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Validation of HPLC analysis method of a novel antihypertensive agent MS23 in rat plasma

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Abstract

MS23 is a vasodilator with unique dual action pharmacological profile to inhibit type 4 PDE and antagonize L-type calcium channels. We validated an analytical protocol for MS23 in rat plasma using high performance liquid chromatography (HPLC). A C18 column and a phosphate/acetonitrite buffer were used for chromatographic separation. UV detection was performed at 307 nm. The calibration curve for MS23 was linear in the range from 50 to 10,000 ng/ml. The limit of quantification (LOQ) was 50 ng/ml. The results demonstrate that the method has linearity ($R = 0.9989$), specificity, and acceptable precision/accuracy. This method is simple, economic, and sufficient for in vivo pharmacokinetic studies on the compound.

Keywords: Validation; MS23; Antihypertensive; PDE4 inhibitor; Dual action; Calcium channel antagonist

1. Introduction

Blood pressure control is crucial in reducing the morbidity and mortality associated with cardiovascular diseases, such as stroke and congestive heart failure [1]. Among clinically used antihypertensives, approximately two thirds of them ultimately lower the blood pressure through vasodilation. Vascular tone is regulated by multiple signaling pathways [2,3], and the cyclic nucleotides (cAMP and cGMP) are potent regulators of vascular smooth muscle relaxation [4–7]. Phosphodiesterases (PDEs) hydrolyze cAMP and cGMP [8]. Inhibition of PDE has been established as an effective and reliable approach to increase intracellular cAMP and/or cGMP [4–6,9,10]. Among the 11 subtypes of PDEs, PDE3, PDE4 and PDE5 are the major targets associated with vasodilation [11,12]. As suggested recently, selective inhibition of PDE4 could be a promising therapy for hypertensive patients [10].

MS23 was reported as a dual action agent that selectively inhibits PDE4 and antagonizes L-type calcium channels [13,14]. This compound could be developed as a preferable antihypertensive drug by enhancing the blood pressure-lowering efficacy and reducing the adverse response associated with targeting each individual mechanism [10].

There is no reported analytical method available for quantifying the concentration of MS23 in biological fluids, such as plasma. Therefore, we developed an analytical protocol to measure MS23 in rat plasma. MS23 has a pyrrole ring structure that possesses a characteristic UV absorption peak at 307 nm (as shown in Fig. 1). This property of MS23 warrants the use of high performance liquid chromatographic (HPLC) method to quantify the compound in biological fluids. The validation study indicates that the protocol is appropriate for in vivo evaluating the pharmacokinetics of MS23 in rats or human subjects.

2. Methods

2.1. Chemicals and reagents

Rat plasma, potassium phosphate dibasic, acetonitrile, methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA), sodium hydroxide from Sigma (St. Louis, MO, USA), perchloric acid (70%) from EM science (Darmstadt, Germany). All solvents were of HPLC grade. MS23 and internal standard S22 were synthesized in our laboratory and characterized with H-NMR, C13-NMR and high-resolution mass spectrometry.

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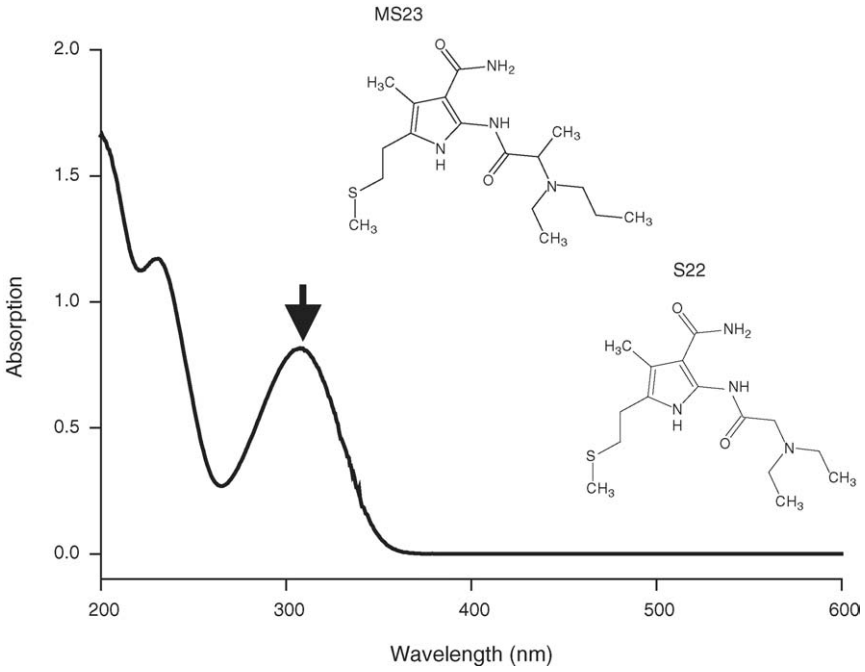

