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CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR THE NIGROSTRIATAL TOXICANT 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE AND ITS MONOAMINE OXIDASE B GENERATED METABOLITES IN BRAIN TISSUES

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SUMMARY

This paper describes a sensitive (1 pmol/mg tissue) and selective bioanalytical method for the quantitative estimation of the nigrostriatal toxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its monoamine oxidase B generated metabolites, the 1-methyl-4-phenyl-2,3-dihydropyridinium species $MPDP^+$ and the 1-methyl-4-phenylpyridinium species MPP^+. The method is based on initial separation of the analytes after treatment of brain tissue homogenates with 5% trichloroacetic acid. The soluble fraction is analyzed directly by cation-exchange high-performance liquid chromatography employing a diode array UV detector. Results obtained with this assay have provided the first evidence for the presence of $MPDP^+$ in the mouse brain following intravenous administration of MPTP.

INTRODUCTION

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (I in Fig. 1) destroys the dopaminergic cells of the substantia nigra and thereby causes a syndrome in man and subhuman primates which resembles that of idiopathic Parkinson's disease[1,2]. The neurotoxic effects of this cyclic tertiary allylamine are dependent on its monoamine oxidase B (MAO B) catalyzed biotransformation to the 1-methyl-4-phenyl-2,3-dihydropyridinium species $MPDP^+$ (II), an intermediary metabolite which undergoes further oxidation to the 4-electron oxidation product, the 1-methyl-4-phenylpyridinium species MPP^+ (III)[3–5]. Although studied extensively during the past two years[6], the molecular events ultimately responsible for the cell death caused by MPTP remain poorly understood.

A major effort in this field has focused on characterizing the toxic properties of the chemically stable pyridinium metabolite MPP^+[7–9]. By contrast, little is known about $MPDP^+$, the primary MAO B generated metabolite of MPTP. Its inherent chemical instability[5] suggests that this dihydropyridinium species may be only a transient intermediate in the overall conversion of MPTP to MPP^+. On the other hand,

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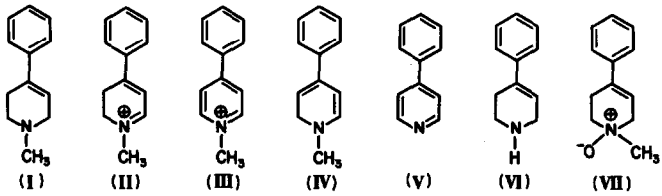

Fig. 1. Chemical structures of MPTP, metabolites and related compounds.

at physiological pH, $MPDP^+$ should exist in part as its lipophilic conjugate free base, 1-methyl-4-phenyl-1,2-dihydropyridine (IV). Therefore, the biodisposition characteristics and potential interactions of $MPDP^+$ with membrane bound macromolecules may influence the sequence of events leading to nigrostriatal cell damage. These considerations led us to undertake a series of studies designed to evaluate the *in vivo* behavior of metabolically generated $MPDP^+$. Since reported assays for MPTP and its metabolites [gas chromatography–mass spectrometry (GC–MS)[7,10], reversed-phase high-performance liquid chromatography (HPLC) with UV[3,4], electrochemical[11] or radiometric detection[12]] do not measure $MPDP^+$ directly, we have developed the present cation-exchange HPLC–diode array assay. With the aid of this assay we have obtained preliminary data on the biodisposition of MPTP, $MPDP^+$ and MPP^+ in mice treated intravenously with MPTP.

EXPERIMENTAL

Chemicals and reagents

MPTP hydrochloride (MPTP–HCl), 4-phenylpyridine (4-PP, V) the hydrochloride salt of 4-phenyl-1,2,3,6-tetrahydropyridine (PTP, VI), sodium acetate and trichloroacetic acid (TCA) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Triethylamine hydrochloride (TEA–HCl) was obtained from Eastman Kodak (Rochester, NY, U.S.A.) and 10% Intralipid from Kabivitrum (Alameda, CA, U.S.A.). All other chemicals were of reagent grade or, in the case of solvents, HPLC grade. Syntheses of MPTP N-oxide (VII)[13], the perchlorate salt of $MPDP^+$ (ref. 5), and the iodide salt of MPP^+ (ref. 14) have been described previously.

