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Development and Validation of a HPLC Method for MS-153 Quantification: Assessment of its Stability in Rat Plasma and Brain Homogenate

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Abstract

MS-153 is a novel pyrazoline compound that serves as a potential neuroprotective therapeutic agent during ischemia. Development of a convenient, quick, and robust analytical method to quantify MS-153 in biological samples is necessary to understand it's *in vivo* pharmacokinetic/pharmacodynamics (PKPD) profiles. An isocratic reverse-phase HPLC method was developed and validated for quantification of MS-153. Chromatographic separation was achieved with a C18 column. Mobile phase consisting of water/acetonitrile (85/15, v/v) was pumped at a flow rate of 1.0 mL/min. The retention time of MS-153 (λ_{max}=260 nm) was found to be 7.15 minutes. A calibration curve established over a range of 0.78125 ng to 500 ng showed a correlation coefficient of 1.0. The LOD and LOQ were found to be 0.164 and 0.496 ng, respectively. The accuracy, intra-day precision, and inter-day precision were found to be 99.97% to 101.66% (recovery), 0.21% to 0.55% (RSD), and 0.32% to 0.82% (RSD), respectively. MS-153 was analysed from biological samples by adding methanol to remove proteins in the biological matrix prior to HPLC analysis. The extraction efficiency was found to be 100%. The developed method was also used to analyse the stability of MS-153 in diluted blank rat plasma and brain homogenate samples. Results indicated that no significant degradation of MS-153 was observed at 37°C for 6 h.

Keywords: MS-153; HPLC; Ischemia

Introduction

MS-153 ((R)-(−)-5-methyl-1-nicotinoyl-2-pyrazoline) (Figure 1A) is a pyrazoline compound found to have neuroprotective effects in the rat focal cerebral ischemia model [1]. MS-153 is able to suppress the extracellular glutamate accumulation increased during ischemia [2-4]. Research suggests that this compound acts as an activator of glutamate transporter 1 (GLT1), which is a Na^+/K^+ dependent glial glutamate trans membrane protein expressed in COS-7 cells [5,6]. MS-153 has been studied extensively by researchers to gain a better mechanistic understanding about its neuroprotective functions. A recent study revealed that MS-153 interacts with protein kinase C gamma and inhibits high voltage-gated calcium channels, which further prevents the release of glutamate from neurons under ischemic conditions [7,8]. It has also been reported that MS-153 regulates glutamate transmission for potential attenuation of alcohol disorders [9-11]. Among all the studies in which MS-153 was tested *in vivo* using rat and mice, only a few have performed a quantitative analysis of MS-153 in the plasma and/or cerebrospinal fluid (CSF). This is probably due to the lack of validated analytical methods reported in the literature. However, the analytical data is crucial for understanding the pharmacokinetic and pharmacodynamic (PKPD) properties of MS-153 in animal models. Moreover, there is little information on the stability of MS-153 in biological fluids. Therefore, the present study aims to develop, optimize and validate a robust analytical method for the quantification of MS-153 in biological samples. The developed method could also assist future researchers on *in vivo* PKPD analysis of MS-153.

Materials and Methods

Materials

MS-153 [(R)-(−)-5-methyl-1-nicotinoyl-2-pyrazoline] was synthetized in house at Columbia University using a protocol described previously [12]. All HPLC-grade solvents were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). HPLC water was obtained by filtering deionized water through nylon membrane filters (0.45 µm, GE Healthcare, UK). Protein estimation kit was procured from Bio-Rad, Hercules, CA.

Chromatographic apparatus and analytical conditions

A high-performance liquid chromatography system (HPLC) (Waters Alliance 2695 separation module, Milford, MA) equipped with a Kinetex C18 column (250 X 4.6 mm, 5 µm particle size, Phenomenex) and UV/Visible detector was used for analysis. MS-153 samples were analyzed using an isocratic method with a mobile phase containing water/acetonitrile (85/15, v/v) pumped at a flow rate of 1.0 ml/min. The wavelength used for the analysis was 260 nm. All the data acquired were processed using Empower 3 software (Waters, Milford, MA).

