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Short communication

High performance liquid chromatographic analysis of MS23 piperidine analog MSP001 in rat plasma

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Abstract

MSP001 is a newly synthesized piperidine analog of the lead antihypertensive compound MS23 that dually targets cAMP-specific type 4 phosphodiesterase and L-type calcium channels. We validated an analytical protocol for MSP001 in rat plasma using high performance liquid chromatographic method. A C18 column and a phosphate/acetonitrile buffer were used to perform the chromatographic separation. UV detection was carried out at 307 nm, a wavelength at which an absorption peak was detected for this group of compounds. The calibration curve for MSP001 was linear in the range from 25 to 10,000 ng/ml. The limit of quantification (LOQ) was 25 ng/ml. The results demonstrate that this method has high linearity ($R = 0.99995$), compound specificity, and acceptable precision/accuracy. The protocol is suitable for in vivo pharmacokinetic studies on the compound.

Keywords: Antihypertensive; MS23; MSP001; Validation; Dual action

1. Introduction

Hypertension (persistent high blood pressure, BP) affects approximately 50 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]. Currently, six categories and more than 70 antihypertensive drugs are prescribed in antihypertensive therapy [2]. However, many factors, such as ethnic-related poor response to certain category of drugs (e.g., the ACEIs are less effective for lowering BP in African–Americans) [3], coexisting medical conditions (e.g., heart failure) and the disruption of the quality of life (e.g., causing male sexual impotence) limit the efficacy of some antihypertensives [2,4]. Therefore, pharmaceutical companies are greatly investing on the R&D of newer and better antihypertensive agents.

Recently, a new group of agents was discovered with unique dual action of mechanism, including antagonizing L-type calcium channels and inhibiting type 4 phosphodiesterase (PDE4) [5,6]. These compounds could be developed into preferable new antihypertensive drugs by increasing their efficacy of lowering blood pressure and reducing the adverse responses associated with targeting each individual mechanism [7]. In spite of the lead compound MS23 showing sufficient efficacy, the newly designed and synthesized analog MSP001 demonstrated better therapeutic index and potency (unpublished data). As there were no previous assay methods reported to determine the concentration of MSP001 in any biological fluid, we developed an assay method for measuring MSP001 level in rat plasma. As shown in Fig. 1, MSP001, similar to MS23, has a chemical structure with UV absorption that made it suitable for quantification using HPLC protocol. The validation study indicates that the protocol is appropriate for in vivo evaluating the pharmacokinetics of MSP001 in rats or human subjects.

2. Experimental methods

2.1. Chemicals and reagents

Rat plasma, potassium phosphate monobasic, acetonitrile, methanol were purchased from Fisher Scientific (Pittsburgh,

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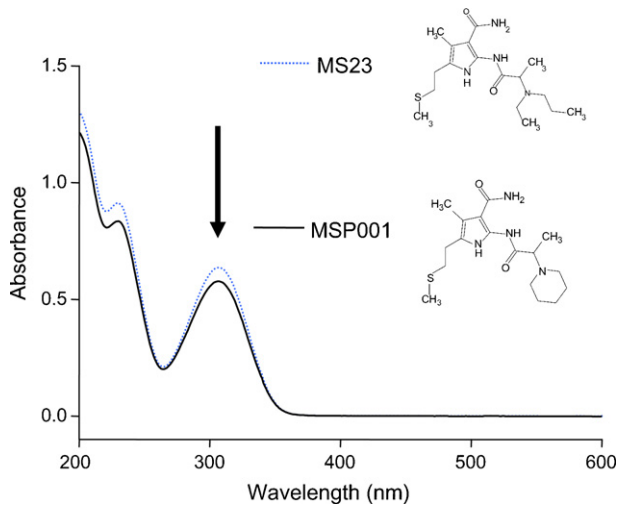

Fig. 1. Chemical structures and UV absorption spectrums of MSP001 and MS23. MSP001 or MS23 25 μg/ml aqueous solution was scanned in a quartz cuvette cell by a UV–vis spectrophotometer (Lambda EZ210, Perkin-Elmer, Norwalk, CT) with a velocity of 800 nm/min. The spectrums were generated after subtraction of that of solvent in the same cuvette.

