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Simultaneous determination of amodiaquine and its active metabolite in human blood by ion-pair liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive and selective ion-pair liquid chromatography–tandem mass spectrometric method (IP-LC–MS/MS) for the simultaneous determination of amodiaquine (AQ) and its active metabolite, *N*-desethylamodiaquine (AQm), in human blood has been developed and validated. Pentafluoropropionic acid (PFPA) was applied as ion-pairing reagent in reversed-phase chromatographic separation. The effects of PFPA concentrations and the volume fraction of acetonitrile in the mobile phase on the retention of analytes were investigated on a Venusil MP-C_{18} column, and the mobile phase was finally optimized as acetonitrile:water (23:77, v/v) with 0.0667% PFPA in the aqueous phase. The results proved that PFPA as an ion-pairing reagent could provide desirable chromatographic performance in the IP-LC–MS/MS determination of 4-aminoquinoline compounds. Blood samples were protein precipitated with acetonitrile using hydroxychloroquine (OHCQ) as the internal standard. The detection was carried out in multiple reaction monitoring (MRM) mode via positive atmospheric pressure chemical ionization (APCI) interface. The lower limits of quantification were established at 0.150 and 1.50 ng/mL for AQ and AQm, respectively. The validated IP-LC–MS/MS method was applied to a clinical pharmacokinetic study of AQ and AQm in human blood after an oral administration of 600 mg AQ hydrochloride (459 mg base).

Keywords: Amodiaquine; *N*-desethylamodiaquine; Pentafluoropropionic acid; Ion-pair liquid chromatography–tandem mass spectrometry; Pharmacokinetics

1. Introduction

Amodiaquine (AQ) is a 4-aminoquinoline antimalarial drug that has remained at the forefront of antimalarial chemotherapy in the treatment of *Plasmodium falciparum*, and retains therapeutic activity against chloroquine resistant parasites [1]. In addition, the combined therapy with artesunate and AQ is widely used in the treatment of *P. falciparum* malaria [2]. After oral administration, AQ is rapidly and extensively metabolized mainly to the pharmacologically active derivative, *N*-desethylamodiaquine (AQm), which is probably responsible for the most of the therapeutic effect [3,4]. It has been reported that AQ targets hematin, and blood cells have a strong uptake of AQm [5], so it is necessary to determine the concentrations of AQ and AQm in human blood to better characterize the pharmacokinetic profile of the drug.

AQ and AQm are organic bases, the pK_a values of AQ are reported to be 8.14 (pK_{a1}, terminal side chain diethylamine nitrogen) and 7.08 (pK_{a2}, quinoline ring heteroatom nitrogen) [6]. Several methods have been reported for the simultaneous quantification of AQ and AQm in biological samples which were based on HPLC separation with UV [7–12] or EC detection [13]. The limit of quantification of HPLC–UV methods were above 5 ng/mL for AQ and 200 ng/mL for AQm. The retention of these ionogenic bases in liquid chromatography is strongly dependent upon the pH and acidic modifiers in the mobile phase [14]. In the methods previously reported, phosphoric acid [7–9], perchloric acid [10,12] and sodium pentanesulfonate [13] were used as acid additives of the mobile phase. However, these HPLC mobile phases are not compatible with mass spectrometry. Alkyl chain perfluorinated carboxylic acids are volatile ion-pairing reagents, which are compatible with MS, however, most of the applica-

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tions have been limited to biomolecules such as peptides and amino acids [15,16], and applications to polar pharmaceuticals have not been extensively explored.

In the present study, PFPA was evaluated as an ion-pairing reagent in reversed-phase HPLC separation of AQ, AQm and OHCQ. The influences of PFPA concentration as well as the volume fraction of organic solvent in the mobile phase on the retention of analytes were investigated. The optimized ion-pair liquid chromatography–tandem mass spectrometry method was validated for the simultaneous determination of AQ and AQm in human blood and was applied in a pharmacokinetic study after a single oral administration of 600 mg AQ hydrochloride (459 mg base) to healthy human volunteers.

2. Experimental

2.1. Materials

Amodiaquine hydrochloride (99.5% purity) was supplied by Guilin Pharmaceutical Co., Ltd. (Guilin, China). *N*-desethylamodiaquine was purchased from BD Gentest, Woburn, MA, USA. Hydroxychloroquine obtained from Chongqing Kangle Pharmaceutical Co., Ltd. (Chongqing, China) was used as the internal standard (IS). Two hundred milligrams amodiaquine hydrochloride tablets (Flavoquine, 153 mg base per tablet) were obtained from Aventis (Paris, France). Acetonitrile and methanol (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Pentafluoropropionic acid was purchased from Alfa Aesar (Karlsruhe, Germany). Blank (drug free) human blood was obtained from Shanghai Shuguang Hospital (Shanghai, China). Milli-Q water (18.2 mΩ and TOC ≤ 50 ppb) from Milli-Q system (Millipore SAS, Molsheim, France) was used.