Procedures for bioanalysis

Extraction procedures. Male C 57 black mice (7–9 months old) purchased from Jackson Labs. (Bar Harbor, ME, U.S.A.) were treated intravenously with 10 mg/kg MPTP–HCl dissolved in Intralipid–ethanol (90:10). The animals were sacrificed at various times after injection and the brains removed rapidly. The following three brain regions were dissected according to literature procedures[15] and placed immediately on dry ice: two 25-mg sections (right and left brain halves) containing the neostriatum; a 50-mg section containing the substantia nigra; and a 50-mg section of cortex. The corresponding tissues isolated from two mice were combined and homogenized in five volumes of cold 5% TCA. To each homogenate was added 4-PP (25 ng/mg tissue) as internal standard. After standing 1 h at 0–2°C to allow complete precipitation of protein, the homogenate was centrifuged at 13000 *g* for 5

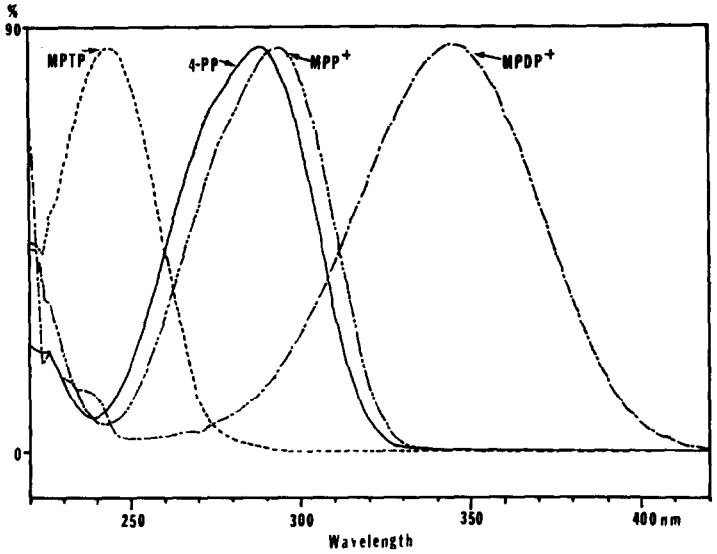

Fig. 2. A composite of the UV spectra obtained with the 4-analytes by HPLC–diode array analysis.

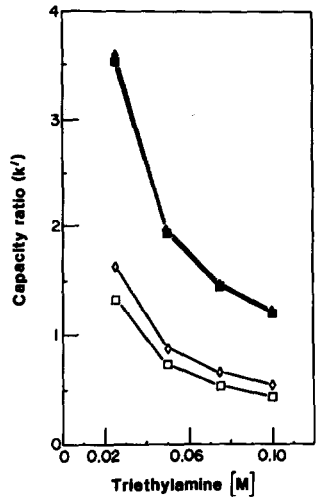

Fig. 3. Separation possibilities of analytes: relationship between capacity ratios (k') and triethylamine–HCl content of mobile phase. Column: Ultrasil SCX (10 μm, 25 cm × 4.6 mm I.D.); eluent: 90% 0.1 *M* acetic acid, 10% acetonitrile, and triethylamine, adjusted to pH 2.3 with formic acid; flow-rate: 1.5 ml/min. □, MPTP; ◆, $MPDP^+$; ■, MPP^+; ◇, 4-PP.

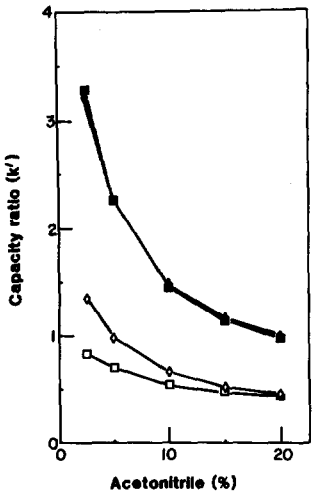

Fig. 4. Separation possibilities of analytes: relationship between capacity ratios (k') and acetonitrile content of mobile phase. Column: Ultrasil SCX (10 μm, 25 cm × 4.6 mm I.D.); eluent: 0.1 *M* acetic acid (80–100%), 0.075 *M* TEA–HCl, and acetonitrile, adjusted to pH 2.3 with formic acid; flow-rate: 1.5 ml/min. □, MPTP; ◆, $MPDP^+$; ■, MPP^+; ◇, 4-PP.

