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Sensitive and selective liquid chromatography/tandem mass spectrometry methods for quantitative analysis of 1-methyl-4-phenyl pyridinium (MPP^+) in mouse striatal tissue

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ABSTRACT

The systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to mice produces a reliable and selective degeneration of the nigrostriatal pathway, a hallmark feature of Parkinson's disease (PD). Determining the brain concentrations of 1-methyl-4-phenyl pyridium (MPP^+), the neurotoxic metabolite of MPTP, is critical for evaluating drugs designed to potentially treat PD. We have developed sensitive and specific quantitative methods for the determination of MPP^+ in mouse striatal tissue by liquid chromatography/tandem mass spectrometry. The separations were carried out based on reversed phase chromatography or cation exchange chromatography with volatile elution buffer. Neutralizing the brain sample with 0.2 M phosphate buffer successfully solved a high-performance liquid chromatography (HPLC) peak tailing of MPP^+ in brain extracts with 0.4 M perchloric acid ($HClO_4$) under the reversed phase HPLC conditions, which significantly improved the sensitivity of the method. The HPLC peak shape of MPP^+ using cation exchange chromatography was not affected by the pH of the samples. Optimization of electrospray ionization (ESI) conditions for the quaternary ammonium compound MPP^+ established the limits of detection (LOD) (S/N = 3) at 0.34 pg/mg tissue and 0.007 pg/mg tissue (5 μl of injection) using the reversed phase liquid chromatography/tandem mass spectrometry (LC/MS/MS) and the cation exchange LC/MS/MS, respectively. Both methods were selective, precise (%R.S.D. < 6%), and sensitive over a range of 0.001–1 ng/mg tissue. The cation exchange method showed greater sensitivity and tolerance to low pH samples than the reversed phase method. The developed methods were applied to monitoring changes in MPP^+ concentrations *in vivo*. Two reference agents, *R*-(−) Deprenyl and MK-801, known to alter the concentration of MPP^+ in MPTP treated mice were evaluated.

1. Introduction

Parkinson's disease (PD) is a very common neurodegenerative disorder most typically characterized by a combination of well defined motor dysfunctions. A prominent neuropathological feature of PD, which underlies some of the motor disturbances, is the degeneration of dopaminergic neurons located in the midbrain, namely the substantia nigra compacta, that gives rise to the nigrostriatal pathway. Quite by accident, it was discovered that the systemic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in humans recapitulates many of the defining clinical features of idiopathic PD as well as the destruction of the nigrostriatal pathway. Consequently the administration of MPTP to experimental animals has been used extensively to study the underlying molecular events of degeneration as well as to evaluate new therapies designed to treat PD [1]. It is well established that the primary step in MPTP toxicity involves the formation of the toxic metabolite 1-methyl-4-phenyl pyridium (MPP^+) by striatal monoamine oxidase B (MAO-B) [2,3], and subsequent uptake of MPP^+ via dopamine transporters into dopaminergic neurons [4]. In the striatal dopaminergic terminal, MPP^+ inhibits mitochondrial respiration, increases the production of free radicals, and activates DA release [5–7]. Accordingly, the brain concentration of MPP^+ is a critical determinant of the extent of neurodegeneration in this model. Therefore, a necessary control, in the evaluation of a putative neuroprotective agent, is to demonstrate that nigrostriatal protection occurs in the absence of lowering MPP^+ concentrations. The majority of analytical techniques reported for the measurement of MPP^+ are based upon use of high-performance liquid chromatography (HPLC) with ultraviolet detection systems (UV) [8–13], in

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which, detection of MPP^+ relies on its chromatographic retention time only. Coupling mass spectrometry with chromatographic separation techniques provides the advantage of detecting MPP^+ by both its chromatographic retention time and the molecular ion. Gas chromatography coupled with mass spectrometry (GC/MS) has been reported for quantification of MPP^+ [14,15]. However, disadvantages of GC/MS assays for detecting MPP^+ in brain tissue include time-consuming sample purification, need for chemical derivatization, and low sensitivity. Several investigators reported the use of LC/MS techniques for MPP^+ measurements [16–18]. However, low sensitivity and matrix interference were the major limitations in these studies.

The most sensitive assay for the analysis of MPP^+ was reported by Hows et al. using LC/MS/MS [19]. The multiple reaction monitoring (MRM) scan mode on a triple quadrupole mass spectrometer monitors specific daughter ions from dissociation of parent molecular ions. This approach combined with the specific LC retention time of analytes provides a sensitive and selective analysis that is unique for individual analytes. In Hows's report [19], the separation of MPP^+ was based on reversed phase chromatography. However, serious peak tailing of MPP^+ (or possible formation of an isomeric form of MPP^+) in the brain extracts with 0.4 M perchloric acid ($HClO_4$) was observed while reproducing this method in our lab, resulting in low sensitivity for detection of MPP^+.

