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Short communication

Development and validation of a sensitive and selective UHPLC–MS/MS method for quantitation of an investigational anti-malarial compound, 2-*tert*-butylprimaquine (NP-96) in rat plasma, and its application in a preclinical pharmacokinetic study

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ABSTRACT

An ultra-high performance liquid chromatographic tandem mass spectroscopy (UHPLC–MS/MS) method was developed and validated for the quantification of an investigational anti-malarial entity, 2-*tert*-butylprimaquine (NP-96), in rat plasma. Simple protein precipitation by acetonitrile was used for the sample preparation. Effective separation of NP-96, internal standard (IS) and matrix components were achieved on an UHPLC column (Hypersil Gold C18, 50 mm × 2.1 mm, 1.9 μm) using a mobile phase composed of acetonitrile and 20 mM ammonium acetate, which was pumped in a gradient mode at a flow rate of 450 μl/min. Selective reaction monitoring (SRM) was utilized for quantitation of the molecules. To increase sensitivity of the method, two ions of *m*/*z* 299 and *m*/*z* 231 were selected for NP-96, while IS was monitored for an ion of *m*/*z* 489. The method was validated according to FDA guideline on bioanalytical method validation and showed good compliance. The intra-day and inter-day precision expressed as R.S.D. was lower than 15% at all the tested quality control levels, including upper and lower limits of quantification. The calibration range was 2.5–500 ng/ml. Total runtime for the method was 5 min, which was suitable to produce high-throughput results for pharmacokinetic evaluation.

1. Introduction

Malaria, which is one of the most serious and widespread parasitic diseases encountered by mankind, is caused by protozoa of the genus *Plasmodium* [1,2]. The attributes like prevalence, virulence, and drug resistance make this disease even more dreadful [3]. Some of the important drugs that are active against the blood forms of the parasite and thereby terminate clinical attacks of malaria include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracyclines, and artemisinin. Primaquine and, to a lesser extent, pyrimethamine are known for their action against the primary tissue forms of the *Plasmodium*. In fact, primaquine is the only prototype anti-relapse anti-malarial drug available, which acts on hypnozoites of *Plasmodium vivax* and *Plasmodium ovale* in the liver. Among other primaquine derivatives, tafenoquine is a newer molecule that is considered as a potential anti-relapse drug in malaria, based on its early preclinical studies [4]. Similarly, a newly synthesized compound, 2-*tert*-butyl primaquine (NP-96), which is yet in early preclinical studies, also showed better *in vitro* and *in vivo* anti-malarial efficacy [5–7]. It proved to be a potent blood schizonticidal and was found to act by inhibiting heme crystallization [8]. The major benefit of this compound is that it is devoid of toxicity associated with primaquine metabolism [5]. The same is attributed to the presence of a bulky *tert*-butyl group on the second carbon of 8-aminoquinoline ring of primaquine (Fig. 1).

There is no report yet on the bioanalytical method for NP-96. Hence, the present study was undertaken as a part of preclinical evaluation of this new promising anti-malarial candidate using a sensitive ultra-high performance liquid chromatographic tandem mass spectroscopy (UHPLC–MS/MS) method. The validation was done as per FDA guideline on bioanalytical method validation [9], and it was employed to analyze pharmacokinetic (PK) profile of the NP-96 in rats at two different doses, *viz.* 5 mg/kg and 10 mg/kg.

