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Sensitive and rapid liquid chromatography/tandem mass spectrometric assay for the quantification of chloroquine in dog plasma

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Abstract

A simple, sensitive and rapid liquid chromatography/tandem mass spectrometric (LC–MS/MS) method was developed and validated for quantification of chloroquine, an antimalarial drug, in plasma using its structural analogue, piperazine bis chloroquinoline as internal standard (IS). The method is based on simple protein precipitation with methanol followed by a rapid isocratic elution with 10 mM ammonium acetate buffer/methanol (25/75, v/v, pH 4.6) on Chromolith SpeedROD RP-18e reversed phase chromatographic column and subsequent analysis by mass spectrometry in the multiple reaction monitoring mode (MRM). The precursor to product ion transitions of *m/z* 320.3 → 247.2 and *m/z* 409.1 → 205.2 were used to measure the analyte and the IS, respectively. The assay exhibited a linear dynamic range of 2.0–489.1 ng/mL for chloroquine in dog plasma. The limit of detection (LOD) and lower limit of quantification (LLOQ) were 0.4 and 2.0 ng/mL, respectively in 0.05 mL plasma. Acceptable precision and accuracy were obtained for concentrations over the standard curve range of 2.0–489.1 ng/mL. A run time of 2.0 min for a sample made it possible to achieve a throughput of more than 400 plasma samples analyzed per day. The validated method was successfully used to analyze samples of dog plasma during non-clinical study of chloroquine.

Keywords: Antimalarial; Chloroquine; Liquid chromatography/tandem mass spectrometry; Pharmacokinetic study; Plasma

1. Introduction

Chloroquine (7-chloro-4-[-4-diethylaminol-methyl butylamino] quinoline), has been the most prescribed drug for the treatment of malaria during past 50 years [1–3] and is also used in the treatment of rheumatoid arthritis.

Several methods have been reported for the quantification of chloroquine in biological fluids. These methods are based on high-performance liquid chromatography (HPLC) [4–29], radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [30] or high performance thin layer chromatography (HPTLC) [31]. Till date, no simple chromatographic tandem mass (LC–MS/MS) method has been reported for chloroquine quantitation in plasma. Quantification of drugs in biological matrices by liquid chromatography/tandem mass spectrometry (LC–MS/MS) is becoming more common, owing to the improved sensitivity and selectivity of this technique [32]. LC–MS/MS requires less extensive sample preparation, hence chloroquine can be detected directly without solid phase extraction [4,12] or liquid–liquid extraction [5,6,11,13,14] techniques, which are expensive, time consuming and tedious. Recently, Samanidou et al. [4] has reported an HPLC method for the quantification of quinine and chloroquine in plasma using solid phase extraction (SPE) with satisfactory sensitivity but it required long analysis time. In the present method, the plasma sample was precipitated with methanol and the supernatant was directly injected onto the LC–MS/MS system for quantification.

The purpose of this investigation was to utilize the high selectivity and sensitivity of LC–MS/MS method in multiple reaction monitoring mode (MRM) for the quantification of chloroquine in plasma. The method was developed and validated to determine low levels of chloroquine in dog plasma during comparative cardiac safety study of antimalarials. It was essential to establish

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an assay capable of quantifying chloroquine at concentrations as low as to 2.0 ng/mL. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, and toxicokinetic studies of chloroquine. The method was validated according to published USFDA guidelines [34].

2. Experimental

2.1. Chemicals

Chloroquine phosphate (99.94% w/w, on dry basis) was obtained from Mangalam drugs and organics Ltd. (Vapi, India) and piperazine bis chloroquinoline (98.69%, % area by HPLC), used as internal standard (IS), was obtained from our NCE Scale up division, R&D (Gurgaon, India). Chemical structures are presented in Fig. 1. HPLC-grade methanol was purchased from SD Fine Chem. Ltd. (Mumbai, India). Formic acid and ammonium acetate were purchased from Fukla (Fluka Chemie, GmbH, Germany). Milli-Q water (18.2 mΩ and TOC ≤ 50 ppb) from Milli-Q system (Millipore SAS, Molsheim, France) was used. All other chemicals were of analytical grade.

