4, 285, 760 ng/ml) levels were selected to cover the entire range of calibration curve. Analysis was done by preparing five samples at each concentration level and estimated concentrations were calculated based on calibration curves. To assess the inter-day accuracy and precision, the intra-day assay was repeated on three different days.

2.6.3. Recovery

For the recovery experiment, recovery samples were made by spiking pyronaridine at three different concentration levels, but without amodiaquine prior to extraction. After extraction and evaporation of sample solvent, amodiaquine was added. For recovery controls, blank blood was extracted. After evaporation of sample solvent, the appropriate amount of pyronaridine and amodiaquine were added. The analysis was done in triplicate twice at each concentration levels. Recovery of amodiaquine was carried out by comparing the area obtained from an extracted sample and standard.

2.6.4. Calibration and sample quantification

The calibration curve extended from 5.7 to 855 ng/mL with pyronaridine standards concentration at 5.7, 28.5, 114, 285, 570, 855 ng/ml ($n = 1$, at each level). The analysis was done using amodiaquine as an internal standard and ratio of parent compound to internal standard was plotted against concentration per ml of blood. The calibration curve was linearly fitted and weighted by concentration. Concentrations of compounds in samples were calculated from the calibration curve of pyronaridine.

2.6.5. Stability

2.6.5.1. Freeze/thaw analysis. Analysis in pentuplate was performed on 11.4, 285 and 760 ng/mL quality control (QC) samples. QC samples were stored at −80 °C for 24 h. Aliquots were thawed unassisted at room temperature. When completely

thawed, the samples were refrozen for approximately 24 h at −80 °C. These freeze/thaw samples were analyzed along with QC's to see if there is any variation due to thawing of the samples. The stability data was used to support request for repeat analysis.

2.6.5.2. Bench stability. Samples were prepared at three different levels (11.4, 285 and 760 ng/ml) and kept on working bench for 24 and 48 h before analysis. These studies were carried out to predict the extent of degradation when samples are kept on bench for more than 24 h. Analysis was done in triplicate at each concentration levels.

2.6.5.3. Autoinjector stability. Stability of samples in autoinjector was carried out for over 16 h by injecting the same extracted blood sample, spiked with 760 ng/mL of pyronaridine and 0.5 μg/mL of amodiaquine, at intervals of 2 h. The stability was carried over a period of 16 h.

2.6.5.4. Long term storage stability. The stability of pyronaridine in human blood was investigated over a period of 7 months to find out the acceptable storage condition for clinical samples. Spiked samples were prepared with drug free blood at three different concentrations: 17.1, 285 and 769.5 ng/mL ($n = 5$). Spiked human blood samples were stored frozen at −80 °C. Aliquots at each level were thawed and analyzed at 0, 1, 3.5, and 7 months. A calibration curve was freshly prepared on the day of analysis and used to determine concentration levels.

2.6.6. Over the range dilution

Concentration of pyronaridine obtained from blood may be higher than the calibration range used for the validation analysis. In such cases *in vitro* dilution analysis was carried out to analyze samples that are above the range of the calibration curve. Partial and over the range dilution methods were used for the analysis. Over the range dilutions (1:1) were carried out with blank blood. Samples were diluted based upon the estimated concentration obtained earlier. Analysis was carried out in pentuplate.

For partial volume analysis 150 μL of blood was used for instead of 300 μL. Analysis was done in pentuplate and calibration curve was freshly prepared on the day of analysis and used to determine concentration of diluted samples.

3. Results

3.1. Mass spectral analysis

When pyronaridine was injected directly in the mass spectrometer along with the mobile phase with a positive ion interface, protonated molecules $(MH)^+$ of pyronaridine were seen in abundance having a mass of *m/z* 518.20. For internal standard we were able to detect the parent mass of *m/z* 356.10. So the analysis was carried out based on parent mass of the compound. The analysis temperature, nebulizer gas, and APCI temperature were selected to optimize specificity and sensitivity of *m/z* 518.20 and *m/z* 356.10 ion detection. The full scan mass spectra for amodiaquine and pyronaridine are shown in Fig. 2A and B, respectively.