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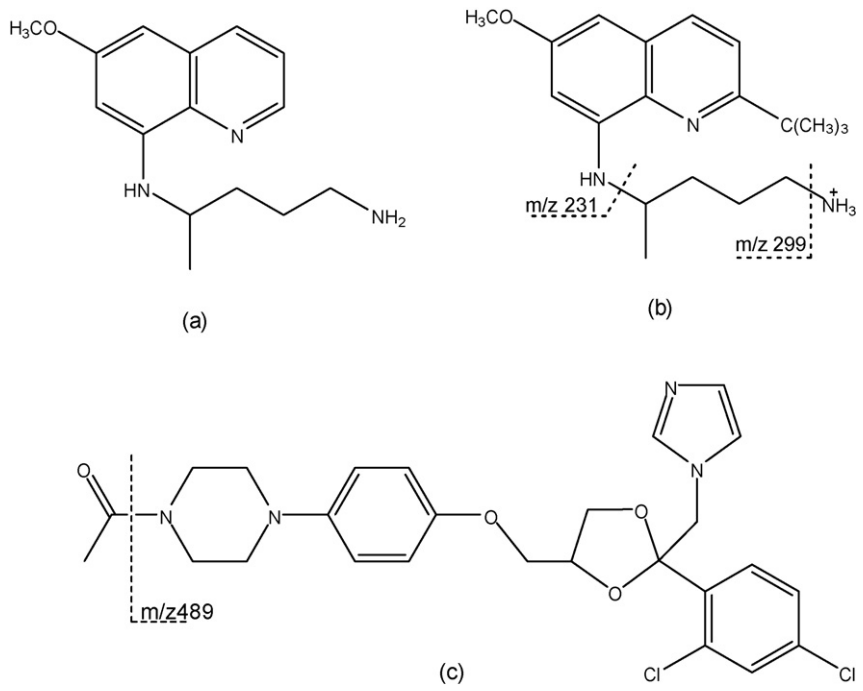

Fig. 1. Structures of primaquine (a), NP-96 (b), and IS (c). Dotted lines show prominent sites of fragmentation of NP-96 and IS in ESI positive mode.

2. Experimental

2.1. Materials and chemicals

NP-96 was synthesized by the Department of Medicinal Chemistry, NIPER (S.A.S. Nagar, India) as a dihydrochloride salt to a purity of 97.8%, as determined by HPLC–UV peak area normalization method. The internal standard (IS), ketoconazole, was purchased from Himedia Laboratories (Mumbai, India). Acetonitrile (ACN) of HPLC grade was purchased from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical-reagent grade. Water obtained from ELGA water purification unit (Elga Ltd., Bucks, England) was utilized to prepare all the solutions.

2.2. Animals

The PK study was performed on adult male Wistar rats (230–280 g) obtained from the central animal facility of the institute. The rats were housed under controlled environmental conditions (temperature, $23 \pm 1\,^{\circ}C$; humidity, $55 \pm 5\%$ and 12 h light/dark cycle) with normal diet and water freely available. Animal experiments were carried out according to institutional guidelines for the care and the use of laboratory animals, and the protocol was approved by the Institutional Animal Ethics Committee (Protocol No. IAEC/08/27). Before commencing the PK study, the animals were kept on fasting for 6 h. After 15 min of dosing, food and water were supplied freely throughout the study.

2.3. UHPLC–MS/MS analyses

The UHPLC–MS/MS analyses were performed on Accela™ (Thermo Electron Corporation, San Jose, USA) ultra-high performance liquid chromatography (UHPLC) system having a quaternary pump and temperature-controlled autosampler connected with LTQ XL™ linear ion trap mass spectrometer (Thermo Electron Corp, San Jose, USA) via an electrospray (ESI) interface. Instrument control and data collection were handled by Xcalibur (version 2.0.7 SP1) and LCquan software (version 2.5.6, Thermo Fisher Scientific Inc., San Jose, USA). The chromatographic separation was carried out on a Hypersil Gold column C18 (50 mm × 2.1 mm, 1.9 μm). Ketoconazole was selected as IS based on its similar polarity to that of NP-96. A gradient LC method was developed to separate drug and IS from the matrix components, and to avoid ion suppression [10] by the latter during quantitation in the mass spectrometer. The mobile phase consisted of 20 mM ammonium acetate buffer (pH 4.8) and the flow rate was 450 μl/min. The advantage of gradient was further potentiated by the use of a divert valve, which was programmed to divert the eluent with matrix from column to waste, while the portion with analytes was subjected to ionization source. This protected mass source from any contamination and interference during ionization (Table 1).