We have developed sensitive and selective quantitative methods for the determination of MPP^+ in brain tissue by LC/MS/MS. The separations were carried out either by reversed phase chromatography or by cation exchange chromatography with volatile elution buffer. In the present work, neutralizing the samples with 0.2 M phosphate buffer solved the chromatographic peak tailing of MPP^+ in low pH samples observed using the reversed phase chromatographic method. The developed methods were applied *in vivo* to detecting changes of MPP^+ concentrations in mouse striatal tissues after administration of MPTP, and co-treatment with reference agents, *R*-(−) Deprenyl or MK-801, which were known to alter the concentration of MPP^+ in MPTP treated mice.

2. Experimental

2.1. Reagents and chemicals

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride, 1-methyl-4-phenyl pyridinium (MPP^+), *R*-(−) Deprenyl hydrochloride, MK-801, and acetyl-β-methylcholine chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Perchloric acid, phosphate, formic acid, LC grade water and acetonitrile were obtained from EM Science (Gibbstown, NJ, USA). Ammonium acetate and ammonium formate were purchased from Sigma (St. Louis, MO, USA).

2.2. Animal and dosing

Animal protocols were performed in accordance with NIH's Guide for the Care and Use of Laboratory Animals and approved by Wyeth's IACUC. Male C57bl/6N mice (Taconic Labs, Hudson, NY) between 8 and 12 weeks old and weighing 20–30 g were housed in groups of 4 at temperature of 20 °C ± 5 °C and were kept on a 12:12-h light:dark cycle with free access to food and water for at least 1 week prior to testing.

All compounds were prepared in saline on the day of the administration. In the study, MPTP was administered ip at a single dose of 40 mg/kg. Vehicle treated mice received saline. Animals were treated with *R*-(−) Deprenyl (10 mg/kg, ip) or MK-801 (1 mg/kg, ip) 30 min prior to MPTP administration.

At 2, 3, or 4 h post MPTP treatment, mice were deeply anesthetized with isoflurane and then perfused through the ascending aorta with saline to remove traces of blood from the brain. Brains were harvested and sliced into 1 mm coronal sections. Striatal regions were dissected from the brain and weighed. Samples were kept on dry ice before storage at −80 °C.

2.3. Mouse brain tissue sample preparation

The mouse brain tissue sample preparations were modified from methods reported in the literature [10–13,17–19]. Mouse striatal samples were homogenized in 0.4 M $HClO_4$ (100 mg tissue/ml). An aliquot of 90 μl of the brain homogenate, 5 μl of water and 5 μl of internal standard (IS) were mixed. An additional neutralization step was added by 10-fold dilution of the above mixture with 0.2 M phosphate buffer. The mixture was centrifuged (14,000 rpm, 4 °C) and filtered (0.2 μm) for 10 min using centrifuge vials with build-in filters. The supernatant was transferred into HPLC vials for subsequent analysis of MPP^+ levels by LC/MS/MS.

2.4. Preparation of standard and QC samples

Stock solutions of MPP^+ were prepared by dilution of a 1 mg/ml of water solution with water. A stock solution of an IS (acetyl-β-methylcholine) was prepared at a concentration of 2 μg/ml in water. The stock solutions were stored at 4 °C. Control brain tissues from mouse striata were homogenized in 0.4 M $HClO_4$ (100 mg tissue/ml). Standard and QC samples were prepared by spiking 5 μl of corresponding standard stock solution and 5 μl of IS into 90 μl of the brain homogenate derived from control brain tissues, and then neutralized by a 10-fold dilution with 0.2 M phosphate buffer. The mixtures were centrifuged (14,000 rpm, 4 °C) and filtered (0.2 μm) for 10 min. The supernatants were transferred into HPLC vials for analysis. Seven-point calibration curves were constructed for MPP^+ in the concentration range of 0.001–1 ng/mg tissue. QC samples at four different concentration levels were used in method validation.

2.5. Liquid chromatography

An Agilent 1100 series HPLC system (Hewlett–Packard GmbH, Waldbronn, Germany) was used in this study. The system consisted of two quaternary pumps, a vacuum degasser, a temperature controlled autosampler, and a thermostated column compartment.