2.2. Instrumentation

The HPLC, Perkin Elmer 200 Series (Perkin Elemer Instruments LLC, Shelton CT, USA) was equipped with a quartenary pump, a degasser, autosampler equipped with a thermostatted column compartment. The compounds were analyzed on a Chromolith SpeedROD RP-18e reversed phase chromatographic column (50 mm × 4.6 mm i.d.) using a mobile phase containing a mixture of 10 mM ammonium acetate buffer/methanol (25/75, v/v, pH adjusted to 4.6 with formic acid), which was pumped at a flow-rate of 0.8 mL/min. Mass detection was performed on an API 4000 triple quadrupole instrument (Applied Biosystems MDS SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.4 software package (Applied Biosystems MDS SCIEX).

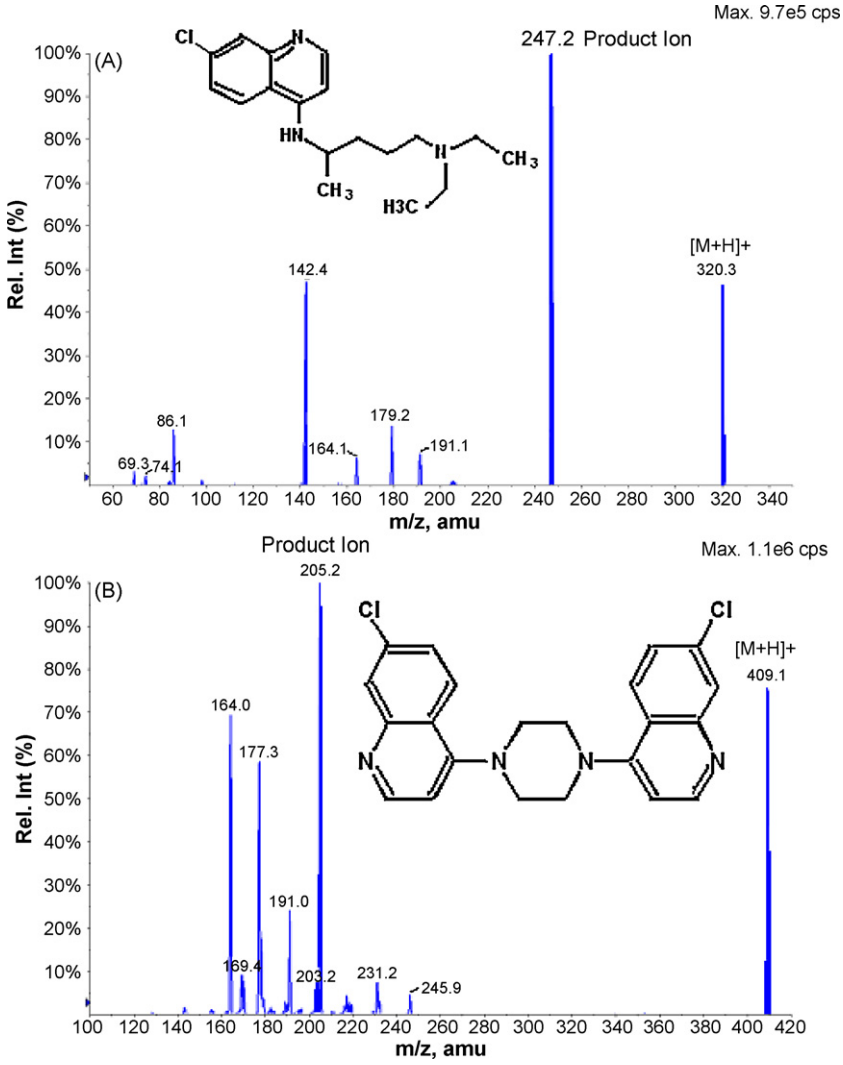

Fig. 1. Non-optimised product ion mass spectra of (A) chloroquine and (B) IS in positive ionization mode.

Table 1
Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature (°C)	400
Dwell time per transition (ms)	250
Ion source gas (gas 1) (psi)	60
Ion source gas (gas 2) (psi)	40
Curtain gas (psi)	25
Collision gas (psi)	6
Ion spray voltage (V)	5500
Entrance potential (V)	10
Declustering potential (V)	60 (analyte) and 130 (IS)
Collision energy (V)	25 (analyte) and 54 (IS)
Mode of analysis	Positive
Collision cell exit potential (V)	6 (analyte) and 15 (IS)
Ion transition for chloroquine (*m/z*)	320.3/247.2
Ion transition for bis quinoline (*m/z*)	409.1/205.2

2.3. Sample preparation

Sample preparation involved a simple protein precipitation with methanol. Aliquots (50 μL) of plasma were subjected to precipitation with the addition of 300 μL of working solution of internal standard (prepared in methanol). The samples were vortexed for 20 s and centrifuged at 12000 rpm for 10 min. The upper layer (200 μL) was transferred into autosampler vials and a 10 μL aliquot was injected into the chromatographic system.

