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Liquid chromatographic determination of pyronaridine in human plasma and oral dosage form

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Abstract

A new procedure for the determination of pyronaridine in plasma by reversed-phase high performance liquid chromatography (HPLC) with UV detection at 278 nm is described. The method involves liquid–liquid extraction of the drug with diethyl ether following basification of the deproteinized plasma with alkaline phosphate buffer. Chromatographic separation was achieved using a microbore C-18 column and a mobile phase consisting of 0.1% aqueous trifluoroacetic acid (TFA)–acetonitrile (75:25% (v/v)), pH 2.2, at a flow rate of 0.07 ml/min. Papaverine was used as internal standard. The response was linear between 50 and 1500 ng/ml. The limit of quantitation (LOQ) after plasma extraction was 50 ng/ml, the intra- and inter-day precision ranged from 2.5 to 13.8% (CV). The recovery of the drug from plasma and accuracy were >90%. Preliminary application of the method for monitoring pyronaridine in humans upon oral administration of the tablet demonstrated the principal usefulness of the assay for clinical trial studies. The method can also be used to analyze the compound in pharmaceutical formulations.

Keyword: Pyronaridine

1. Introduction

Pyronaridine, 7-chloro-2-methoxy-10-[3′,5′-bis(pyrolidinyl-1-methyl)-4′-hydroxyanilino]benzo[*b*]-1,5-naphthyridine tetraphosphate (Fig. 1) is an anti-malarial drug synthesized in China in the early 1970's [1,2] and introduced for the therapy of malaria [2]. Pyronaridine is effective against chloroquine and multi-drug resistant *Plasmodium falciparum* malaria [3–5]. In order to investigate the pharmacokinetics of pyronaridine in humans it is necessary to develop a simple, sensitive and specific method for the analysis of the drug in biological fluids such as plasma or urine. Only few reports have been published on the analysis of the drug from plasma or whole blood [6–10]. The spectrofluorimetric method described for early pharmacokinetic studies of the drug in China is relatively non-specific, laborious and time-consuming

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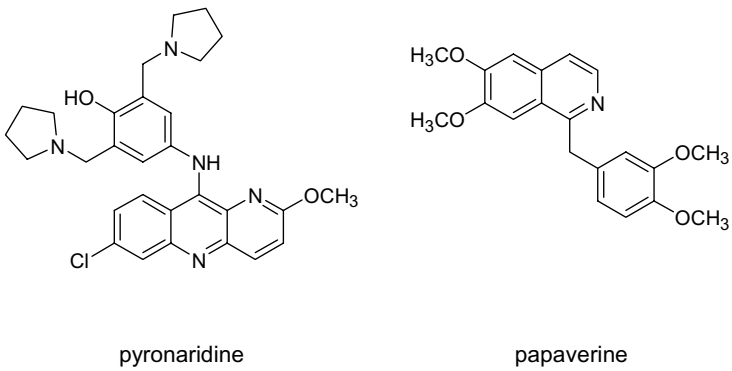

Fig. 1. Structure of pyronaridine and papaverine.

[6]. While the HPLC methods proved to be sensitive, the method by Wages et al. [7] was time-consuming and laborious and utilized electrochemical detection, which is not always convenient and accessible. The sensitivity of the assay reported by Saleh and Loh [8] was not sufficient for the analysis of the drug in plasma. Sensitive methods reported by Jayaraman et al. [9] and Chen and Fleckenstein [10] utilized the anti-malarial drugs amodiaquine or quinine as internal standard (ISTD). Such drugs can interfere with analysis of pyronaridine in patients who have taken these antimalarials prior to a therapy with pyronaridine. In addition, the above mentioned phosphate buffer-based HPLC procedures could not be adapted in our laboratories.

A specific assay of the new anti-malarial drug pyronaridine is necessary for its clinical trials especially in tropic countries where malaria is endemic. Therefore, a new method was developed that is simple, specific and sensitive for the analysis of pyronaridine in human plasma. Papaverine (Fig. 1) was used as internal standard. The mobile phase consisted of a mixture of aqueous trifluoroacetic acid and acetonitrile. Preliminary data of the application of the new method to pharmacokinetics of the drug upon oral administration are presented.