In the reversed phase chromatographic method, the chromatographic separation was carried out using a Waters XTerra MS C18 column (50 mm × 2.1 mm i.d., 3.5 μm particle size) (Waters, Beverly, MA, USA), maintained at 40 °C. The mobile phase consisted of solvent A: 0.1% formic acid in water–acetonitrile (H_2O:ACN = 95:5, v/v) and B: 0.1% formic acid in acetonitrile. The HPLC analysis started with 0% B for 0.5 min, and then followed a gradient from 0 to 80% B in 2.5 min. The flow rate was 0.7 ml/min. The HPLC flow was split before the MS and ~0.2 ml/min effluent was directed into the electrospray ionization (ESI) source of the mass spectrometer. The injection volume was 5 μl.

In the cation exchange chromatographic method, the analytes were separated using a Supelco LC–SCX column (150 mm × 2.1 mm i.d., 5 μm particle size) (Supelco, Bellefonte, PA, USA) at an oven temperature of 40 °C. The mobile phase consisted of A: 15 mM ammonium acetate and 10 mM ammonium formate in water–acetonitrile, pH 4.0 (H_2O:ACN = 80:20, v/v) and B: 15 mM ammonium acetate and 10 mM ammonium formate in water–acetonitrile, pH 4.0 (H_2O:ACN = 20:80, v/v). The HPLC analysis started with a rapid gradient from 0 to 100% B in 2 min, and then held at 100% B for 3 min. The flow rate was 0.6 ml/min. The 0.6 ml/min effluent from the LC column was split before the MS,

and ~0.2 ml/min effluent was directed into the ESI source of the mass spectrometer. The injection volume was 5 μl.

2.6. Mass spectrometry

On-line LC-MS/MS analyses were performed using a Micromass Quatro Micro tandem quadrupole mass spectrometer (Waters, Beverly, MA, USA) operated in positive electrospray ionization mode with the ion source temperature of 120 °C. The ESI ionization of MPP^+ was optimized to a desolvation temperature of 350 °C, a spray voltage of 0.9 kV, and a cone voltage of 40 V. Nitrogen was used as both desolvation (1000 l/h) and nebuliser gas (fully open). The pressure of the argon collision gas was set at 5 psi and adjusted to an analyzer pressure of $2.0–3.0 \times 10^{-4}$ mbar. MRM conditions for analytes were used for analysis. The MRM analyses were preformed by passing molecular ions through the first quadrupole (Q1) and collision dissociating the molecular ions in the second quadrupole (collision cell – Q2). A selected product ion, based on intensity and structure characteristics, was isolated by the third quadrupole (Q3) and detected with the photomultiplier set at 650. The MRM transitions of m/z 170 → 128 for MPP^+ and 160 → 101 for acetyl-β-methylcholine were simultaneously monitored. This approach provided a sensitive and selective analysis that is unique for individual analytes. Acetyl-β-methylcholine was chosen as an IS in the analysis. The concentration of MPP^+ was determined by its area ratio to that of the IS using a linear fit weighting.

3. Results and discussion

3.1. Optimization of mass spectrometry conditions

The majority of organic molecules form protonated molecular ions under positive electrospray ionization. MPP^+ has a quaternary amine functional group, which carries a permanent positive charge. All of the molecules of MPP^+ are ionized, resulting in good sensitivity. The molecular ion (M^+) was the predominant ion in the positive ESI spectrum of MPP^+. The optimized ESI spray voltage for detection of M^+ of MPP^+ was lower than that commonly used ESI spray voltages (typically in a range of 3.0–3.5 kV) for formation of protonated molecular ions of small organic compounds. The spray voltage of 0.9 kV and the cone voltage of 40 V were selected to achieve the optimized ion signal of the molecular ions of MPP^+. Acetyl-β-methylcholine, which also has a quaternary amine functional group, was chosen as an IS because of the same optimal spray voltage required as MPP^+ for electrospray ionization.

Collision-induced dissociation of the molecular ion at m/z 170 of MPP^+ generated more than seven product ions. The substructures of these product ions are assigned as shown in Fig. 1. The product ion at m/z 128 was the most abundant ion and was also a structurally specific product ion (a quinolinium ion) from the dissociation of MPP^+. Therefore, the MRM transition of m/z 170 → 128 for MPP^+ was chosen for quantitative analysis. The MRM transition of 160 → 101 for acetyl-β-methylcholine was simultaneously monitored. These structurally specific MRM transitions associated with the specific HPLC retentions times were used to confirm the identity of MPP^+ and IS in the samples.