MS/MS data for the drug and IS were collected in positive ESI mode at optimized capillary voltages of 25 eV and 30 eV, respectively. Both standard solutions and plasma samples were analyzed in selective reaction monitoring (SRM) mode by scanning the abundant fragments of NP-96 and IS. Different time programs used in UHPLC–MS/MS method are shown in Table 1.

Table 1
Different time programs used in UHPLC–MS/MS method.

Time program	Time (min)	Programs
Gradient (% ACN:buffer)	0.0–0.5	30:70
	0.5–1.7	30:70 → 65:35
	1.7–2.5	65:35
	2.5–3.0	65:35 → 30:70
	3.0–5.0	30:70
Divert flow	0.0–1.4	Waste
	1.4–4.1	Ionization source
	4.1–5.0	Waste
MS/MS scan segment	0.00–2.70	For IS (m/z 531 → 489)
	2.70–5.00	For NP-96 (m/z 316 → 299 and 231)

2.4. Preparation of stock and working solutions for calibration curve

The stock solution of NP-96 was prepared by dissolving 1.7 mg of NP-96 dihydrochloride (corresponding to 1.38 mg/ml of free base) in 1 ml of diluent (ACN:water, 2:1). Using stock solution of NP-96, serial dilutions were made to prepare primary aliquots for calibration curve (CC) and quality control (QC) samples. Similarly, stock solution of IS was also prepared to a concentration of 1.0 mg/ml in ACN, which was taken in sufficient quantity and diluted up to 100 ml with the same diluent to prepare a working solution of 20 ng/ml.

2.5. Preparation of calibration curve (CC) and quality control (QC) samples

10 μl of each primary aliquot was spiked in 190 μl of blank plasma to yield CC samples ranging from 2.5 ng/ml to 500 ng/ml. Similarly, QC samples at three concentration levels (7.82 ng/ml, 208.52 ng/ml and 417.16 ng/ml) were prepared as low, middle and high QC samples (LQC, MQC and HQC), respectively. Blank sample (plasma sample processed without IS) and zero standard (plasma sample processed only with IS) were also prepared and analyzed. All solutions were stored at 4 °C during experimentation.

2.6. Sample preparation

Aliquots of 200 μl sample were placed in microcentrifuge tubes for processing and 50 μl of dilute ammonia was added to convert the drug salt to free base. The mixtures were thoroughly vortexed and then 600 μl of diluted IS solution was added. The processed samples were again vortexed and then centrifuged at 9000 rpm for 15 min to settle down the precipitate. 100 μl of supernatant layer was taken in UHPLC vials. Injection volume was 5 μl.

2.7. Method validation

2.7.1. Selectivity and specificity

For selectivity and specificity, six different batches of drug-free rat plasma (without IS and analyte) were analyzed for the exclusion of any endogenous co-eluting interference at the peak region of both analyte and IS.

2.7.2. Linearity and sensitivity

The calibration standards (2.5–500 ng/ml) were assayed in triplicate to demonstrate linearity of the method. The lower limit of quantification (LLOQ) was determined as the lowest concentration of sample spiked with NP-96, where signal-to-noise (S/N) ratio was ≥10 and both precision and accuracy were <20%.

2.7.3. Precision and accuracy

The precision and accuracy of the method were assessed by performing replicate analyses of samples at three concentration levels of QC and at LLOQ. The inter-day ($n = 5$) and intra-day ($n = 3$) precision were determined at three levels of QC samples and expressed as relative standard deviation (R.S.D.). The accuracy was calculated as percent difference in the observed and nominal concentrations of QC samples.

2.7.4. Recovery and matrix effect

For the determination of matrix effect and recovery, first blank plasma was spiked with known concentration of NP-96 at three QC levels, and analyzed using the developed method. Then, neat solutions of NP-96 of same concentrations were prepared in the diluent, and analyzed. The peak areas of the extracted samples and neat solutions were obtained, and recovery was calculated using the following equation:

$$\%\,\text{recovery} = \frac{\text{peak area of extracted sample}}{\text{peak area of neat solution}} \times 100$$

Moreover, the matrix effect was determined ($n = 6$) by comparing response of neat solutions and processed samples at LLOQ level.