Preparation of calibration curve

One mg of MS-153 was accurately weighed using Micro Balance (Mettler Toledo, Columbus, OH) and was dissolved in 1 ml of acetonitrile. This yielded a stock solution with a concentration of 1 mg/ml. The stock solution was diluted using mobile phase to yield

concentrations of 10 and 4 µg/ml. The calibration sample (4 µg/ml) was serially diluted with a dilution factor of four until the least concentrated calibration sample (0.015625 µg/ml) was obtained. This yielded calibration samples with a series of concentrations of 10, 4, 1, 0.25, 0.0625 and 0.015625 µg/ml. Each calibration sample was injected six times and was analyzed using HPLC. The calibration curve was constructed by plotting the average peak area (Y-axis) versus the corresponding drug amount (X-axis).

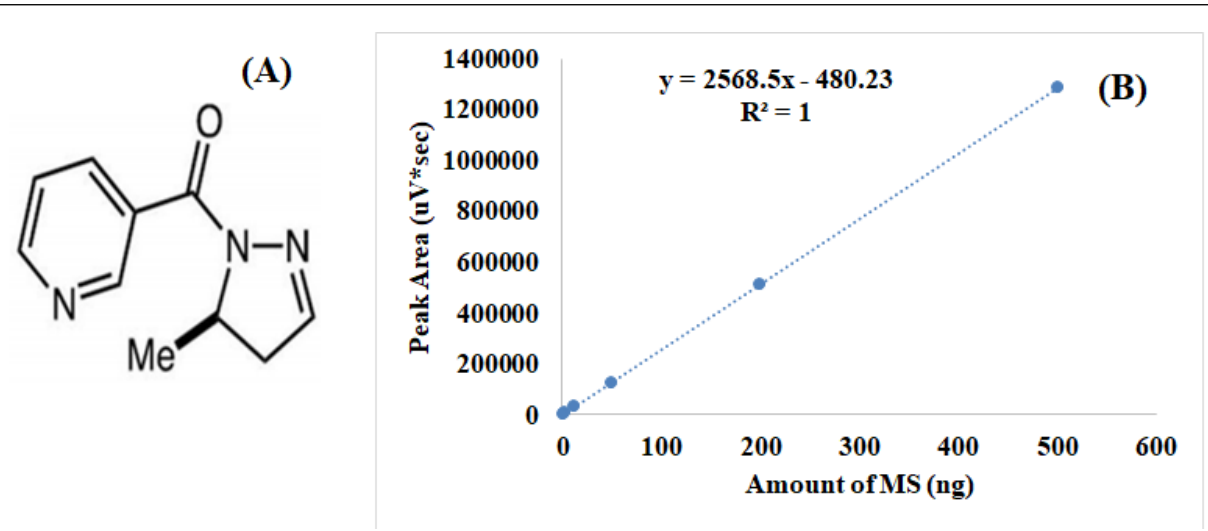

Figure 1: (A) Chemical structure of (R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153), (B) Calibration curve of MS-153 showing linearity over the concentration range of 0.015625 to 10 µg/ml (equivalent to the amount range of 0.78125 to 500 ng when using a volume of 50 µl).

Method validation

Validation of the HPLC analysis method was performed regarding to precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) as per ICH Q2 (R1) method validation guidelines [13].

Accuracy

Samples with three different levels of concentrations were prepared, and accuracy was assessed by analyzing the recovery of these samples. Six replications were performed for each concentration. The recovery was calculated using the following equation: Recovery (%) = (calculated amount of MS-153/known amount of MS-153) x 100.

Precision

Concentration samples of 4 µg/ml, 1 µg/ml and 0.25 µg/ml were used for determining precision. Intra-day precision was assessed as relative standard deviation (RSD) by analyzing the samples with six replicates. Inter-day precision was assessed as RSD by analyzing the same set of samples on two different days.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the signal-to-noise ratio as per ICH Q2 (R1) validation guidelines [13]. Using the six different concentrations (10, 4, 1, 0.25, 0.0625, 0.015625 µg/ml) analyzed with six replicates, six calibration curves were plotted for each replicate. The standard deviation of y-intercepts (peak area) and the slopes of six regression lines were calculated. They were used to determine LOD and LOQ using the following formula: LOD = 3.3*σ/S; LOQ = 10*σ/S, where σ is the standard deviation of y-intercepts of six regression lines; S is the slope of the calibration curve.