3.2. Optimization of chromatographic conditions

MPP^+ is a polar molecule with low molecular weight. The selection criteria of HPLC columns and mobile phase systems are important for detection of MPP^+. Hows et al. [19] reported a LC/MS/MS method for the determination of MPP^+ in brain tissue homogenates using reversed phase chromatography with a Synergie Hydro RP column (150 mm × 2.1 mm, i.d., 4 μm) and a

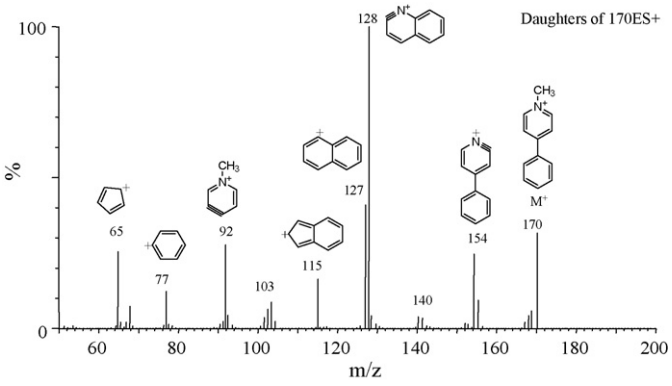

Fig. 1. The product ion spectrum of the molecular ion (M^+) at m/z 170 of MPP^+.

mobile phase consisting of formic acid and acetonitrile. In Hows's paper, the ion chromatogram of MPP^+ in a striatal extract sample contained a matrix peak, which eluted after the MPP^+ peak at 6.2 min. When repeating Hows's HPLC conditions in our lab, we found that the matrix peak in the ion chromatogram of MPP^+ was only observed in brain homogenate samples with 0.4 M perchloric acid ($HClO_4$), not in brain homogenated samples with water. Further investigation found that this matrix peak produced the same molecular ion and the same product ions as MPP^+. These observations suggested that this matrix peak was a serious peak tailing of MPP^+ (or a possible isomeric form of MPP^+) in brain homogenate samples with 0.4 M $HClO_4$, resulting in reduced detection sensitivity for MPP^+.

The use of perchloric acid has been commonly reported in MPP^+ tissue extraction protocols [10–13,17–19]. In order to develop a modified reversed phase chromatography method [19] for analysis of MPP^+ in brain samples, five different reversed phase columns with short column lengths were evaluated. The XTerra MS C18 column (50 mm × 2.1 mm i.d., 3.5 μm) provided the best performance, including the optimal retention time and peak shape of MPP^+ in a water solution, which was subsequently selected for the analysis. Next, the MPP^+ peak shapes in brain samples under different pH conditions were investigated. The results suggested that sample pH significantly affected the MPP^+ peak shapes. As shown in Fig. 2, a symmetric peak of MPP^+ was obtained in a water sample spiked with MPP^+ (2A), but a significant peak tail of MPP^+ was observed in a brain sample with 0.4 M $HClO_4$ (2B). Neutralization of the brain samples with 0.2 M phosphate buffer in a 1:10 ratio (pH = 7.0) sufficiently solved the peak tailing of MPP^+ on the column (2C). As presented in Table 1, the area ratios of the MPP^+ peak in the brain samples to that in the water sample using the XTerra MS C18 column was 11, 90 and 99% at the sample pH of 0.8 (in 0.4 M $HClO_4$), 5.4 (in 1:10 of 0.4 M $HClO_4$:0.1 M K_3PO_4 buffer) and 7.0 (in 1:10 of 0.4 M $HClO_4$:0.2 M K_3PO_4 buffer), respectively. The neutralization method significantly improved the sensitivity of the reversed phase LC/MS/MS method.

The cation exchange chromatographic technique has been commonly used for the separation of small polar molecules. Mobile phase systems containing alkanesulphonates are routinely used in cation exchange chromatography. However, these mobile phase systems are not ideal for electrospray ionization, since they could significantly reduce the ionization efficiency due to ion suppression. A cation exchange chromatographic method using a Supelco LC–SCX column and a mobile phase system with elution buffer, consisting of a mixture of ammonium acetate, ammonium formate and acetonitrile, was developed for the analysis of MPP^+ in the present work. This system was suitable for MS detection. In addition, the MPP^+ peak shape was not significantly affected by the