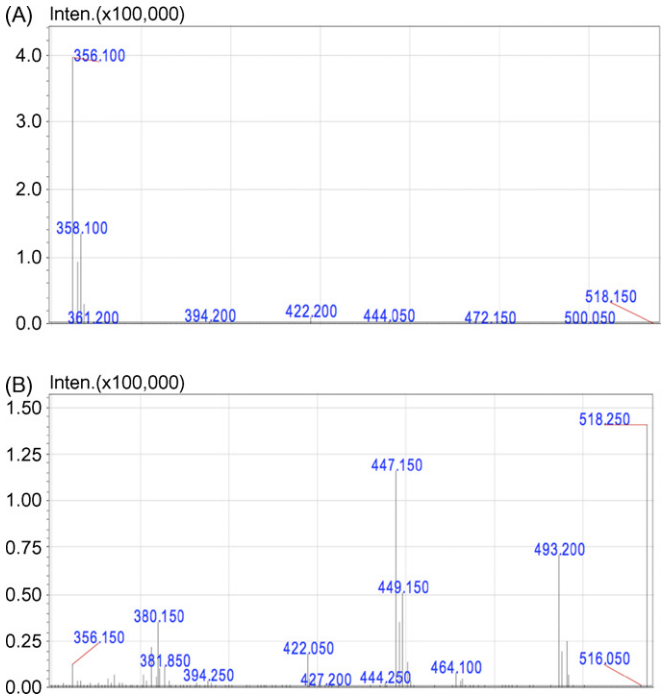

Fig. 2. Full mass spectra scan for amodiaquine (A) and pyronaridine (B).

3.2. Separation and retention time

Observed retention times were 9.2 and 8.2 min for pyronaridine and amodiaquine respectively, with a total run time of 14 min. Blank blood was tested for endogenous interference. No additional peaks due to endogenous substances were observed that would interfere with the detection of compounds of interest. Typical chromatograms are shown in Fig. 3. A representative chromatogram of blank blood is shown in Fig. 3(A). Fig. 3(B) shows a chromatogram calibration standard containing 570 ng/ml of pyronaridine and amodiaquine. Fig. 3(C) shows a chromatogram calibration standard containing 5.7 ng/ml of pyronaridine and amodiaquine The LC–MS procedure results in much cleaner chromatograms then reported by other methods (1, 3, and 4).

3.3. Specificity and matrix effects

The apparent response at the retention time of pyronaridine and amodiaquine was compared to the lower limit of quantification. None of the six lots of blood had any interference at the retention time of pyronaridine and amodiaquine. Signal to noise ratio was greater than 5:1 when compared with lower limit of quantification.

Matrix effect was determined by comparing the LC–MS response (peak area) of pyronaridine at a concentration of 285 ng/mL (spiked postextraction into a blood extract) to the LC–MS response of the analyte present in reconstitution solution. The mean absolute matrix effect calculated was 96.3%

