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Development and validation of a liquid chromatography–mass spectrometry assay for the determination of pyronaridine in human urine

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Abstract

A reliable method has been developed for the determination of pyronaridine in human urine using amodiaquine as an internal standard. Liquid–liquid extraction was used for sample preparation. Analysis was performed on a Shimadzu LCMS-2010 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. Chromatography was carried out using a Gemini 5 μm C_{18} 3.0 mm × 150 mm column using 2 mM perflurooctanoic acid and acetonitrile mixture as a mobile phase 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 9.1 and 8.1 min respectively, with a total run time of 14 min. The assay was linear over a range of 14.3–1425 ng/mL for pyronaridine ($R^2 \geq 0.992$, weighted 1/Concentration). The analysis of quality control samples for pyronaridine at 28.5, 285, 684 and 1140 ng/mL demonstrated excellent precision with relative standard deviation of 5.1, 2.3, 3.9 and 9.2%, respectively ($n = 5$). Recoveries at concentrations of 28.5, 285, 684 and 1140 ng/mL were all greater than 85%.This LC–MS method for the determination of pyronaridine in human urine has excellent specifications for sensitivity, reproducibility and accuracy and can reliably quantitate concentrations of pyronaridine in urine as low as 14.3 ng/mL. The method will be used to quantify pyronaridine in human urine for pharmacokinetic and drug safety studies.

Keywords: Pyronaridine; Antiparasitic; Liquid chromatography–mass spectroscopy

1. Introduction

The worldwide spread of strains of *Plasmodium falciparum* that are resistant to chloroquine has highlighted the urgent need for development of new antimalarial drugs, particularly in less developed tropical countries. The constraints of antimalarial chemotherapy underscore the need for the development of novel compounds. Pyronaridine, in combination with artesunate is being developed as a 3-day treatment for acute uncomplicated malaria caused by *P. falciparum* or *vivax*.

Pyronaridine (7- chloro - 2- methoxy- 10- [3′, 5′- bis (pyrolidinyl-1-methyl) - 4′ -hydroxyanilino] benzo [*b*] - 1, 5 - naphthyridine tetraphosphate,) is an acridine type mannich base schizontocide active against multiple chloroquine-resistant malaria *in vitro* and *in vivo* models. Originally synthesized in China in the 1970s, it was used for almost 20 years as monotherapy to treat malaria in the Hunan and Yunan Provinces. Studies conducted by Looareesuwan et al. [1], Ringwald and co-workers [2], and others demonstrated that pyronaridine was an effective antimalarial in both Asian and African patient populations.

Pyronaridine tetraphosphate is a hydroscopic yellow powder, odourless with a bitter taste. It is soluble in water, methanol, very sparingly soluble in ethanol, and insoluble in chloroform and other organic solvents [3,4]. Pyronaridine has both acidic and basic functional groups and cannot be obtained in an uncharged form. Structure of pyronaridine is shown in Fig. 1.

Similar to chloroquine, pyronaridine targets hematin, as demonstrated by its ability to inhibit *in vitro* β-hematin formation to form a complex with hematin with a stoichiometry of 1:2, enhances hematin-induced red blood cell lysis and inhibits glutathione dependent degradation of hematin [5]. Growth studies of *P. falciparum* K1 in culture showed antagonism of pyronaridine in combination with antimalarials that inhibit β-hematin formation (chloroquine, mefloquine and quinine).

Preliminary animal studies conducted in rats have shown that a substantial amount of pyronaridine is excreted unchanged in urine. To measure the extent of excretion of unchanged pyronaridine in human urine an accurate analytical method is required.