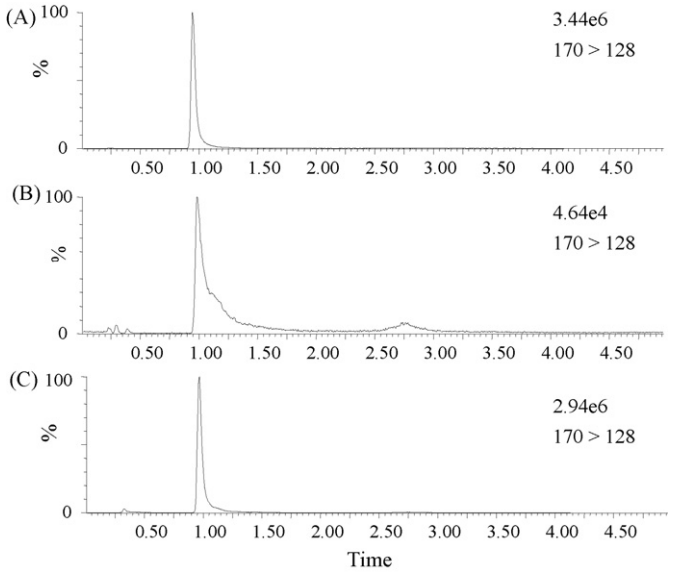

Fig. 2. The MRM chromatograms of MPP^+ using a XTerra MS C18 column and a mobile phase system consisting 0.1% formic acid and acetonitrole in the samples spiked with MPP^+ in: (A) water solution, (B) brain homogenate in 0.4 M $HClO_4$, and (C) brain homogenate in 0.4 M $HClO_4$ after neutralization with 0.2 M phosphate buffer (1:10).

sample pH. The HPLC retention times, the peak shapes and the peak areas of MPP^+ were the same under pH 0.8 and 7.0. These data suggested that sample neutralization is unnecessary using the cation exchange chromatographic method. The cation exchange method showed greater sensitivity and tolerance of the sample pH than the reversed phase method.

3.3. Sensitivity and linearity

The selected mobile phase systems allowed MPP^+ to be ionized with high ionization efficiency under (+)ESI. The MRM mode provided a sensitive and selective analysis that was unique for individual compounds. The limits of detection (LOD) (5 μl injection) were 0.34 pg/mg tissue and 0.007 pg/mg tissue (S:N = 3:1) using the reversed phase and the cation exchange methods, respectively. Based on the brain concentration range of MPP^+ in these studies, the lowest quantification level (LOQ) was selected at 0.001 ng/mg tissue (S:N > 10:1) using both reversed phase and cation exchange methods.

A seven-point standard curve at levels of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1 ng/mg tissue was prepared to establish the calibration range, and the linear regression analysis with $1/x^2$ weighting was applied. Calibration curves of MPP^+ were linear in the concentration range of 0.001–1 ng/mg tissue with the correlation coefficients >0.997 under both reversed phase and cation exchange chromatographic conditions. The quantification range was selected based on the brain concentration range of MPP^+ in the study. A dilution factor of 100 was used to calculate the MPP^+ concentrations in brain tissue. The calculated final concentration range was 0.1–100 ng/mg brain tissue.

3.4. Precision and accuracy

Precision and accuracy measurements were acquired for the QC samples at four different concentrations from three sets of samples, and analyzed on three different days. The intra-day and in inter-day precision and accuracy are summarized in Table 2. The intra-day precision (%R.S.D.) was better than 5.2% at all three concentrations. The value of the inter-day accuracy was in a range of 91.6–105.6%. The inter-day precision and accuracy were determined by pooling all validation data from all QC samples at each concentration. The inter-day precision was better than 6.2%. The value of the inter-day accuracy was in a range of 90.8–106.6%. These results indicate good precision and accuracy.

3.5. Selectivity, matrix effect and stability

In the present work, the standards were prepared using control brain tissues. This could minimize the potential deviations between

Table 1
The peak areas of MPP^+ in the samples with different pH using a XTerra MS C18 column with the mobile phase consisting of 0.1% formic acid and acetonitrile

Sample solution of MPP^+	Sample pH	Peak area of MPP^+	Percent area ratio of the MPP^+ peak in brain sample to that in H_2O sample
1:10 of 1 μg/ml of MPP^+ spiked in H_2O:H_2O (v/v)	7.1	96987	100
1:10 of 1 μg/ml of MPP^+ spiked in brain extract contained 0.4 M $HClO_4$:0.2 M K_3PO_4 buffer (v/v)	7.0	96295	99
1:10 of 1 μg/ml of MPP^+ spiked in brain extract contained 0.4 M $HClO_4$:0.1 M K_3PO_4 buffer (v/v)	5.4	87183	90
1:10 of 1 μg/ml of MPP^+ spiked in brain extract with 0.4 M $HClO_4$:H_2O (v/v)	0.8	10959	11