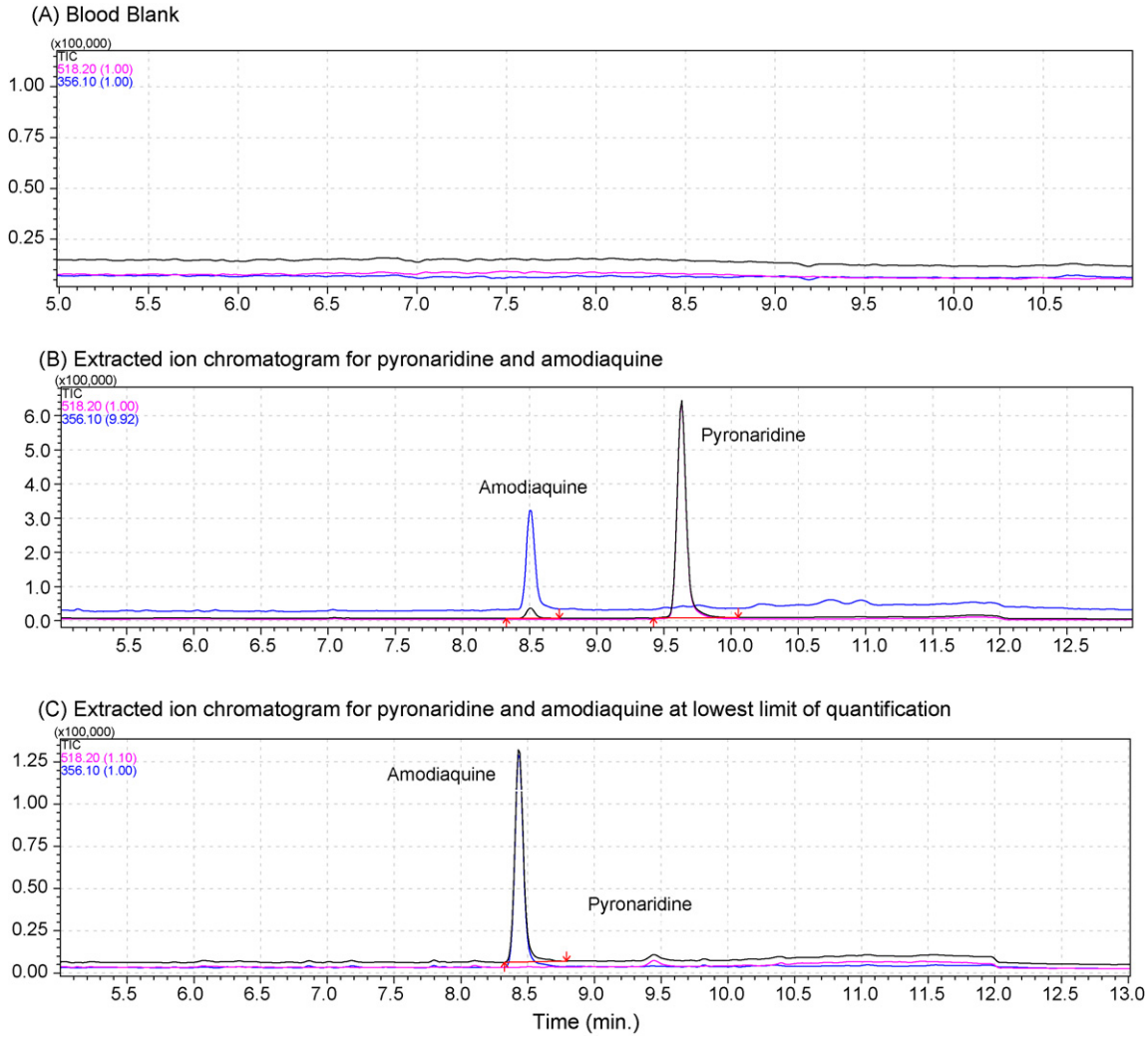

Fig. 3. Chromatograms of 75 μl injection blank blood (A), 15 μl calibration standard containing 570 ng/mL of pyronaridine and internal standard amodiaquine in human blood (B). Extracted ion chromatogram for pyronaridine and amodiaquine at lowest limit of quantification (C).

($n = 5$), indicating responses in the reconstitution solution and blood extract are same and no absolute matrix effect was observed. Thus, no ion suppression was observed. In addition, a sample run was also carried out including various reported metabolite masses of pyronaridine. No suppression or enhancement of the intensity was observed.

3.4. Linearity

Calibration curves for pyronaridine were linear using weighted (1/Concentration) linear regression in the concentration range of 5.7–855 ng/ml on all five days, with a correlation coefficient greater than or equal to 0.991 for all curves. The calibration curves accuracy is presented in Table 1, demonstrating that measured concentration is within ±15% of the actual concentration except for the lower calibration point. Mean value for slope and intercept for heptuplate analysis were 0.02593 and −0.01138, respectively. Results were calculated using peak area ratios. The lower limit of quantitation has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 20% ($n = 5$) and signal to noise ratio of 5:1 for a pentuplate analysis for pyronaridine. Results at lowest concentration studies (5.7 ng/mL) met the criteria for lower limit of quantitation. Data for lower limit of quantification are shown in Table 2.

