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Journal of Chromatography B, 859 (2007) 24–29
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

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

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Received 3 July 2007; accepted 2 September 2007
Available online 21 September 2007

Abstract

A simple, sensitive and rapid liquid chromatography/tandem mass spectrometric (LC–MS/MS) method was developed and validated for quantification of piperaquine, an antimalarial drug, in human plasma using its structural analogue, piperazine bis chloroquinoline as internal standard (IS). The method involved a simple protein precipitation with methanol followed by rapid isocratic elution of analytes with 10 mM ammonium acetate buffer/methanol/formic acid/ammonia solution (25/75/0.2/0.15, v/v) on Chromolith SpeedROD RP-18e reversed phase chromatographic column and quantification by mass spectrometry in the multiple reaction monitoring mode (MRM). The precursor to product ion transitions of *m/z* 535.3 → 288.2 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 1.0–250.2 ng/mL for piperaquine in plasma. The limit of detection (LOD) and lower limit of quantification (LLOQ) in plasma were 0.2 and 1.0 ng/mL, respectively. Acceptable precision and accuracy (±20% deviation for LLOQ standard and ±15% deviation for other standards from the respective nominal concentration) were obtained for concentrations over the standard curve ranges. A run time of 2.5 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 applied to analyze human plasma samples from phase-1 clinical studies. The mean pharmacokinetic parameters of piperaquine following 1000 mg oral dose: observed maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}) and elimination half-life ($T_{1/2}$) were 46.1 ng/mL, 3.8 h and 13 days, respectively.

Keywords: Antimalarial: Piperaquine; Liquid chromatography/tandem mass spectrometry; Human plasma

1. Introduction

Piperaquine, 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane (Fig. 1A), is a bisquinoline antimalarial compound belonging to the 4-aminoquinoline group. It is currently being used as partner drug in artemisinine based combination therapy [1–5].

Very few methods have been reported for the quantification of piperaquine in biological fluids. These methods are based on high-performance liquid chromatography (HPLC) [6–11]. Malm et al. reported an automated solid-phase extraction (SPE) method for the determination of piperaquine in capillary blood using HPLC with UV detection at 345 nm [9]. Recently, Lindegardh et al. reported an HPLC-UV method for the quantification of piperaquine in plasma using solid phase extraction (SPE) [10]. This sample processing technique involves expensive, time consuming and tedious steps compared to protein precipitation. The major limitations of protein precipitation are sample dilution, lower sensitivity and ion suppression [12] as well as frequent clean up of injection port and mass spectrometer (MS) detector. So far there is no report on application of LC–MS/MS in quantification of piperaquine in plasma and the lowest limit of quantification of most sensitive HPLC methods reported till date is 2.5 ng/mL with 250 μL plasma sample.

Quantification of drugs in biological matrices by LC–MS/MS is a method of choice, owing to the improved sensitivity and selectivity of this technique [13]. In the method described here

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doi:10.1016/j.jchromb.2007.09.021

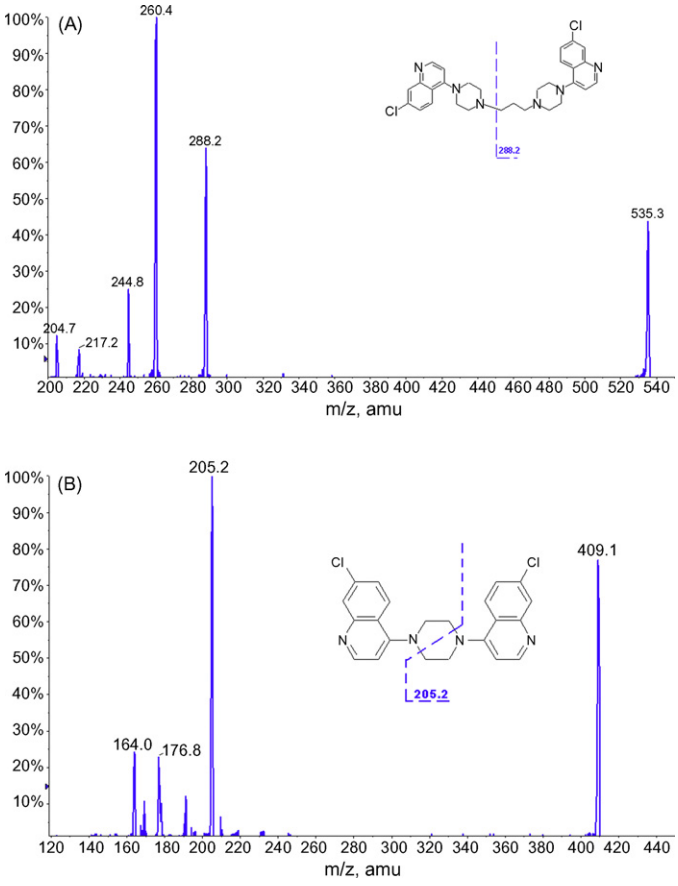

Fig. 1. (A) Product ion mass spectra of piperaquine in positive ionization mode. (B) Product ion mass spectra of IS in positive ionization mode.

