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HPLC/MS/MS analysis of 3-carbamyl-4-methylpyrrole analog MNP001, a highly potent antihypertensive agent, in rat plasma

Binfeng Xia[a], Ting Wang[b], Laura M. Fox[a], Desuo Wang[a,*]

[a] Department of Basic Pharmaceutical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, SC 29208, USA
[b] Department of Medicine, University of Chicago, Chicago, IL 60637, USA

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ABSTRACT

Chemically synthesized 3-carbamyl-4-methylpyrroles were characterized as a group of antihypertensive agents with dual-targeting mechanism to simultaneously inhibit type 4 phosphodiesterase (PDE4) and L-type calcium channels. A 5-butyl analog of the pyrrole family, MNP001, was found to have high potency in reducing animal blood pressure and heart rate. A method for measuring MNP001 using high performance liquid chromatography combined with tandem mass spectrometry (HPLC/MS/MS) was developed. The calibration curve for MNP001 showed good linearity with the value of correlation coefficient greater than 0.987 over the range of 0.25–500 ng/mL. The results for inter-day and intra-day precision as well as accuracy were acceptable according to the criteria established by FDA. The lower limit of quantification was 0.25 ng/mL. This method was quick, sensitive and sufficient for *in vivo* pharmacokinetic and pharmacodynamic studies on this novel antihypertensive pyrrole compound.

1. Introduction

Hypertension (high blood pressure, BP) affects more than 70 million Americans and is responsible for significant cerebro- and cardio-vascular morbidity and mortality, which impose an enormous economic burden to the health care system in the United States [1,2]. Currently, six categories of drugs, including diuretics, adrenergic agents (β- and α1-antagonists or α2-angonists), ACE inhibitors, angiotensin II antagonists, calcium channel antagonists, and direct vasodilators, are prescribed in antihypertensive therapy. However, only one third (<35%) of hypertensive patients are managed to have their BP lowered to target levels despite the emphasis from health care communities on the effectiveness and importance of controlling BP in reducing stroke and cardiovascular diseases [2]. The poor control rate is largely due to patients' unawareness and ignorance for treatment [2], nevertheless, substantial number of patients failed to lower their BP to optimal levels, even after vigorously modifying their therapy by maximizing the doses, switching between regimens, or adding a third antihypertensive drug to their prescription. The unsatisfactory control of high blood pressure is associated with deficiencies of the antihypertensive agents available, which includes poor ethnic-related pharmacological responses [3], co-existing medical conditions, and severe side effects. Therefore, pharmaceutical scientists continue to investigate new classes of antihypertensive agents with new mechanisms of action.

Two 3-carbamyl-4-methylpyrrole analogs, MS23 [4] and MSP001 [5], have been reported as a class of unique agents with dual-targeting mechanism of type 4 phosphodiesterase (PDE 4) inhibition and L-type calcium channel antagonism [6]. Animal study showed that a newly synthesized 5-butyl pyrrole analog, MNP001, has significantly improved antihypertensive potency in comparison with the previously reported pyrrole compounds. It was found that the marginal blood pressure lowering action of MNP001 (0.5 μg/kg body weight) in rats was ~80 times more potent than MSP001 (40 μg/kg body weight) [5]. Therefore, it was expected that the minimum effective concentration of MNP001 in plasma would be much lower than that of MS23 and MSP001. Indeed, a standard HPLC/UV-based quantification method was not sufficient to assess the low plasma levels of MNP001 due to sensitivity limitations. In order to determine the concentration of MNP001 in rat plasma for future pharmacodynamic and pharmacokinetic evaluation, a more sensitive bio-analytical method must be developed. This study is to validate a high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) method developed for measuring pharmacologically relevant concentrations of MNP001 in biological fluids.

* Corresponding author. Tel.: +1 803 777 7101; fax: +1 803 777 8356.
E-mail address: wang@sccp.sc.edu (D. Wang).