2.7.5. Stability

The stability of NP-96 in plasma was determined under different storage and handling conditions. It was evaluated at two QC levels, *i.e.*, LQC and HQC. Short-term temperature stability, *i.e.*, bench-top stability, was assessed by analyzing samples kept at ambient temperature for 6 h, while stability of samples in autosampler was conducted by re-analyzing processed samples kept in the autosampler conditions at 4 °C for 24 h. The long-term stability was checked by keeping spiked plasma samples at −20 °C freezer conditions for 15 days. And finally for freeze–thaw stability, samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for over 24 h under the same conditions and again thawed unassisted at room temperature. The freeze–thaw cycle was repeated three times, and samples were analyzed after the third cycle. The obtained results were compared with the nominal concentrations of the analyte and it was considered stable if the calculated concentration was less than the nominal concentration by 15% [9].

2.7.6. Application to pharmacokinetic study

Six rats were randomly divided into two equal groups of three each. Group 1 received oral administration of NP-96 at a dose of 5 mg/kg, while group 2 was given dose of 10 mg/kg through oral gavage. Serial blood samples were collected before dosing, and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h after drug administration. Blood samples were centrifuged at 9000 rpm at 4 °C for 15 min, and the plasma was collected and stored at −20 °C until analysis.

3. Results and discussion

3.1. UHPLC–MS/MS method development

The objectives for bioanalytical method development in this study were: (i) high-throughputness using simple sample preparation and UHPLC approaches, (ii) good sensitivity up to 5 ng/ml or better, (iii) good selectivity and specificity, (iv) no or minimal ion suppression, and (v) reduced mass source contamination by complex biomatrix.

For the same, first mass fragmentation scan of NP-96 was recorded (Fig. 2a) and based on the ion map (Fig. 2b), fragments of high intensities were selected for ion monitoring. The structural formulae of analyte and IS with prominent sites of fragmentation are shown in Fig. 1. For selectivity and specificity of the method, detection of eluted peaks was done in SRM mode, wherein two most abundant fragmentation ions of NP-96, *i.e.*, m/z 299 and m/z 231, were selected for sensitive quantitation of the drug, while IS response was monitored using single most abundant fragment of m/z 489.

Representative chromatograms of blank plasma and real plasma samples are shown in Fig. 3. UHPLC–MS/MS analysis of blank and spiked plasma samples showed no matrix peak interference during quantification of both the analyte and IS. As shown in Fig. 3(a′) and (b′), IS and NP-96 were well separated from sample matrix in the chromatography procedure with retention times of 2.2 min and 3.1 min, respectively. Acidic mobile phase improved peak shape of analytes and their ionization efficiency.