for analysis of piperaquine, the plasma sample was precipitated with methanol and the supernatant was directly injected onto the LC–MS/MS system for quantification.

The present work utilizes high selectivity and sensitivity of triple quadrupole MS system with an electrospray interface for the development and validation of a robust LC–MS/MS method in MRM mode for the quantification of piperaquine in human plasma. It was essential to establish an assay capable of quantifying piperaquine at concentrations down to 1.0 ng/mL [14]. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained from clinical studies of piperaquine. The advantages of this new method presented in this paper in comparison to earlier methods are: (1) less plasma volume; 50 μL, allowing inclusion of additional points and pediatric population, (2) higher sensitivity; sensitivity of 1.0 ng/mL was achieved, (3) shorter run time and higher through put (up to 400 samples per day). The sensitivity could be further improved by sample concentration and increasing the injection volume. The method has been validated according to published FDA guidelines [15].

2. Experimental

2.1. Chemicals

Piperaquine (CAS No. 4085-31-8) and piperazine bis chloroquinoline, used as IS, were obtained from NCE Scale up department, R&D, Ranbaxy (Gurgaon, India). Chemical structures are presented in Fig. 1A and B. HPLC-grade methanol was purchased from SD Fine Chem. Ltd. (Mumbai, India). Formic acid and ammonium acetate were purchased from Fluka (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-Elmer Instruments LLC, Shelton, CT, USA) consisted of a quaternary pump, a degasser and an autosampler equipped with a thermostatted column compartment. The compounds were analyzed on a Chromolith SpeedROD RP-18e (Merck KGaA, Germany) reversed phase chromatographic column (50 mm × 4.6 mm i.d.). The column was maintained at ambient temperature (25 ± 2 °C). The mobile phase was a pre-mixed mixture of 10 mM ammonium acetate buffer, methanol, formic acid and ammonia solution (25:75:0.2:0.15, v/v) pumped at a flow rate of 0.8 mL/min. Mass detection was performed on an API 4000 Q-Trap 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).

2.3. Sample preparation

Sample preparation involved a simple protein precipitation with methanol. Aliquots (50 μL) of plasma were subjected for precipitation with addition of 300 μL of working solution of IS (prepared in methanol). The samples were vortex mixed for 20 s and centrifuged (Eppendorf 5810 R, Eppendorf AG, Germany) at 10,640 g for 10 min. The upper layer (200 μL) was transferred into injector vials and a 10 μL aliquot was injected into chromatographic system.

Table 1
Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature (°C)	400
Dwell time per transition (ms)	200
Ion source gas (gas 1) (psi)	60
Ion source gas (gas 2) (psi)	40
Curtain gas (psi)	20
Collision gas (psi)	6
Ion spray voltage (V)	5500
Entrance potential (V)	10
Declustering potential (V)	130
Collision energy (V)	45 (analyte) and 47 (IS)
Mode of analysis	Positive
Collision cell exit potential (V)	15
Ion transition for piperaquine (*m/z*)	535.3/288.2
Ion transition for bisquinoline (*m/z*)	409.1/205.2

2.4. Bioanalytical method validation

Standard stock solutions of piperaquine (1 mg/mL) and the IS (1 mg/mL) were separately prepared in 10 mL polypropylene 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 (0.1% formic acid/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 human plasma to obtain piperaquine concentration levels of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.1 and 250.2 ng/mL. Quality control (QC) samples were prepared as a bulk based on an independent weighing of standard drug, at concentrations of 1.0 ng/mL (LLOQ), 3.0 ng/mL (low), 99.9 ng/mL (medium) and 199.8 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 sample (a plasma sample processed without the IS), a zero sample (a plasma sample processed with the IS) and eight non-zero samples covering the range 1.0–250.2 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 a calibration curve were, a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification. The within-batch precision and accuracy were determined by analyzing five sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing five sets of quality control samples in five different batches. The acceptance criteria for within and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and that for the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for other concentrations.