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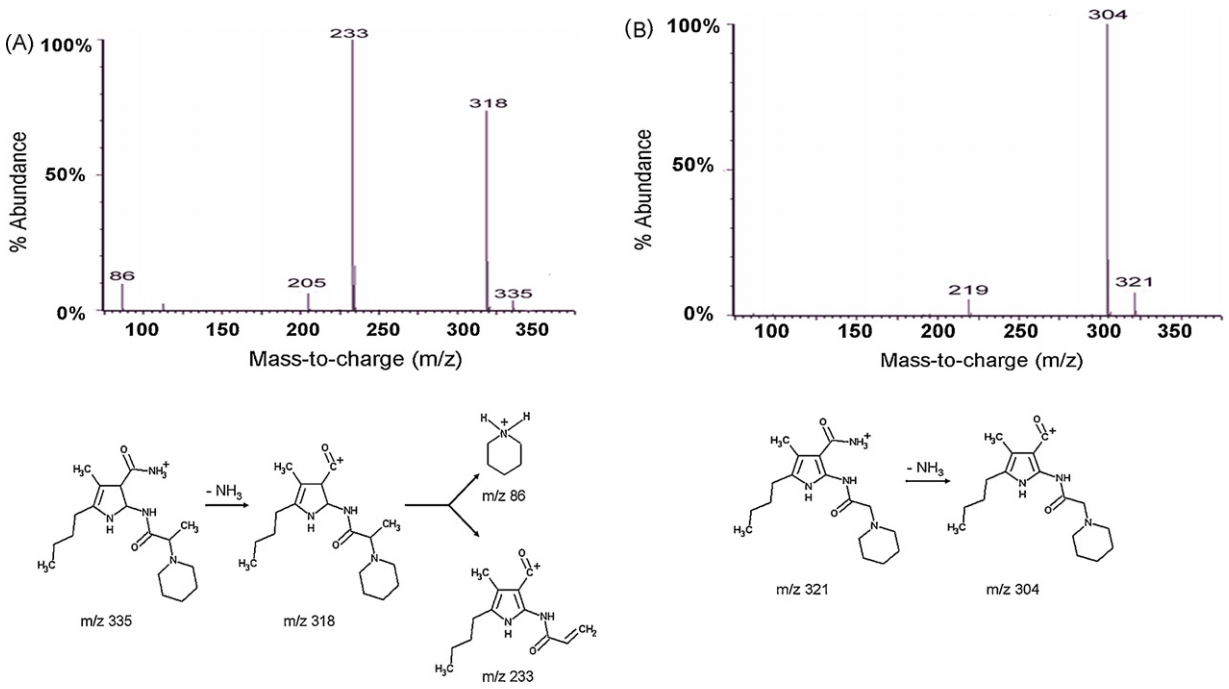

Fig. 1. MS/MS spectra and schematic fragmentation of MNP001 and NP06. A: spectrum of MNP001 from precursor ions at *m/z* 335 and proposed fragmentation schema; B: spectrum of NP06 from precursor ions at *m/z* 321 and proposed fragmentation schema.

2. Methods

2.1. Chemicals and reagents

Drug-free rat plasma, ammonium acetate, glacial acetic acid, acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid and acetone was purchased from Sigma–Aldrich Inc. (St. Louis, MO). All solvents were of HPLC grade. MNP001 and internal standard NP06 were synthesized in our medicinal chemistry laboratory, with their chemical structures confirmed by ^{1}H-NMR, ^{13}C-NMR, and high-resolution mass spectrometry.

2.2. Preparation of stock solutions, calibration samples, and quality control samples

Stock solutions of drug (MNP001) and internal standard (NP06) were independently prepared by dissolving 2.5 mg of MNP001 or NP06 in 100 mL of DD–H_2O containing 0.5% acetic acid to achieve a concentration of 25 μg/mL. A series of working solutions of MNP001 were prepared over a range from 2.5 to 5000 ng/mL by diluting the 25 μg/mL stock solution with DD–H_2O. Ten μL of the appropriate working solution of MNP001 were added to 90 μL of drug-free rat plasma on each day of assay to make eight levels of calibration standards at the following concentrations: 0.25, 0.5, 2, 10, 25, 50, 250 and 500 ng/mL. Quality control (QC) samples were independently prepared in the same manner at concentrations of 0.25, 10 and 500 ng/mL, representing the lower, middle, and upper concentration range, respectively. Internal standard working solution (50 ng/mL) was prepared by diluting internal standard stock solution with DD–H_2O.