2.2. Chromatographic conditions

An Agilent 1100 liquid chromatographic system equipped with a G1379A vacuum degasser, a G1311A quaternary pump, a G1316A column oven, a G1313A autosampler and a G1313A variable wavelength detector (Agilent, Waldbronn, Germany) was used. Data were collected and processed using Chemstation software Version Rev.A.10.02 (Agilent Technologies). The analytes were dissolved in the mobile phase to obtain sharp peak shapes. Mobile phase A was water with PFPA in the concentration range of 0.003–0.4% and mobile phase B was acetonitrile. The injection volume was 20 μL and the flow-rate was 0.5 mL/min. UV-vis detection was performed at 340 nm. A Venusil MP-C_{18} column (50 mm × 2.1 mm ID., 5 μm, Agela, Newark, DE, USA) protected by a SecurityGuard C_{18} column (4 mm × 3.0 mm ID., 5 μm, Phenomenex, Torrance, CA, USA) was used for all measurements. The column hold-up volume (V_m) was determined as the elution volume of uracil (non-retained marker) at the wavelength of 254 nm. The column was thermostated at 25 °C.

2.3. IP-LC–MS/MS analysis

An IP-LC–MS/MS method for the determination of AQ and AQm in human blood was developed using the column mentioned above. Mobile phase A was water with PFPA at the concentration of 0.0667% and mobile phase B was acetonitrile. Chromatography was performed by running 23% B isocratically at the flow rate of 0.5 mL/min. The column temperature was maintained at 25 °C.

Mass spectrometric detection was conducted on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) equipped with APCI interface operated in the positive ionization mode. The tuning parameters were optimized for AQ, AQm and IS separately by infusing solutions of AQ, AQm and IS at a flow-rate of 20 μL/min into the mobile phase (0.5 mL/min) using a post-column "T" connection. The nebulizer, auxiliary and curtain gases (nitrogen) were set at 50, 50 and 10 psi, respectively. The optimized nebulizer current (NC) and temperature were set at 3.5 μA and 400 °C, respectively. For collision activated dissociation (CAD), nitrogen was used as the collision gas set at 4 psi. The values of collision energy (CE) and declustering potential (DP) were set at 25 eV and 50 V, respectively for AQ, AQm and IS. Multiple reaction monitoring (MRM) was used to quantify AQ and AQm using the transitions m/z 356 → m/z 283 for AQ, m/z 328 → m/z 283 for AQm and m/z 336 → m/z 247 for IS, respectively, with a dwell time of 200 ms per transition. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3. Data were collected and processed using Analyst 1.4.1 software (Applied Biosystems).

2.4. Preparation of standard and quality control (QC) samples

Standard stock solutions of AQ and AQm were prepared individually in methanol at the concentrations 400 μg/mL (calculated as the free base) and 200 μg/mL, respectively. Stock solutions were further diluted with methanol to obtain a combined stock solution of 5.00 μg/mL of AQ and 50.0 μg/mL of AQm. Serial working solutions were prepared by further dilutions of combined stock solution with 1% formic acid aqueous solution to obtain the desired concentrations of 0.750, 1.50, 5.00, 15.0, 50.0, 150 and 500 ng/mL for AQ and 10-fold higher than that of AQ for AQm at each level. The working solution (400 ng/mL) of IS was prepared by diluting the 400 μg/mL stock solution of OHCQ with methanol–water (50:50, v/v). All the solutions were stored at 4 °C and were brought to room temperature before use.

QC solutions were prepared by a separate weighing of the standard reference and the QC samples were prepared at the concentrations of 0.240/2.40, 20.0/200 and 90.0/900 ng/mL for AQ/AQm by spiking appropriate amount of QC solutions into blank human blood. QC samples were aliquoted into 1.5 mL polypropylene vials and stored at 78 °C.

2.5. Sample preparation

Frozen unknown samples and QCs were thawed at room temperature and vortexed thoroughly. To 200 μL of blood sample, 40 μL of 1% formic acid aqueous solution and 40 μL of the internal standard (400 ng/mL OHCQ) were added. The sample mixture was vortexed briefly and 600 μL of acetonitrile was added to precipitate the protein. The mixture was vortexed for 1 min on a vortex mixer, and left standing at room temperature for about 5 min, then centrifuged at 11,300 × *g* for 5 min. The supernatant was transferred into a glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. Residues were dissolved in 150 μL of the mobile phase and vortexed briefly. A 20 μL aliquot of the resulting solution was injected onto the IP-LC–MS/MS system for analysis.