Recovery of piperaquine from the extraction procedure was determined by a comparison of the peak area of piperaquine in spiked plasma samples (five low, medium and high quality controls) with the peak area of piperaquine in aqueous samples prepared by with the same amounts of piperaquine 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 prepared by with the same amounts of IS at the step immediately prior to chromatography.

The stock solutions stabilities of piperaquine and IS were tested at room temperature and under refrigeration (~4 °C). The stock solutions of piperaquine and IS were diluted to 25 ng/mL with diluent (0.1% formic acid/methanol, 50/50, v/v) prior to analysis. The stability of piperaquine in human plasma after three freeze-thaw cycles was investigated by comparing quality control samples that have been frozen and thawed three times with freshly prepared quality control samples. Furthermore, the stability of piperaquine in human plasma under processing (ambient temperatures) and storage conditions (−50 °C) was evaluated. Finally, stability in the final extract for human plasma was determined in the autosampler.

3. Results and discussion

3.1. Method development

In order to develop a method with the desired LLOQ (1.0 ng/mL), it was necessary to use MS–MS detection, as this provides improved limit of detection (LOD). 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 piperaquine and the IS in positive ionization mode are shown in Fig. 1A and B, respectively. The predominant $[M]^+$ and $[M+H]^+$ ion for piperaquine and IS, respectively were used as the precursor ion to obtain the product ion spectra. The most sensitive mass transition was from m/z 535.3 to m/z 288.2 for piperaquine and m/z 409.1 to m/z 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 for the analyte and the IS. 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/formic acid/ammonia solution (25/75/0.2/0.15, v/v) 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 the good peak shape whereas formic acid was necessary to lower the pH to protonate piperaquine and enhance the resolution. The percentage of ammonia and formic acid was optimized to maintain this peak shape whilst being consistent with good ionization and fragmentation in the mass spectrometer. The development of the chromatographic system was focused on achieving shorter run time to assure high throughput, paying attention to matrix effects as well as good peak shapes. The high proportion of organic solvent in mobile phase [10 mM ammonium acetate/methanol (25/75, v/v)] eluted the analyte and the IS at retention times of 1.1 and 1.2 min, respectively. A flow-rate of 0.8 mL/min produced good peak shapes with in 2.5 min run time. A structural analogue of piperaquine, piperazine bis chloroquinoline (Fig. 1B) was used as internal standard.

3.2. Assay performance and validation

The calibration curve was linear over the concentration range 1.0–250.2 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 $y = 0.00460(\text{S.D. } \pm 0.00085)x + 0.00124(\text{S.D.}$

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

Concentration added (ng/mL)	Concentration found (ng/mL) (mean ± S.D.; $n = 5$)	Precision (%)	Accuracy (%)
1.0	1.0 ± 0.10	9.9	95.8
2.5	2.3 ± 0.12	5.0	92.9
5.0	4.5 ± 0.62	13.7	90.5
10.0	9.7 ± 0.76	7.8	97.3
25.0	25.2 ± 1.28	5.1	100.8
50.0	51.3 ± 2.57	5.0	102.5
100.1	104.9 ± 3.41	3.3	104.9
250.2	254.5 ± 12.40	4.9	101.7

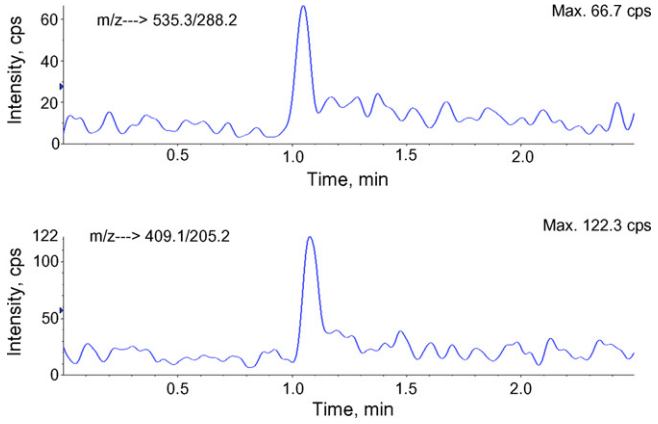

Fig. 2. MRM ion-chromatograms resulting from the analysis of blank (drug and internal standard free) human plasma for piperaquine and internal standard.