2.3. Sample processing and extraction

A simple protein precipitation procedure by acetone with further quick filtration through solid-phase extraction disk cartridges (3 M, St. Paul, MN) was applied to extract MNP001 and internal standard. Aliquots of rat plasma samples (100 μL) with known (standard and QC samples) or unknown (plasma samples obtained from *in vivo* animal study) concentrations of MNP001 were mixed with 50 μL of internal standard working solution (final plasma concentration 25 ng/mL NP06) in polypropylene micro-centrifuge tubes. Protein precipitation was performed by adding 850 μL of cold acetone. After vortex-mixing for 1 min, samples were incubated at −80 °C for 15 min. Then, the mixtures were centrifuged for 15 min at 18,300 × *g* and 4 °C. Solid-phase extraction disk cartridges were pre-conditioned with 0.5 mL of acetone before loading the samples. Extraction supernatant (900 μL) was forced through the cartridge installed at a vacuum manifold by applying negative pressure generated from a vacuum pump. Subsequently, the cartridge was rinsed twice with 150 μL of acetone. Eluted liquids were collected in a 1.5-mL micro-centrifuge tube and evaporated to dryness using a Centrivap Concentrator System with Cold Trap (Labconco Corporation, Kansas City, MO). Residues were reconstituted in 100 μL of resuspension buffer containing 25% acetonitrile and 75% of 0.025 M ammonia acetate buffer with pH value adjusted to 3.5 by formic acid. After 30 s of ultra-sonication and vortexing, the reconstituted mixture was centrifuged for 10 min at 18,300 × *g* and 4 °C. A 15-μL aliquot of supernatant was injected onto HPLC/MS/MS for analysis.

2.4. Chromatographic separation

Liquid chromatographic separation was performed with a Waters ACQUITY Ultra Performance Liquid Chromatograph system (Waters Corporation, Milford, MA) equipped with a binary pump, an autosampler and a solvent degasser. The mobile phase was freshly made daily and degassed before use. Consisting of (A) DD–H_2O containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid, the mobile phase was delivered according to a gradient program through a Chromegabond WR C_{18} Column (15 cm × 2.1 mm, particle size 5 μm 120 Å, ES Industry, West Berlin, NJ) at room temperature and a flow rate of 0.2 mL/min, using the following gradient program: 25% B for 1 min, linear gradient to 50% B between 1 and 7.5 min, held at 50% B until 10 min, then re-equilibrated to initial conditions between 10 and 15 min. The solvent delay time was 2.5 min to reduce

the matrix effect from the endogenous polar interferences in the plasma.

2.5. Mass spectrometry instrumentation

The LC post-column effluents were introduced into a MICROMASS® Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA) equipped with an electrospray interface operated in the positive ion mode (ESI^+) in conditions: desolvation temperature, 350 °C; source temperature, 100 °C; cone voltage, 25 V; and capillary voltage, 3.0 kV. Two channels of mass-to-charge (m/z) ratio transitions were selected for the protonated analytes of MNP001 and internal standard in the mode of multiple reactions monitoring (MRM) as follows: (1) MNP001, m/z 335 → 233; (2) NP06, m/z 321 → 304. Argon was used as the collision gas at a flow rate of 0.25 mL/min. The collision energy for dissociation of the parent ions was set at 21 and 15 eV for MNP001 and NP06, respectively. The dwell time for both channels was set at 0.1 s and mass span was set at 0.3 amu. The chromatograms from these two channels were recorded for quantification using Masslynx program (Version 4.1, Waters Corporation, Milford, MA).

2.6. Method validation

Linearity, intra- and inter-day precision, accuracy, stability and extraction recovery were assessed to determine the validity of this method. Eight levels of calibration samples were analyzed by HPLC/MS/MS. Standard curves were calculated by least-squares linear regression using the measured peak area ratio (MNP001/internal standard) versus the known MNP001 concentrations of calibration samples. A weighting factor of $1/x^2$ (the reciprocal of the squared concentration) was used to increase goodness of fit. Intra-day precision was determined as the relative standard deviation (RSD) calculated from measured values of five replications of QC samples at the nominal concentrations of 0.25, 10 and 500 ng/mL, with the samples processed in the same day. Intra-day accuracy was determined by the percentage differences of measured concentrations versus nominal concentrations of the QC samples with five replications in the same measurement of intra-day precision. Inter-day precision and accuracy were calculated using the values measured for QC samples at each nominal concentration on five different days. The lower limit of quantification (LLOQ) was determined as the lowest concentration detected with a signal-to-noise ratio greater than 5.