2. Experimental

2.1. Chemical and reagents

Pyronaridine (as base) and pyronaridine tetraphosphate tablets were donated by Professor C. Chen (Institute of Parasitic Diseases, China). Papaverine hydrochloride, 1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-isoquinoline (Fig. 1) was obtained from Caelo (Hilden, Germany). All solvents and chemicals were of HPLC or analytical grade. Trifluoroacetic acid (TFA), methanol, acetonitrile and disodium phosphate were from Sigma–Aldrich (Taufkirchen, Germany) and diethyl ether was from E. Merck (Darmstadt, Germany). All chemicals were used as received except for diethyl ether, which was double distilled.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Shimadzu LC-10 DVP dual pump system, a Shimadzu SPD-10A UV detector set at 278 nm and a Shimadzu SCL-10A system controller (Shimadzu AG, Duisburg, Germany). The analysis was performed using a GROM-SIL ODS-4 microbore column (5 μm, 125 mm × 2 mm i.d.; Grom, Herrenberg, Germany). Other columns tested were Zorbax Extend-C18 (5 μm, 150 mm × 2.1 mm i.d.; Agilent, Waldbronn, Germany) and LiChrospher RP Select B (5 μm, 125 mm × 2 mm i.d.; E. Merck, Darmstadt, Germany). The final mobile phase consisted of a 0.1% aqueous trifluoroacetic acid containing 25% acetonitrile (v/v), pH 2.2, the flow rate was 0.07 ml/min. The mobile phase was filtered and degassed prior to use. The experiments were performed at ambient temperature.

2.3. Preparation of standard solutions

The 1 mg/ml stock solutions of pyronaridine were prepared in methanol and stored in a refrigerator at

4 °C. Standard solutions containing between 10 and 1500 ng/ml were obtained by dilution of the stock solution in the mobile phase. The 1 mg/ml stock solutions of the internal standard (ISTD) papaverine were also prepared in methanol and stored at 4 °C. Prior to the construction of the calibration curves standard solutions containing 1, 10 and 50 μg/ml of pyronaridine and 25 μg/ml of ISTD were prepared in methanol for spiking drug-free plasma.

2.4. Plasma analysis and calibration

Extraction of the drugs from human plasma was carried out in 100 mm × 16 mm glass centrifuge tubes with polytetrafluoroethylene lined screw caps. Five hundred microlitres drug-free plasma were spiked with known amounts of the drug from the standard solutions of pyronaridine comprising of 10, 25, 50, 100, 250, 500, 1000 and 1500 ng/ml and 30 μl of the ISTD solution containing 25 μg/ml (375 ng/ml). The buffer solution for basification was prepared from 0.2 M disodium hydrogen phosphate adjusted to pH 11 with 5 M NaOH. Five hundred microlitres of this buffer were added to the plasma and vortexed briefly. Six millilitre of diethyl ether were added to the mixture and vortexed for 90 s followed by centrifugation for 10 min at 3000 rpm. The upper organic layer was aspirated into a clean tube and evaporated to dryness under a gentle stream of nitrogen. The dried extract was reconstituted in 100 μl of the mobile phase, vortex-mixed for 60 s and a 5 μl aliquot injected into the HPLC.

Calibration graphs were constructed by using the peak area ratio of drug/internal standard. Unweighted regression analysis was used to determine slope, intercepts and correlation coefficient of the calibration curve. The concentrations of pyronaridine in the test samples were calculated using regression parameters obtained from the standard curves.

2.5. Precision and recovery

Inter- and intra-day assay precision, accuracy and recovery of the method were determined by carrying out repeated analysis of drug-free plasma spiked with different concentrations of pyronaridine ($n \geq 5$ for each concentration). The coefficient of variation (CV) was used to determine the precision.

Absolute recovery of the extraction procedure was determined by comparing the peak area of an extracted spiked sample with the peak area of a direct injection of a solution containing the same concentration of the drug.

2.6. Pharmacokinetics of pyronaridine

Pyronaridine tetraphosphate tablets (produced and provided by the Institute of Parasitic Disease, China) was administered as a single oral dose (400 mg, 6 mg/kg) to a 65 kg healthy male subject. Venous blood samples (5 ml) were withdrawn at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, 216, 288 and 336 h. The blood was centrifuged at 3000 rpm for 10 min and the resulting plasma was stored at −20 °C until analysis. The protocol was approved by University of Ibadan and University College Hospital (UI/UCH) Joint Ethics Committee. Work-up of the samples was conducted as described above.