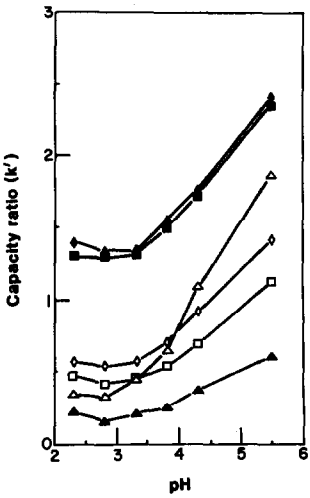

Fig. 5. Separation possibilities of analytes: relationship between capacity ratios (k') and pH of mobile phase. Column: Ultrasil SCX (10 μm, 25 cm × 4.6 mm I.D.); eluent: acetic acid–acetonitrile (85:15), 0.05 *M* TEA–HCl, adjusted to various pH values with 0.1 *M* acetic acid, formic acid or sodium acetate; flow-rate 1.5 ml/min. □, MPTP; ◆, $MPDP^+$; ■, MPP^+; ◇, 4-PP; ▲, PTP; △, MPTP-N-oxide.

min. The supernatant fraction was filtered through a 0.2-μm nylon filter and 50–200 μl of this filtrate was analyzed by HPLC.

Chromatography of biological extracts. HPLC analyses were performed with a Beckman model 114M chromatograph equipped with a Rheodyne 7125 injection valve and Altex Ultrasil SCX column (10 μm, 25 cm × 4.6 mm I.D.). The mobile phase was delivered at a flow-rate of 1.5 ml/min and consisted of a mixture of 90% 0.1 *M* acetic acid and 0.075 *M* TEA–HCl (adjusted to pH 2.3 with formic acid) and 10% acetonitrile. HPLC effluents were analyzed with the aid of a Hewlett-Packard model 1040A diode array detector. Each analyte was monitored at the wavelength which corresponded to its maximum absorption as follows: λ_{max} = 244 nm (ε = 12000) for MPTP; 295 nm (ε = 18000) for MPP^+; and 345 nm (ε = 17000) for $MPDP^+$. The HPLC–diode array UV spectra obtained for these compounds are displayed in Fig. 2. The 4-PP internal standard [λ_{max} = 288 nm, (ε = 17000)] was monitored at 295 nm. The influence of the mobile phase concentrations of TEA–HCl, acetonitrile and H^+ on the chromatographic behavior of each analyte was examined (see Figs. 3–5).

Quantitation. The assay was standardized with the aid of calibration curves which were constructed by analyzing 0.5 ml of drug-free brain tissue homogenates (10% in 0.32 *M* sucrose) spiked with known amounts of the analytes and processed as described above. Peak height ratios of analytes (1–25 ng/mg tissue) *vs.* the 4-PP internal standard (25 ng/mg tissue) were used. Tissue concentrations of the three analytes in the unknown samples were determined by comparing measured peak height ratios to those of the calibration curves. Recoveries were determined by comparing peak height ratios of spiked brain samples with samples prepared in an identical way but without the addition of brain homogenate.

RESULTS AND DISCUSSION

Chromatographic behavior

The chromatographic behavior of MPTP, $MPDP^+$, MPP^+ and 4-PP standards was examined with a variety of columns including various reversed-phase and normal-phase systems. Preliminary results indicated that the best peak profiles could be realized with a cation-exchange column (Altex Ultrasil SCX) employing an aqueous acetonitrile mobile phase which initially was buffered at pH 4.0 with 0.1 *M* sodium acetate/acetic acid. Subsequently, we discovered that the quality of the chromatograms could be improved by adding TEA–HCl to the mobile phase. Additional improvements could be made by varying the percentage of acetonitrile and by adjusting the pH of the final solution to 2.3 with formic acid. The effect of each of these parameters on the observed chromatographic characteristics of the three analytes and internal standard was examined systematically. The results of these studies are summarized in plots of k' (the capacity ratio) *vs.* each factor (Figs. 3–5). We found that acceptable peak shape characteristics were obtained at k' values of less than 2. On the other hand, overlap of the analyte peaks with early eluting compounds derived from the biological matrix required selection of k' values greater than 0.3. The relative retention volumes of MPTP, $MPDP^+$, MPP^+ and 4-PP as well as those of MPTP N-oxide and PTP (MPTP metabolites formed in the liver[13]) were quite sensitive to pH. The best separation was achieved at pH 2.3.