The stability of MNP001 in plasma was assessed by subjecting QC samples to three conditions: three freeze-thaw cycles, keeping them at room temperature for 24 h (short term stability), and storing them at −80 °C for 40 days (long term stability). The measurement of MNP001 concentration was performed at the end of each treatment. The percent MNP001 remaining was calculated for each test sample. All of the stability samples were processed in triplicate at the extremes of the concentration range, 0.25 and 500 ng/mL.

Extraction efficiency of the assay was evaluated by a standard recovery test. Drug-free rat plasma sample was processed as described above and the dry residues were reconstituted with 100 μL of MNP001 buffer solution at concentrations of 0.25, 10 and 500 ng/mL. The resuspended mixture was centrifuged for 10 min at 18,300 × g and 4 °C and the supernatant was regarded as the nominal solution. The peak area of the MNP001 measured from the nominal solution represented 100% recovery. Recovery for MNP001 was measured by comparing the peak area of QC samples with the peak area of the nominal solution. Extraction efficiency for the internal standard, NP06, was performed in the same way but at a plasma concentration of 25 ng/mL.

2.7. Application of method to in vivo pharmacodynamic experiment

Female Sprague–Dawley rats (200–225 g, Harlan, Indianapolis, IN) were anesthetized with pentobarbital (50 mg/kg body weight) plus ketamine (25 mg/kg body weight). The anesthetized rats were placed on a heated plate to maintain body temperature at 36.5 °C. Animals were ventilated through a tracheal cannula at a rate of 75–80 breaths/min (Kent Scientific, Torrington, CT). Carotid blood pressure was recorded via a pressure transducer (Capto SP844, Norway) connected to a data acquisition system (ML110 bridge amplifier, 16SP interface, Chart 5.1 software; Power Lab, AD Instrument, Colorado Springs, CO) [4,7]. An infusion line was established in the left femoral vein and 0.1 mL of saline (0.9% NaCl) or saline containing MNP001 to achieve doses of 0.25, 0.5, and 1 μg/kg body weight was injected into the vein over 5–10 s. A 250 μL of blood sample was collected from the carotid catheter after the animal's blood pressure decreased and reached steady state following the injection. The blood sample was centrifuged at 10,000 × g for 10 min and plasma (100 μL) was collected and analyzed using the method described above.

2.8. Application of method to pharmacokinetic study

Male Sprague–Dawley rats implanted with right jugular vein cannulas, weighing from 200 to 250 g, were purchased from Harlan Inc. (Indianapolis, IN) and individually housed in a temperature-controlled room with 12 h light/dark cycle for at least 48 h before the experiment. Food was withheld 12 h before drug dosing while water was available *ad libitum*. Rats (n = 5) were intravenously given MNP001 at a single dose of 0.05 mg/kg. Blood samples (0.3 mL) were collected from the cannula into heparinized tubes prior to and at various times (0.08, 0.25, 0.5, 1, 3, 4, 6, 8, 12, 24 h) after dosing, and centrifuged at 5600 × g for 5 min. The plasma samples were separated and stored at −80 °C until analysis. A non-compartmental analysis (NCA) was used to calculate the pharmacokinetic parameters using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA).

3. Results

3.1. Chromatography, mass spectrometry and specificity

MNP001 and NP06 formed protonated ions ($[M+H]^+$) in the positive electrospray ion mode (Fig. 1). The first quadrupole (Q1) mass spectra of MNP001 and NP06 showed their protonated ions at m/z 335 and 321, respectively. After the precursor ions were introduced into the collision cell (Q2) and then the third quadrupole (Q3), the MS/MS spectra of their daughter ions were detected and the suggested defragmentation scheme is presented in Fig. 1. Between the two daughter ions of MNP001 (m/z 318 and m/z 233), fragment ion of m/z 233 was selected to monitor the transition because it was more abundant and reliable to measure in terms of signal to noise ratio. For the internal standard, NP06, only one species of major Q3 daughter ion (m/z 304) was recorded and therefore, selected for monitoring its transition. With a simple chromatographic gradient program, NP06 and MNP001 were well separated (Fig. 2). In drug-free control plasma samples, no endogenous interference peaks were detected near the retention time of MNP001 or internal standard NP06 (Fig. 2).