2.4. Bioanalytical method validation

A full validation according to USFDA guidelines was performed for the assay in dog plasma [34]. Partial validation evaluating precision and accuracy, specificity and recovery was performed in human plasma.

Standard stock solutions of chloroquine (1 mg/mL) and the IS (1 mg/mL) were prepared separately in 10 mL volumetric flasks with diluent (1% formic acid/methanol, 50/50, v/v). Working solutions for calibration and quality controls were prepared from the stock solution by adequate dilution using diluent (water/methanol, 50/50, v/v). The IS working solution (50 ng/mL) was prepared by diluting its stock solution with methanol. Working solutions (50 μL) were added to 950 μL drug-free plasma to obtain chloroquine concentration levels of 2.0, 4.9, 9.8, 19.6, 48.9, 97.8, 244.6 and 489.1 ng/mL. Quality control (QC) samples were prepared in bulk based on an independent weighing of standard drug, at concentrations of 2.0 ng/mL (LLOQ), 5.8 ng/mL (low), 194.1 ng/mL (medium) and 388.2 ng/mL (high) as a single batch at each concentration. These samples were divided into aliquots in microcentrifuge tubes (Tarson, 1.5 mL) and stored in the freezer below −50 °C until analysis. A calibration curve consisted of a blank dog plasma sample (plasma sample processed without the IS), a zero sample (plasma processed with the IS) and eight non-zero samples covering the range 2.0–489.1 ng/mL including LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression on five consecutive days. The acceptance criteria for calibration curve standards and quality control samples were as per USFDA guidelines [34].

Recovery of chloroquine from the extraction procedure was determined by a comparison of the peak area of chloroquine in spiked plasma samples (five low, medium and high quality controls) with the peak area of chloroquine in samples prepared by aqueous samples with the same amounts of chloroquine at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted quality control samples ($n = 15$) to mean peak areas of IS in aqueous samples with the same amounts of IS at the step immediately prior to chromatography.

The chloroquine and IS stock solutions stability was tested at room temperature and under refrigeration (∼4 °C). The stock solutions of chloroquine and IS were diluted to 50 ng/mL with diluent (water/methanol, 50/50, v/v) prior to analysis. The stability of chloroquine in dog plasma after three freeze–thaw cycle was investigated. These samples were quantified against the freshly spiked calibration curve standards over the concentration range equivalent to that used for the calculation of precision and accuracy. The stability of chloroquine in dog plasma under processing (ambient temperatures) and storage conditions (−50 °C) was also evaluated. Finally, stability in the final extract for dog plasma was determined in the autosampler.

3. Results and discussion

3.1. Method development

In order to develop a method with the desired LLOQ (2.0 ng/mL), it was necessary to use MS–MS detection, as this provides improved limit of detection (LOD) for trace-mixture analysis [33]. The inherent selectivity of MS–MS detection was also expected to be beneficial in developing a selective and sensitive method. The product ion mass spectra of chloroquine and the IS in positive ionization mode are shown in Fig. 1, respectively. The predominant $[M + H]^+$ and $[M]^+$ ion in the Q1 spectrum for chloroquine and IS were used as the precursor ion to obtain the product ion spectra. The most sensitive mass transition was from *m/z* 320.3 to 247.2 for chloroquine and *m/z* 409.1 to 205.2 for the IS. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the results of this optimization.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes, as well as shorter run time. Modifiers such as formic acid and ammonium acetate alone or in combination at different concentrations were added. It was found that a mixture of 10 mM ammonium acetate buffer/methanol (25/75, v/v; pH 4.6) could achieve this purpose and was finally adopted as the mobile phase. The ammonium hydroxide was found to be necessary to reduce the peak tailing and thus deliver good peak shape whereas formic acid was necessary to lower the pH to protonate chloroquine and enhance the resolution. The percentage of ammonia and formic acid were optimized to obtain this peak shape whilst being consistent with good ionization and fragmentation in the mass spectrometer.