±0.00091) 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 piperaquine was above 0.996. Calibration curve results are summarized in Table 2.

The specificity of the method was examined by analyzing ($n = 6$) blank plasma extract (Fig. 2). No significant interference in the blank plasma traces was observed from endogenous substances in drug-free plasma at the retention time of the analyte. Fig. 3 depicts a representative MRM ion-chromatogram for the LLOQ (1.0 ng/mL). The selectivity of this method was

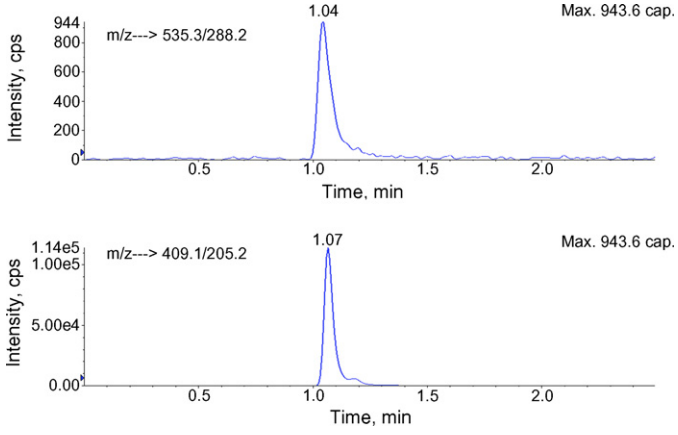

Fig. 3. Representative MRM ion-chromatograms resulting from the analysis of 1.0 ng/mL (LLOQ) of piperaquine spiked with the internal standard.

investigated by spiking other antimalarials (i.e. chloroquine, artemether, lumefantrine) and metabolites of piperaquine (cleavage product, oxidation product and dioxidation product) in quality control samples. Neither interference nor ion suppression was observed in presence of other antimalarial compounds and metabolites. Within precision and accuracy ranged from 2.90% to 5.24% and 96.15% to 99.73%, respectively. The extraction recoveries of piperaquine and IS were 93.4 ± 10.2% and 92.11 ± 9.0%, respectively in human plasma and the dependence on concentration is negligible. Recoveries of the analyte and IS were high and with the consistency in the recovery of piperaquine, the assay has proved to be robust in high-throughput bioanalysis.

The matrix effect was determined by spiking low (3.0 ng/mL) and high (199.8 ng/mL) quality control samples in duplicate from each of the six different batches of human plasma. The within-batch precision and accuracy ranged from 2.28% to 2.41% and 100.43% to 112.01%, respectively hence the matrix effect on estimation of piperaquine is negligible.

The LOD demonstrated that the analyte gave a signal-to-noise ratio (S/N) of ≥3 at a concentration of 0.2 ng/mL. The LLOQ, the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision was found to be 1.0 ng/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit (1.0 ng/mL) was ∼25-fold greater than the mean response for the peak in six blank human plasma samples at the retention time of the analyte. Excellent sensitivity was observed for a 10 μL injection volume; the LLOQ corresponds to 1.4 pg on-column. The between-batch precision at the LLOQ was 12.0% and the between-batch accuracy was 97.4% (Table 3). The within-batch precision was 10.9% and the accuracy was 93.1% for piperaquine.

The lower and upper quality control levels of piperaquine were 3.0 and 199.8 ng/mL in human plasma. For the between-batch experiments, the precision ranged from 7.5% to 12.0% and the accuracy ranged from 96.1% to 101.6% (Table 3). For the within-batch experiments, the precision and accuracy met the acceptance criterion (±15%). The upper concentration limits can be extended with acceptable precision and accuracy by a 10-fold dilution with control plasma. For the within batch dilution integrity experiment, the precision and accuracy at two concentration levels 99.9 ng/mL and 199.8 ng/mL ($n = 5$) ranged from 3.00% to 7.56% and 98.86% to 108.70%, respectively. These results suggest that sample with concentrations greater than 10-

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

Concentration added (ng/mL)	Within-batch precision ($n=5$)			Between-batch precision ($n=3$)		
	Concentration found (ng/mL) (mean ± S.D.)	Precision (%)	Accuracy (%)	Concentration found (ng/mL) (mean ± S.D.)	Precision (%)	Accuracy (%)
1.0	1.0 ± 0.11	10.9	93.1	1.0 ± 0.12	12.0	97.4
3.0	2.8 ± 0.3	10.4	94.1	3.0 ± 0.34	11.4	101.6
99.9	100.5 ± 2.1	2.1	100.6	100.0 ± 7.5	7.5	100.0
199.8	194.5 ± 30.5	5.8	97.4	192.1 ± 15.7	8.2	96.1