3.2. Linearity and sensitivity

The peak area ratio of MNP001 to internal standard indicated a linear relationship with the nominal concentrations of MNP001 in the tested range of 0.25–500 ng/mL. The reciprocal of the squared

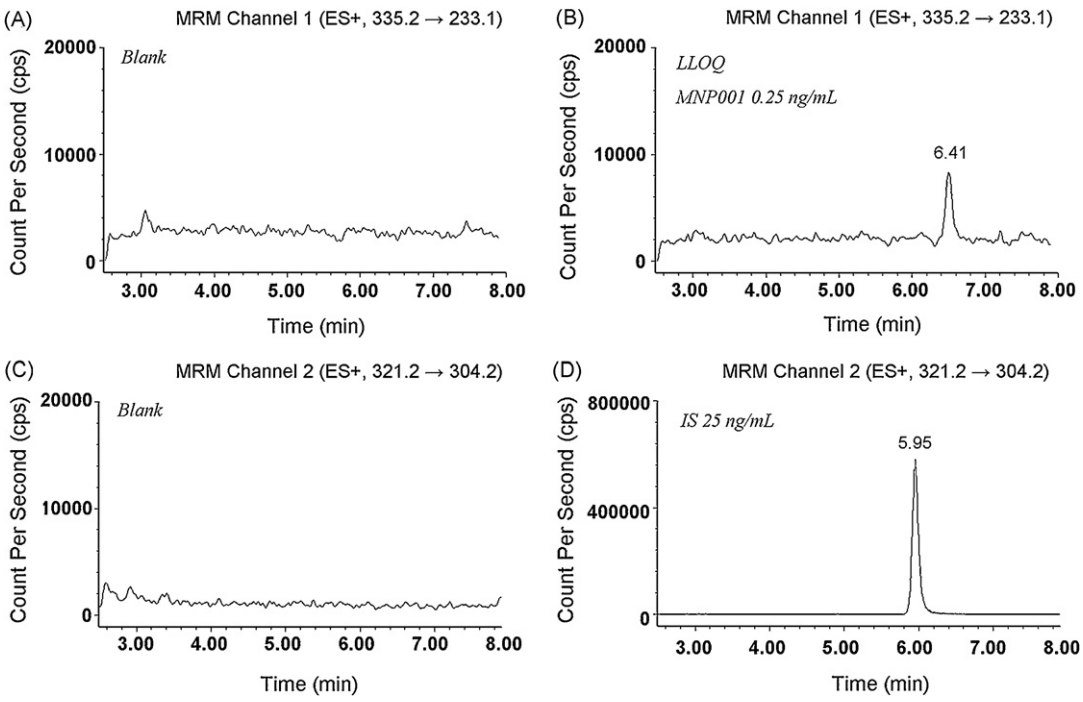

Fig. 2. Representative multiple reaction monitoring chromatograms of MNP001 (m/z 233) and NP06 (m/z 304) in rat plasma. A: control plasma measurement for channel detecting MNP001. B: calibration sample at LLOQ level (0.25 ng/mL MNP001). C: control plasma measurement for channel detecting NP06. D: plasma sample of 25 ng/mL NP06.

concentration ($1/x^2$) was used as a weighting factor in the linear regression model to increase goodness-of-fit. The least-squares linear regression correlation coefficient (R) was greater than 0.987 in all analytical tests. The assay results demonstrated linearity over the concentration range set for this validation study and had a lower limit of quantification (LLOQ) of 0.25 ng/mL.

3.3. Precision, accuracy and recovery

The intra- and inter-day precision and accuracy are summarized in Table 1. The intra-day and inter-day precision of MNP001 ranged from 4.3% to 5.3% and from 3.3% to 5.7%, respectively. Likewise, the intra-day and inter-day accuracy of MNP001 ranged from 94.0% to 96.2% and from 95.5% to 96.7%, respectively. The extraction recovery of MNP001 was 64.0 ± 5.3% at 0.25 ng/mL (n = 3), 66.1 ± 1.5% at 10 ng/mL (n = 3), and 59.6 ± 2.3% at 500 ng/mL (n = 3). The recovery of internal standard was 62.3 ± 3.3% at 25 ng/mL (n = 9).