Table 2
Precision and accuracy data of back-calculated concentrations of calibration samples for chloroquine in dog plasma

Concentration added (ng/mL)	Concentration found (ng/mL) (mean ± SD; $n = 5$)	Precision (%)	Accuracy (%)
2.0	2.13 ± 0.11	5.4	108.9
4.9	4.60 ± 0.30	6.5	95.0
9.8	9.53 ± 0.40	4.2	97.4
19.6	19.37 ± 0.52	2.7	99.0
48.9	46.92 ± 1.18	2.5	95.9
97.8	99.27 ± 1.96	2.0	101.5
244.6	255.48 ± 6.42	2.5	104.5
489.1	495.06 ± 10.3	2.1	101.2

The development of the chromatography method was focused on shorter run time in order to assure high throughput, paying attention to matrix effects as well as good peak resolution. The high proportion of organic solvent (10 mM ammonium acetate/methanol (25/75, v/v; pH 4.6)) eluted the analyte and the IS at retention times of 0.9 and 1.1 min, respectively. A flow-rate of 0.8 mL/min produced good peak shapes and permitted a run time to 2.0 min. Internal standard is necessary for determination of analyte in biological samples. Several compounds were investigated to find a suitable internal standard by spiking blank plasma extracts at the low and high QC levels and finally piperazine bis chloroquinoline (Fig. 1B) was found to be best IS for the present purpose.

3.2. Assay performance and validation

The calibration curve was linear over the concentration range 2.0–489.1 ng/mL. The best linear fit and least squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of $y = 0.01060x - 0.0053$ where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The correlation coefficient (r) for chloroquine was above 0.994. Calibration curve results are summarized in Table 2.

The specificity of the method was established with pooled and individual plasma samples from six different sources. Representative chromatograms of extracted blank dog plasma (Fig. 2), blank dog plasma fortified with IS (Fig. 3), blank human plasma (Fig. 4) and chloroquine (Fig. 5), demonstrated the specificity and selectivity of the method. As shown in Figs. 2 and 3, no significant interference in the blank plasma traces was seen from endogenous substances in drug-free plasma at the retention time of the analyte. Fig. 3 shows the absence of interference from the IS to the MRM channels of the analyte. Fig. 5 depicts a representative ion-chromatogram for the LLOQ (2 ng/mL). The representative MRM ion-chromatogram of an extracted dog plasma sample is depicted in Fig. 6.

The matrix effect in the LC–MS/MS method was determined by spiking low and high quality control samples in duplicate from each of the six different batches of dog plasma. The within-batch precision and accuracy ranged from 4.70% to 12.77% and 88.14% to 96.90%, respectively for dog plasma. Hence the matrix effect on estimation of drug is negligible.

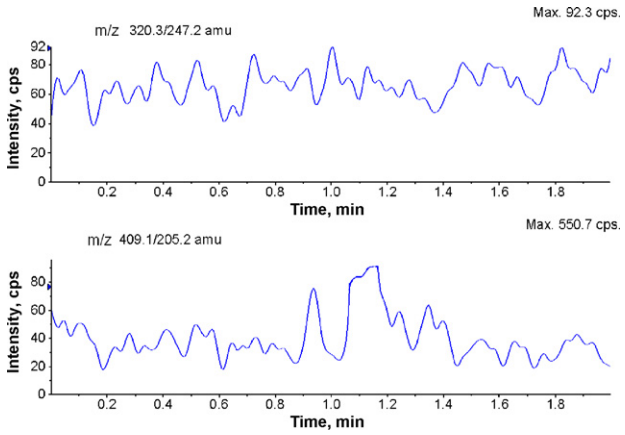

Fig. 2. MRM ion-chromatograms resulting from the analysis of blank (drug and internal standard free) dog plasma for chloroquine and internal standard.

The extraction recoveries of chloroquine in dog and human plasma were 18.6 ± 3.3% and 19.0 ± 2.8%, respectively. The extraction recoveries of IS were 102.52 ± 4.3% and 104.31 ± 10.0% in dog and human plasma, respectively and the dependence on concentration is negligible. Recovery of the analyte was low but it was consistent, precise and reproducible. There were no significant differences between recoveries of chloroquine and IS from human and dog plasma, the quality control samples prepared in dog plasma can be quantified using calibration standard prepared in human plasma or vice versa.