2.6. Method validation

The selectivity of the method was evaluated by analyzing six blank blood samples and six spiked blood samples at LLOQ level from six different sources. The MRM chromatograms of these blank blood samples were compared with MRM chromatograms obtained when spiking blank blood samples from the same source with the analytes and the internal standard. Peak areas of endogenous compounds coeluting with the analytes should be less than 20% of the peak area of the LLOQ standard.

The matrix effect was investigated by comparing the extracted blank blood from six different sources spiked with low (1.20/12.0 ng/mL, AQ/AQm), medium (100/1000 ng/mL, AQ/AQm) and high (450/4500 ng/mL, AQ/AQm) concentrations with those of standard solutions that had been prepared in the same way as the QC samples, except that water was substituted for blank blood. The corresponding peak areas of the analytes in spiked blood post-extraction samples (A) were then compared to those of the water-substituted samples (B) at equivalent concentrations. The ratio (A/B × 100) is defined as the matrix effect.

Calibration curves were constructed by analyzing spiked calibration samples (each concentration in duplicate) on three separate days. The calibration samples were prepared by adding 40 μL of the AQ/AQm standard solutions to 200 μL of blood. Samples were quantified using the ratio of the peak area of each analytes to that of IS. Peak area ratios were plotted against analytes concentrations, and standard curves were calculated using weighted ($1/x^2$) least squares linear regression.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (low, 0.240/2.40 ng/mL; medium, 20.0/200 ng/mL; and high, 90.0/900 ng/mL for AQ/AQm) in three validation days. The precision was expressed by relative standard deviation (RSD) and the accuracy by relative error (RE). The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ± 15%, except at the lower limit of quantification (LLOQ), where precision should be below 20% and accuracy within ± 20%.

The extraction recovery was estimated by comparing the peak areas of the analytes in the extracted QC samples ($n = 6$) with those obtained from the extracted blank blood samples post-spiked with corresponding neat solutions in six replicates. The extraction recovery of the IS was determined in a similar way using the QC samples at medium concentration as a reference.

The stabilities of AQ and AQm in human blood were evaluated by analyzing replicates ($n = 3$) of blood samples at the concentrations of 0.240/2.40 and 90.0/900 ng/mL for AQ/AQm, which were exposed to different conditions (time and temperature). These results were compared with those obtained from freshly prepared blood samples. The analytes were considered stable in the biological matrix when 85–115% of the initial concentrations were found. The short-term stability was determined after the exposure of the spiked samples at room temperature for 6 h, and the ready-to-inject samples (after extraction) in the HPLC autosampler at room temperature for 24 h. The long-term stability was assessed after storage of the standard spiked blood samples at −78 °C for 20 days. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−78 to 25 °C) on consecutive days. The stabilities of standard solutions were also investigated at 4 °C for 19 days and for 6 h at room temperature.

2.7. Application of the method

The developed IP-LC–MS/MS method was used to investigate the blood profiles of AQ and AQm after a single oral dose of three 200-mg AQ hydrochloride tablets (153 mg base per tablet) to 12 healthy Chinese male volunteers. The pharmacokinetic study was approved by the Medical Ethics Committee of Shanghai Shuguang Hospital. Venous blood samples (3 mL) were collected in heparinized tubes before and 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 48, 72, 96 and 336 h after the dose. Blood samples were stored at −78 °C until analysis. The pharmacokinetic parameters of AQ and AQm were calculated by non-compartmental analysis using the computer program WinNonlin (WinNonlin V5.0.1, Pharsight Corporation, Mountain View, CA, USA). The maximum blood concentrations (C_{max}) and their time of occurrence (T_{max}) were both obtained directly from the measured data. The area under the blood concentration–time curve from time zero to the time of the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal method; the terminal elimination rate constant (k_e) was estimated by linear least squares regression of the terminal portion of the blood concentration–time curve, and the corresponding elimination half-life ($T_{1/2}$) was then calculated as $0.693/k_e$.

3. Results and discussion

3.1. Investigation of the effects of ion-pairing reagent on the retention of analytes

Several HPLC methods have been reported for the determination of AQ and AQm, including normal phase HPLC method using dichloromethane:methanol:1 M perchloric acid

(100:10:0.9, v/v/v) as mobile phase [11], and reversed-phase HPLC methods which typically employed phosphoric acid [7–9], perchloric acid [10–12] or ion-pairing reagent alkyl sulfonates [13] as acid modifiers to achieve the baseline separation and improve retention of protonated AQ and AQm.

Mass spectrometric detection provides high sensitivity and specificity, but it is not compatible with the mobile phases reported previously [7–13]. At the present study, a reversed-phase HPLC condition compatible with MS detection was developed. When the chromatographic separation was carried on a Zorbax SB-C_8 column (150 mm × 4.6 mm ID, 5 μm), acetonitrile:water:acetic acid (40:60:0.1, v/v/v) was used as mobile phase. Although good peak shapes and reasonable retention times of the analytes and IS could be achieved, a retention time drift was observed after multiple injections. When a short column (Venusil MP-C_{18}, 50 mm × 2.1 mm ID, 5 μm) was used, the analytes and IS were all eluted at the dead time.