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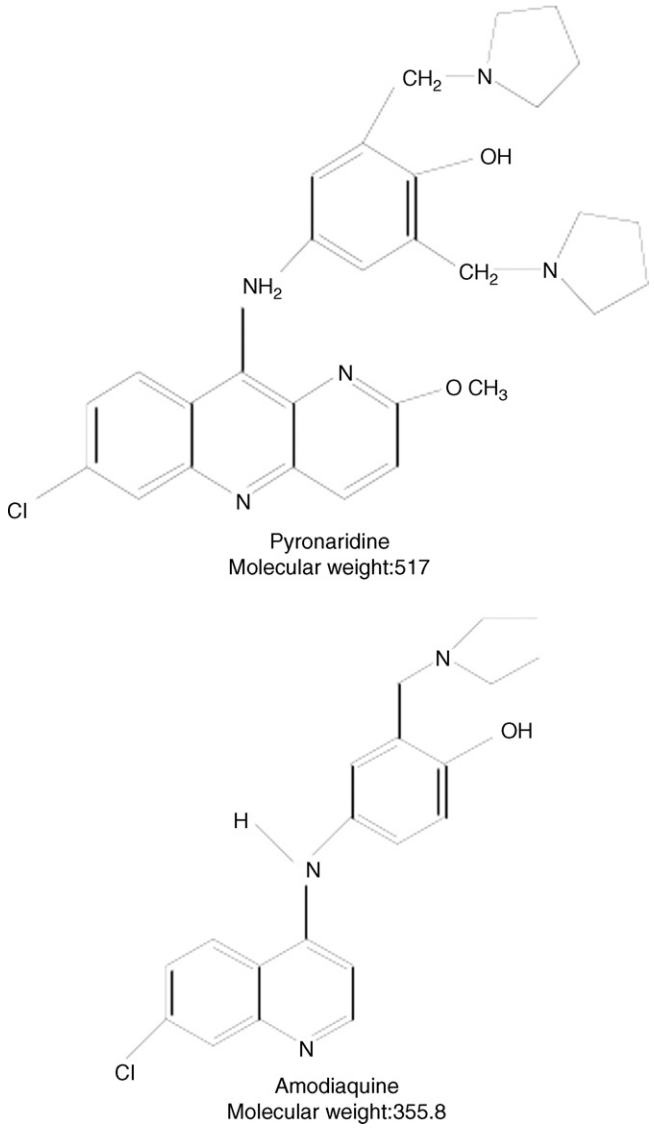

Fig. 1. Chemical structures of pyronaridine (top) and amodiaquine (bottom).

Other reported analytical methods used for the quantification of pyronaridine in blood and plasma were unsuited for clinical pharmacokinetic studies in urine because they lack specificity and are unable to measure pyronaridine with good separation, or have biological interferences [3,6–9]. Pharmacokinetics of pyronaridine has been previously characterized in blood or plasma by high-performance liquid chromatography (HPLC) using spectrofluorimetric [6,7] or by electrochemical detection [3]. However these methods are relatively non-specific, laborious and time-consuming and have long retention times. Liquid chromatography-mass spectroscopy (LC–MS) gives definite compound identification and provides high sensitivity and selectivity for all analytes of interest with shorter retention time.

We describe a specific and reliable method, which uses amodiaquine as an internal standard, for the determination of pyronaridine in human urine for clinical trials with this antimalarial agent.

2. Experimental

2.1. Solvents and chemicals

Pyronaridine (Lot # PYROG-03006) was obtained from Shin Poong Pharm Ltd (South Korea) and amodiaquine was obtained from Sigma–Aldrich (St Louis, MO, USA). Acetonitrile, methanol, ether, ortho-phosphoric acid 85% were obtained from Fischer Scientific (Fair Lawn, NJ, USA). 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. Standards

Standard stock solution 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. For calibration curves concentrations of 14.3, 57, 114, 285, 570, 855, 1140 and 1425 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 was also prepared in methanol. The stock solution was stored at −20 °C.

2.3. Instrumentation

Chromatographic analysis was carried out on a Shimadzu Model 2010 liquid chromatograph and mass spectrometer (Shimadzu, Columbia, MD, USA) using a LC-10AD Solvent Delivery system (Pump: A, B). The injection was made with a Shimadzu SIL-10AD automatic injector and analysis uses Shimadzu model 2010 data analysis software Lab Solutions Version 3.04. Samples were stored in a ULT 2586-5-A14 freezer (Revco scientific, Asheville NC, USA) at −80 °C.