PA), sodium hydroxide from Sigma (St. Louis, MO), and perchloric acid (70%) from EM science (Darmstadt, Germany). All solvents are of HPLC grade. MSP001 and internal standard MS23 were synthesized in our laboratory and characterized with H NMR, C13 NMR and high resolution mass spectrometry.

2.2. HPLC system

The analyses were carried out using a System Gold HPLC system (Beckman Coulter, Fullerton, CA) equipped with a 125 solvent module, a 166 UV detector and a 507 autosampler. A compound-specific wavelength of 307 nm was chosen for the detection of MSP001. The separation was carried out using a Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent, Palo Alto, CA) proceeded by a Pinnacle II C18 guard column (4.0 mm × 10 mm) (Resteck, Bellefonte, PA).

2.3. Mobile phase

Isocratic HPLC mobile phase consisted of potassium phosphate (50 mM, pH 3.47) and acetonitrile (75:25, v/v) was used, which was freshly mixed and filtered with NL17 polyamide membrane filter (0.45 μm, Schleicher & Schuell, Germany) and degassed before used to run the samples. All the assays were performed at room temperature and with a flow rate of 1 ml/min.

2.4. Sample preparation and extraction procedures

MSP001 was extracted from reconstituted samples of rat plasma using a solid phase extraction method with C18-HD Empore High Performance Extraction Disk Cartridges (3 M, St. Paul, MN). The cartridges were prepared immediately before sample processing. Methanol (0.4 ml) and de-ionized distilled H_2O (DD-H_2O, 0.4 ml) were used sequentially to condition the cartridges by applying positive pressure. Residual DD-H_2O was left to keep the surface of the disk wet. Rat plasma samples (100 μl) with known (for reconstituted samples) or unknown (for samples obtained from in vivo animal experiments) MSP001 concentration were mixed with 100 μl of internal standard solution (500 ng/ml MS23) and 400 μl of 10% perchloric acid. After vortex-mixing and sonication (15 s), the mixture was centrifuged for 5 min at 12,000 × *g*. Then, 550 μl of supernatant was mixed with 230 μl 2 M NaOH, loaded, and pushed through the cartridge by positive pressure. DD-H_2O (0.5 ml) and 0.3 ml of 20% methanol was added to rinse the disc cartridge. Finally, MSP001 and internal standard MS23 were eluted by 150 μl acetonitrile twice into a collecting micro-centrifuge tube and dried under streams of nitrogen gas at room temperature inside a fume hood. The dried samples were re-suspended in 120 μl of mobile phase and 50 μl was subsequently injected for quantification analysis.

2.5. Validation of the analytical method

MSP001 was added to blank rat plasma to achieve a range of concentrations 10, 25, 50, 100, 200, 400, 1000, 2000, 5000, and 10000 ng/ml, respectively, and extracted using the protocol described above. The standard calibration curves used to quantify the MSP001 concentration in a given sample were constructed with the spike area ratios of MSP001 and the internal standard MS23. The linearity, intra- and interday precision, accuracy, recovery and stability were validated to show the reliability of the analytical method [8,9]. Standard calibration curves were fitted using linear regression algorithm (Origin 5.0, Microcal Software Inc., Northampton, MA). Intraday precision was defined as relative standard deviation (R.S.D.) calculated from the values measured from five samples at concentrations of 50, 500, and 5000 ng/ml, respectively, in the same day ($n = 5$). Intraday accuracy was defined as relative value on the same measurements. Interday precision and accuracy were calculated using the values measured from five different samples (one sample from each different day) at the concentrations of 50, 500, and 5000 ng/ml, respectively.

