Simultaneous Quantification of Ciprofloxacin, Quinine and 3-hyrdoxyquinine in Human Plasma using a HPLC Method

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Abstract

Malaria has been shown to strongly predispose patients in areas of malaria endemicity to bacteremia with severe outcomes, thus justifying the use of antibiotics in combination with antimalarial therapy in patients with severe malaria. This study describes a High-Performance Liquid Chromatographic (HPLC) method for simultaneous determinations of Ciprofloxacin (CPN), Quinine (QN), and its major metabolite, 3-Hydroxyquinine (3-HQN), in human plasma. Following a simple precipitation with acetonitrile, chromatographic separation was achieved on a reversed-phase Agilent Zorbax (CN) column (5 µm, 150 X 4.6 mm i.d) using a mobile phase consisting of acetonitrile: potassium dihydrogen phosphate (pH = 2.8; 0.02 M) (42:58, v/v). Retention times for CPN, 3-HQN, IS and QN were 2.7, 3.3, 3.6 and 4.9 minutes respectively. The limits of detection and validated lower limits of quantitation were 30 and 70 ng/ml for both QN and 3-HQN while the corresponding values were 50 and 100 ng/ml for CPN, respectively. The new HPLC method here developed, when compared with previous methods for the analysis of either or both drugs is simple, rapid, selective, reproducible and cost-effective. It is also suitable for conducting a simultaneous therapeutic monitoring of quinine and ciprofloxacin in patients when concomitantly administered as demonstrated in five healthy volunteers.

Keywords: 3-Hydroxyquinine, Ciprofloxacin, HPLC Analysis, Quinine

1. Introduction

Co-morbidity of malarial and bacterial infections is a common phenomenon in the world today particularly in the tropics. WHO malaria report for year 2014 revealed that up to 198 million cases of malaria were reported in 2013 and out of this, the death toll was up to 584,000[1]. Similarly, bacterial infections are ravaging in this same region since a large number of hospital visits results from bacterial infections. A study in Kenya provided evidence that *Plasmodium falciparum* infection increases the risk for bacteraemia, particularly for gram-negative invasive infections[2,3]. Co-morbidity of malaria and bacterial infections could warrant co-administration of antimalarial and antibacterial drugs.

Artesunate and Quinine (QN) injections are the drugs of choice for severe or cerebral malaria and oral dosage form of these drugs are to be considered for follow-up treatment when the patient is fully conscious. QN is a more popular option in paediatrics and in resource-limited regions such as sub-Saharan Africa essentially because of availability challenges couple with uneven distribution of the available artesunate finished products and high cost associated with artesunate therapy in the region[4]. Children and some adults with severe malaria are likely to receive QN either as injection, oral or rectal.

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Ciprofloxacin (CPN) is a fluoroquinolone often prescribes especially for lower respiratory tract infections probably because of its good tissue penetration and safety profile, and a broad spectrum of action that covers a number of clinically important pathogens, especially Gram-negative bacteria including *Pseudomonas aeruginosa*, against which very few effective oral antibiotics are available[5]. The use of CPN in paediatrics has been limited due to the fear that it caused arthropathy in weight-bearing joint of juvenile animals. However, a recent review depicted that different investigators have used CPN in paediatric patients and the general experience is that it is safe and effective in children also[6].

Cinchonism and cardiac arrhythmias are common toxicity of QN therapy and this toxicity may be connected with the plasma concentration of the drug. In the same vein, cardiac arrhythmias and arthropathy are postulated to be associated with quinolones administration. Thus, there may be increased manifestations of these toxicities in a circumstance where CPN and QN are to be administered concurrently. This is adduced to the fact that toxicity is related to the drugs exposure in the plasma. In order to achieve a good clinical outcome particularly in paediatrics, therapeutic drug monitoring of the plasma levels of QN and CPN when they are co-administered will be of great benefit. This will help to adjust the dosage regimens of the drugs appropriately in order to meet the therapeutic targets or needs without or with minimal unpleasant effects.