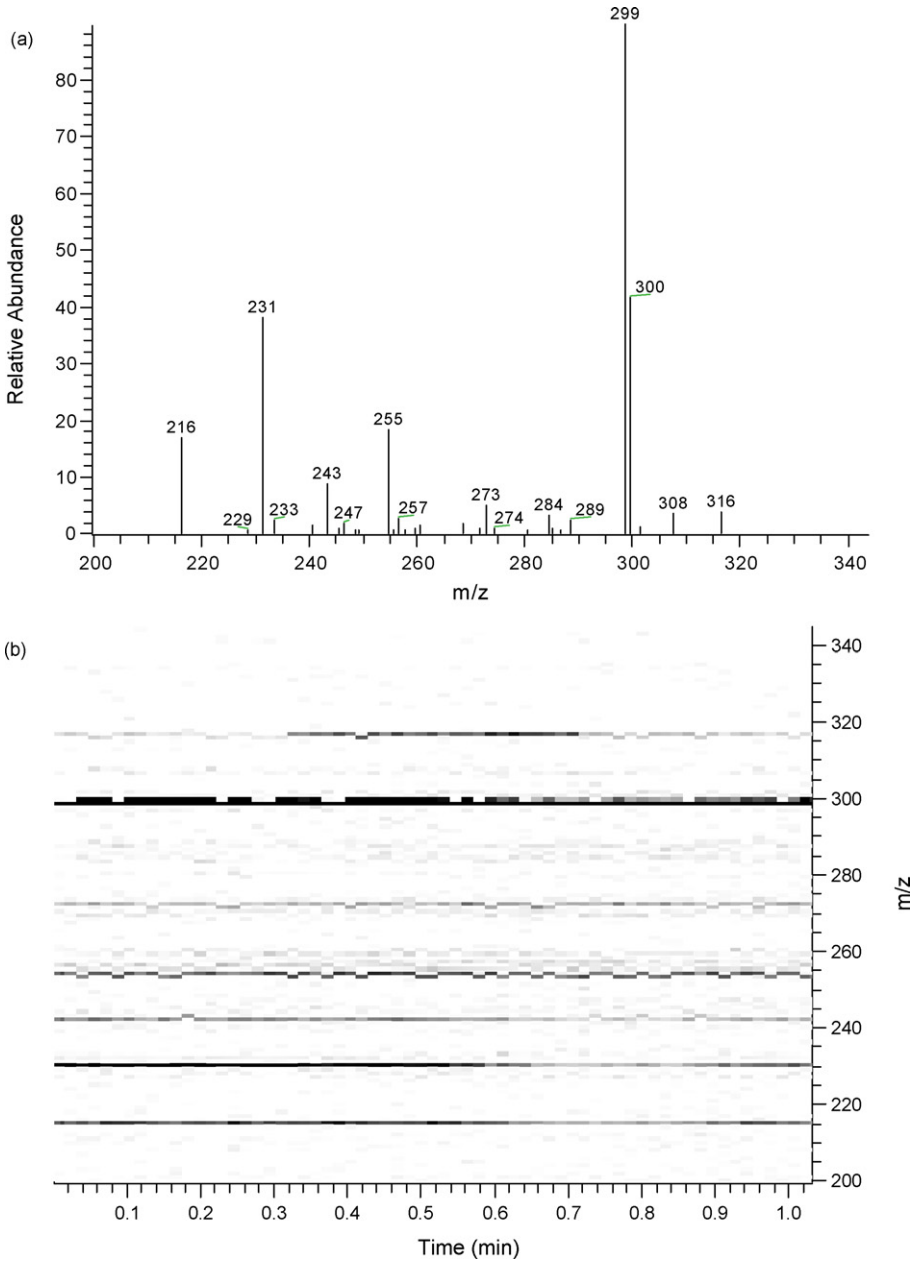

Fig. 2. Mass spectra of NP-96 (a) and its ion map from *m*/*z* 200–350 (b).

3.2. Method validation

3.2.1. Linearity and LLOQ

The calibration data were analyzed by linear regression. The linear equation was $y = 0.079x - 0.083$ ($r^2 = 0.9995$). Each CC standard was back calculated with the help of calibration equation, and all the non-zero samples showed <15% deviation. The LLOQ of NP-96 was 2.5 ng/ml.

3.2.2. Accuracy and precision

The upper limit of intra-day and inter-day precision was ≤15%, except at LLOQ, where it was fixed at ≤20%, as per the FDA guideline

Table 2
Precision and accuracy data of quality control samples of NP-96 in rat plasma.