Table 2
Accuracy and precision of the reversed phase and the cation exchange chromatography/tandem mass spectrometric methods for MPP^+ in QC samples

	Reversed phase chromatography				Cation exchange chromatography			
	0.005[a,b]	0.050[a]	0.200[a]	0.500[a]	0.005[a,b]	0.050[a]	0.200[a]	0.500[a]
Intra-day (N = 5)								
Mean	0.0046	0.0455	0.1937	0.5278	0.0046	0.0466	0.2089	0.4857
Accuracy (%)	91.6	91.0	96.4	105.6	92.2	93.2	104.5	97.14
S.D.	0.00025	0.00155	0.01002	0.02692	0.00019	0.00228	0.00731	0.02089
%R.S.D.	5.0	3.4	5.2	5.1	3.8	4.9	3.5	4.3
Inter-day (N = 15)								
Mean	0.0045	0.0465	0.1935	0.5331	0.0046	0.0473	0.2102	0.4786
Accuracy (%)	90.8	93.0	96.8	106.6	92.2	94.6	105.1	95.7
S.D.	0.00027	0.00172	0.01103	0.02932	0.00023	0.00156	0.0103	0.0235
%R.S.D.	5.4	3.7	5.7	5.5	4.6	3.3	4.9	4.9

[a] Nominal concentration (ng/mg tissue).
[b] Intra-day (N = 3), Inter-day (N = 12).

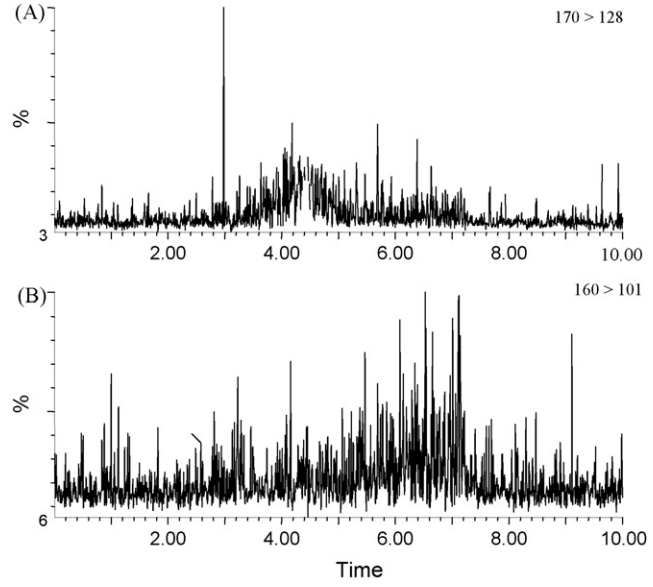

Fig. 3. The MRM ion chromatograms of (A) MPP^+ and (B) IS in mouse brain extracts derived from mice treated with vehicle using the cation exchange method.

the standards and the analyte brain samples caused by the matrix effects on ESI ionization and by the brain sample preparation. Representative MRM ion chromatograms of MPP^+ and IS, obtained from the brain extracts derived from vehicle treated animals using the cation exchange method are shown in Fig. 3. No chemical interference was observed for MPP^+ or IS using the reversed phase or the cation exchange chromatographic method. The MRM ion chromatograms of MPP^+ and IS from the brain tissue homogenates spiked with MPP^+ (0.005 ng/mg tissue) and IS (0.1 mg/mg tissue) using cation exchange chromatographic method are presented in Fig. 4. Utilization of structurally specific MRM transitions associated with the specific HPLC retention times were used to confirm the identity of MPP^+ and IS.

The stability of MPP^+ spiked in control brain homogenates was evaluated at both 4 °C and 25 °C for 24 h. Our observations showed that MPP^+ was stable under these conditions.

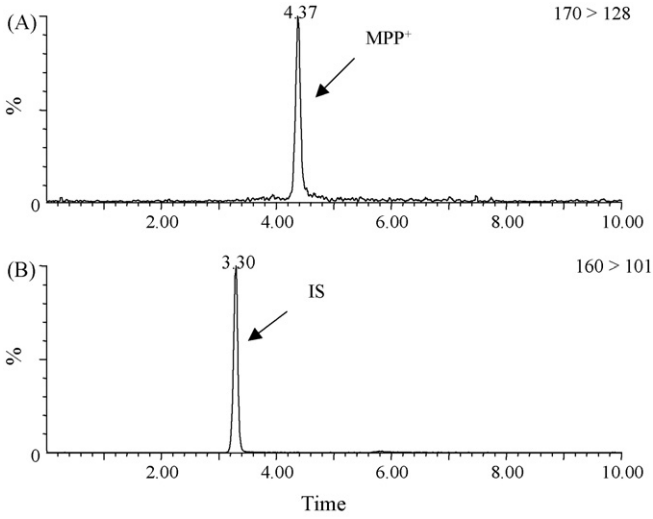

Fig. 4. The MRM ion chromatograms of (A) MPP^+ and (B) IS in mouse brain extracts spiked with MPP^+ (0.005 ng/mg tissue) and IS (0.01 ng/mg tissue) using the cation exchange method.