In severe *P. falciparum* malaria, a peak plasma quinine level of 10–15 μg/ml was recommended for effective parasite clearance and tolerable side effects, although, these levels were reported as toxic in non-malaria subjects[7]. Also, monitoring the plasma levels of 3-HQN along with QN is necessary since this major metabolite is reported to have a higher toxicity than QN, but much less potent as an antimalarial[8]. To conduct a successful therapeutic drug monitoring, a suitable simple, rapid and cost-effective analytical procedure to quantify the analytes, is of paramount important.

A number of bioassay techniques have been described in literatures to quantify CPN and QN separately from biological fluids. For instance HPLC determination of quinine with UV detector developed by Babalola et al and Soyinka et al[9,10], HPLC with fluorescence to detect quinine by Wanwimolruk et al and Mirghani et al[11,12]. Similarly, a number of HPLC methods have been described for determination of ciprofloxacin in biological samples but some of them are limited in application owing to the long run time, requirement for large plasma volume and cumbersome extraction procedure[13–16]. In addition to all of these shortcomings, to our knowledge, an assay method to determine both QN and CPN simultaneously in the plasma has not been described. Thus, the objective of this study is to develop and validate a modest, easily operable and reliable HPLC method for the concurrent quantifications of QN, CPN and 3-HQN in human plasma.

2. Experimental

2.1 Chemical and Reagents

Ciprofloxacin (Cyplox® from Medreich, Pharmaceutical Company) and quinine tablets (Quinmax®) were purchased locally from a retail pharmacy outlet. Ciprofloxacin hydrochloride (Figure 1) was sourced from AK Scientific Inc. Union City (USA) while quinine sulphate (Figure 1) was purchased from BDH chemicals (Poole, UK). 3-Hydroxyquinine (Figure 1) was donated by Prof. James M. Cook, University of Wisconsin, USA. Acetonitrile (HPLC grades), potassium dihydrogen phosphate (analar grade) and hydrochloric acid were all purchased from Sigma–Aldrich Chemicals (Steinheim, Germany). Perchloric acid (Hopkins and Williams, Essex, UK) was of analar grade. Pyrimethamine was obtained from Swiss Pharma Nig Ltd.

2.2 Instrumentation

The chromatographic system consisted of an Agilent 1100 series liquid chromatography (Agilent technologies, Palm alto, USA) using a reversed-phase Agilent Zorbax Cyano (CN) column (5 μm, 150 X 4.6 mm i.d) fitted with a quaternary pump and a Diode array UV detector (DAD, 190-900 nm). DAD was connected to a computer and the signals were recorded therein as chromatograms. Sample injection was through a Rheodyne model 7725 valve (California; USA) fitted with a 20 μl loop. Vortex mixer (Gallenkamp, London, UK) and centrifuge (Gallenkamp) were used in the extraction procedure.

2.3 Chromatographic Conditions

Chromatographic separation was achieved at ambient temperature on a reversed-phase Agilent Zorbax (CN) column (5 μm, 150 X 4.6 mm i.d). Isocratic elution was performed using a mobile phase consisting of acetonitrile and potassium dihydrogen phosphate buffer (pH =2.8 ;

Figure 1. Chemical Structures of Ciprofloxacin, Pyrimethamine, Quinine, Quinine salt and its metabolite.

0.02 M) pH was adjusted with a few drops of 60% v/v perchloric acid (42:58 v/v) and the effluent was monitored by UV detection at 254 nm for quinine and 3-hydroxyquinine and 275 nm for ciprofloxacin. The buffer solution was filtered through 0.45μm membrane filter. The mobile phase was pumped through the column at a flow rate of 1.0 mL/min and the injection volume was 20 μL.

2.4 Preparation of Stock Solution

1.0 mg/ml of quinine base, 3-hydroxyquinine and ciprofloxacin were prepared in 0.1 M hydrochloric acid and various concentrations of working solutions were prepared from the stock solutions

2.5 Sample Preparation and Extraction Procedure

To 250 μl of plasma sample placed in a 2-ml eppendorf tube was added 50 μl of 10μg/mL pyrimethamine solution (internal standard). 700 μl of acetonitrile was added and the content was vortex-mixed for 1 min and centrifuged for 5 min at 3000 g. 20 μl of a clean supernatant was injected into HPLC column.