Extraction of MS-153 from biological samples

Organic solvents (acetonitrile or methanol) were used to precipitate proteins in biological samples in order to extract MS-153 for analysis. Compared to acetonitrile, methanol produced a better elution profile with less tailing. Therefore, methanol was chosen as the precipitation solvent for drug extraction. Biological samples such as rat plasma or cerebrospinal fluid (CSF) were mixed with an equal volume of methanol, followed by vortexing for 30 seconds in order to fully precipitate out proteins present in samples. The mixture was then centrifuged at 5,000 rpm for 10 min, and the supernatant was analyzed by HPLC.

Extraction efficiency of MS-153 from biological samples

Three known concentrations of MS-153 (4, 1, and 0.25 µg/ml) were prepared in blank rat plasma. Subsequently, they were subjected to the extraction method and were analyzed using the developed HPLC method. The extraction efficiency of MS-153 from blank rat plasma was assessed by looking at the recovery of MS-153. Three replicates were performed for each concentration.

Stability of MS-153 in rat plasma and brain homogenate

Protein contents in rat plasma and brain homogenate were determined using the BioRad protein estimation kit (BioRad, Hercules, CA). Suitable dilutions were made to achieve a final protein concentration of 1 mg/ml. An aliquot (1 ml) of diluted blank rat plasma and brain homogenate was incubated separately with MS-153 (10 µg/ml) at 37°C in a shaking water bath (60 RPM). A one hundred microliter sample was withdrawn at predetermined time intervals, and an equal volume of methanol was added to stop the reaction. The stability studies were performed for up to 6 h, and both the experiments were performed in quadruplicate. The concentration of

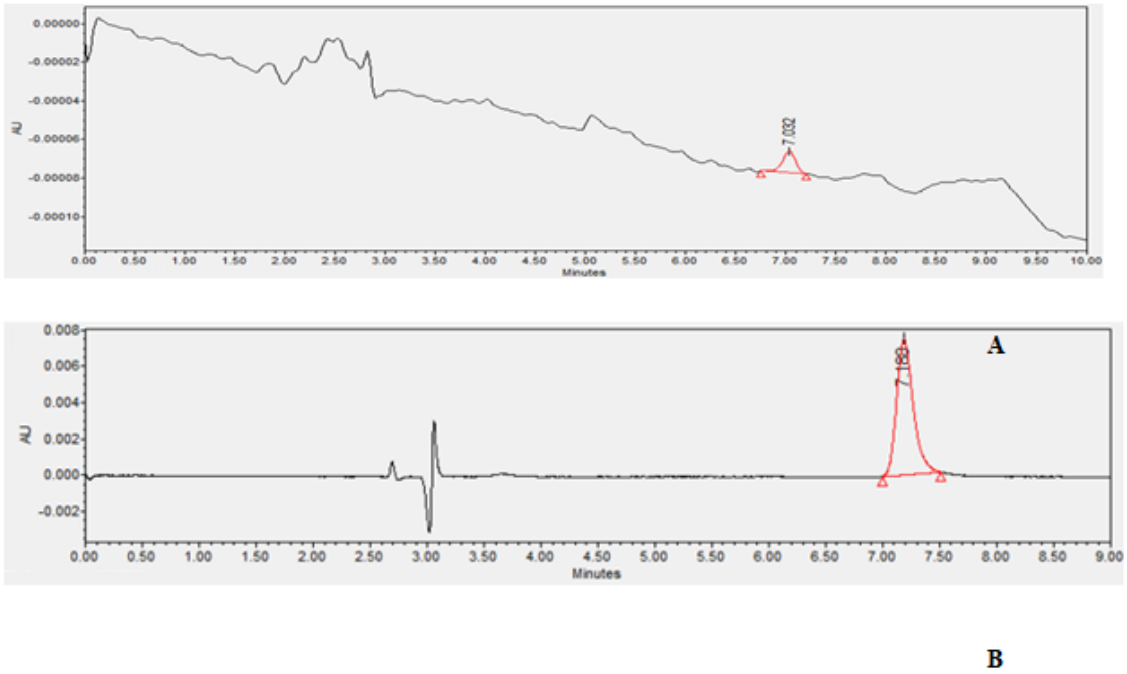

Figure 2: A sample chromatogram of blank (A) and 4 µg/ml calibration standard (B) of MS-153.