2.7. Analysis of pyronaridine tetraphosphate tablets

From the stock solution of pyronaridine standard solutions ranging from 0.13 to 130 μg/ml were prepared by dilution with the mobile phase and calibration graphs were constructed from these concentrations. Pyronaridine tetraphosphate tablets were weighed, crushed and an aliquot (233.3 mg) containing 100 mg of pyronaridine phosphate was dissolved in the mobile phase. The suspension was centrifuged, a part of the supernatant was filtered and used for analysis. The filtrate was diluted to obtain an approximate concentration of 100 μg/ml. The accuracy and precision of the assay method were also determined. The peak area of pyronaridine was used for analyzing the data.

3. Results and discussion

3.1. Method development

Three chromatographic columns were evaluated in initial experiments using mixtures of acidic phosphate buffers (pH 2–4) and acetonitrile to identify a suitable

stationary phase. While the GROM-SIL ODS-4 and Zorbax Extend-C18 produced sharp peaks, only broad peaks were observed when using the LiChrospher RP Select B column. The Zorbax column was also evaluated using ammonia- and pyrrolidine-based buffers in the pH range 9–11 and acetonitrile as organic modifier. These conditions resulted however in tailing peaks and low sensitivity. In addition, poor reproducibility was observed under these conditions. Overall the GROM-SIL ODS column gave the best peak shape and subsequently best sensitivity. The mobile phase was further optimized employing this column.

In addition to the pH of the phosphate buffer, further variations of the mobile phase included the concentration of the phosphate buffer (0.02–0.08 M), using methanol as organic modifier, and evaluation of perchloric acid or sodium hexane sulfonate and sodium octane sulfonate as ion pair reagents. While sharp peaks were observed in the presence of perchloric acid, these conditions resulted in short retention times and interference of pyronaridine with the peaks of the solvent front or endogenous peaks from plasma. Methanol as organic modifier or the use of alkyl sulfonates as ion pair reagents resulted in broad and/or strongly tailing peaks. Eventually, a mobile phase consisting of a mixture of 0.1% aqueous trifluoroacetic acid (TFA)–acetonitrile (75:25% (v/v)), pH 2.2, was found suitable to separate pyronaridine from any endogenous peaks from plasma and the solvent front. Papaverine was selected as internal standard (ISTD). The retention times were 4.4 min for pyronaridine and 10.5 min for ISTD. Fig. 2 shows the chromatograms obtained from standard solutions (Fig. 2(A)) and following extraction of blank plasma (Fig. 2(B)) as well as spiked plasma (Fig. 2(C)).

An ISTD having a retention time longer than pyronaridine was selected for this reversed-phase method because no interference with possible polar metabolites from the parent drug can be expected. Papaverine also has the advantage compared to the use of antimalarials as reported in earlier papers [9,10] because it cannot interfere with the analysis of pyronaridine in individuals who have taken such antimalarials prior to the ingestion of pyronaridine. Papaverine has also been used as ISTD for the HPLC analysis of chloroquine [11].

Several liquid–liquid extraction procedures were tested in order to obtain a suitable, sensitive and specific method for pyronaridine. Pyronaridine proved difficult in many systems as it is an amphoteric substance. After spiking the drug into plasma, several parameters were investigated such as precipitation of plasma proteins by methanol, acetonitrile, perchloric acid or TFA, varying pH of the sample by basification with different phosphate buffers, ammonia, or sodium hydroxide, as well as extraction with a number of organic solvents such as diethyl ether, ethyl acetate and hexane alone or in various combinations. Reconstitution of the drug after the liquid–liquid extraction was evaluated using methanol, acetonitrile, 0.1 M HCl or the mobile phase as solvents. However, most of the methods gave poor recovery. Eventually, the best recovery of the drug from spiked plasma was achieved by basification of 500 μl of plasma with 500 μl of 0.2 M Na_2PO_4 adjusted to pH 11 with 5 M NaOH and extraction with ethyl ether. Upon evaporation of the organic solvent the residue was reconstituted in the mobile phase and 5 μl were injected onto the column.

The specificity of the method was measured by injecting the common antimalarials such as amodiaquine, quinine, chloroquine, primaquine and halofantrine onto the column. None of them interfered with pyronaridine or the ISTD. Injecting a solution of the unfiltered tablet matrix produced a single peak characteristic of pyronaridine with no interference of any excipient or impurity.