3.5. Recovery data

Percentage recovery of pyronaridine was measured by dividing the ratio of concentration levels with that of controls. The mean recoveries ($n = 6$) for pyronaridine (11.4, 285, 760 ng/ml) were 76.0, 80.3 and 78.7%, respectively. The mean recovery of internal standard amodiaquine was 97%.

Table 1
Back calculated concentration from calibration curves ($n = 7$)

	Theoretical concentration (ng/mL)					
	5.7	28.5	114	285	570	855
Pyronaridine						
Average	7.2	25.3	96.5	266.9	597.0	865.3
S.D.	0.6	0.5	10.5	15.0	45.8	60.9
C.V. (%)	8.5	2.2	10.8	5.6	7.7	7.0

Table 2
Accuracy and precision of pyronaridine in blood at lower limit of quantification (LLOQ)

Sample no.	Predicted concentration (ng/mL)
LLOQ 1	6.9
LLOQ 2	7.4
LLOQ 3	6.5
LLOQ 4	5.4
LLOQ 5	5.6
Mean	6.4
S.D.	0.85
C.V. (%)	13.4
Mean bias (%)	11.6

3.6. Accuracy and precision

The intra-day coefficients of variation for pyronaridine samples (11.4, 285, 760 ng/ml) were 11.1, 4.8 and 2.2%, respectively. Coefficients of variation of inter-day analysis of pyronaridine samples (11.4, 285, 760 ng/ml) were 15.9, 9.7 and 7.8%, respectively. The data obtained for the pyronaridine was within the acceptable limits to meet guidelines for bioanalytical methods guidelines for bioanalytical validation [9]. Data for accuracy and precision are shown in Table 3.

3.7. Autoinjector stability

Stability of samples stored in the autoinjector was carried out over a period of 16 h by injecting same sample at an interval of 2 h. The R.S.D. for the peak area ratio of pyronaridine measurements was 3.4%. Concentration of pyronaridine varied from 662.3 to 731.6 ng/mL. These results demonstrate that extracted pyronaridine samples are stable up to 16 h in the autoinjector.

3.8. Freeze/thaw analysis

The freeze/thaw stability of pyronaridine was determined by measuring the accuracy and precision for samples that underwent three freeze/thaw cycles. The results showed that pyronaridine was stable in human blood through three freeze/thaw cycles. The precision ranged from 2.2 to 9.9% and the accuracy ranged from 80.8 to 104.4% for pyronaridine. These data are shown in Table 4.

Table 3
Intra-day and inter-day precision and accuracy for pyronaridine in human blood

	Theoretical concentration (ng/mL)		
	11.4	285	760
Intra-day run			
Overall mean ($n=5$)	11.6	244.4	788.9
S.D.	1.3	11.6	17.4
C.V. (%)	11.1	4.8	2.2
DMT (%)	1.9	−14.3	3.8
Inter-day run			
Overall mean ($n=15$)	11.3	269.6	756.5
S.D.	1.8	26.0	59.2
C.V. (%)	15.9	9.7	7.8
DMT (%)	−0.7	−5.4	−0.5

S.D., standard deviation; C.V., coefficient of variation; DMT, deviation of mean value from nominal.

Table 4
Freeze/thaw precision and accuracy for pyronaridine in human blood

	Theoritical concentration (ng/mL)		
	11.4	285	760
Pyronaridine (Cycle 1)			
Overall mean ($n=5$)	11.6	281.3	788.9
S.D.	1.2	8.6	17.4
C.V. (%)	11.1	3.1	2.2
DMT (%)	1.9	−1.3	3.8
Pyronaridine (Cycle 3)			
Overall mean ($n=5$)	9.2	283.9	793.6
S.D.	0.9	7.5	17.7
C.V. (%)	9.9	2.7	2.2
DMT (%)	−19.2	−0.4	4.4

S.D., standard deviation; C.V., coefficient of variation; DMT, deviation of mean value from nominal.