2.6 Calibration Curve

Calibration curves were constructed based on the relationship between the peak area ratios and the standard solutions of ciprofloxacin, quinine and its metabolite. 250 μL of drug-free plasma samples were spiked with 50 μL of internal standard solution (10 μg/mL pyrimethamine) and standard solutions (between the range of 0.05 –10 μg/mL) of ciprofloxacin, quinine and its metabolite. To each sample, acetonitrile was added to make up to one-mL and the samples were sequentially vortex-mixed and centrifuged for 1 and 5 minutes respectively and the supernatant (20 μl) was injected into HPLC column. The Peak Area Ratios (PAR) were generated from the ratio of response (peak area) of the analytes to that of the internal standard. A plot of PARs versus concentrations of the standard solutions was done.

2.7 Recovery, Accuracy and Precision Studies

Blank plasma samples were spiked with standard solutions of ciprofloxacin, quinine and 3-hydroxyquinine to yield concentrations of 0.5, 5.0 and 10.0 μg/ml for ciprofloxacin and quinine and 0.2, 2.0 and 10.0 μg/ml for 3-hydroxyquinine. Then, the samples were taken through extraction procedure. The absolute recovery, expressed as percentage, was determined by comparing the PAR obtained from the extracted samples with the neat injection of reference samples of the same concentration. The PAR was converted to concentration using calibration curves for each of the analytes. Accuracy of the analytical method for

each of the three analytes was determined by the percentage ratio of the estimated concentrations to that of the actual concentrations of the reference standard prepared. Also the coefficients of variation of the estimated concentrations were determined and used for the assessment of precision.

2.8 Application of Analytical Method

Ethical clearance was sought and obtained from the Health Research Ethics Committee (HREC No: IPHOAU/12/124)), Institute of Public Health, Obafemi Awolowo University, Ile Ife. Five healthy volunteers with body weight ranging from 45 to 85 Kg (63.2 ± 14.5 Kg) and age ranging from 25 to 40 years, received 500 mg ciprofloxacin twice daily for one week and 600 mg quinine was administered to each of the volunteers as oral dose concurrently with 11th dose of ciprofloxacin. Then, venous blood samples were collected into EDTA bottles just before the concurrent drug administration and at 1, 2, 4, 6, 8, 12 and 24 hours after drug administration. The blood samples were centrifuged at 3000 x g for 5 min to obtain the plasma which was analysed for ciprofloxacin, quinine and 3-hydroxyquinine concentrations following the procedures described above. The maximum plasma drug concentration C_{max} and T_{max} were obtained by visual inspection of the concentration–time data. The area under plasma concentration-time curve (AUC_{0-t}) was computed using the log linear trapezoidal method where $t_0 = 0$ corresponded to $C_0 = 0$ for QN and 3-HQN but this was considered as the trough for ciprofloxacin. $AUC_{0-\infty}$ was obtained from the sum of AUC_{0-t} and $C_{t/Lz}$. L_z is the elimination rate constant obtained by linear regression analysis of the terminal phase of the curve.