3.4. Stability

After three freeze-thaw cycles, MNP001 was shown to be stable with 107.1 ± 5.9% MNP001 remaining for high concentration (500 ng/mL) and 104.0 ± 5.9% for low concentration (0.25 ng/mL). Short-term stability studies also indicated that MNP001 is stable for 24 h at room temperature, which is ample time for large batches of sample pretreatment. After MNP001 solutions were left for 24 h at room temperature, the remaining amount of MNP001 detected were 96.5 ± 14.5% and 104.2 ± 2.8% for high (500 ng/mL) and low (0.25 ng/mL) concentrations, respectively. Long-term stability tests showed no significant degradation (<15%) of MNP001 after 40 days of storage at −80 °C, giving remaining amounts 99.3 ± 7.5% and 90.4 ± 1.3% for high (500 ng/mL) and low (0.25 ng/mL) concentrations, respectively.

3.5. Application to pharmacodynamic study

It was found that intravenous bolus injection of MNP001 at a dose of 0.25 μg/kg produced a plasma concentration of 0.371 ± 0.088 ng/mL (n = 4) that failed to cause any measurable blood pressure lowering action (Fig. 3A). When the dose was increased to 0.5 μg/kg, a ∼10 mmHg decrease of mean arterial pressure (MAP) (Fig. 3B) was recorded and the corresponding plasma concentration of MNP001 was measured as 0.573 ± 0.045 ng/mL (n = 4). At a still higher intravenous dose (1 μg/kg) that resulted in a plasma concentration of 0.753 ± 0.16 ng/mL (n = 4), MNP001 decreased the MAP by ∼40 mmHg and the heart rate by 30

Table 1
Intra- and inter-day precision and accuracy (n = 5).

Nominal concentration (ng/mL)	Detected concentration (ng/mL) (mean ± SD)	Precision (RSD%)	Accuracy
Intra-day			
0.25	0.265 ± 0.011	4.33	94.0 ± 4.4
10	10.2 ± 0.54	5.33	96.2 ± 3.8
500	436.9 ± 23.1	4.75	95.9 ± 3.0
Inter-day			
0.25	0.260 ± 0.0087	3.34	96.1 ± 3.3
10	9.78 ± 0.56	5.71	95.5 ± 3.4
500	490.8 ± 22.5	4.59	96.7 ± 3.3

SD, standard deviation; RSD, relative standard deviation.

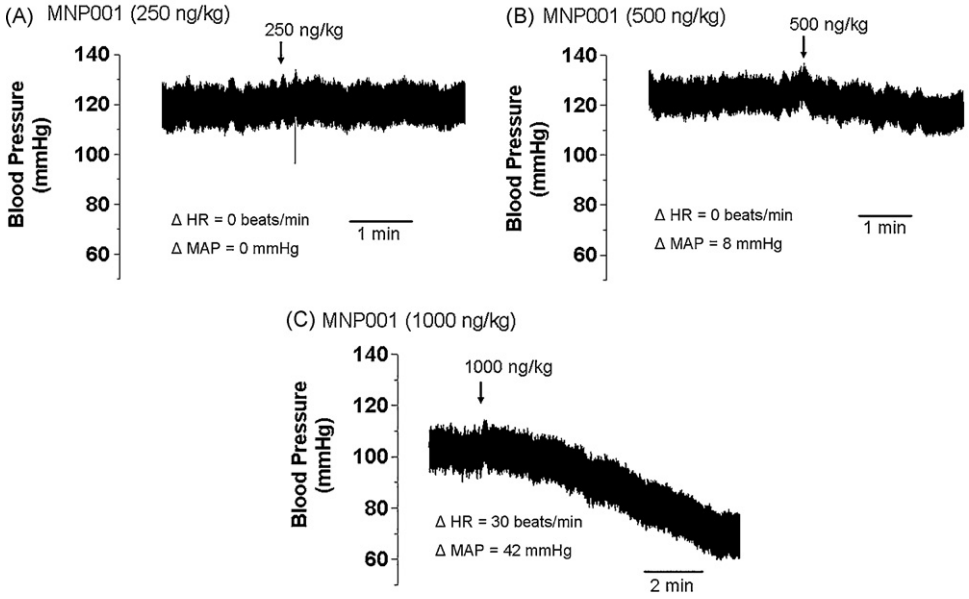

Fig. 3. Blood pressure decrease caused by an intravenous bolus injection of MNP001 at indicated doses (Panel A, B, and C) in anesthetized rats. The corresponding plasma concentration of the agent in samples collected at the end of the recording traces were measured and reported in the text. The delta changes of heart rate (HR) and mean arterial blood pressure (MAP) were indicated.

beats/min (Fig. 3C). All of the concentrations measured were confidently greater than the assay's defined LLOQ (0.25 ng/mL).