Fig. 1. UV absorption spectrum of MS23. The arrow indicates the MS23 specific UV absorption peak at 307 nM. Method: MS23 was dissolved in aqueous solution at a concentration of 30 μM. The solution was scanned with Lambda EZ210 UV–vis Spectrophotometer (Perkin-Elmer, Norwalk, CT) in a quartz cuvette cell and absorption spectrum was generated after subtraction of that of water in the same cuvette cell. The scanning starts from 600 nm and ends till 200 nm with scanning velocity 800 nm/min. S22 has similar UV absorption spectrum. The chemical structures of MS23 and S22 are illustrated in the insets.

2.2. Apparatus

The analyses were carried out using a System Gold HPLC system (Beckman Coulter, USA) 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 MS23. The separation was carried out using a Pinnacle II 5 μm C18 column (150 mm × 4.6 mm) proceeded by a Pinnacle II C18 guard column (4.0 mm × 10 mm) (Resteck, PA).

2.3. Mobile phase

An isocratic HPLC mobile phase consisted of 25% acetonitrile and 75% phosphate buffer containing 6.8 g potassium phosphate monobasic and 150 μl 85% phosphoric acid in 1 liter volume (pH 3.47). The mobile phase was made fresh daily and filtered with NL17 polyamide membrane filter (0.45 μm, Schleicher & Schuell, Germany) and degassed before use. All the assays were performed at room temperature and a flow rate of 1 ml/min.

2.4. Sample preparation

MS23 was isolated and purified from reconstituted samples of rat plasma by a solid-phase extraction method using C18-HD Empore High Performance Extraction Disk Cartridges (3M, MN, USA) prepared immediately before sample processing. Methanol (0.4 ml) and deionized distilled H_2O (0.4 ml, DD-H_2O) 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. Samples (100 μl) with known or unknown MS23 concentration were mixed with 100 μl of internal standard solution (500 ng/ml S22) and 400 μl of 7% 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 2M NaOH, loaded, and pushed through the cartridge by positive pressure. 0.5 ml DD-H_2O and 0.3 ml of 20% acetonitrile was added to rinse and remove the non-specifically bound substances. Finally MS23 and internal standard were eluted by 150 μl methanol 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 resuspended in 120 μl of mobile phase and 50 μl was subsequently injected for quantification analysis.

2.5. Validation

MS23 was added to blank rat plasma to achieve a range of concentrations 20, 50, 100, 200, 400, 1000, 2000, 5000, 10,000 ng/ml, respectively, and extracted using the protocol described above. The standard calibration curves used to quantify the MS23 concentration in a given sample were constructed with the spike area ratios of MS23 and the internal standard. The linearity, intra- and interday precision, accuracy, recovery and stability were validated to show the reliability of the analytical method [15,16]. Standard calibration curves were fitted using linear regression (Origin 5.0, Microcal Software Inc., MA, USA). 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 one day ($n = 5$). Intraday accuracy was defined as relative value on the same measurements of intraday precision.

Interday precision and accuracy were calculated using the values measured from five different samples (one sample for each day) at the concentrations of 50, 500 and 5000 ng/ml, respectively.

2.6. Animal test

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, USA). 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, CA, USA) [17]. An infusion line was established in the left femoral vein and control or MS23 containing saline was infused through a syringe pump (SP101i, WPI, FL, USA) at various infusion rates (5–20 μl/min). When animal's blood pressure was decreased to approximately 10–20 mmHg and reached a steady state during MS23 infusion, a 250 μl blood sample was colleted from the carotid catheter. Plasma was separated by centrifugation at 1000 g for 10 min and 100 μl plasma was collected and analyzed via the method described above.

3. Results

3.1. Chromatographic separation and UV detection of MS23

MS23 and its related compounds have a structure-specific UV absorption peak at the wavelength of 307 nm (Fig. 1). As shown in Fig. 2, the internal standard S22 and the assay compound MS23 have specific and independent chromatographic spikes when measured using the 307 nm wavelength. Assay of the plasma samples containing 50 ng/ml MS23 resulted in a clear and quantifiable chromatographic spike (Fig. 2C). The retention time for MS23 and S22 were 6.2 and 3.7 min, respectively (Fig. 2C and D). The ratio of the integrated peak areas between MS23 and the internal standard linearly increased as the concentration of MS23 was increased.