Based on these data, a mobile phase consisting of 90% 0.1 *M* acetic acid and 0.075 *M* TEA–HCl (pH adjusted to 2.3 with formic acid) and 10% acetonitrile was selected. The chromatographic characteristics of MPTP, $MPDP^+$ and MPP^+ are illustrated with a mixture of standards in Fig. 6A. It will be noted that under these chromatographic conditions, MPP^+ and $MPDP^+$ have overlapping retention volumes. As shown in Fig. 6B, however, MPP^+ does not absorb light at 345 nm (the λ_{max} value for $MPDP^+$) and therefore the height of the peak monitored at 345 nm provides a direct measure of $MPDP^+$ extracted from the tissue. Since the chromophore for $MPDP^+$ extends below 295 nm (Fig. 6C), estimations for MPP^+ at 295 nm must be corrected by substracting 20% of the height of the 345 nm peak from

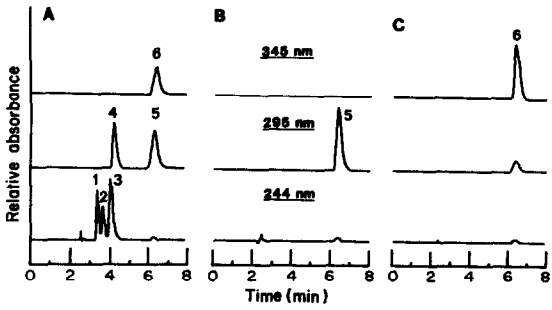

Fig. 6. Liquid chromatographic trace from the injection of a mixture of MPTP and its metabolites. Column: Ultrasil SCX (10 μm, 25 cm × 4.6 mm I.D.); eluent: 90% 0.1 *M* acetic acid, 10% acetonitrile, 0.075 *M* TEA–HCl, adjusted to pH 2.3 with formic acid; flow-rate: 1.5 ml/min. (A) mixture of PTP (1), MPTP-N-oxide (2), MPTP (3), 4-PP (4, internal standard), MPP^+ (5), $MPDP^+$ (6). (B) MPP^+. (C) $MPDP^+$.

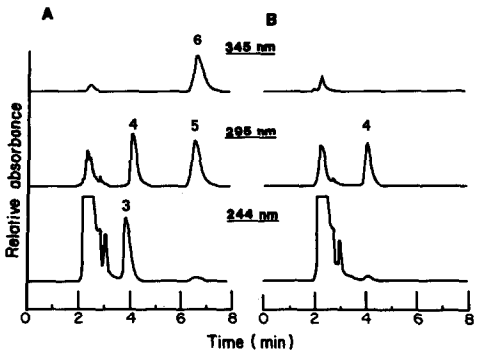

Fig. 7. Liquid chromatographic trace from the injection of an extract from mouse brain tissue isolated 30 min after administration of 10 mg/kg MPTP i.v. (A), or administration of saline (B). Chromatographic conditions were as described.in Fig. 6.

the height of the 295 nm peak. Although 4-PP and MPTP have similar elution volumes, no significant overlap of these two species occured under these conditions. The lower limits of detection for on column injections of standard MPP^+, $MPDP^+$ and MPTP were found to be 5, 5 and 10 ng, respectively. Additionally, we found that MPTP N-oxide and PTP have retention volumes of 0.35 and 0.22, respectively (Fig. 5), and do not interfere with the present assay. A typical chromatographic tracing of a brain extract obtained from a mouse treated with MPTP is shown in Fig. 7A. The corresponding "background" tracing obtained with a brain sample isolated from an untreated animal (Fig. 7B) is included to show that no interfering substances are extracted from the tissue.

Sample preparation

$MPDP^+$ is an unstable compound which, at neutral pH, undergoes autoxi-

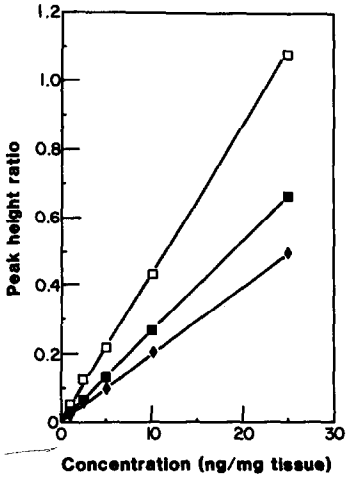

Fig. 8. Calibration curves for MPTP (□), $MPDP^+$ (◆) and MPP^+ (■). See legend to Fig. 6 for chromatographic conditions.