Table 4
Stability of piperaquine in human plasma

Sample concentration (ng/mL) ($n=5$)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability for 21 h			
3.0	3.2 ± 0.15	4.8	108.6
199.8	217.2 ± 14.5	6.7	108.7
Autosampler stability for 24 h			
3.0	3.2 ± 0.28	8.6	107.9
199.8	189.5 ± 14.2	14.8	94.8
Stability for 64 days at ≤50 °C			
3.0	3.3 ± 0.46	14.1	110.3
199.8	213.2 ± 11.5	5.4	106.7
Three freeze-thaw cycles			
3.0	3.1 ± 0.1	2.4	104.5
199.8	196.9 ± 2.9	1.5	98.6

fold of the upper limit of the calibration curve can be assayed to obtain acceptable data.

3.3. Stability studies

Knowing that piperaquine is highly unstable in glass, only plastic ware (polypropylene material) was used during sample processing from solution preparation to reconstitution. The stability of the analyte and IS in human 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 (3.0 and 199.8 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 21 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 24 h was also assessed. The results indicated that processed plasma sample of piperaquine and IS can remain in the autosampler for at least 24 h, without showing significant loss in the quantified values (Table 4). The data representing the stability of piperaquine in human plasma at two QC levels over three freeze-thaw cycles are given in Table 4. These tests indicated that the analyte is stable in human 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 piperaquine in plasma samples stored for a period of 64 days at below −50 °C. The stability study of piperaquine in human plasma showed reliable stability behavior, as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicated that storage of piperaquine in human plasma samples at below −50 °C was adequate, and no stability related problems would be expected during routine analysis for clinical trial samples. The stability of stock solutions was tested and established at room temperature for 6 h and under refrigeration (∼4 °C) for 62 days. The recoveries for piperaquine and IS were 98.1 (CV 2.5%), 101.9 (CV 2.5%) and 106.5 (CV 1.6%), 91.1 (CV 2.2%), respec-

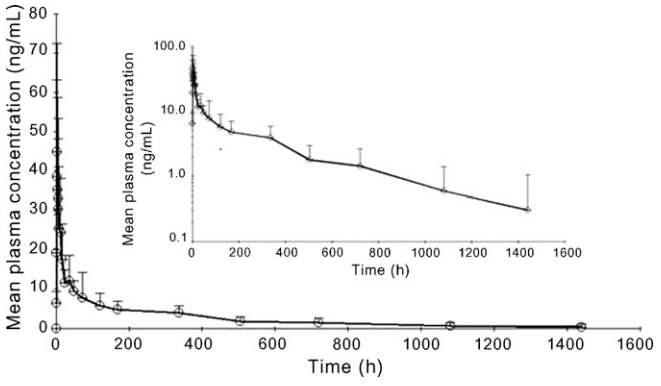

Fig. 4. Mean plasma concentration profile of piperaquine following 1000 mg of piperaquine phosphate oral administration to healthy human volunteers ($n=6$).

tively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

3.4. Application

The validated method was successfully used to quantify piperaquine concentrations in the human plasma samples during phase-1 clinical trials. A representative mean plasma concentration profile of piperaquine following 1000 mg of piperaquine phosphate oral administration to healthy human volunteers ($n = 6$) is presented in Fig. 4.

4. Conclusions

In summary, LC–MS/MS method for the quantification of piperaquine in human plasma was developed and fully validated as per FDA guidelines [15]. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (2.5 min) and lower sample requirements. Thus the volume of samples is reduced significantly, allowing inclusion of additional points. With dilution integrity up to 10-fold, we have established that the upper limit of quantification is extendable up to 2502.0 ng/mL.

Piperaquine was shown to be stable in routine analysis conditions and in plasma for up to 64 days when stored at below −50 °C. The simplicity, protein precipitation and sample turnover rate of 2.5 min per sample, make it an attractive procedure in high-throughput bioanalysis of piperaquine. The validated method allows quantification of piperaquine in the 1.0–250.2 ng/mL range.

Acknowledgements

Authors wish to acknowledge Medicines for Malaria Venture (MMV) as cosponsor and providing financial support for this project and NCE Scale up department, R&D, Ranbaxy (Gurgaon, India) for providing working standards of piperaquine phosphate and piperazine bis chloroquinoline (IS).

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