Sample concentration (µg/ml)	Injection Volume (µl)	Amount (ng)	No. of Injection	Peak Area (µV*sec)	Calculated Amount (ng)	Recovery (%)
4	50	200	1	518194	201.94	100.97
			2	519301	202.37	101.18
			3	519376	202.40	101.20
			4	519573	202.47	101.24
			5	521724	203.31	101.66
			6	521609	203.27	101.63
1	50	50	1	127904	49.98	99.97
			2	128098	50.06	100.12
			3	128220	50.11	100.21
			4	128350	50.16	100.32
			5	128711	50.30	100.60
			6	128745	50.31	100.62
0.25	50	12.5	1	31621	12.50	99.98
			2	31615	12.50	99.97
			3	31638	12.50	100.04
			4	31830	12.58	100.64
			5	31693	12.53	100.21
			6	31843	12.58	100.68

Table 1: Evaluation of accuracy of the proposed HPLC method for MS-153 quantification.

MS-153 in the samples was analyzed using the developed HPLC method.

Results and Discussion

Development and optimization of HPLC method

The UV absorption spectrum of MS-153 showed maxima (λ_{max}) at 260 nm. This wavelength produced the best signal to noise ratio and was therefore chosen as the wavelength for HPLC analysis. The mobile phase composition was optimized to obtain a well-resolved MS-153 elution peak within a reasonable period. A composition of water/acetonitrile (85/15, v/v) at a flow rate of 1.0 mL/min was found to elute MS-153 at approximately 7.15 minutes. Also, the elution peak of MS-153 was not interfered by impurities present in biological matrix (e.g., plasma and brain homogenate). The peak area was plotted as a function of six different concentrations of MS-153, ranging from 0.015625 to 10 µg/ml (equivalent to the range of 0.78125 to 500 ng when using an injection volume of 50 µl). As shown in Figure 1B, the following calibration equation was obtained: Y=2568.5 X- 480.23, where Y is the peak area of the elution peak and X is the amount of MS-153 in ng. A sample chromatogram of calibration standard (4 µg/ml) is shown in Figure 2. The chromatographic response of MS-153 was linearly proportional to the drug concentration (or drug amount) over the range studied with a correlation coefficient (R^2) of 1.000.

Validation of HPLC method

A set of characteristics of the proposed HPLC method, including precision, accuracy, LOD, and LOQ were further validated to determine the suitability of this method for the quantification of MS-153. The accuracy of an analytical method indicates the closeness between the experimental value and the true value. The accuracy of this proposed HPLC method was evaluated by measuring the recovery of MS-153. The accuracy data is summarized in Table 1. All the runs showed a good recovery, ranging from 99.97% to 101.66%. Precision of

Sample concentration (µg/ml)	Amount (ng) in 50 µl injection volume	No. of Injection	Peak Area (uV*sec)	Ave. Peak Area (uV*sec)	SD	Intra-day RSD(%)	Ave. Peak Area (uV*sec)	SD	Inter day RSD(%)
4	200	1-1	511039	512165.2	1065.26	0.21	516064	4242.21	0.82
		1-2	511552						
		1-3	511452						
		1-4	512175						
		1-5	512868						
		1-6	513905						
4	200	2-1	518194	519962.8	1405.33	0.27			
		2-2	519301						
		2-3	519376						
		2-4	519573						
		2-5	521724						
		2-6	521609						
1	50	1-1	127119	127522.5	703.81	0.55	127930.25	676.67	0.53
		1-2	126944						
		1-3	126918						
		1-4	127416						
		1-5	128660						
		1-6	128078						
1	50	2-1	127904	128338	336.10	0.26			
		2-2	128098						
		2-3	128220						
		2-4	128350						
		2-5	128711						
		2-6	128745						
0.25	12.5	1-1	31645	31674.67	102.27	0.32	31690.67	99.91	0.32
		1-2	31502						
		1-3	31634						
		1-4	31750						
		1-5	31769						
		1-6	31748						
0.25	12.5	2-1	31621	31706.67	104.35	0.33			
		2-2	31615						
		2-3	31638						
		2-4	31830						
		2-5	31693						
		2-6	31843						

Table 2: Evaluation of precision of the proposed HPLC method for MS-153 quantification.

an analytical method reflects the variation of measurements among a series of samplings of the same homogeneous sample. Precision can be considered at different levels, such as intra-day precision, inter-day precision, and precision between different analysts, equipment, or even laboratories. In this study, the inter-day and intra-day precision of the proposed analytical method was determined and was reported as the relative standard deviation (RSD) within runs. As shown in Table 2, the intra-day precision was found to be in the range of 0.21% to 0.55% (% RSD); whereas, the inter-day precision ranged from 0.32% to 0.82% (% RSD). The LOD and LOQ of an analytical method are the lowest quantities of analyte that can be detected and quantified, respectively. As shown in Table 3, the LOD and LOQ of MS-153 were found to be 0.164 and 0.496 ng, respectively.