3.6. Application to pharmacokinetic study

The plasma concentration versus time curve in animals receiving a single intravenous dose (0.05 mg/kg) was represented in the Fig. 4. Following the intravenous administration, the systemic plasma clearance (CL) was 4.29 ± 1.91 mL/min/kg. The volume of distribution was 4.26 ± 2.33 L/kg, which is greater than the total body water volume [8]. The estimated terminal half life was 22.58 ± 14.46 h and mean residence time (MRT) was 33.57 ± 22.14 h. The maximum mean plasma concentration of MNP001 was 69.89 ng/mL and decreased to 5.43 ng/mL at 24 h after dosing, higher than the concentration needed to produce the pharmacodynamic effect (see Section 3.4). All the concentrations measured in the pharmacokinetic study were greater than the assay's defined LLOQ (0.25 ng/mL) and were within the linearity range (0.25–500 ng/mL) of the method.

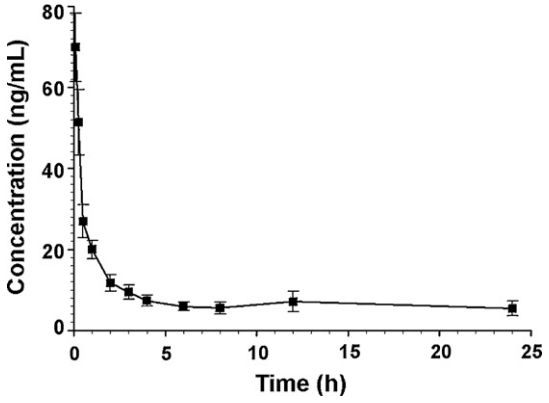

Fig. 4. Mean concentration–time curve in rats after a single i.v. dose of MNP001 (0.05 mg/kg). Each point represents the plasma concentration (mean ± SE) for five animals.

4. Discussion

NP06, the demethylated analog of MNP001 (Fig. 1B), was selected as the internal standard because it has a structure similar to MNP001. The chemical properties of the two compounds are comparable in terms of their stability and extraction recovery as well as their ionization efficiency. The chromatographic peaks of the test drug MNP001 and the internal standard NP06 were separated from each other with a fixed retention time interval of 0.5 min, which warrants a sufficiently independent ionization and a reliable/reproducible quantification of MNP001 using this HPLC/MS/MS assay.

Sample processing procedure (acetone protein precipitation followed by C_{18} cartridge filtration) was sufficient to provide clean filtrates for the highly sensitive HPLC/MS/MS assay. It was reported that matrix effects caused by endogenous interferences in the biological specimens can be typically minimized if the HPLC eluants are diverted to waste in first 10–20% of the total sample running time [9], therefore a 2.5-min solvent delay procedure was applied in the HPLC/MS/MS gradient program. The measured mean matrix suppression (the peak responses of MNP001 and IS of the post-extraction spiked plasma samples versus that of the QC samples prepared in the mobile phase) is 4.36% for MNP001 and 4.44% for IS, respectively. Thus, the matrix effect should have been canceled out in this method because the quantification of MNP001 was based on IS in each run. In addition, spiked plasma samples were always used for preparing calibration and QC samples, which minimizes the matrix differences between the samples for method validation and PK/PD samples.

A relatively large amount of rat plasma volume (100 μL) was tested in this assay to compare with our previous studies [5]. We realized that there is room to improve the sensitivity in order to determine the drug concentrations using less amount of plasma for future pharmacokinetic and pharmacodynamic studies if necessary. Compared with the assay of MSP001 [5], the quantification limit of this protocol was at least 80-fold lower, which serves well for our current PK/PD studies.