An effective way to increase the retention of polar compounds is ion-pair chromatography. Houze et al. [17] and Zhong et al. [18] have reported ion-pair chromatography methods for the determination of chloroquine, which is an analogue of AQ, in biological samples by UV detection, with alkyl sulfonate being used as ion-pairing reagent. However, for the MS detection, volatile perfluorinated carboxylic acids are preferred, which have been successfully applied in the LC–MS/MS analysis of underivatized small peptides and amino acids [15,16].

In the present study, PFPA was used to increase the retention of AQ, AQm and OHCQ on the Venusil MP-C_{18} column. There might be two processes in ion-pair chromatographic separation: RP retention and ion-exchange retention. These processes might be altered by adjustment of the amount of ion-pairing reagent that is taken up by the stationary phase. In the experiment, HPLC–UV was used as a supplementary method to systematically investigate the effects of ion-pair reagent on the retention of the three 4-aminoquinoline compounds, AQ, AQm and OHCQ. Plot of retention factor (k) versus the ion-pairing reagent concentrations was generated, where $k = V_R/V_m - 1$, V_R is the elution volume of the analytes and V_m is the dead volume of the system, determined to be 0.23 mL using uracil.

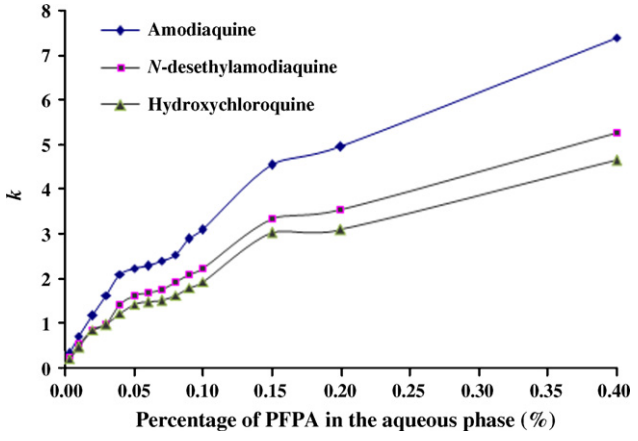

Fig. 1. Retention factors of amodiaquine, *N*-desethylamodiaquine and hydroxychloroquine in relation to the percentage of PFPA in the aqueous phase. Chromatographic column: Venusil MP-C_{18} (50 mm × 2.1 mm ID, 5 μm); mobile phase: 80% aqueous with PFPA in the concentration range of 0.003–0.4% and 20% acetonitrile; column temperature: 25 °C; UV wavelength: 340 nm. $k = V_R/V_m - 1$ is the retention factor.

Fig. 1 demonstrates the retention for the three compounds in relation to the amount of PFPA in the mobile phase. It was observed that the analytes were unretained ($k = 0$) without ion-pairing reagent. The increase of k was more pronounced in the PFPA concentration range of 0.003–0.04% and tended to level off in the concentration range of 0.05–0.07%, which was a suitable PFPA concentration range for choosing. In view of a reasonable retention factor value, reproducible retention behavior and column life, PFPA concentration in the aqueous phase was optimized to be 0.0667%, which fell in the plateau range of the k–PFPA percentage curve. Retention times for AQ, AQm and OHCQ were 2.7, 2.1 and 1.9 min, respectively, using the mobile phase of acetonitrile:water (20:80, v/v) with 0.0667% PFPA in the aqueous phase.

3.2. Effects of the concentration of acetonitrile in the mobile phase on the retention of analytes

During the optimization of chromatographic condition, the influence of acetonitrile on the retention factor was investigated by adjusting the percentage of acetonitrile in the mobile phase from 18 to 27% with 0.0667% PFPA in the aqueous phase. Fig. 2 shows good linearity of the plots of the logarithms of retention factors, log k, versus the volume fraction of acetonitrile in the mobile phase for AQ, AQm and OHCQ, which is in agreement with the equation [19]:

$$\log k = a - m\phi \quad (1)$$

The parameters a, m of the regression equation and statistical results were provided in Table 1. The higher is m, the greater change in retention is induced by changing the concentration of acetonitrile by 1%. The results indicated that the retention of AQ, AQm and OHCQ were sensitive to the percentage of acetonitrile in the mobile phase, and the effects of the volume fraction of acetonitrile on the retention of the three compounds were similar. For the purpose of improving throughput of the method,