2.4. Sample preparation

Extraction of pyronaridine and amodiaquine was carried out by liquid–liquid extraction [8]. Extraction procedure was validated by spiking human urine with known concentrations of pyronaridine and internal standard amodiaquine. Human urine was obtained from individuals of the laboratory and was used for control preparation. Urine aliquots (0.2 mL) were spiked with 10 μL of 10 μg/mL amodiaquine solution. For standard preparation accurate amount of pyronaridine and internal standard were added to screw cap glass test tubes using working solution. A 0.2 mL aliquot of urine was added to these tubes and solution was mixed on a vortex mixer for 15 min. A volume of 500 μL 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 3 min. To this mixture 3 mL of ether was added and solution was vortexed for 5 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 (27:73, 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 auto injector vial. A 30 μL aliquot of this solution was injected onto the chromatographic system.

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 mm × 150 mm column and a guard column (Phenomenex, USA) with C-12 max-RP cartridges.

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. Column and autoinjector were kept at room temperature and daily temperature was recorded using a temperature recorder. The flow was directed to mass spectrometer 7.5 min after the start of the run and diverted from mass spectrometer after 12 min. The total run time was 14 min.

2.6. Validation

2.6.1. Selectivity

Six human urine 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 in to ensure that precision, selectivity and sensitivity was not compromised. Five different lots of urine were used and matrix effects were determined as referred in the Matuszewski et al. [10]. 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. Four different concentrations (28.5, 285, 684, 1140 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 concentrations were calculated from calibration curves. The upper limit of quantification (ULOQ) was given by highest level of the calibration curve. 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 the four different concentration levels, but without amodiaquine prior to extraction. After extraction and evaporation of sample solvent, amodiaquine was added. For recovery controls, blank urine was extracted. After evaporation of sample solvent, the appropriate amount of pyronaridine and amodiaquine were added. The analysis was done in quadruplets at each concentration levels. Recovery of amodiaquine was carried out by comparing the area obtained from an extracted sample and a standard.

2.6.4. Freeze/thaw analysis

Analysis in pentuplate was performed on 28.5 ng/mL, 285 ng/mL and 684 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. Calibration and sample quantification

The calibration curve extended from 14.3–1140 ng/mL with pyronaridine standards concentration at 14.3, 57, 114, 285, 570, 855, 1140, 1425 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 urine. The calibration curve was linearly fitted and weighted by concentration. Concentrations of compounds in samples were calculated from the calibration curve of pyronaridine.

2.6.6. Stability

2.6.6.1. Bench stability. Samples were prepared at three different levels (28.5, 285 and 684 ng/ml) and kept on the laboratory work bench for 24 and 48 h before analysis. These studies were carried out to predict the extent of degradation when samples are kept on the work bench for more than 24 h. Analysis was done in triplicate at each concentration levels.

2.6.6.2. Autoinjector stability. Stability of samples in autoinjector was carried out for over 16 h by injecting the same extracted urine sample, spiked with 684 ng/mL of pyronaridine, at intervals of 2 h. The stability was carried over a period of 16 h at ambient temperature (nominally 25 °C).

2.6.6.3. Long term storage stability. The stability of pyronaridine in human urine was investigated over a period of 130 days

to find out the acceptable range storage condition for clinical samples. Spiked samples were prepared with drug free urine at three different concentrations: 114, 684 and 1140 ng/mL ($n = 5$). Spiked human urine samples were stored frozen at −80 °C. Aliquots at each level were thawed and analyzed at 0, 50, 80 and 130 days. A calibration curve was freshly prepared on the day of analysis and used to determine concentration levels.

2.6.7. Over the range dilution

Pyronaridine concentration obtained from actual urine specimens may be higher than the calibration range used for the validation analysis. In such cases the sample will be diluted 1:1 or higher using control urine. Over the range dilution experiment was carried out by preparing a concentration of 2280 and 6840 ng/mL. The prepared samples were diluted 1:1 (2280 ng/mL) and 1:8 (6840 ng/mL) with blank urine. Analysis was done in a set of five replicates and calibration curve was used to determine concentration levels of the 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, then protonated molecules $(MH)^+$ of pyronaridine was 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 analysis was carried out based on these masses. 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. 2(A) and (B), respectively.