3.2. Method validation

The calibration curves ($n = 6$) obtained by plotting the peak area ratio of pyronaridine/ISTD versus the concentration of the drug spiked into the plasma were linear over the range of 50–1500 ng/ml. The correlation coefficients (r) were over 0.995 from each of the six standard curves ranging from 0.9950 to 0.9994 (mean, 0.997 ± 0.001). The equation of the calibration plot was $y = 0.0058x - 0.0529$. The coefficient of variation (CV (%)) obtained for regression parameters after repeated runs were 9.05% for the slope (mean 0.00584 ± 0.00053), 0.17% for the correlation coefficient (mean, 0.997 ± 0.001) and 12.2% for the intercept (mean, 0.0529 ± 0.0065). The results indicate good linearity after unweighted simple linear regression

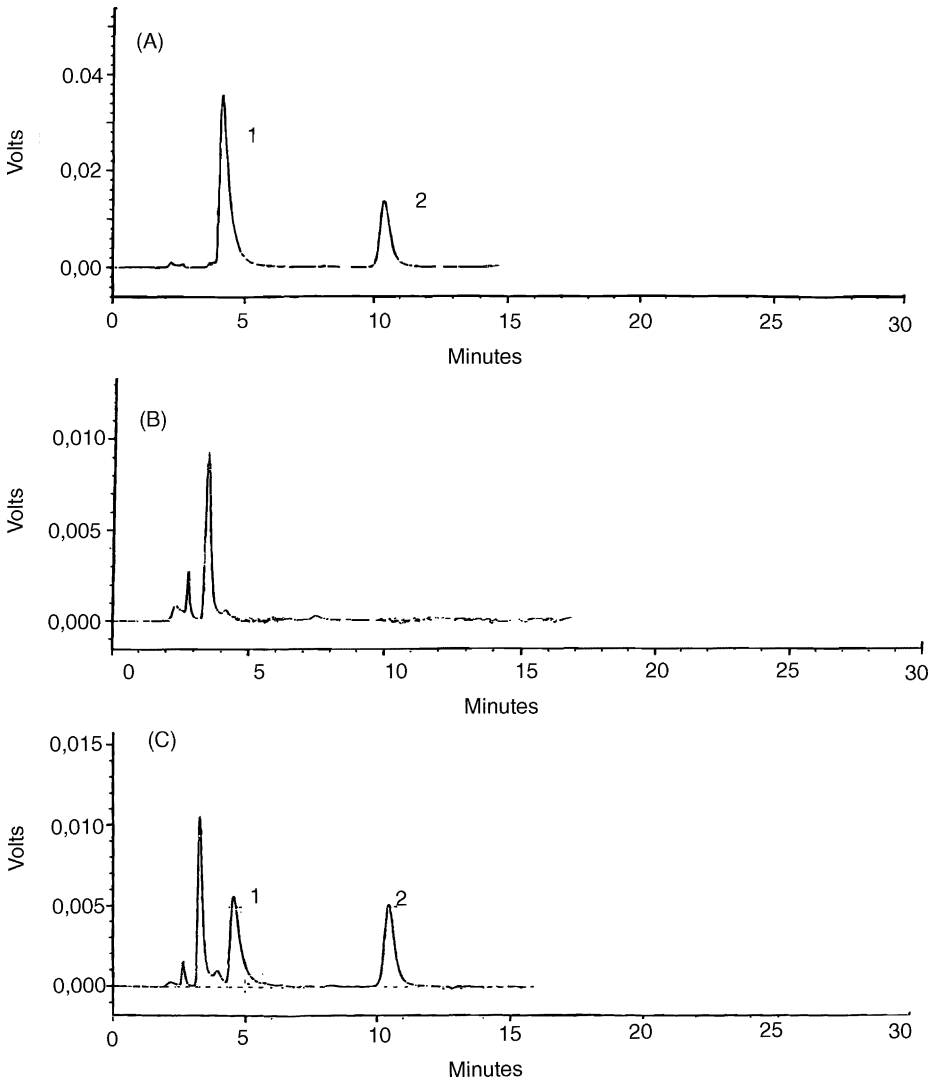

Fig. 2. Typical chromatograms of: (A) a standard solution containing 500 ng/ml pyronaridine and 1000 ng/ml papaverine, (B) drug-free plasma and (C) plasma spiked with 250 ng/ml pyronaridine and 375 ng/ml papaverine. (1) pyronaridine; (2) papaverine.

analysis. The linear regression obtained with standard solutions of pyronaridine (0.13–130 μg/ml) and used for assaying the tablets was 0.99998 and the equation of the calibration graph was $y = 0.000003x + 0.1924$.