TABLE I

EXTRACTION RECOVERY

Mouse brain homogenates (10%, w/v) were prepared in 0.32 *M* sucrose. Known amounts (20, 50 and 100 ng) of the test compounds were added to 0.5-ml aliquots of the homogenate. To each sample cold 5% TCA (0.5 ml) and 4-PP (internal standard, 200 ng) were added and the resulting mixtures processed as described in the Experimental section. The recovery efficiencies were estimated by comparing the peak height ratios of these tracings with those obtained using 0.32 *M* sucrose solutions.

Test compound	$\bar{x}$ *(%)*	*S.D. (%)*	*n*
MPTP	88.4	6.1	3
$MPDP^+$	95.3	8.3	4
MPP^+	89.0	6.7	3

dation[16] and concentration-dependent disproportionation[5]. At low pH, however, $MPDP^+$ is relatively stable. Thus, the 5% TCA used to precipitate protein also stabilizes the $MPDP^+$ present in the tissue extract. Analysis of extracts of freshly prepared brain homogenates spiked with the perchlorate salt of $MPDP^+$ showed no MPP^+ or MPTP even after standing at room temperature for 1 h.

Recovery data

Calibration curves (Fig. 8) displayed good linearity. Recovery values for all three compounds (Table I) averaged about 90%. Using this method, 0.1 ng/mg of tissue (0.6 pmol/mg) of $MPDP^+$ and MPP^+, and 0.5 ng/mg tissue (3 pmol/mg) of MPTP could be estimated accurately using pooled brain sections isolated from two treated mice.

Brain levels of MPTP and its metabolites

The time-dependent changes of the concentrations of MPTP and MPP^+ in the

TABLE II

QUANTITATIVE ANALYSIS OF MPTP, $MPDP^+$ AND MPP^+ (ng/mg TISSUE) IN MOUSE BRAIN NEOSTRIATUM (NS), SUBSTANTIA NIGRA (SN) AND CORTEX (C) FOLLOWING INTRAVENOUS ADMINISTRATION OF MPTP (10 mg/kg)

Time (min)	*MPTP*			*$MPDP^+$*			*MPP^+*		
	NS	*SN*	*C*	*NS*	*SN*	*C*	*NS*	*SN*	*C*
10	3.35	3.70	3.30	6.00	5.48	4.18	3.08	3.30	2.30
	3.63	3.28	3.05	6.10	5.80	4.23	2.45	2.90	1.85
30	0	0	0	1.40	0.79	0.59	6.08	5.00	2.85
	1.33	0.78	1.03	2.88	1.20	1.30	6.60	6.10	4.08
60	0	0	0	0.20	0	0	6.45	5.05	3.03
	0	0	0	0.68	0.28	0.25	8.00	5.95	3.80
120	0	0	0	0	0	0	3.78	2.25	1.15

various brain regions examined (Table II) are similar to those published by Rollema *et al.*[11] and Shih and Markey[10]. MPTP is detectable only at the earliest time point while MPP^+ concentrations peak at about 60 min. Also of interest is the apparent localization of MPP^+ in the dopamine-containing neostriatum and substantia nigra regions of the brain compared to the cortex. The limited data available suggest that this property is not shared by MPTP.

Results from these studies also establish that $MPDP^+$ is not a transient brain metabolite of MPTP. Although it appears that $MPDP^+$ does not accumulate in mouse brain, the relatively high brain concentrations found at 10 min suggests that the rate limiting step in the conversion of MPTP to MPP^+ is that involving the oxidation of $MPDP^+$ to MPP^+. This conclusion is consistent with the reported relative rates of MAO B catalyzed conversion of MPTP to $MPDP^+$ *vs.* $MPDP^+$ to MPP^+ (about 30:1)[16].

CONCLUSIONS

The bioanalytical method described in this paper extends currently available capabilities in the MPTP field to include quantitative estimations of $MPDP^+$, the primary metabolite produced in the MAO B catalyzed bioactivation of of MPTP. Preliminary applications of this technique have led to the identification of $MPDP^+$ in brain tissues isolated from mice which have been treated with MPTP. In fact, 10 min after administration of MPTP the concentrations of this intermediary metabolite are considerably higher in all brain regions analyzed than both the parent compound itself and MPP^+. It also is of interest to note that the half lives of all three compounds in mouse brain appear to be quite short which may account for the high doses of MPTP required to cause nigrostriatal lesions in this species[17]. Comparison of these biodisposition characteristics with those in animals known to be sensitive to MPTP could lead to a better understanding of the remarkable species selectivity associated with the neurotoxic properties of this compound.

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