3.2. Separation and relative retention time

Observed retention times were 9.1 and 8.1 min for pyronaridine and amodiaquine respectively, with a total run time of 14 min. Blank urine 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 the urine blank is shown in Fig. 3(A). Fig. 3(B) shows a chromatogram calibration standard containing 570 ng/ml of pyronaridine. Fig. 3(C) shows a chromatogram calibration standard containing 14.3 ng/ml of pyronaridine The LC–MS procedure results in much cleaner chromatograms.

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 urine 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.

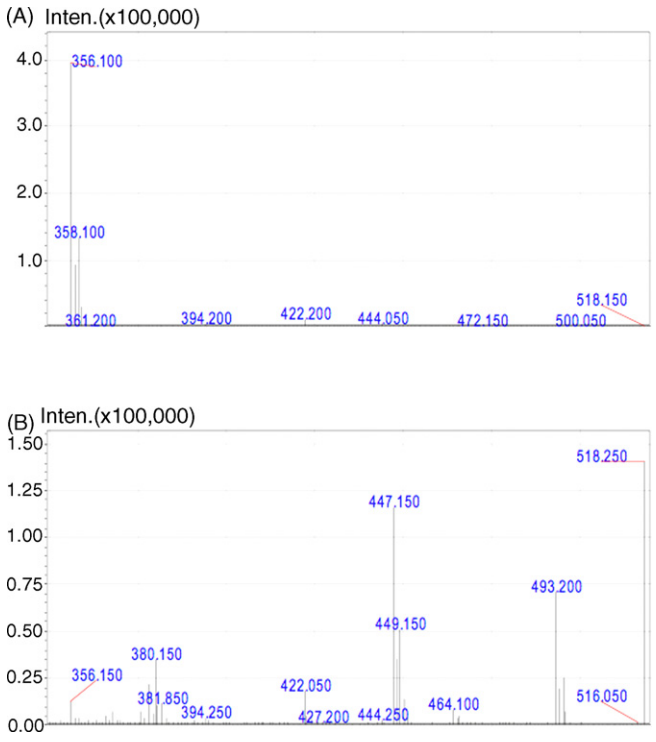

Fig. 2. Full mass spectra scan for amodiaquine (A) and pyronaridine (B).

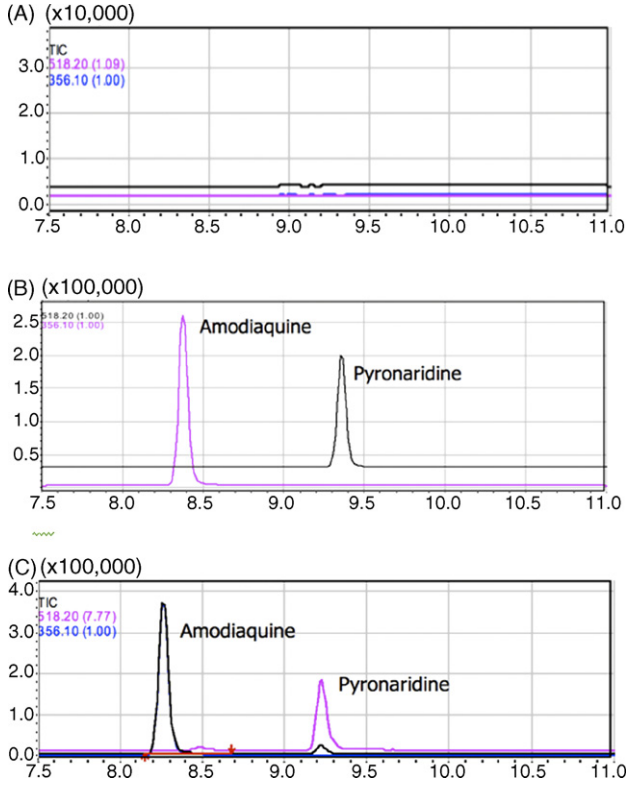

Fig. 3. Chromatograms of 30 μl injection blank urine (A), calibration standard containing 570 ng/mL of pyronaridine and internal standard amodiaquine 0.5 μg/mL in human urine (B). Calibration standard containing 14.3 ng/mL of pyronaridine and internal standard amodiaquine 0.5 μg/mL in human urine (C).