Using an injection volume of 5 μl, the limit of quantitation (LOQ) of the drug from plasma was 50 ng/ml corresponding to 0.25 ng on-column. The criteria for determination of LOQ are based on the S/N ratio, the reproducibility and the variability of back-calculation concentration [12]. Spiked plasma samples ($n = 6$) with a concentration of 50 ng/ml were found to have a S/N ratio of >10. The mean ± S.D. of the peak area ratio were 0.115 ± 0.014 and this ratio was >3S.D.s with a CV of 12.1%. The CV and the percent relative error of the back-calculation of the LOQ were 11.27 and 19.55, respectively (Table 1). Thus, the LOQ of the present method was 50 ng/ml with both the variability and accuracy of <20%. This indicates that the LOQ has acceptable precision, accuracy and reproducibility.

Table 1
Inter-day precision and accuracy of the method in plasma ($n = 6$)

Theoretical conc. (ng/ml)	Precision		Accuracy	
	Observed conc. (ng/ml)	CV (%)	Observed percent (%)	Relative error (%)
50	59.7 ± 6.7	11.27	119.5 ± 13.5	19.5
100	91.2 ± 11.3	12.40	91.2 ± 11.3	8.8
250	253 ± 28	10.90	101.4 ± 11.0	1.4
500	537 ± 74	13.82	107.4 ± 14.8	7.4
1000	945 ± 90	9.54	94.5 ± 9.0	5.5
1500	1559 ± 39	2.58	101.6 ± 2.6	1.5

The limit of detection (LOD) using standard solutions of the drug based on signal-to-noise (S/N) ratio of >3 was 25 ng/ml corresponding to 0.125 ng on-column. Based on the on-column sensitivity this method appeared to be more sensitive than the previous reported methods whose on-column detection limits were calculated to be 0.4 ng for Wages et al. [7], 0.5 ng for Jayaraman et al. [9] and 3 ng for Chen and Fleckenstein [10].

The inter- and intra-day variations of the method after repeated analyses at different concentrations are shown in Tables 1 and 2. The inter-day precision ($n = 6$) ranged between 2.5 and 13.8% (CV) for concentrations between 50 and 1500 ng/ml, respectively, while the intra-day precision ($n = 5$) were 9.4 and 10.6% at 200 and 500 ng/ml, respectively. The accuracy of the method was calculated as percentage of the nominal concentration and the relative error. The relative error calculated as the percent difference of the amount of the drug added to the plasma and the amount found in the experiments ranged from 6.04 to 8.80% for the inter-day and 1.38 to 19.5% for intra-day analysis (Tables 1 and 2). At all concentrations, at least 90% of the added drug were recovered showing that the method is suitable for the analysis of pyronaridine in plasma.

Table 2
Intra-day precision, accuracy and recovery of the method in plasma ($n = 5$)

Theoretical conc. (ng/ml)	Precision		Accuracy	
	Observed conc. (ng/ml)	CV (%)	Observed %	Relative error (%)
200	182 ± 17	9.38	91.1 ± 8.6	8.9
500	530 ± 56	10.57	106.0 ± 11.2	6.0

The assay precision of the standard solutions used for the assay of the tablet ranged between 0.75 and 0.88% (<1%) and accuracy of check samples were 103 ± 13.21% with a CV of 12.6%.

3.3. Application of the method

In order to test the applicability of the present method for the analysis of pyronaridine, plasma samples obtained upon single oral administration of 400 mg of pyronaridine tetraphosphate (6.15 mg/kg) to a 65 kg healthy male volunteer were analyzed. Fig. 3 illustrates the time dependence of the observed plasma concentration of the drug. The maximum plasma concentration (C_{max}) of 76.2 ng/ml occurred at 1 h (t_{max}) after the administration of the drug. The area under the curve ($AUC_{0–12}$) was 662.85 ng h/ml. After 24 h the drug levels were below the LOQ.