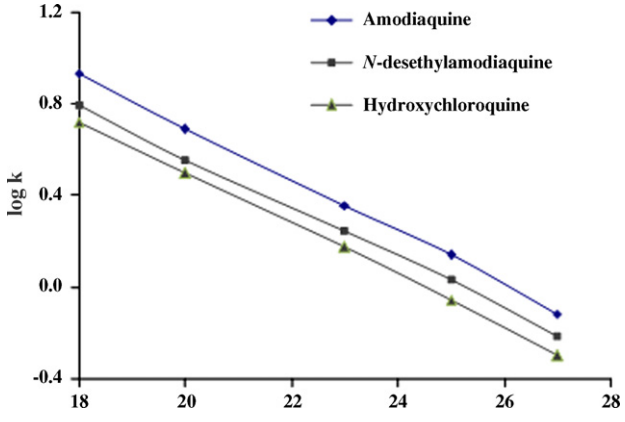

Fig. 2. Effects of the volume fraction of acetonitrile on the retention, log k, of amodiaquine, *N*-desethylamodiaquine and hydroxychloroquine. Mobile phase B was water containing 0.0667% PFPA. Other conditions are same as in Fig. 1.

Table 1
Parameters of Eq. (1), intercepts *a*, slopes *m*, and statistic results for AQ, AQm and OHCQ

Compound	Parameters		Statistics[a]		
	a	*m*	*R*	SE	*P*
Amodiaquine	3.01	11.5	0.9993	0.015	0.0007
N-desethylamodiaquine	2.77	11.0	0.9992	0.016	0.0008
Hydroxychloroquine	2.76	11.3	0.9996	0.012	0.0004

[a] *R*, correlation coefficient; SE, standard error; *P*, value of *f*-statistic.

the mobile phase was finally chosen as acetonitrile:water (23:77, v/v) with 0.0667% PFPA in the aqueous phase, which was delivered using binary pump at the flow rate of 0.5 mL/min, and the retention time for AQ, AQm and IS were 1.27, 1.06 and 0.96 min, respectively.

3.3. Mass spectrometry

The possibility of using electrospray ionization (ESI) or APCI source under positive ion detection mode was evaluated. Results showed that APCI could offer higher stability of signal response for the analytes than that of ESI, and thus improve the reproducibility of the assay. Consequently, APCI was chosen in this study. Full-scan Q1 mass spectra of AQ and AQm in the positive-ion mode formed protonated molecule $[M+H]^+$ at *m/z* 356 and 328, respectively as base peak ions. The internal standard, OHCQ, also gave rise to abundant protonated molecule $[M+H]^+$ at *m/z* 336. The base peak ions of the analytes and IS were therefore selected as the precursor ions to obtain their major fragment ions for MRM analysis. The structural similarity of AQ and AQm leads to a same abundant fragment ion at *m/z* 283, which is corresponding to the elimination of the amino side chain from precursor ions. This fragment was observed as the most abundant ion when the CE was increased from 10 to 50 eV. The fragment ion at *m/z* 247 was chosen for IS. The optimal CE value was found to be 25 eV for AQ, AQm and IS. The corresponding product ion scan spectra are shown in Fig. 3.

3.4. Sample preparation

The adsorption of AQ on the glassware has been reported in the literature [7], for this reason, the working solutions were prepared with 0.1% formic acid to minimize AQ adsorption.

Either plasma or blood has been chosen for the pharmacokinetic study of AQ. Considering blood cells have a strong uptake of AQm [5], it is preferable to determine simultaneously AQ and AQm in human blood to characterize the pharmacokinetic profile of the drug. The biological sample preparation procedures reported in the literature were liquid–liquid extraction (LLE) [7–10,12,13] or solid-phase extraction (SPE) [11]. In this study, protein precipitation was used in sample preparation and acetonitrile was selected as precipitant due to its efficiency in protein precipitating. The simple preparation procedure can save time and simplify the operating process.