Table 1
Back calculated concentration from calibration curves ($n = 5$)

Theoretical concentration (ng/mL)	14.3	57	114	285	570	855	1140	1425
Pyronaridine								
Average	19.2	47.2	97.7	259.6	582.1	889.7	1151.3	1385.3
S.D	3.1	4.4	14.5	42.3	24.9	54.8	59.6	83.4
C.V (%)	15.9	9.4	14.8	16.3	4.3	6.2	5.2	6.0

Matrix effect was determined by comparing the LC–MS response (Area) of pyronaridine at a concentration of 1140 ng/mL (spiked postextraction into a urine extract) to the LC–MS response of the analyte present in reconstitution solution. The mean absolute matrix effect calculated was 108% ($n = 5$), indicating responses in the reconstitution solution and urine 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 14.3–1425 ng/ml on all five days, with a correlation coefficient greater than or equal to 0.99 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. All concentrations of pyronaridine have been calculated as the free base. Linear calibration curves were obtained with a coefficient of correlation usually higher than 0.992 for pyronaridine. The mean values for slope and intercept for pentuplate analysis were 0.00144 and −0.01914, respectively. Results were calculated using peak area ratios. The limit of quantification has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 15% ($n = 5$) and signal to noise ratio of 5:1 for a pentuplate analysis for pyronaridine. Results at lowest concentration studies (14.3 ng/mL) met the criteria for lower limit of quantification. Accuracy and precision (pentuplate analysis) at LLOQ were −7.2% and 12.0%, respectively.

3.5. Recovery data

Percentage recovery of pyronaridine was measured by dividing the ratio of concentration levels with that of controls. The mean recoveries ($n = 4$) for pyronaridine (28.5, 285, 684, 1140 ng/ml) were 93.8, 85.8, 93.3 and 99.9% respectively. Mean recovery ($n = 5$) for internal standard was $100 \pm 4\%$. Data for pyronaridine recovery are shown in Table 2.

3.6. Accuracy and precision

The intra-day coefficients of variation for pyronaridine samples (28.5, 285, 684, 1140 ng/ml) were 5.1, 2.3, 3.9 and 9.2%, respectively. Coefficients of variation of inter-day analysis of pyronaridine samples (28.5, 285, 684, 1140 ng/ml) were 13.1, 7.1, 12.8 and 6.7%, respectively. The data obtained for the pyronaridine was within the acceptable limits to meet guidelines for bioanalytical methods guidelines for bioanalytical validation [11]. Data for accuracy and precision are shown in Table 3.

Table 2
Recovery data for pyronaridine

Theoretical concentration (ng/mL)	Pyronaridine (%)
28.5 ($n = 4$)	93.8
285 ($n = 4$)	85.8
855 ($n = 4$)	93.3
1140 ($n = 4$)	99.9

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 RSD for the peak area ratio of pyronaridine measurements was 8.0% with a mean ratio of pyronaridine/I.S was 1.07 ± 0.09. Concentration of pyronaridine varied from 558.3 to 735.8 ng/mL. These results demonstrate that 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 urine through three freeze–thaw cycles. The precision ranged from 3.0 to 8.6% and

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

	Theoretical concentration (ng/mL)			
	28.5	285	684	1140
Intra-day run				
Overall mean ($n = 5$)	30.0	268.4	668.1	1169.17
S.D.	1.5	6.1	26.1	107.67
CV (%)	5.1	2.3	3.9	9.2
DMT (%)	5.3	−5.8	−2.3	2.6
Inter-day run				
Overall mean ($n = 15$)	27.7	262.4	677.3	1206.02
S.D.	3.6	18.7	86.7	81.19
CV (%)	13.1	7.1	12.8	6.7
DMT (%)	−2.8	−7.9	−1.0	5.8