3. Results

Sample chromatogram is shown in Figure 2 which represents an output from the HPLC method described above. It clearly indicated that the peaks ascribed to QN, 3-HQN and CPN were well separated from one another and they were not in any way interfered with the endogenous compounds. The retention times were 2.7, 3.3 and 4.9 minutes for CPN, 3-HQN and QN respectively and the run time was 7 minutes. The method was specific for these analytes because there was no interference with the peaks of compounds such as paracetamol, trimethoprim, sulphamethoxazole, proguanil and chlorpheniramine. The lowest detectable concentration or Limit of Detection (LOD) was taken as a concentration producing a peak
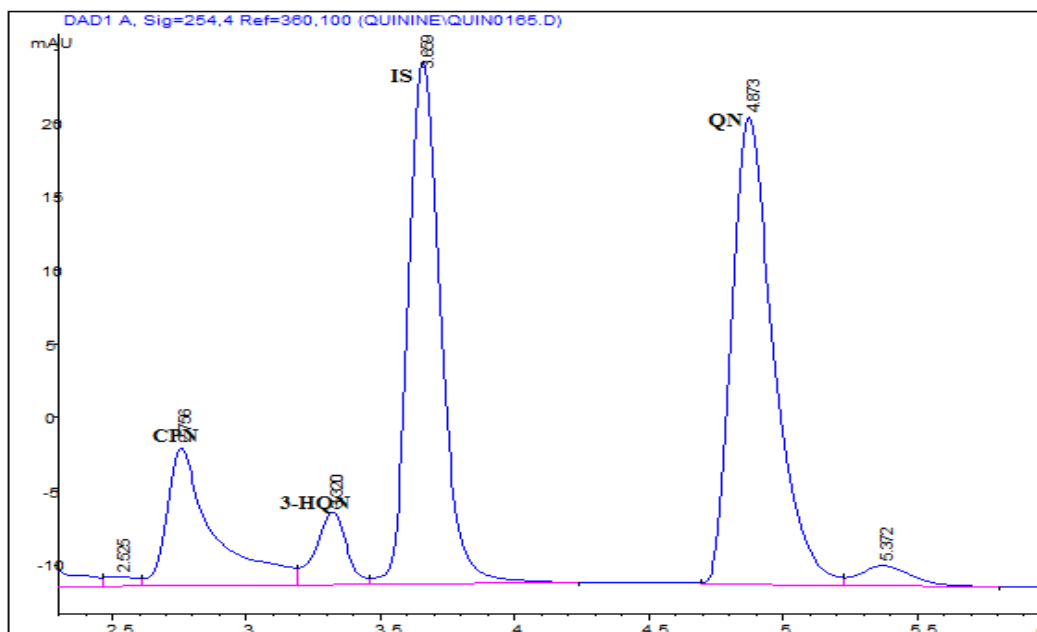

Figure 2. High-performance liquid chromatogram of extracted plasma sample obtained from a volunteer at 2 h following concurrent administration of a single 600mg oral dose of quinine sulphate with multiple oral doses of ciprofloxacin (500mg 12 hourly for one week). The quinine dose was co-administered with the 11th ciprofloxacin dose. The sample was spiked with 50 μl of 10 μg/ml pyrimethamine. CPN = Ciprofloxacin, 3-HQN = 3-Hydroxyquinine, IS = Internal Standard (pyrimethamine) and QN = Quinine.

of three times the baseline noise and this parameter was found to be 10 ng/ml for QN, 3-HQN and 40 ng/ml for CPN. The lowest concentrations of the analytes which are assayable with this method with an acceptable level of precision and accuracy[1] were 30 ng/ml for QN and 3-HQN while that of CPN was 70 ng/ml. Linear curves were obtained for QN and 3-HQN in the plasma with correlation coefficients of 0.999 with the range of linearity between 50 ng/ml to 10,000 ng/ml while the linearity for CPN is between 100 ng to 10,000 ng/ml. The results for the accuracy, recovery and precision for the drugs are indicated in Table 1. All the values for precision and accuracy were within the normal limits. The mean recovery for the three analytes were between 90.8 to 99.2 % at

a lower, a middle and higher concentration within the range of linearity.

The average plasma concentration-time profiles of QN, 3-HQN and CPN following concomitant administration of multiple doses of CPN (500mg 12 hourly for 1 week) and a single 600mg oral dose of quinine sulphate to each of seven volunteers are illustrated in Figure 3. The plasma exposure of ciprofloxacin was determined within a dosing interval of 12 hour after the 11th oral dose as this is acceptable measures for evaluation of pharmacokinetics of a drug when steady-state drug levels are reached in a multiple dose regimen. The derived pharmacokinetic parameters for QN, 3-HQN and CPN are shown in Table 2.