3.2. Limit of quantification (LOQ) and Limit of detection (LOD)

Two criteria are used to define 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%) [15]. LOD is defined as the lowest concentration of MS23 at which the signal is larger than three times of the baseline noise [15]. The measured LOQ and LOD values (Table 1) for MS23 using this method are 50 and 20 ng/ml, respectively, which well meet the requirements of validation for a new assay protocol.

3.3. Linearity of the calibration curve

Over the plasma concentration range from 50 to 10,000 ng/ml, regression analysis indicated that there was an excellent linearity between UV absorption and MS23 concentrations ($r \geq 0.9989$). High repeatability (R.S.D. of slope = 1.92%) was observed. The mean and standard deviation (mean ± S.D.) for the slope and intercept were 0.00217 ± 0.0000416 and -0.00275 ± 0.00298, respectively (Table 1). The range of lin-

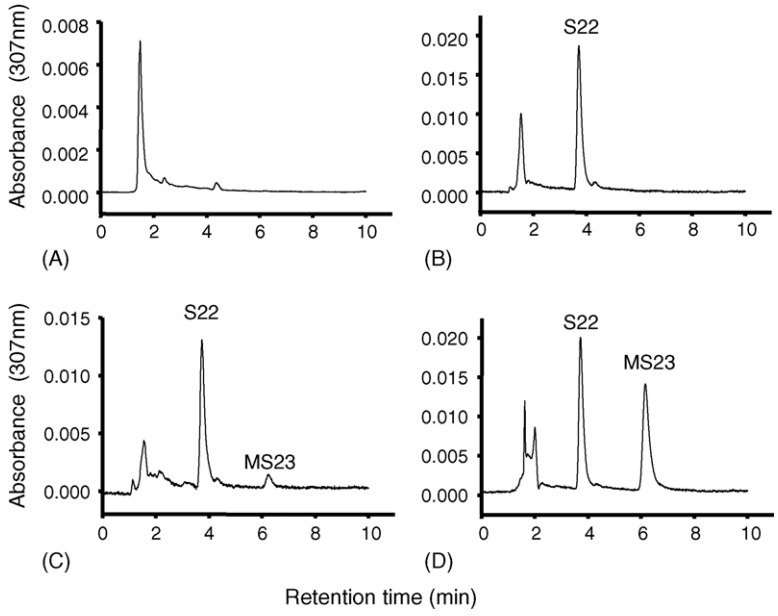

Fig. 2. Representative chromatograms of MS23 and internal standard S22 from plasma samples. Panel A is a blank chromatogram from plasma. Panel B is from a sample only containing internal standard S22 (500 ng/ml). Panel C and D are from samples containing, respectively, 50 and 500 ng/ml MS23 as well as 500 ng/ml S22. The retention times for MS23 and S22 are 6.2 and 3.7 min, respectively.

Table 1
Interday slopes, intercepts and R values of the calibration curves as well as the mean ± S.D. ($n = 5$)

Days	Slope	Slope	Correlation (R)
1	0.00213	−0.00293	0.9989
2	0.00217	−0.0058	0.9998
3	0.00224	0.00221	0.9993
4	0.00218	−0.00339	0.9994
5	0.00215	−0.00384	0.9989
Mean ± S.D.	0.00217 ± 0.0000416	−0.00275 ± 0.00298	0.9993 ± 0.00038
R.S.D. (%)	1.92	108	0.04

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

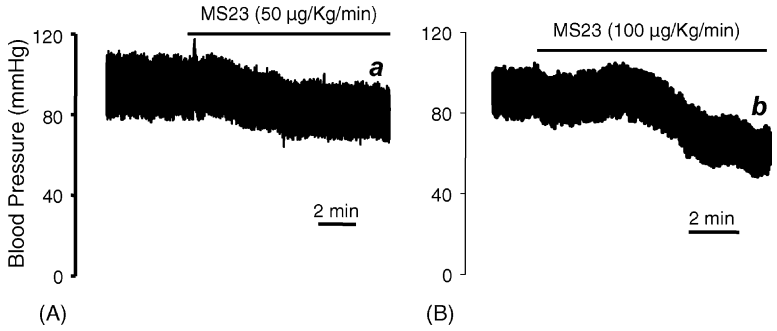

Fig. 3. MS23 infusion-caused blood pressure decrease in anesthetized rats. The corresponding plasma concentrations of MS23 that caused BP decrease at points a and b were 662.5 ± 53.1 and 1171.2 ± 52.5 ng/ml ($n = 4$), respectively.

earity of the calibration curve covers the lowest and highest MS23 concentrations in the plasma that produce meaningful reduction in animal blood pressure in vivo studies (see Fig. 3).