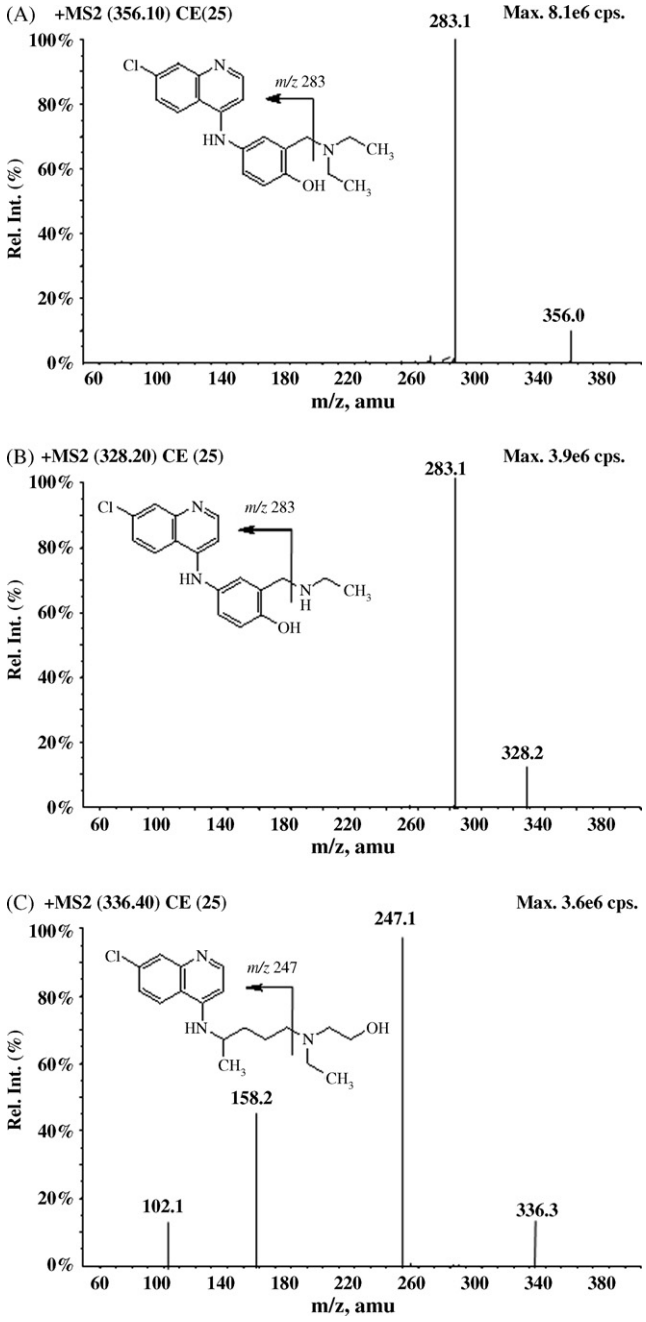

Fig. 3. Full scan product ion spectra of $[M+H]^+$ of amodiaquine (A), *N*-desethylamodiaquine (B) and hydroxychloroquine (IS, C) dissolved in the mobile phase.

3.5. Method validation

3.5.1. Selectivity and matrix effect

Selectivity was assessed by comparing the chromatograms of six different batches of blank human blood with the corresponding spiked blood samples. Fig. 4 shows the typical chromatograms of a blank blood sample, blank blood sample spiked with AQ and AQm at the LLOQ and IS, and a blood sample obtained at 24 h after a single oral administration of 600 mg AQ hydrochloride (459 mg base) to a volunteer. No interfering endogenous substances were observed at the retention times

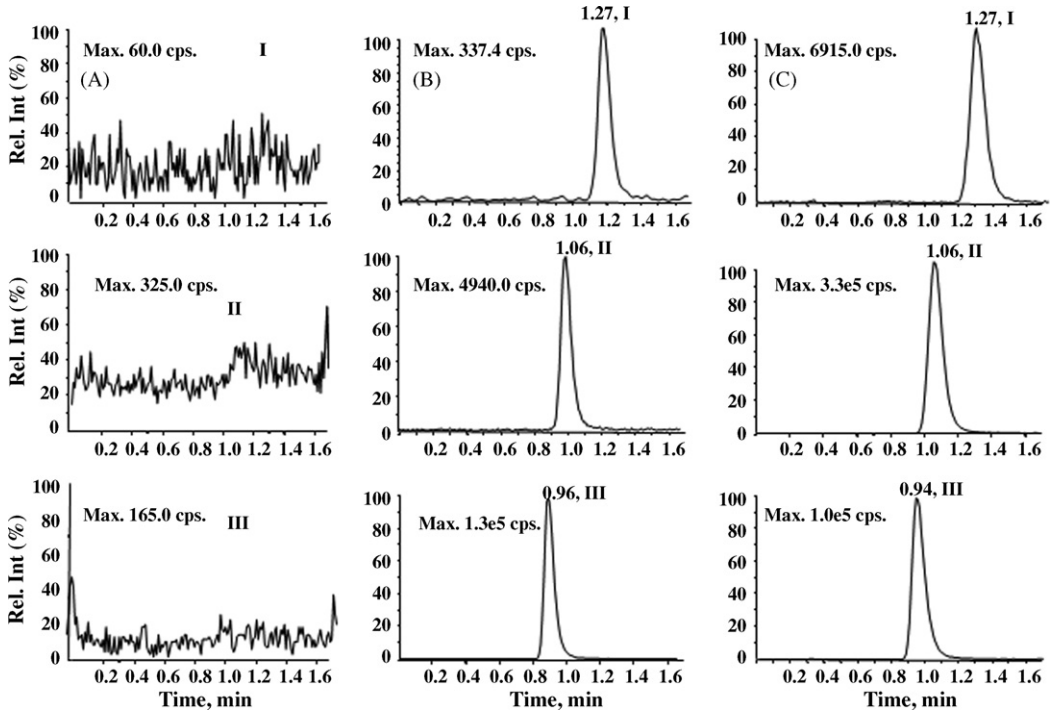

Fig. 4. Typical MRM chromatograms of (A) a blank human blood sample; (B) a blank human blood sample spiked with amodiaquine and *N*-desethylamodiaquine at LLOQ (0.150 and 1.50 ng/mL, respectively) and IS at 80.0 ng/mL; (C) a human blood sample obtained at 24 h after a single oral administration of 600 mg amodiaquine hydrochloride (459 mg base). Peak I, amodiaquine; Peak II, *N*-desethylamodiaquine, Peak III, IS.

of the analytes and IS. The chromatograms presented in Fig. 4 indicated that the method was selective.