Extraction efficiency of MS-153 from biological samples

Ideally, the recovery efficiency of MS-153 from biological samples should be near 100%; otherwise, an internal standard could be used to account for the loss of MS-153 during extraction. The recovery of MS-153 for samples prepared in blank rat plasma (4, 1 and 0.25 µg/ml) was found to be between 96.6 and 102.2%, suggesting a complete recovery of the analyte. A sample chromatogram of MS-153 in plasma is shown in Figure 3.

Stability of MS-153 in rat plasma and brain

Stability of MS-153 (10 µg/ml) was carried out in diluted blank rat plasma and rat brain homogenate (with a protein concentration of 1 mg/mL) at 37°C for 6 h. No significant degradation of MS-153 was found in either sample (Figure 4), indicating that MS-153 is stable in diluted rat plasma and brain homogenate at 37°C for a period of 6 h.

Calibration curve #	Calibration curve parameter			SD of intercept	Average of slope
	slope	intercept	R^2		
1	2564.08	-347.87	1.00	127.46	2567.03
2	2567.7	-286.16	1.00		
3	2564.7	-365.51	1.00		
4	2570.9	-145.42	1.00		ng
5	2565.4	-374.48	1.00	LOD	0.164
6	2569.4	-71.27	1.00	LOQ	0.496

Table 3: Assessment of LOD and LOQ of the proposed HPLC method for MS-153 quantification.

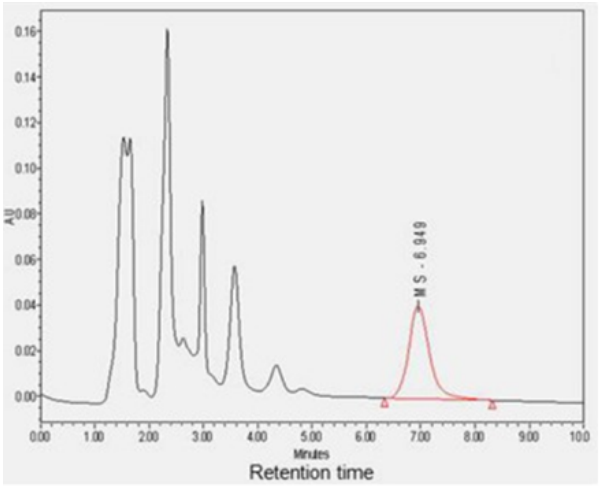

Figure 3: Sample chromatogram of MS-153 in rat plasma.

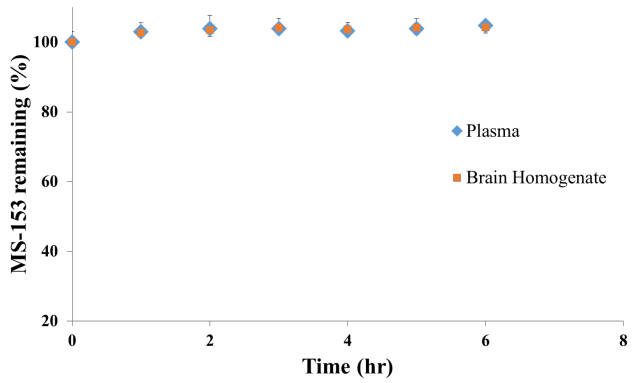

Figure 4: Stability of MS-153 in blank rat plasma and brain homogenate at 37°C.

Conclusion

This study describes an efficient sample extraction method using methanol and a robust HPLC-UV method for the quantification of MS-153 in biological samples. The linearity, LOD, LOQ, precision, and accuracy were evaluated according to the ICH guidelines. The data showed that the proposed HPLC method was suitable to quantify MS-153 with high sensitivity, great accuracy, and reasonable precision. The extraction efficiency was found to be 100%, and the analyte peak was not interfered by impurities in the biological matrix. A stability study on MS-153 in diluted blank rat plasma and CSF using the developed method showed no significant degradation of MS-153 at 37°C for at least 6 h.

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Declaration of Interest

None

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