The LOD demonstrated that the analyte gave a signal-to-noise ratio (S/N) of ≥3 at the concentration of 0.4 ng/mL. The LLOQ, the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision was found to be 2.0 ng/mL in dog plasma. The mean response for the analyte peak at the assay sensitivity limit (2.0 ng/mL) was ∼ 25-fold greater than the mean response for the analyte peak in six blank dog plasma samples. Excellent sensitivity was observed with a 10 μL injection volume; the LLOQ corresponds to 20 pg on-column. The between-batch precision for chloroquine at LLOQ was 9.5% and the accuracy was 107.5% (Table 3). The within-batch precision was 8.8% and the accuracy was 105.4% for chloroquine at LLOQ.

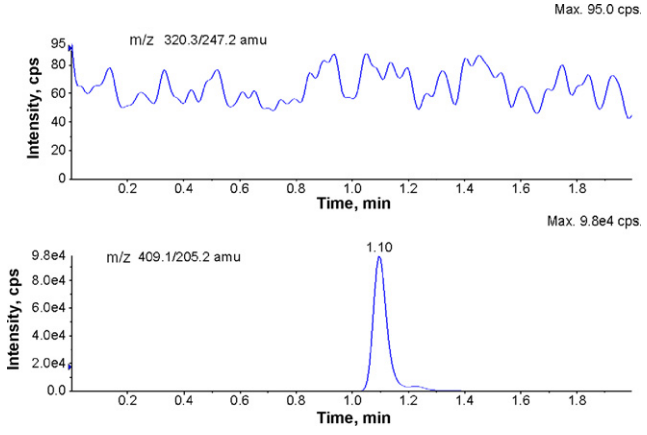

Fig. 3. MRM ion-chromatograms resulting from the analysis of blank (drug-free spiked with IS) dog plasma for chloroquine and internal standard.

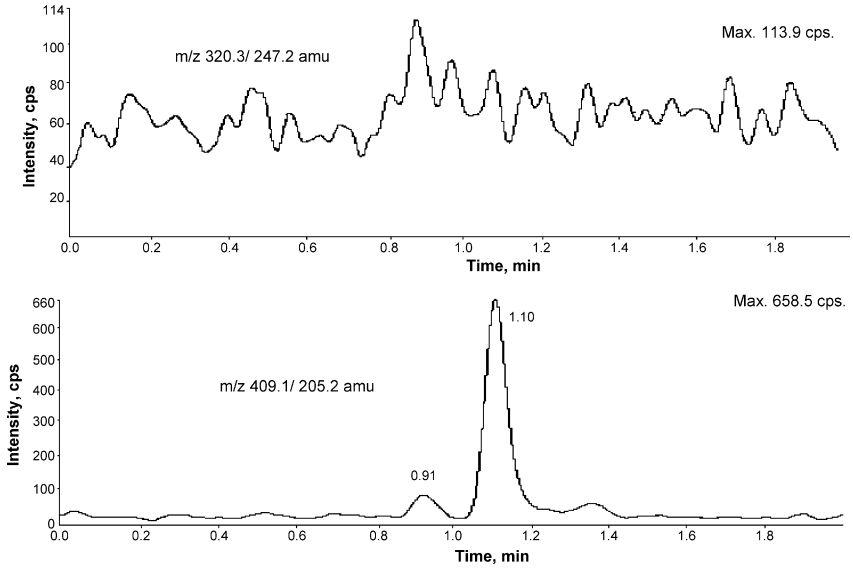

Fig. 4. MRM ion-chromatograms resulting from the analysis of blank (drug and internal standard free) human plasma for chloroquine and internal standard.

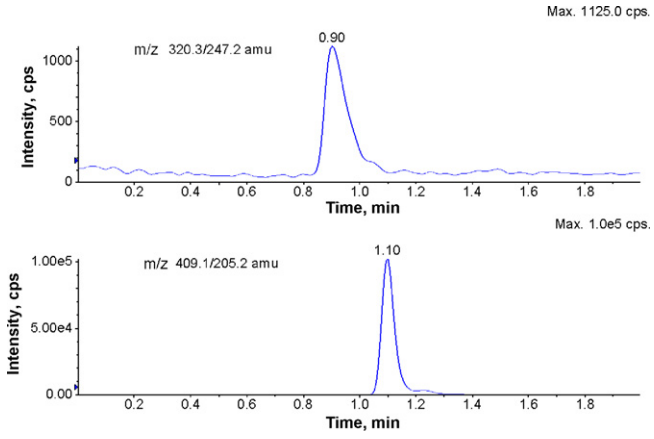

Fig. 5. Representative MRM ion-chromatograms resulting from the analysis of 2.0 ng/mL (LLOQ) of chloroquine spiked with the internal standard.