2.6. Animal study

Female Sprague–Dawley rats (200–225 g) were anesthetized with 50 mg/kg pentobarbital plus 25 mg/kg ketamine. The anesthetized rats were placed on a heated plate (36.5 °C) to maintain the body temperature. Animals were ventilated through a tracheal cannula at a rate of 75–80 breath/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) [8,10]. An infusion line was established in the left femoral vein and saline or MSP001 containing saline (0.1 ml) was injected into the vein within 5–10 s. When animal's BP was decreased and reached a steady state after MNP001 injection, a 250 μl blood sample was collected from the carotid catheter.

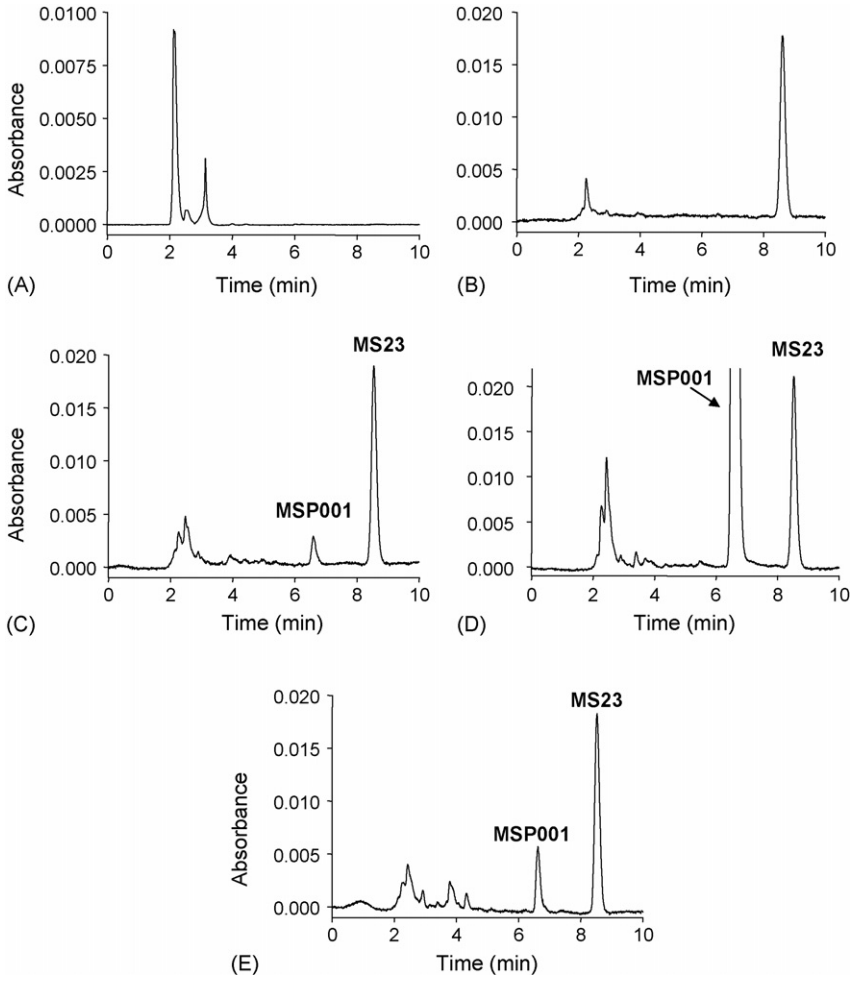

Fig. 2. Representative chromatograms of MSP001 and the internal standard MS23 from plasma samples. The retention time is 6.6 min for MSP001 and 8.6 for the internal standard MS23. (A) A blank plasma; (B) a blank plasma with internal standard MS23; (C) a plasma sample at 50 ng/ml of MSP001; (D) a plasma sample spiked with 5000 ng/ml MSP001; (E) a plasma sample from a rat after an intravenous injection of 100 μg/kg MSP001, the concentration was determined to be 127.4 ng/ml in the plasma that reduced BP significantly (see Fig. 3).

Plasma was separated by centrifugation at 1000 × *g* for 10 min and 100 μl plasma was collected and analyzed using the method described above.