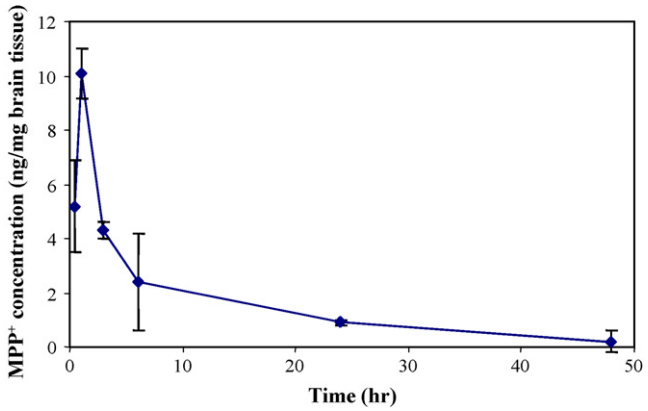

Fig. 5. MPP^+ concentrations in mouse brain striatal extracts ($N = 3$) following intraperitoneal administration of MPTP at the dose of 40 mg/kg measured using the cation exchange method.

The matrix effects of MPP^+ and IS from brain extracts were evaluated by comparing the area ratios of MPP^+ spiked in brain extracts to that in water. Using the cation exchange chromatographic method, the peak area ratios of MPP^+ in the brain extracts to that in water were 89.5 ± 7%, 94.7 ± 8% and 93.2 + 6% at levels of 0.005, 0.02 and 0.5 ng/mg tissue, respectively. The peak area ratio of the IS (0.1 ng/mg tissues) in the brain extract to that in water was 96.8% ± 6%. The data also suggested that there were no significant matrix effects for MPP^+ and IS in the brain extracts.

4. Application

4.1. Measurement of MPP^+ in mouse striatal tissue after treatment with MPTP

The developed sample preparation method and the LC/MS/MS methods were used to assess the temporal profile of MPP^+ concentrations in the mouse striatal tissue during the 48 h after treatment with MPTP (40 mg/kg, ip) (Fig. 5). The MPP^+ concentrations measured by the reversed phase method and the cation exchange method were in a good agreement. As previously described, MPTP administration resulted in the formation of MPP^+, the neurotoxic metabolite of MPTP, which reached the C_{max} of 10.1 ± 0.9 ng/mg tissue at 1 h after MPTP injection and rapidly decreased to 4.3 ± 0.3 ng/mg tissue at 3 h measured using the cation exchange method. The observation of a rapid decrease in MPP^+ concentrations after administration of MPTP is consistent with the previous report by Vaglini et al. [10] using HPLC with UV detection. One of the advantages of the LC/MS/MS methods described in the present work over the HPLC/UV methods is that LC/MS/MS methods allow unequivocal identification of MPP^+. In addition, the LC/MS/MS methods provided higher sensitivity than the LC/UV methods. The present study showed that the MPP^+ concentration further decreased to 2.4 ± 0.8 ng/mg tissue and 0.9 ± 0.1 ng/mg tissue at 6 and 24 h post MPTP administration, respectively. The MPP^+ concentration was below 0.1 ng/mg tissue after 48 h post dosing of MPTP. In comparison, the MPP^+ levels after 6 h post dosing of MPTP were undetectable using LC/UV method [10].