Table 1. Precision, recovery and accuracy of the analytical method

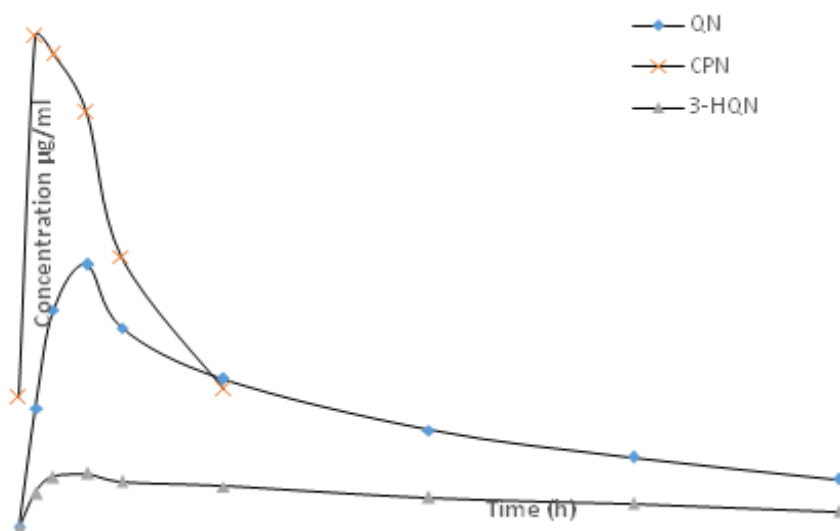| Analytes	N	Concentration (µg/ml)	Intra-day precision (CV) (%)	Inter-day precision (CV) (%)	Accuracy mean±S.D. (%)	Recovery mean±S.D. (%)
QN	6	0.5	3.3	6.0	94.7±5.7	96.7±4.6
	6	5.0	4.9	4.4	96.3±4.3	94.6±4.6
	6	10.0	1.5	2.3	101.2±2.3	95.9±3.3
3-HQN	6	0.5	7.7	3.2	94±3.0	92±7.4
	6	5.0	3.9	2.6	98.0±2.6	93.6±4.2
	6	10.0	3.6	2.9	98.7±2.9	95.3±3.9
CPN	6	0.5	5.6	11.6	98.2±7.2	99.2±4.5
	6	5.0	6.1	10.2	102.9±10.5	90.8±11
	6	10.0	6.1	3.1	99.3±4.5	99.2±3.6

Figure 3. Mean Plasma Concentration vs. time Profiles of Quinine (QN), 3-Hydroxyquinine (3-HQN) and Ciprofloxacin (CPN) after concurrent administrations of a single 600mg oral dose and multiple oral doses of 500 mg 12 hourly to each of sevenhealthy volunteers.

Table 2. Some derived pharmacokinetic parameters for quinine, ciprofloxacin and 3-hydroxyquinine

PK parameters	QN	CPN	3-HQN
C_{max} (µg/ml)	1.64 ± 0.11	3.6± 1.9	0.39 ± 0.06
T_{max} (h)	3.5 ± 0.36	2.14± 1.34	4.92 ± 1.05
$AUC_{(0-t)}$ (µg/ml.h)	32.38 ± 2.85	22.14± 14.65	9.54 ± 1.22
$AUC_{(0-\infty)}$ (µg/ml.h)	41.62 ± 4.04	29.17± 18.4	14.83 ± 2.04
$t_{(\frac{1}{2})(h)}$	21.43 ± 0.93	5.68± 0.47	31.58 ± 3.18