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 urine

	Theoritical concentration (ng/mL)		
	28.5	285	684
Pyronaridine (Cycle 1)			
Overall mean ($n=5$)	28.88	245.45	747.80
S.D.	4.04	18.01	100.26
C.V. (%)	14.0%	7.3	13.4
DMT (%)	1.3%	−13.9	9.3
Pyronaridine (Cycle 3)			
Overall mean ($n=5$)	24.7	281.1	594.2
S.D.	0.9	15.6	50.9
C.V. (%)	3.0	6.0	8.6
DMT (%)	−13.4	−1.4	−13.6

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

the accuracy ranged from 86.4 to 98.6% for pyronaridine. Data for freeze/thaw analysis are shown in Table 4.

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

The samples long term stability at −80 °C was evaluated to establish acceptable storage condition for the 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 concentration ranged from 85 to 112%. Thus the results indicated that the drug was stable at −80 °C for at least 130 days (4 months). Stability data are shown in Table 6.

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 and 1:8 dilution with blank urine. The results showed that dilution of the over the range samples can be carried out with good accuracy and precision. The precision for 1:1 and 1:8 dilution method was 5.1 and 6.7% respectively and the accuracy in ranged from 91.2–94.9%. Over range dilution data for pyronaridine are reported in Table 7.

Table 5
Bench stability for pyronaridine

Time (h)	Theoretical concentration (ng/ml)		
:	28.5 (PDB)	285 (PDB)	684 (PDB)
: 0 ($n=3$)	100	100	100
.24 ($n=3$)	92.5	115.0	94.8
.48 ($n=3$)	109.4	102.2	82.0

PDB: percentage deviation from baseline (0 h).

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

Time (days)	Theoretical concentration (ng/ml)		
	114	684	1140
0	98.1	663.6	1092.0
50	108.9	744.3	1120.9
80	128.2	576.5	970.4
130	117.8	584.3	973.5

Table 7
Over range precision and accuracy for Pyronaridine in human urine

	1:1 Dilution	1:8 Dilution
Pyronaridine		
Prepared Concentration (ng/mL)	2280	6840
Expected Concentration (ng/mL)	1140	855
Overall mean ($n=5$)	1198.1	930.4
S.D.	61.3	62.6
C.V. (%)	5.1	6.7
DMT (%)	5.1	8.8

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

3.12. Application to clinical sample analysis

The method was applied to clinical pharmacokinetic study of pyronaridine in healthy volunteers. An oral dose of 12 mg/kg pyronaridine was administered to the volunteers. Thirteen samples were collected over a period of 11 days and analyzed using the proposed method. The sample run was analyzed in a batch of 28 samples which include 13 patient samples, 8 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%. A plot of the amount of pyronaridine remaining to be excreted versus mid-point time (T_{mid}) is shown in Fig. 4 for a healthy volunteer who received the drug.

4. Discussion

Several columns were investigated at the outset of this project to identify a suitable column and mobile phase to optimize the chromatography. The Gemini 5 μm C_{18} was found to give sharp, symmetrical peaks and good sensitivity using a mobile phase consisting of perfluorooctanoic acid and acetonitrile in a gradient mode. Various other columns were also evaluated, but the Gemini 5 μm C_{18} column gave the best separation and chromatography under the conditions testified.

Different mobile phase compositions were evaluated to increase the sensitivity, obtain better separation and sharper peaks. Formic acid, trifluroacetic acid and acetic acid in the