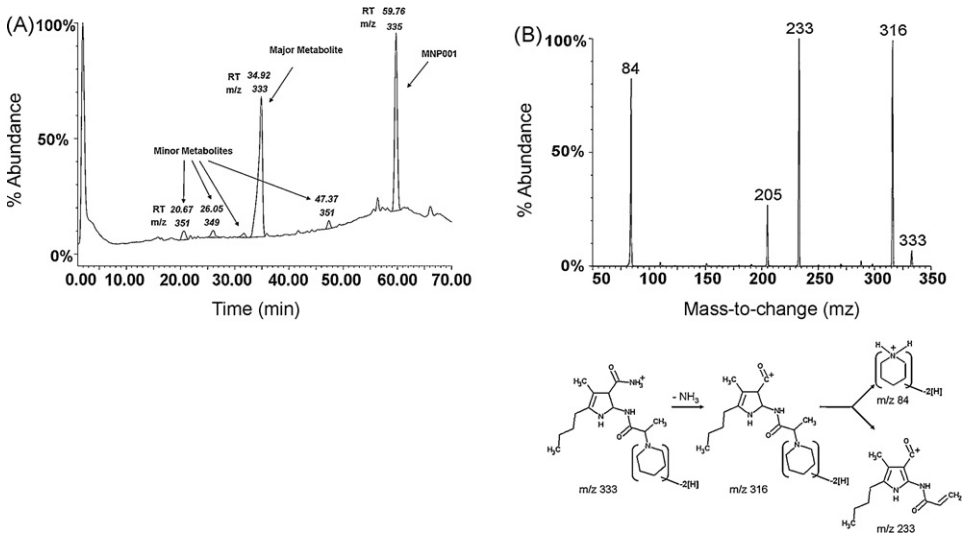

Fig. 5. A: HPLC/MS chromatograms of an extraction from the supernatant of rat microsomal incubation of MNP001. The incubations were conducted at 37 °C in a cocktail containing MNP001 (20 μM), rat liver microsome protein (0.5 mg/mL), and the NADPH regeneration system. The major metabolite MNP-M4 was eluted using an HPLC protocol through a C_{18} Column with a mobile phase containing acetonitrile and ammonium acetate (details were omitted). Mass chromatograms were performed in ESI^+ mode with a scan range from 150 to 600 *m*/*z*. The spike at 35 min was designated as the major metabolite of MNP-M4. Other minor metabolites were identified as the hydroxylation products of MNP001 (or MNP-M4). B: The MS/MS spectrum of MNP-M4 from precursor ions at *m*/*z* 333 and proposed fragmentation schema. The collision energy for dissociation of the protonated MNP-M4 ion was 17 eV.

Our preliminary study showed that the major P450-catalyzed metabolic pathway is a dehydrogenation reaction by loss of two hydrogen atoms on the piperidine ring of MNP001 (Fig. 5A). The dehydrogenation metabolite ($[M+H]^+$, *m*/*z* 333) of MNP001 (MNP-M4) was found to have a similar MS/MS spectrum to MNP001 except for the fragment ion of *m*/*z* 84 (compare to Fig. 1A), indicative of a tetrahydropyridine moiety (Fig. 5B), which is similar to the P450-catalyzed dehydrogenation reactions of felodipine [10] and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [11]. We obtained a small amount of MNP-M4 (~ 20 μg) from the HPLC effluence fraction of a collected-supernatant of rat microsomal incubation of MNP001 and ran an analysis on a sample that contains MNP001 (500 ng/mL), IS (25 ng/mL) and MNP-M4 (~500 ng/mL) using the validated HPLC/MS/MS method. By monitoring the transition channel of *m*/*z* 333 → 233 (MNP-M4), we found that including MNP-M4 in the samples does affect neither IS NP06's nor MNP001's peak areas. It suggests that MNP-M4 does not interfere in the detection and quantification of MNP001. Up to now, the amount of the metabolite produced in our microsomal reaction is not sufficient for an NMR analysis to define the exact chemical structures of MNP-M4. An alternative approach is to synthesize the proposed isomers and then compare the retention time in HPLC/MS chromatograms.

According to the FDA Guidance for Bio-analytical Method Validation, the mean accuracy should be within ±20% for the lower limit of quantification (LLOQ) concentration and ±15% for all other concentrations and the mean precision should be within 20% for the LLOQ concentration and 15% for all other concentrations [12]. All the parameters measured in this study meet the criteria well, indicating that the developed HPLC/MS/MS assay is satisfactory for the determination of MNP001 concentrations in biological samples. The validation results show that the method has good linearity, specificity, high sensitivity, and acceptable accuracy and precision. In addition, preliminary animal studies indicate that this analytical method can satisfactorily measure concentrations of MNP001 both within and below the compound's pharmacologically effective concentration range. The preliminary pharmacokinetic study indicates that the reported method can meet the requirements of pharmacokinetic study on MNP001 followed intravenous administration and assure sufficient sensitivity even if the administration dose is low (e.g. 0.05 mg/kg).

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