This data show poor absorption of the drug from the tablet formulation used. Similar results have been reported in the literature [7,10,13]. Chang et al. [13] reported that after a single dose of 204 mg of pyronaridine by intra-muscular injection, 600 mg as a capsule and 600 mg as a tablet, the amount of drug absorbed was 100% for the injection (C_{max}, 525 ng/ml), 32.7% for the capsule (C_{max}, 225 ng/ml) and 19.7% for the tablet (C_{max} 127 ng/ml). Wages et al. [7] were unable to detect pyronaridine in plasma after a first and a second oral dose of 50 mg/kg pyronaridine applied as the free base to a monkey. Chen and Fleckeinstein [10] did not detect pyronaridine in plasma 24 h after a single intra-muscular injection of 20 mg/kg of the free base into a rabbit. They also observed that the concentrations of pyronaridine in plasma were <10% of the concentrations in whole blood suggesting an uptake of the drug by blood cells. In the present study, the 400 mg

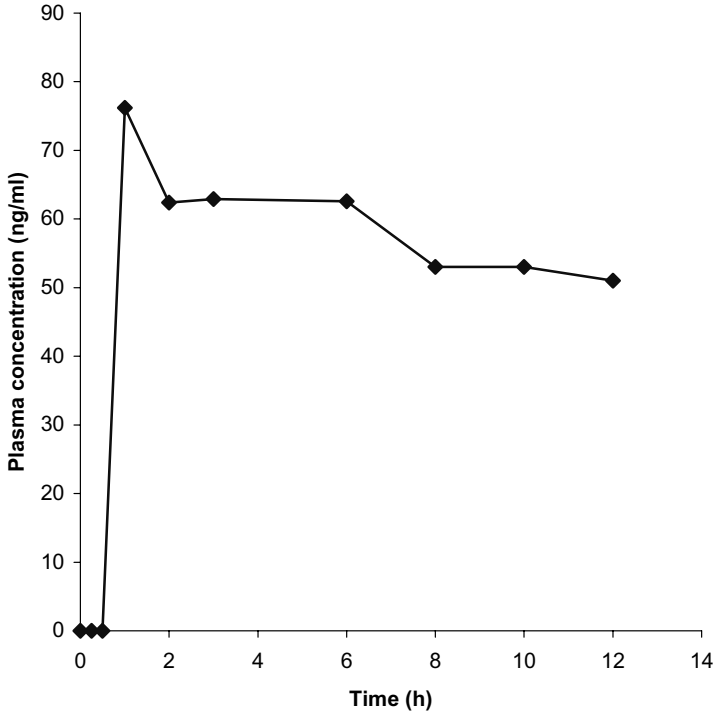

Fig. 3. Plasma profile of pyronaridine after a single oral dose of 400 mg of the tablet to a healthy male subject.

pyronaridine tetraphosphate tablet given to a 65 kg male is equivalent to 3.5 mg/kg of the free base, which is a much more lower dose than the doses used in the animal studies described above. Although Jayaraman et al. [9] reported a C_{max} of 496 ng/ml at t_{max} = 0.5 h after oral administration of a capsule containing 400 mg to a volunteer, it is not clear from the paper if the dose was based on the free base or the salt. In addition, the bioavailability of the capsule was reported to be higher than that of the tablet [13]. Thus, higher doses of the drug have to be applied if the currently available tablets are administered or oral formulations with a better bioavailability are needed for proper pharmacokinetic elucidation of pyronaridine in humans.

The method was also applied to quantify pyronaridine in the tablets used in the present study. We found that one tablet with a declared content of 100 mg of pyronaridine tetraphosphate contained a quantity corresponding to 53.77 mg of the free pyronaridine base. This amounts to a purity of the compound of 94.5% in the tablets as the tetraphosphate salt corresponds to 56.9% of free base.

4. Conclusions

A sensitive, selective, reproducible and accurate high performance liquid chromatographic method for the analysis of pyronaridine in human plasma and tablet formulations has been developed. The liquid–liquid extraction process is simple and rapid. Preliminary pharmacokinetic data demonstrate the principal applicability of the method for clinical trials of the drug, especially in malaria endemic regions such as Africa. The assay can also be applied to the analysis of pyronaridine formulations without extraction processes.

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