3. Results

3.1. Chromatographic separation and specificity

MSP001 and its related compounds have a structure-specific UV absorption peak at the wavelength of 307 nm (Figs. 1 and 2). Separation of the mixture of MSP001 and the internal standard MS23 was performed and optimal retention times were determined. Fig. 2 shows the representative chromatograms of a blank plasma sample and plasma samples containing 50 and 5000 ng/ml MSP001. The interfering peaks from the rat plasma was successfully removed by the extraction procedures and the clean spikes of MSP001 and MS23 were independent of each other with a fixed retention time interval of 2.0 min. The ratio of the integrated peak areas between MSP001 and the internal standard linearly increased as the concentration of MSP001 was increased.

3.2. Linearity of the calibration curve

Over the plasma concentration range from 10 to10,000 ng/ml, regression analysis indicated that there was an excellent linearity between UV absorption and MSP001 concentrations ($R > 0.9999$). High repeatability (R.S.D. of slope = 3.62%) was observed. The mean and standard deviation (mean ± S.D.) for the slope and intercept were 0.00217 ± 0.0000724 and 0.00444 ± 0.0159, respectively (Table 1). The range of lin-

Table 1
Interday precision in the slopes and intercepts of calibration curves ($n = 5$)

Days	Slope	Intercept	*R*
1	0.00190	−0.00265	0.99999
2	0.00196	−0.01591	0.99993
3	0.00203	0.02762	0.99992
4	0.00209	0.00695	0.99998
5	0.00202	0.00621	0.99993
Mean ± S.D.	0.00200 ± 0.0000724	0.00444 ± 0.0159	0.99995 ± 0.00003
R.S.D. (%)	3.62	358	0.003

S.D.: standard deviation; R.S.D.: relative standard deviation.

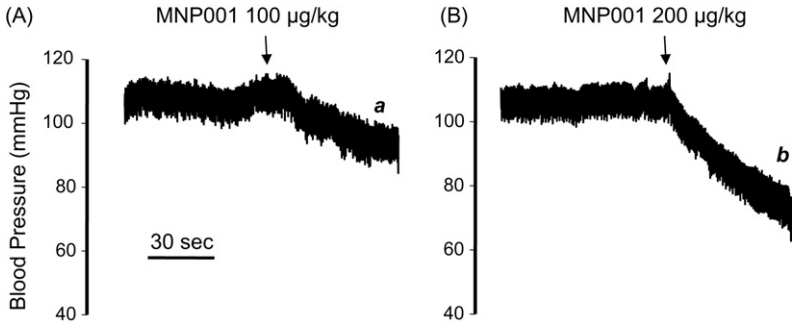

Fig. 3. Intravenous bolus injection of MSP001 caused blood pressure decrease in anesthetized rats. The corresponding plasma concentrations of MSP001 that caused BP decrease at point *a* and *b* were 141.1 ± 23.9 and 270.2 ± 21.3 ng/ml, respectively ($n = 4$).

earity of the calibration curve covers the lowest and highest MSP001 concentrations in the plasma that produce meaningful reduction in animal blood pressure in vivo studies (Fig. 3.)

3.3. Precision and accuracy

The acceptable interday and intraday precision (relative standard deviation, R.S.D.) and accuracy were set as <10% and between 95 and 105%, respectively [8]. The precision and accuracy for MSP001 plasma concentration measurement is summarized in Table 2. The precision (R.S.D.) of MSP001 was ranged from 1.51 to 1.97% for intraday and from 1.29 to 3.10% for interday, respectively. The accuracy of MSP001 was ranged from 98.79 to 102.84% for intraday and from 99.74 to 102.00% for interday, respectively.

3.4. Sensitivity

Two criteria are used to define limit of quantification (LOQ), i.e., (1) the analytical response at LOQ must be five times of the baseline noise; and (2) the analytical response at LOQ can be detected with sufficient precision (10%) and accuracy (90–110%) [8]. Limit of detection (LOD) is defined as the lowest concentration of MSP001 at which the signal is larger than three times of the baseline noise [8]. The measured LOQ and LOD values (Table 1) for MSP001 using this method are 25 and 10 ng/ml, respectively, which well meet the requirements of validation for a new assay protocol.