4. Discussion

A simple protein precipitation applied in order to extract the analytes was efficient. A clear and clean supernatant from plasma was produced after a single step extraction with acetonitrile and there was no interference with the endogenous compounds. QN, 3-HQN and CPN were completely resolved to the baseline with a short run-time of 8 min. In this work, the retention times for CPN, 3-HQN and QN were 2.7, 3.3 and 4.9 minutes respectively. This offers an excellent efficiency in terms of the time it will require to work on both the extraction and chromatographic procedures. The method also requires low plasma volume of 250 µl compared with higher plasma volume used in other methods which makes it to be suitable for most patients particularly paediatrics. Similarly, most other methods described for QN and CPN involved cumbersome liquid-liquid extraction, whereas, a-step protein precipitation approach was adopted in this method which removed a more laborious extraction procedure. On this basis, the newly developed method has an edge of simplicity, rapidity and economical when compared to some of the previously developed HPLC methods to determine ciprofloxacin, quinine and its principal metabolite[9–17]. The percentage recovery and evidence of accuracy of over 90% for each of the analytes also lend credence to the effectiveness of this method. The composition of the mobile phase 52% acetonitrile and 48% 10 nm of potassium dihydrogen phosphate buffer also make the method very affordable to run.

Therapeutic drug monitoring (TDM) is suggested for ciprofloxacin where the ratio between the minimum inhibitory concentration (MIC) and the plasma concentration or area under concentration-time profile is considered as a predictor of its effectiveness[19,20].

MIC is an index to measure or predict clinical efficacy of antibacterial and this factor depends on both the effective plasma concentration and the ability of the antibacterial to penetrate the site of infection[19]. Difficult-to-predict pharmacokinetics of ciprofloxacin can occur in critically-ill patients and because of this variability in pharmacokinetics; TDM may be of a great importance to avoid treatment failure and emergence of resistance[19]. For this purpose, sampling times that correspond with the trough and peak concentrations are usually considered and both were measurable with this method. Accumulation of ciprofloxacin in the plasma or serum necessitate dose reduction whereas rapid clearance from the biological fluids may call for upward review of dosage regimen. The ratio of AUC to MIC of 125 is usually considered for treatment of gram-negative organism. Similarly, Cmax- to-MIC ratio of 8-10 is considered as a surrogate value for effectiveness of ciprofloxacin. Clinicians may want to rely on this parameter in order to prevent exposure to excess plasma concentration especially in paediatrics where the risk of developing musculoskeletal toxicity is probable.

This method was applied to pharmacokinetic studies on ciprofloxacin and quinine after administrations of a single dose of 600 mg quinine and 11th 500 mg oral dose of ciprofloxacin to five healthy volunteers. The average area under the curve ($AUC_{0-\infty}$), C_{max}, T_{max} and half-life ($t_{1/2}$) for ciprofloxacin are 22.1 µg/ml*hr, 3.6 µg/ml, 2 h and 5.7 h respectively. Ciprofloxacin pharmacokinetics is dose-dependent where the values for pharmacokinetic parameters vary with doses. The values obtained from this study were comparable with the established figures[15,17,19,21]. Derived pharmacokinetic parameters harvested for quinine after a single oraladministration in the presence of ciprofloxacin were 41.62 µg/ml*hr, 1.64 µg/ml, 3.5 h and 21.4 h for AUC, C_{max}, T_{max} and half-life ($t_{1/2}$) respectively and a clearance of 16 L/h. There was a reduced clearance and prolonged $t_{1/2}$ when the data were compared with the established figures[22,23]. Also the plasma exposure of 3-HQN was low when compare to literature values obtained from healthy volunteers. A recent report revealed that CPN is capable of inhibiting the metabolism of QN in healthy volunteers which resulted in about 39% increase in plasma concentration of QN[24]. CPN depresses the formation of 3-hydroxyquinine (3-HQN) through the inhibition of cytochrome

P450 3A4 (CYP3A4). This is suggestive of metabolic interaction between quinine and ciprofloxacin. This type of interaction would result in increased QN plasma levels with associated toxicities

5. Conclusion

In conclusion, the HPLC method described is very simple, reproducible, and rapid. The method is also accurate, selective and cost-effective. It will help in conducting either individual or simultaneous determination of quinine and ciprofloxacin for TDM pursuit based on their C_{max} and AUC. It will also facilitate the monitoring of quinine metabolite.

6. Acknowledgement

7. References

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