The matrix effect data in six different batches of human blood were 103.6 ± 5.3%, 101.7 ± 1.3%, and 100.9 ± 1.2% for AQ at concentrations of 0.240, 20.0 and 90.0 ng/mL, respectively, and 101.4 ± 2.7%, 101.7 ± 1.5% and 102.3 ± 0.6% for AQm at concentrations of 2.40, 200 and 900 ng/mL, respectively. The matrix effect for the IS was 101.4 ± 1.2%. The results indicated that no coeluting substances significantly influenced the ionization of AQ, AQm and IS.

3.5.2. Linearity of calibration curve and lower limit of quantification

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 0.150–100 ng/mL for AQ and 1.50–1000 ng/mL for AQm in human blood. Typical equations of the calibration curves were as follows: AQ: $y = 2.21 \times 10^{-2}x - 2.60 \times 10^{-4}$, $r^2 = 0.9991$; AQm: $y = 2.50 \times 10^{-2}x + 5.66 \times 10^{-4}$, $r^2 = 0.9985$. Where y represents the ratios of AQ or AQm peak area to that of IS and x represents the blood concentrations of AQ or AQm.

The LLOQ was 0.150 ng/mL for AQ and 1.50 ng/mL for AQm. The precision and accuracy at LLOQ were 10.4% and 4.6% for AQ, while the values were 13.6% and 8.0% for AQm. Under the present LLOQ, the AQ concentration could be determined in blood samples up to 96 h and the concentration of AQm could be determined up to 336 h after a single oral dose of 600 mg AQ hydrochloride (459 mg base), thus indicated that the method was sensitive enough to investigate the pharmacokinetics of the drug.

3.5.3. Precision and accuracy

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days using a one-way analysis of variance (ANOVA) [20]. Intra-day precision was 5.2% or less for AQ and 4.5% or less for

Table 2
Precision and accuracy data for the analysis of amodiaquine and *N*-desethylamodiaquine in human blood (in prestudy validation, 3 days, six replicates per day)

Analyte	Concentration (ng/mL)		RSD (%)		Relative
	Added	Found	Intra-day	Inter-day	Error (%)
Amodiaquine	0.240	0.229	5.2	1.0	−4.7
	20.0	20.6	0.9	4.9	3.0
	90.0	91.8	1.1	4.5	2.0
N-desethylamodiaquine	2.40	2.39	2.4	5.4	−0.3
	200	199	0.8	5.8	−0.3
	900	875	4.5	3.5	−2.8

Table 3
Summary of stability of amodiaquine and *N*-desethylamodiaquine under various storage conditions ($n = 3$)

Condition	Concentration (ng/mL)				Amodiaquine		*N*-desethylamodiaquine	
	Amodiaquine		*N*-desethylamodiaquine					
	Added	Found	Added	Found	RSD (%)	RE (%)	RSD (%)	RE (%)
Ambient, 6 h	0.240	0.224	2.40	2.47	4.2	−4.2	3.0	3.1
	90.0	94.6	900	873	0.7	2.2	4.8	−3.0
−78 °C, 20 days	0.240	0.235	2.40	2.68	10.8	−1.9	5.5	11.6
	90.0	91.4	900	854	0.4	1.6	0.1	−5.1
Three freeze–thaw	0.240	0.237	2.40	2.53	2.3	−1.4	1.4	5.6
	90.0	91.7	900	862	0.9	1.9	0.6	−4.1
Auto sampler	0.240	0.233	2.40	2.51	4.6	−2.8	2.0	4.4
Ambient, 24 h	90.0	90.8	900	865	0.1	0.9	0.5	−3.9

AQm, and the inter-day precision was 4.9% or less for AQ and 5.8% or less for AQm at each QC level (0.240/2.40, 20.0/200, 90.0/900 ng/mL for AQ/AQm, respectively).