3.5. Recovery

Recovery of the analytical method was measured for both MSP001 and internal standard MS23. The internal standard MS23 had an average recovery of 65.2 ± 6.1% ($n = 9$) in the final elute, and MSP001 had an average recovery of 66.1 ± 2.3% at 50 ng/ml ($n = 3$), 64.0 ± 3.1% at 500 ng/ml ($n = 3$), and 64.7 ± 0.2% at 5000 ng/ml ($n = 3$), respectively. We found that there was no extraction partition lost in washing elution and the recovery for MSP001 from the plasma samples was independent of the concentrations of the compound and of the presence of the internal standard MS23.

3.6. Stability

The stabilities of MSP001 and MS23 in aqueous solutions or in plasma were tested following freeze-thaw cycles and after ≥30-day storage [8]. After three freeze-thaw cycles of the stock solutions (10 mg/ml in H_2O), the measured MSP001 and MS23 were 101.7 ± 1.9% ($n = 5$) and 99.9 ± 1.9% ($n = 4$) of their initial concentrations, respectively. After the storage of plasma with MSP001 at 50, 500, and 5000 ng/ml at −20 °C for 30 days, the detected quantities ($n = 5$) were 97.4 ± 2.3%, 99.0 ± 5.0%, and 99.9 ± 1.4% of the added amounts of the compound, respectively.

3.7. Application to animal study

In order to evaluate the applicability of the method for in vivo animal studies, we assayed the plasma MSP001 concentrations

Table 2
Intra- and interday precision and accuracy of quantifying MSP001 in rat plasma samples using the described HPLC method ($n = 5$)

Actual concentration (ng/ml)	Detected concentration (mean ± S.D.) (ng/ml)	Precision (R.S.D. %)	Accuracy (%)
Intra-day			
50	49.39 ± 0.80	1.63	98.79
500	514.23 ± 7.76	1.51	102.84
5000	5009.1 ± 58.8	1.97	100.18
Inter-day			
50	50.37 ± 1.14	2.25	100.74
500	510.00 ± 15.79	3.10	102.00
5000	4987.0 ± 64.4	1.29	99.74

S.D.: standard deviation; R.S.D.: relative standard deviation.