The lower and upper quality control levels of chloroquine were 5.8 and 388.2 ng/mL in plasma. For the between-batch experiments, the precision ranged from 2.1% to 9.5% and the accuracy ranged from 93.4% to 107.5% (Table 3). For the within-batch experiments, the precision and accuracy met the acceptance criterion (<±15%). For human plasma the within-batch precision ranged from 1.2% to 7.7% and the accuracy ranged from 101.2% to 109.2% (Table 3). The upper concentration limits can be extended with acceptable precision and accuracy by a 20-fold dilution with control plasma. For the within-batch experiment, the precision and accuracy at two concentration levels 194.1 and 388.2 ng/mL were 3.5% to 6.8% and 92.6% to 103.5%, respectively. These results suggest that samples with concentrations greater than 20-fold of upper limit of the calibration curve can be assayed to obtain acceptable data.

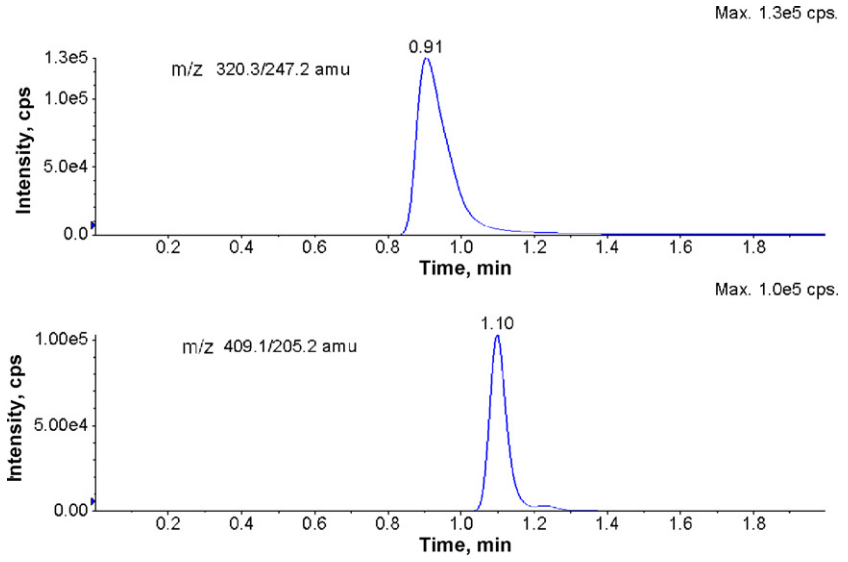

Fig. 6. MRM ion-chromatograms resulting from the analysis of dog plasma sample after the administration of 10 mg/kg oral single dose of chloroquine. The sample concentration was 62.3 ng/mL.

Table 3
Precision and accuracy data of back-calculated concentrations of quality control samples for chloroquine in plasma

Species	Concentration added (ng/mL)	Within-batch precision ($n=5$)			Between-batch precision ($n=3$)		
		Concentration found (ng/mL) (mean ± SD)	Precision (%)	Accuracy (%)	Concentration found (ng/mL) (mean ± SD)	Precision (%)	Accuracy (%)
Dog	2.0	2.2 ± 0.19	8.8	105.4	2.19 ± 0.21	9.5	107.5
	5.8	5.5 ± 0.31	5.6	95.1	5.44 ± 0.35	5.9	93.4
	194.1	198.9 ± 7.9	3.9	102.4	198.27 ± 5.76	2.9	102.1
	388.2	419.1 ± 6.22	1.5	107.9	414.82 ± 8.67	2.1	106.9
Human	2.0	2.2 ± 0.17	7.7	109.2			
	5.8	5.9 ± 0.14	2.4	101.2			
	194.1	196.7 ± 5.12	2.6	101.3			
	388.2	401.2 ± 4.98	1.2	103.4			

3.3. Stability studies

The stability of the analyte and IS in dog plasma under different temperature and timing conditions, as well as the stability in stock solution, was evaluated as follows. All the stability studies were carried out at two concentration levels (5.8 and 388.2 ng/mL as low and high values) with five determinations for each. For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during the routine sample preparation (around 6 h). Samples were extracted and analyzed as described above and the results are given in Table 4. These results indicated reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler for 19 h was also assessed. The results indicated that processed plasma sample of chloroquine and IS can remain in the autosampler for at least 19 h, without showing significant loss in the quantified values (Table 4). The data representing the stability of chloroquine in plasma at two QC levels over three freeze–thaw cycles are given in Table 4. These tests indicated that the analyte was stable in plasma for three freeze–thaw cycles, when stored at below −50 °C and thawed to room temperature. Table 4 also summarizes the long-term stability data for chloroquine in plasma samples stored for a period of 60 days at below −50 °C.