The accuracy of the method, expressed in terms of RE, ranged from −4.7% to 2.0% for AQ, and from −2.8% to −0.3% for AQm at each QC level. Assay performance data are presented in Table 2. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.5.4. Extraction recovery

Mean extraction recoveries of AQ were 93.8 ± 6.2%, 93.0 ± 1.0% and 91.1 ± 0.5% ($n = 6$) at concentrations of 0.240, 20.0 and 90.0 ng/mL, respectively; Mean extraction recoveries of AQm were 90.7 ± 3.5%, 93.3 ± 0.8% and 90.2 ± 5.6% ($n = 6$) at concentrations of 2.40, 200 and 900 ng/mL, respectively. The extraction recovery of the IS was 93.1 ± 3.3% ($n = 6$).

3.5.5. Stability

The stability tests of the analytes were designed to cover anticipated conditions for the preservation of the clinical samples. The stability results showed that AQ and AQm spiked into human blood were stable for 6 h at room temperature, for 20 days at −78 °C, and during three freeze–thaw cycles. Stability of AQ and AQm extracts in the sample solvent on autosampler was also observed over a 24 h period. The stock solutions of the analytes were stable for at least 19 days. The results of stability experiments are shown in Table 3.

3.6. Application of the method to a pharmacokinetic study in healthy volunteers

The validated analytical method was applied to the assay of AQ and AQm in human blood after a single oral administration of 600 mg AQ hydrochloride (459 mg base) to healthy male volunteers. The blood samples were processed based on the proposed extraction protocol for the quantification of AQ and AQm. Mean blood concentration versus time profile is presented in Fig. 5. This method was sensitive enough to monitor the AQ blood concentration up to 96 h while AQm up to 336 h. Table 4 lists the major pharmacokinetic parameters of

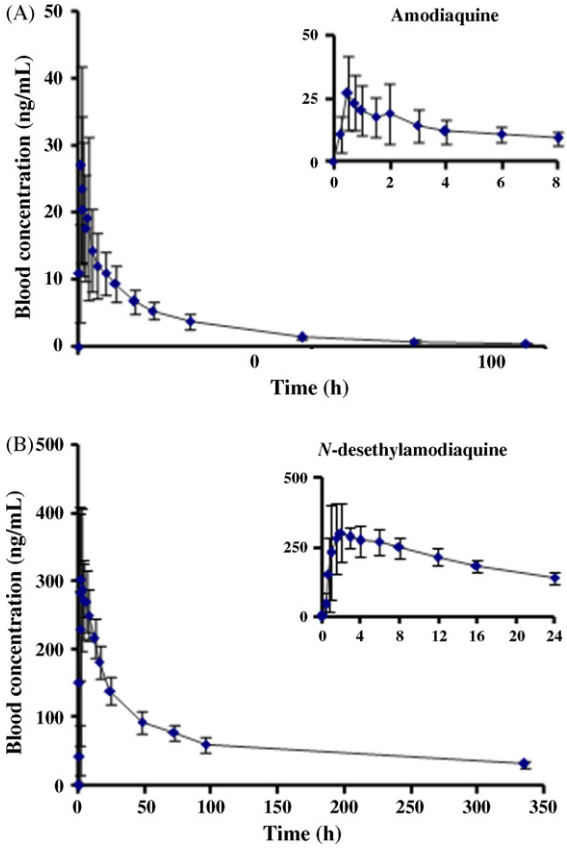

Fig. 5. Mean blood concentrations of amodiaquine (A) and *N*-desethylamodiaquine (B) after a single oral dose of 600 mg amodiaquine hydrochloride (459 mg base) to 12 healthy volunteers.

Table 4
Mean pharmacokinetic parameters for amodiaquine and *N*-desethylamodiaquine after an oral administration of 600 mg amodiaquine hydrochloride tablets to 12 healthy volunteers

Parameters	Amodiaquine	*N* -desethylamodiaquine
T_{max} (h)	0.90 ± 0.60	2.17 ± 1.17
C_{max} (ng mL^{-1})	30.3 ± 10.9	356 ± 82
AUC_{0-t} (ng h mL^{-1})	305 ± 77	22451 ± 4460
$T_{1/2}$ (h)	23.1 ± 5.2	153.7 ± 82.0

AQ and AQm in 12 volunteers. Compared with the pharmacokinetic results reported previously [10,21], it was observed that the value of $T_{1/2}$ in this study was longer, but that T_{max} did not differ significantly among them, which might result from the different sensitivities of the analytical methods or ethnic differences.

4. Conclusions

An ion-pair liquid chromatography–tandem mass spectrometry method for the simultaneous determination of AQ and AQm in human blood was developed and validated. The volatile PFPA as ion-pairing reagent in the mobile phase provided necessary retention enhancement and reproducible chromatographic behavior for the ionizable polar analytes. This method offers advantages of improved sensitivity and selectivity, short run time, and simple blood sample preparation. It was successfully applied to a pharmacokinetic study after a single oral administration of 600 mg AQ hydrochloride (459 mg base) to healthy human volunteers, with AQ and AQm being quantified at lower levels in blood and for longer duration than previously documented.

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