Nominal concentration (ng/ml)	Intra-day precision ($n = 5$)			Inter-day precision ($n = 3$)		
	Concentration found (ng/ml) (mean ± S.D.)	Precision (%)	Accuracy (%)	Concentration found (ng/ml) (mean ± S.D.)	Precision (%)	Accuracy (%)
2.58 (LLOQ)	2.97 ± 0.03	10.97	115.13	2.92 ± 0.03	15.09	113.24
7.82 (LQC)	8.45 ± 0.04	6.51	108.06	7.37 ± 0.01	2.17	94.22
208.58 (MQC)	219.68 ± 1.47	8.51	105.32	201.40 ± 1.05	6.60	96.56
417.16 (HQC)	407.91 ± 2.81	8.72	97.782	408.58 ± 2.90	8.98	97.94

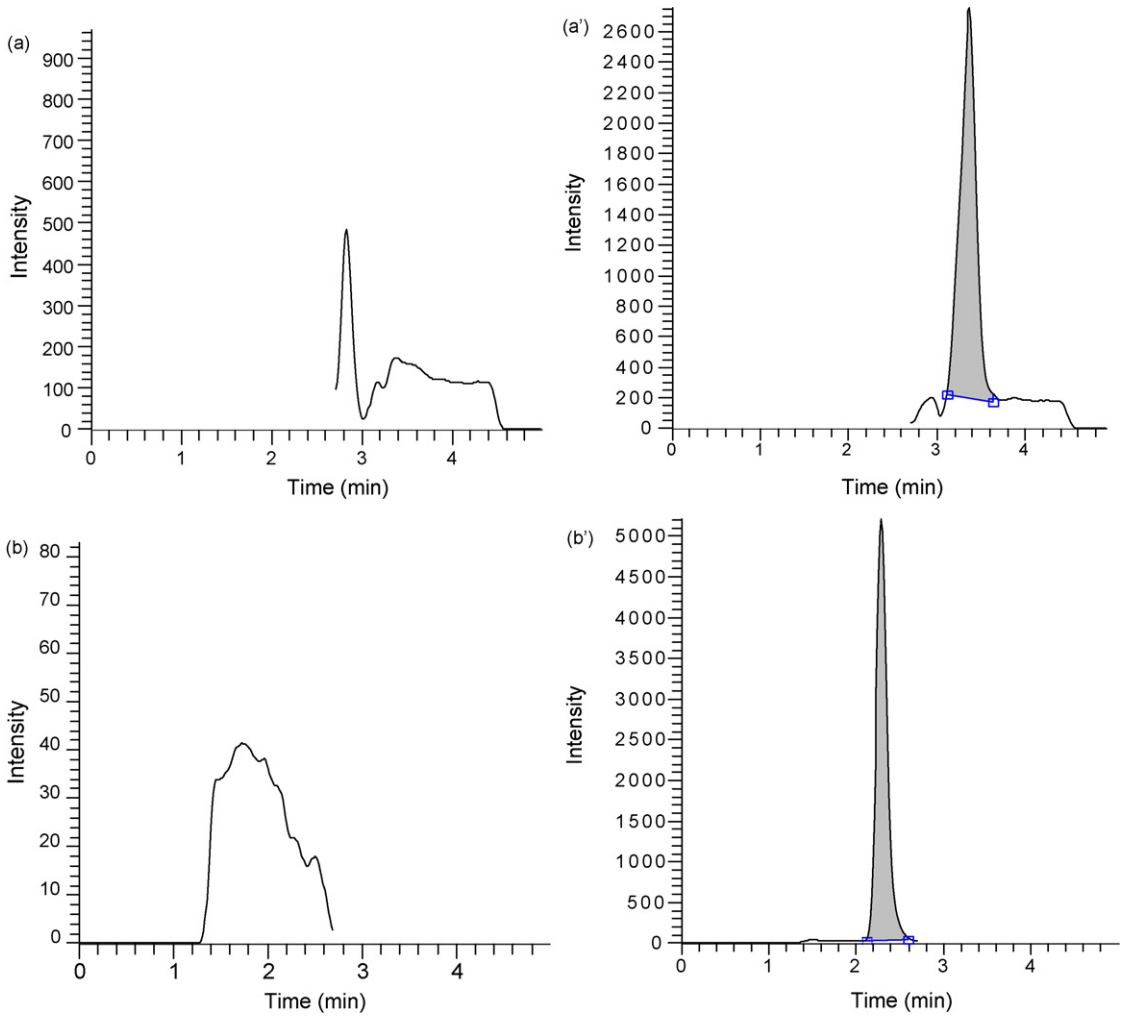

Fig. 3. Representative chromatograms of blank plasma in scan segment of NP-96 (a), NP-96 at LLOQ level (a′), blank plasma in scan segment of IS (b), and IS in a pharmacokinetic sample (b′).