3.4. Precision and accuracy

The acceptable inter- and intraday precisions (relative standard deviation) and accuracy (relative error, RD) were set as <10% and between −5% and 5%, respectively [15]. The calculated R.S.D. and RD values from repeated plasma MS23 measurements are summarized in Table 2. The assay precision was ranged from 0.56% to 6.42% and the accuracy was greater than 96.4% (Table 2).

Table 2
Intra- and interday precision and accuracy of quantifying MS23 (ng/ml) in rat plasma samples using the described HPLC method

Actual concentration	Detected concentration (mean ± S.D., $n = 5$)	Precision (R.S.D.) (%)	Accuracy (RD) (%)
Intraday			
50	49.00 ± 1.80	3.68	98.00
500	504.22 ± 10.21	2.02	100.84
5000	5016.8 ± 322.4	6.42	100.34
Interday			
50	51.76 ± 2.30	4.44	103.53
500	502.56 ± 27.00	5.37	100.51
5000	5016.4 ± 2.8	0.56	100.33

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

3.5. Recovery

Recovery of the analytical method was measured for both MS23 and internal standard. The internal standard had an average recovery of 68.0 ± 2.2% ($n = 9$) in the final elute, and MS23 had an average recovery of 65.6 ± 8.3% at 50 ng/ml ($n = 3$), 64.6 ± 8.1% at 500 ng/ml ($n = 3$), and 66.9 ± 5.2% at 5000 ng/ml ($n = 3$), respectively. We found that there were no other extraction partition lost in washing elution and the recovery for MS23 from the plasma samples was independent of the concentrations of the compound and of the presence of the internal standard S22.

3.6. Stability

The stabilities of MS23 and S22 in aqueous solutions or in plasma were tested following freeze-thaw cycles and after ≥30-day storage [15]. After three freeze-thaw cycles of the stock solutions (10 mg/ml in H_2O), the measured MS23 and S22 were 99.88 ± 1.92% and 100.83 ± 0.21% ($n = 4$) of their initial concentrations, respectively. After the storage of plasma containing 50, 500 and 5000 ng/ml of MS23 and aqueous solution of S22 at −20 °C for ≥30 days, an average of 103.10 ± 7.78% ($n = 9$) of MS23 and of 100.44 ± 0.25% ($n = 4$) of S22 was detected from those samples, respectively.

3.7. In vivo plasma concentrations of MS23

In order to evaluate the applicability of the method for in vivo animal studies, we assayed the plasma MS23 con-

centrations at which the compound caused clinically relevant reduction in blood pressure. Intravenous infusion of MS23 at a rate of 50 μg/kg/min (in a volume of 5 μl/min) for 10 min reduced rat mean arterial blood pressure (MAP) by 11.8 mmHg (Fig. 3A). The measured plasma concentration of MS23 was 662.5 ± 53.1 ng/ml (n = 4). When the infusion rate was increased to 100 μg/kg/min (in a volume of 10 μl/min), the MAP was decreased by 29.0 mmHg. Meanwhile, the plasma concentration of MS23 correspondingly increased to 1171.2 ± 52.5 ng/ml (Fig. 3B).

4. Discussion

We selected S22 as the internal standard because it has a core structure similar to MS23, therefore similar UV absorption properties and stability as MS23. The independent extraction and separation of MS23 from the internal standard S22 warrants the valid quantification of the assay compound using this method.

The solid extraction method yielded reasonable recoveries and clean chromatographic peaks for both MS23 and S22 in plasma samples. MS23 can significantly reduce rat BP with an intravenous injection dose larger than 100 μg/kg [17], although the final elute has an approximate 65% recovery, the well-defined MS23 peaks at low plasma concentrations (20 and 50 ng/ml) demonstrated that the assay protocol has sufficient sensitivity to detect the lowest concentrations of MS23 in animal plasma that produce clinically relevant BP lowering effect.

The perchloric acid concentration used in this assay is 0.467 M, lower than the commonly used 0.6 M for deproteination. We found that this lower concentration of perchloric acid could remove, if not all, most of the protein in the plasma (a sizable centrifuge pellet was formed). Further deproteination was accomplished by the following solid phase extraction using 3M Disk Cartridges (information from 3M's technique support). Thus, the deproteination is sufficient in the protocol. The linear region of the calibration curve (from 50 to 10,000 ng/ml) also envelops the high end of MS23 concentrations in plasma that lowers BP to a level far below the normal range.

The described HPLC assay method needs a plasma volume of 100 μl, which is suitable for carrying out future pharmacokinetic studies in rats or other animals larger than rats. The lower solvent consumption with a short chromatographic run (<8 min), indicates that this method is also suitable for high throughput quantitative analysis, such as monitoring the plasma level of the compound in clinical patients.

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