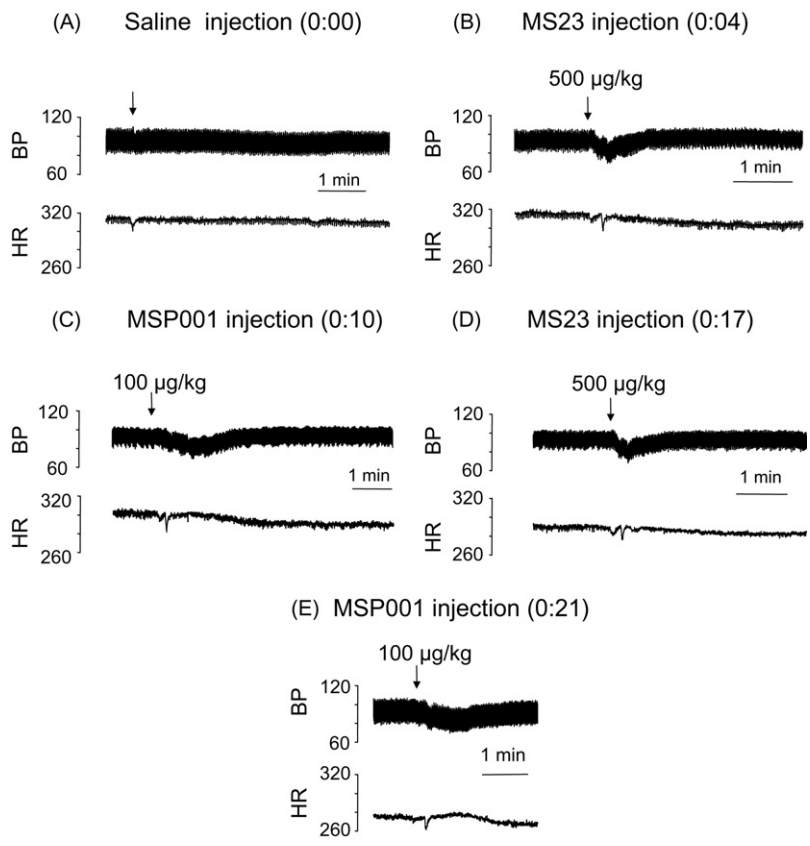

Fig. 4. Comparison of hemodynamic efficacy of MS23 and MSP001. (A–E) BP (blood pressure, mmHg) and HR (heart rate, beats per min) changes following intravenous bolus injection of MS23 and MSP001 (in 0.1 ml saline) to the same rat at indicated time points during the experiment.

at which the compound caused clinically relevant reduction in animals' BP. We found that MSP001 bolus injection at a dose of 30 μg/kg did not cause any measurable BP lowering effects and there was 30.3 ± 4.4 ng/ml ($n = 5$) MSP001 in the plasma samples collected 2 min following the administration. When the dose was increased to 40 μg/kg, a marginal BP lowering action, i.e., a decrease of mean arterial pressure (MAP) by ~5 mmHg (data not shown), was recorded and the plasma concentration of MSP001 was measured to 60.8 ± 11.8 ng/ml ($n = 5$) which was 2.4-fold greater than the defined LOQ value (25 ng/ml). At bolus doses of 100 and 200 μg/kg injection, MSP001 decreased MAP by 16.8 ± 4.0 and 32.5 ± 1.0 mmHg (Fig. 3), respectively, while the measured plasma concentrations of the compound were 141.1 ± 23.9 and 270.2 ± 21.3 ng/ml, respectively ($n = 4$).

4. Discussion

The newly synthesized MSP001 has a piperidine ring and shows increased potency, prolonged action duration, and improved therapeutic index than the lead compound MS23 in controlling animal's BP (Fig. 4). The core structure of MSP001 is similar to that of MS23 and the two chemicals have almost identical UV absorption property (Fig. 1). The independent extraction and separation of MSP001 from the internal standard MS23 warrants the valid quantification of the assay compound using this method.

Several columns were investigated at the outset of this project to identify a suitable column and mobile phase to optimize the chromatography. The Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm) and the isotonic phosphate/acetonitrite were found to produce sharp, narrow, symmetrical peaks and sufficient sensitivity for quantification of MSP001 in plasma samples.

The solid phase extraction with C18 material for drug concentration analysis in biological fluids was widely used previously [8,11,12]. Our protocol yielded reasonable recoveries and clean chromatographic peaks for both MSP001 and MS23 in plasma samples. MSP001 at a plasma level of approximately 60 ng/ml could only produce a marginal BP reduction in animals, which was well above the defined LOQ (25 ng/ml). Therefore, the assay protocol has sufficient sensitivity to quantify the lowest plasma concentrations of MSP001 in animal that produce clinically relevant BP lowering effect. The linear region of the calibration curve (from 25 to 10,000 ng/ml) also envelops the high end of MSP001 concentrations (2000 ng/ml) in plasma that lowers BP to a level far below the normal range (data not shown).

We found that during the solid phase extraction, 20% methanol could wash off most of the impurities without producing any detectable MSP001 in the elution (data not shown), while other liquid extraction approaches such as using ethyl ether, ethyl acetate, or chloroform all gave rise to low recovery and large interfering spikes around MSP001 peaks. The 0.6 M

perchloric acid precipitation [13] and the use of solid phase extraction (3M Disk Cartridge) guarantee the achievement of complete deproteination of plasma samples.

We noted that the HPLC protocol requires a relatively large plasma volume (100 μl), which is only suitable for pharmacokinetic studies in rats or other animals larger than rats. The relative simple sample preparation and the short chromatographic run (<10 min) indicates that this method is suitable for high throughput quantitative analysis such as monitoring the plasma level of the compound in clinical patients.

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