[9]. The results of the accuracy and precision at the four concentrations, *i.e.*, LLOQ and three QC levels, are presented in Table 2. The results showed that accuracy and precision of the quantification method were within acceptable limits.

3.2.3. Recovery and matrix effect

The experimental recovery was found to be more than 90%. Additionally, the results also indicated that there was no matrix effect, *i.e.*, interference on the quantitation of analytes from the plasma matrix, even at LLOQ (Fig. 3).

3.2.4. Stability

On storage at ambient temperature (25 °C) for 6 h, the concentrations of analytes in plasma deviated less than ±15% from their nominal concentrations, showing that the samples were stable during preparation and analytical processes. On storage in the autosampler at 4 °C for 24 h, the analytes showed good stability, wherein, also the concentration varied no more than ±15% of their nominal concentrations. In freeze–thaw stability and long-term stability experiments even, the concentrations obtained were within 15% of their nominal concentrations.

3.3. Pharmacokinetic study

The method was successfully employed for the determination of plasma concentrations of NP-96 in a PK study. The mean plasma concentration time profiles of NP-96 are shown in Fig. 4 for the two doses of 5 mg/kg and 10 mg/kg. From the PK profile, C_{max} and T_{max} values for 5 mg/kg dose were found to be 260 ± 30 ng/ml and 2.5 h, respectively, while at 10 mg/kg dose, the values were 310 ± 20 ng/ml and 2.5 h, respectively. The drug was not found to show significant

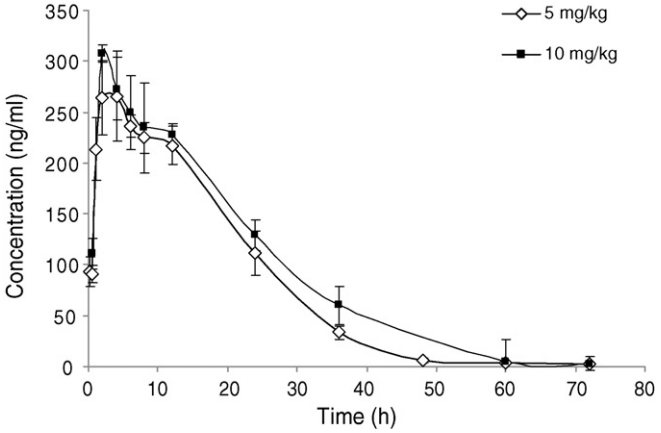

Fig. 4. Mean plasma concentration profiles of NP-96 in rats ($n = 3$) at doses of 5 mg/kg, and 10 mg/kg.

differences in PK parameters, reflecting saturation at the tested doses. The same may be attributed to poor solubility of HCl salts at the intestinal pH [11].

4. Conclusion

A simple, rapid, sensitive, selective and specific UHPLC–MS/MS method involving protein precipitation using ACN was developed and validated for the quantification of NP-96 in rat plasma. The use of shorter UHPLC column with small bore/particle size led to an efficient separation, better resolution, decreased run time and high-throughput. At the same time, the gradient mode helped in elution of matrix components early during the chromatographic run, while retaining acceptable separation of drug and IS. This approach helped in achieving better R.S.D. and less ion suppression normally seen during co-elution of the peaks. The method involved use of SRM mode, wherein simultaneous analysis of two fragments of NP-96 helped in better sensitivity, selectivity and specificity for accurate quantification. The C_{max} and T_{max} values at 5 mg/kg dose were found to be 260 ± 30 ng/ml and 2.5 h, respectively, while the values at the dose of 10 mg/kg were 310 ± 20 ng/ml and 2.5 h, respectively.

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