3.9. Bench stability

Extracted validation samples at three QC levels were kept at room temperature for over 24 and 48 h and were reanalyzed and quantified against freshly made standard curves. The result show a minimal reduction in levels of pyronaridine when compared with samples analyzed at time zero. Data for the percentage reduction in levels of pyronaridine from baseline (0 h) are shown in Table 5.

3.10. Storage stability data

Samples long term stability at −80 °C was evaluated to establish acceptable storage condition for clinical samples. Spiked human samples with known concentration of pyronaridine were analyzed at regular intervals. A standards calibration curve was freshly prepared on the day of analysis, and concentration levels are measured on the basis of calibration curve.

The percentage deviation for pyronaridine from baseline (0 months) concentration at −80 °C baseline concentration ranged from 95.8 to 113.0%. The results indicated that the drug was stable at −80 °C for at least 7 months. The analysis will be carried out over a period of one year to determine the stability of pyronaridine. Long term stability data are shown in Table 6.

Table 5
Bench stability for pyronaridine in blood

Time (h)	Theoretical concentration (ng/ml)		
	11.4 (PDB)	285 (PDB)	760 (PDB)
0 ($n=3$)	100	100	100
24 ($n=3$)	104.1	106.8	84.0
48 ($n=3$)	118.6	115.6	100.1

PDB: percentage deviation from baseline (0 h).

Table 6
Long-term stability of pyronaridine in blood at −80 °C

Time (months)	Temperature (°C)	Theoretical concentration (ng/ml)		
		17.1 (PDB)	285 (PDB)	769.5 (PDB)
0	−80	100	100	100
1		108.1	96.7	102.6
3.5		102.3	113.0	103.8
7		97.8	99.0	95.8

PDB, percentage deviation from baseline (0 months).

Table 7
Over the range precision and accuracy for pyronaridine in human blood

	Dilution	Partial volume
Pyronaridine		
Prepared concentration (ng/mL)	1425	1710
Expected concentration (ng/mL)	712.5	855
Overall mean ($n=5$)	783.8	910.1
S.D.	38.1	37.9
C.V. (%)	4.9	4.2
DMT (%)	10.0	6.4

S.D., standard deviation; C.V., coefficient of variation; DMT, deviation of mean value from nominal.

3.11. Over the range dilution

The over the range dilution analysis of pyronaridine was determined by measuring the accuracy and precision for samples that underwent 1:1 dilution with control blood and by partial volume analysis. The results showed that dilution of the over the range samples by both methods can be carried out with good accuracy and precision. The precision for 1:1 and partial volume dilution method was 4.9 and 4.2%, respectively, and the accuracy ranged from 90.0 to 94.6%. Expected concentration of pyronaridine after dilution was within the acceptance criteria of ±15%. Thus, we conclude that either method can be used for the dilution of over the range samples. Over range dilution data for pyronaridine are reported in Table 7.

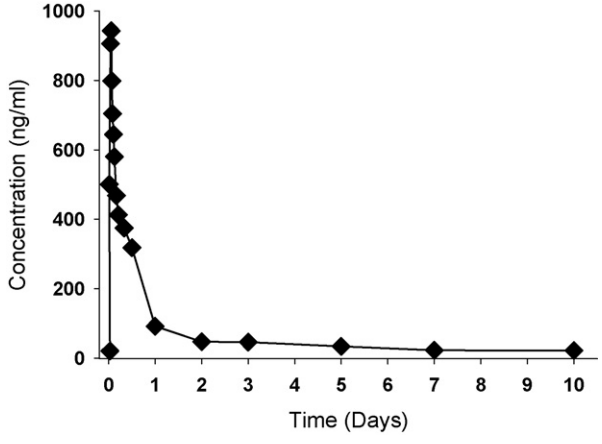

Fig. 4. Concentration–time profile of pyronaridine after oral administration of 12 mg/kg dose of pyronaridine to a healthy adult volunteer.