4.2. Effect of R-(−) Deprenyl and MK-801 on MPP^+ levels in mouse striatal tissue after treatment with MPTP

These analytical methods were applied to detect changes of MPP^+ concentrations in mouse striatal tissues after administration of MPTP, subsequent to the pre-treatment with reference

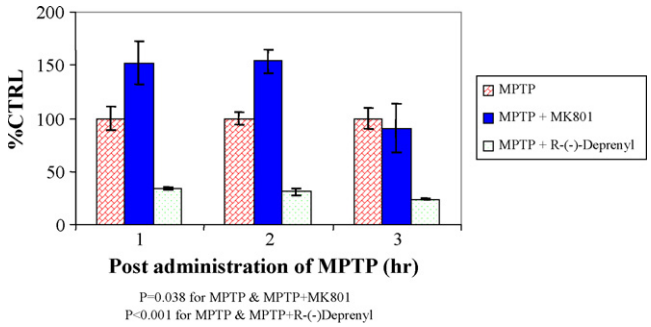

Fig. 6. MPP^+ concentrations in mouse brain striatal extracts ($N = 3$) following treatment of MPTP (40 mg/kg, ip), co-treatment of MPTP (40 mg/kg, ip) and MK-801 (1 m/kg, ip), and co-treatment of MPTP (40 mg/kg, ip) and *R*-(−) Deprenyl (10 mg/kg, ip) measured using the cation exchange method.

agents, *R*-(−) Deprenyl or MK-801, which were known to alter the concentration of MPP^+ in MPTP treated mice. Both methods provided consistent results, however, only the results using the cation exchange method were presented here. *R*-(−) Deprenyl, a MAO-B inhibitor that prevents the metabolism of MPTP to MPP^+, was administered 30 min prior to MPTP. Co-administration of *R*-(−) Deprenyl significantly reduced MPP^+ concentrations in mouse striatum as compared to animals treated with MPTP alone (Fig. 6) ($N = 3$, $P < 0.001$). In particular, *R*-(−) Deprenyl suppressed MPP^+ levels at all time intervals. Conversely, co-treatment with MK-801, a non-competitive NMDA receptor antagonist, significantly enhanced the MPP^+ concentrations in the striatum 2 h after the administration of MPTP, but did not prolong the half-life of MPP^+ (Fig. 6) ($N = 3$, $P = 0.034$). Thus, the concentrations of MPP^+ at 3 h were similar between the MK-801/MPTP group and the vehicle/MPTP group. These results are consistent with previous publications showing that MPP^+ concentrations are reduced with L-Deprenyl and increased with MK-801 [10]. It has been speculated that MK-801, functioning as an NMDA antagonist, may diminish glutamatergic excitation of nigrostriatal terminals [20], thus prolonging MPP^+ storage in terminal vesicles in the striatum [10,21]. L-Deprenyl has been shown to be neuroprotective in the MPTP model [22], and there are conflicting reports regarding the efficacy of MK-801 [23,24]. *R*-(−) Deprenyl reduces concentrations of MPP^+, the neurotoxic metabolite responsible for the loss of dopaminergic terminals in the striatum. However, a wide range of other activities have been postulated that may contribute to its neuroprotective profile, including the prevention of free radical formation, increased antioxidant activities and enhanced synthesis of trophic factors [25]. MK-801, despite transiently increasing MPP^+ concentrations in the striatum, is neuroprotective by presumably blocking downstream events that lead to glutamate toxicity mediated through the NMDA receptor. One of the pathogenic activities of MPP^+ is to inhibit mitochondrial function, thereby reducing ATP production. Ultimately this leads to neuronal depolarization, resulting in the liberation of the voltage dependent Mg^{2+} block of the NMDA receptor [23].

5. Conclusion

A brain sample preparation method, a reversed phase LC/MS/MS method and a cation exchange LC/MS/MS method have been developed for measurement of MPP^+ in mouse striatal tissue. The current method provides higher detection sensitivity than that reported using HPLC methods based on UV detection. In addition, our LC/MS/MS method provides higher accuracy by identifying the analytes using structurally specific MRM transitions associated with the selective HPLC retention times. Neutralizing the brain sample with 0.2 M phosphate buffer successfully solved the peak tailing of MPP^+ in the brain extracts with 0.4 M $HClO_4$, observed under the reversed phase HPLC conditions. The sample neutralization step significantly improved the sensitivity of the method. Both cation exchange and reversed phase methods were suitable for quantification of MPP^+ in brain tissues. The cation exchange method showed greater sensitivity and tolerance of the sample pH than the reversed phase method, which provided the advantages for quantification of MPP^+ in brain tissue, especially for the studies containing low levels of MPP^+. The reversed phase method using 0.1% FA in ACN/H_2O required minimum maintenance of the ion source and the HPLC system, which could be an advantage for the studies containing large number of samples.

The developed methods were applied *in vivo* to detecting MPP^+ concentrations in mouse striatal tissues after administration of MPTP, and co-treatment with *R*-(−) Deprenyl or MK-801. These methods appear to be sensitive and specific and have been successfully applied to measure MPP^+ concentrations in brain tissues in the process of evaluating potential novel agents designed to treat PD.

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