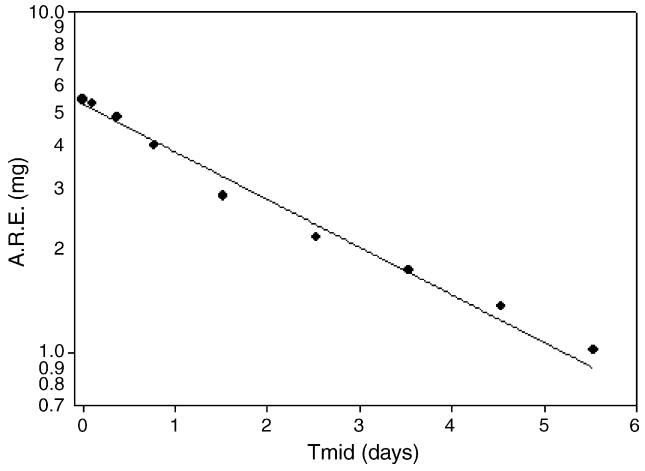

Fig. 4. Amount remaining to be excreted (A.R.E) vs. midpoint time (T_{mid}) profile of pyronaridine after oral administration of 12 mg/kg pyronaridine to a healthy volunteer.

mobile phase were evaluated, but the separation was not as good as obtained with present perfluorooctanoic acid. To see whether pH has an effect on elution and sensitivity of the peaks, the pH of aqueous phase was varied using ammonium acetate, sodium acetate and ammonium formate. Poor separation between the parent and the internal standard was obtained in the pH range of 2.3 to 6.5.

Amodiaquine was found to be suitable internal standard because its structure was similar to pyronaridine, and it was well recovered from urine. 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 chemical ionization (APCI) source was used instead of electro spray ionization (ESI) because it gave good reproducibility and sensitivity.

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 30 μL instead of 50 μL and reconstitution volume was increased to 200 μL instead of 100 μL. The retention time using this methodology is reduced compared to other reported methods to help improve sample throughput. The higher volume of reconstitution provides sufficient volume for reinjection of subject samples. Phosphate buffer was used as reconstitution solution to improve the solubility of pyronaridine compared to mobile phase.

The mass detector response was linear over the range of 14.3–1425 ng/mL. Linear regression analysis performed for pyronaridine in above concentration ranges yielded mean correlation coefficients ($n = 5$) of 0.992. We believe this calibration curve range is appropriate for analyzing the body fluids of subjects given anticipated pyronaridine doses. The quantification limit for pyronaridine was 14.3 ng/mL of urine using an injection volume of 30 μL.

Validation of our method consisted of intra and inter-day reproducibility at 4 concentrations levels: 28.5, 285, 684, 1140 ng/mL ($n = 5$). These concentrations were selected to cover entire range of the calibration curve. The intra day and interday precision for pyronaridine in urine for all levels varied from 2.3–9.2% and 6.7–13.1% respectively. The variation was within the acceptable range required for validation of an assay [11].

A stability study was conducted at −80 °C to determine the storage temperature for urine samples with minimum degradation. The study was conducted at three levels of concentration for a period of 130 days. The analysis shows that the samples were stable up to 130 days when kept at −80 °C. Therefore, all urine samples were stored at −80 °C.

Freeze–thaw analysis results suggested that urine samples could be thawed and refrozen without compromising the integrity of the sample. Bench stability analysis demonstrated that samples are stable when kept on bench for 48 h, except at 684 ng/mL, which was stable for 24 h.

This validated LC–MS method was developed to support the clinical development of the promising antimalarial drug, pyronaridine. The method was developed to determine extent of excretion of unchanged drug in urine. The methodology is also useful to apply to a pharmacokinetic evaluation of the pyronaridine urinary excretion profile. Fig. 4 shows an example of an amount remaining to be excreted (A.R.E) versus time profile for a healthy subject that received pyronaridine.

5. Conclusion

We describe a reliable and selective LC–MS assay procedure using liquid–liquid extraction for the specific and quantitative analysis of pyronaridine in human urine. The assay uses amodiaquine as an internal standard, the extraction procedure is simple and relatively short allowing sufficient sample throughput to be applied to clinical pharmacokinetic studies of pyronaridine. The assay has been validated, and the results of validation show the method is reproducible and accurate. There is excellent linearity of standard curve for pyronaridine over the concentration range 14.3–1425 ng/mL. The analysis requires 0.2 ml of urine and has a detection limit of 14.3 ng/mL for the analytes of interest.

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