3.12. Application to clinical sample analysis

The method was applied to a clinical pharmacokinetic study of pyronaridine in healthy volunteers. An oral dose of 12 mg/kg pyronaridine was administered to the volunteers and samples were collected at constant interval of time. Nineteen samples were collected over a period of 10 days and analyzed using the proposed method. The sample run was analyzed in a batch of 32 samples which include 19 patient samples, 6 calibration levels, blank and 6 QC's. QC's were run at the start and the end of the run. Accuracy of QC's for Pyronaridine was within a range of 85–115%. Concentration-time profile for a healthy volunteer is shown in Fig. 4.

4. Discussion

This analytical method with a simple liquid–liquid extraction procedure and MS detection has several advantages over the other published methods. The limit of detection for pyronaridine in this study is 5.7 ng/mL which is superior to the sensitivity reported by Ramanathan (10 ng/mL), Blessborn (50 nmol/L), Babalola (50 ng/mL) and Jayaraman (10 ng/mL). The method also has the advantage or requiring only 0.3 ml of whole blood. The retention time of the method was reduced to 14 min compared to 25–30 min reported in other methods. This reduction in retention time has approximately doubled sample throughput.

The liquid–liquid extraction method used was similar to that used in our laboratory for the extraction of pyronaridine from blood. However, the injection volume was reduced to 15 μL instead of 50 μL and reconstitution volume was increased to 200 μL instead of 100 μL. Higher volume of reconstitution provides sufficient volume for reinjection of subject samples. Solubility of pyronaridine in phosphate buffer was higher compared to mobile phase solution, for this reason phosphate buffer was used for reconstitution of samples.

A stability study was conducted at −80 °C to determine the storage temperature for blood samples with minimum degradation. The study was conducted at three levels of concentration for a period of 7 months. The analysis shows that the samples were stable up to 7 months when kept at −80 °C. Therefore, all blood samples were stored at −80 °C prior to analysis. Freeze/thaw analysis results suggested that blood samples could be thawed and refrozen without compromising the integrity of the sample. Bench stability analysis demonstrated that samples are relatively stable when kept on bench for 48 h.

The mass detector response was linear over the range of 5.7–855 ng/mL. Linear regression analysis performed for pyronaridine in above concentration ranges yielded mean correlation coefficient of 0.991. The greater sensitivity of the method will help to carry our better pharmacokinetic analysis of the data.

Amodiaquine was found to be suitable internal standard because its structure was similar to pyronaridine, and it was well recovered from blood. Amodiaquine had a retention time which lies ahead of pyronaridine.

Analysis was tested with both positive and negative modes, but the sensitivity obtained with a positive mode was much higher then that of negative mode. Atmospheric pressure chem-

ical ionization (APCI) source was used instead of electro spray ionization (ESI) because it gave good reproducibility and sensitivity.

This validated LC–MS method was developed to support the clinical development of the promising antimalarial drug, pyronaridine. Method uses lower volume of blood and highly sensitive compared to other reported methods thus can be applied to clinical pharmacokinetic studies in children. Fig. 4 shows an example of a concentration vs. time profile for a healthy subject that received a single oral dose of pyronaridine (12 mg/kg). Blood concentration of pyronaridine was detected 20 min after administration, with a maximum concentration in blood (C_{max}) of 600.7 ng/mL. The time to maximum concentration of pyronaridine in blood (T_{max}) was 2.5 h and the elimination half life ($t_{1/2}$) was 7.7 days.

In summary, an LC–MS method was developed and validated for the specific and quantitative analysis of pyronaridine in human blood. The analyte was extracted using liquid–liquid extraction. The assay has been validated and the results of validation show the method is reproducible and accurate. The calibration range for pyronaridine is linear from 5.7 to 855 ng/mL with practically no interference or matrix effects from endogenous blood components. Compared to previously reported methods this method requires a lower sample volume, provides better sensitivity and higher throughput. The method was successfully applied to clinical pharmacokinetics studies of pyronaridine in adult and children with acute malaria. It is estimated that this method can be used from whole blood samples from the lowest clinical dose (6 mg/kg) until at least 28 days after oral administration.

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