Table 4
Stability of chloroquine in dog plasma

Sample concentration (ng/mL) ($n=5$)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability for 6 h			
5.8	5.7 ± 0.32	5.7	97.2
388.2	433.0 ± 13.2	3.0	111.5
Autosampler stability for 19 h			
5.8	5.30 ± 0.62	11.6	91.1
388.2	356.91 ± 18.4	5.2	91.9
Stability for 60 days at ≤−50 °C			
5.8	5.49 ± 38	7.0	94.3
388.2	377.0 ± 17.3	4.6	97.1
Three freeze–thaw cycles			
5.8	6.17 ± 0.39	6.3	106.0
388.2	402.14 ± 26.3	6.6	103.6

The stability study of chloroquine in plasma showed reliable stability behavior, as the mean of the results of the tested samples were within the acceptance criterion of ±15% of the initial values of the controls. These findings indicated that storage of chloroquine in plasma samples at below −50 °C was adequate, and no stability related problems would be expected during routine analysis for pharmacokinetic and toxicokinetic studies. The stability of stock solutions of chloroquine and IS were tested and established at room temperature for 6 h and under refrigeration (∼4 °C) for 55 days. The recoveries were 99.3 (CV 2.0%), 100.8 (CV 1.6%) and 100.6 (CV 1.2%), 96.9 (CV 3.5%), respectively. The results revealed optimum stability of the stock solutions throughout the period intended for their daily use.

3.4. Application

The validated method was successfully used to quantify chloroquine concentrations in the dog plasma samples after the administration of a single 10 mg/kg oral dose of chloroquine phosphate. Mean plasma concentration–time profile of chloroquine following 10 mg/kg oral administration of chloroquine phosphate to Beagle dogs ($n=6$) is presented in Fig. 7. The method was partially validated in human plasma. It can be used for estimation of chloroquine in human plasma from clinical studies and measuring residual chloroquine levels in volunteers/patients being enrolled for new antimalarial drug trials, as chloroquine has long half-life of 106.43 ± 10.13 h [35].

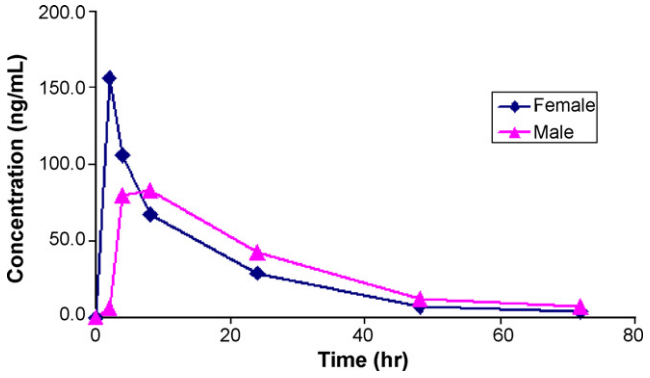

Fig. 7. Mean plasma concentration–time profile of chloroquine following 10 mg/kg oral administration of chloroquine phosphate to Beagle dogs.

4. Conclusions

In summary, LC–MS/MS method for the quantification of chloroquine in dog plasma was developed and fully validated as per USFDA guidelines [34]. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, shorter run time (2.0 min) and lower sample volume requirements. With dilution integrity up to 20-fold, we have established that the upper limit of quantification is extendable up to 9782 ng/mL. The current method has shown acceptable precision and adequate sensitivity for the quantification of chloroquine in dog plasma samples in non-clinical study. The method was partially validated in human plasma and can be used for quantification of chloroquine in human plasma samples from clinical studies.

Chloroquine was shown to be stable in routine analysis conditions and in dog plasma for at least 60 days when stored at below −50 °C. The simple, inexpensive protein precipitation and high sample turnover rate (2.0 min per sample), makes this method an attractive procedure in high-throughput bioanalysis of chloroquine. The validated method allows quantification of chloroquine in the 2.0–489.1 ng/mL range.

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