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Studies on the uptake of N-methylisoquinolinium ion into rat striatal slices using high-performance liquid chromatography with fluorimetric detection

Y. HIRATA* and M. MINAMI
Department of Hygiene and Public Health, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113 (Japan)
and
M. NAOI and T. NAGATSU
Department of Biochemistry, Nagoya University School of Medicine, Nagoya 466 (Japan)
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SUMMARY

A simple and sensitive procedure for the measurement of N-methylisoquinolinium ion ($NMIQ^+$), a putative neurotoxin, was devised using high-performance liquid chromatography (HPLC) with fluorescence detection. Separation of $NMIQ^+$ was carried out by gel filtration and reversed-phase HPLC on a column of hydrophilic polymer gels (Asahipak GS-302H). The method was sensitive enough to measure 50 fmol of $NMIQ^+$. Uptake of $NMIQ^+$ into rat striatal slices was confirmed by this method.

INTRODUCTION

N-Methyl-1,2,3,6-tetrahydropyridine (MPTP) induces the degeneration of dopaminergic neurons, especially in nigro-striatal pathways in humans[1,2] and monkeys[3], resulting in a syndrome similar to Parkinson's disease. MPTP is first oxidized to 1-methyl-4-phenylpyridinium ion (MPP^+) by monoamine oxidase (MAO)[4] and then taken up into dopaminergic neurons through the dopamine reuptake system[5]. Although the molecular basis of this toxicity to destroy the nigro-striatal system has not been well elucidated, the discovery of MPTP has led to extensive studies in attempts to find similar neurotoxic substances that produce parkinsonism.

We have already reported that MPTP and MPP^+ inhibited tyrosine hydroxylation, the rate-limiting step of the biosynthesis of catecholamines, in rat striatal slices[6,7]. In addition to MPP^+, several pyridinium ions structurally related to MPP^+ were found to inhibit tyrosine hydroxylation[8]. Among them, N-methylisoquinolinium ion ($NMIQ^+$) was suggested to be a possible naturally produced neurotoxin that might

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induce parkinsonism. Both $NMIQ^+$ and MPP^+ were found to inhibit monoamine oxidase[9]. Thus, $NMIQ^+$ inhibits MAO-B non-competitively with respect to the amine substrate, whereas it inhibits MAO-A in competition with the substrate. On the other hand, the presence of tetrahydroisoquinoline as a novel endogenous amine in the rat brain and also in the brain of both normal and parkinsonian human subjects has recently been documented[10,11]. In addition, the administration of tetrahydroisoquinoline to mice and marmosets resulted in decreases in dopamine, biopterin, tyrosine hydroxylase activity and/or 3,4-dihydroxyphenylacetic acid, a dopamine metabolite[12,13]. These results led us to study the mechanism of toxicity of isoquinoline compounds.

Tetrahydroisoquinoline and its derivatives were identified by gas chromatography–mass spectrometry (GC–MS)[10,11]. N-Methylated isoquinoline derivatives, however, could not be measured by these methods. In this paper, we describe a procedure for the determination of $NMIQ^+$ in crude samples by use of high-performance liquid chromatography (HPLC) with fluorescence detection. Uptake to $NMIQ^+$ into rat striatal slices was confirmed using this method.

EXPERIMENTAL

Material

N-Methylisoquinolinium iodide was prepared from isoquinoline and methyl iodide[8].

Measurement of amount of $NMIQ^+$ taken up into striatal slices

Rat striatal slices were prepared as described previously[6]. Briefly, male Wistar rats (200–300 g) were decapitated, and striata were immediately dissected and cut into slices (0.22 mm thick) on a McIlwain tissue chopper. The slices were washed extensively before use in Krebs–Ringer hydrogencarbonate medium composed of 118 m*M* NaCl, 4.7 m*M* KCl, 2.5 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$, 0.1 m*M* $MgSO_4$, 1.4 m*M* KH_2PO_4 and 8 m*M* glucose. The medium was saturated with an atomosphere of O_2–CO_2 (95:5). Incubation was carried out at 37°C in 1 ml of Krebs–Ringer hydrogencarbonate medium. The uptake was terminated by the addition of 3 ml of ice-cold Krebs–Ringer hydrogencarbonate medium. After three consecutive washes with the same medium, the slices were homogenized with the medium by sonication and the protein was removed by adding perchloric acid (0.16 *M* final concentration). The mixture was centrifuged at 5000 *g* for 20 min at 4°C. The supernatant was filtered through a Chromatodisc 13A (0.45 μm) and an aliquot was injected into an HPLC apparatus (Hitachi L-6200) with a fluorimetric detector (Hitachi F-1100). The fluorescence intensity at 375 nm was measured by excitation at 225 nm. The column used for multi-mode determination (gel filtration and reversed-phase chromatography) was an Asahipak GS-320H (250 mm × 7.6 mm I.D.) (Asahi Chemical, Kanagawa, Japan). The sample was eluted with 0.1 *M* sodium phosphate buffer (pH 2.6) at a flow-rate of 1.0 ml/min at room temperature. The determination of the amount of $NMIQ^+$ taken up was performed by comparison of the fluorescence intensity (peak height) with that of an external standard ($NMIQ^+$), using a Shimadzu CR-1A Chromatopack integrator. The protein concentration was measured by the method of Bradford[14] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Fig. 1 shows excitation and emision spectra of $NMIQ^+$. $NMIQ^+$ had an excitation maximum at 225 nm and an emission maximum at 375 nm. Fig. 2 shows curves reflecting the relative fluorescence intensity at various pH with overlapping values. The fluorescence intensity was relatively constant over a wide pH range. With increasing pH of the phosphate buffer used for elution in HPLC on the GS-320H column, the elution volume of $NMIQ^+$ was increased and the fluorescnce intensity of $NMIQ^+$ was decreased. Therefore, we used phosphate buffer of pH 2.6 for the determination of $NMIQ^+$ by HPLC with fluorescence detection. Linearity of the concentration of $NMIQ^+$ using HPLC was observed from 50 fmol to 1 pmol, as shown in Fig. 3. The limit of the sensitivity by this assay was 50 fmol taking a signal-to-noise ratio of 5.

As an application of this method, $NMIQ^+$ was incubated with rat striatal slices and the amount of $NMIQ^+$ taken up into striatal slices was determined. Fig. 4 shows typical HPLC patterns. Rat striatal slices were found to take up and accumulate $NMIQ^+$ by a temperature-dependent mechanism. Further, the described procedure allows, after simple deproteinisation, the immediate determination of $NMIQ^+$ in brain homogenates by HPLC. As shown in Fig. 4, no interference by other compounds was observed. Compared with most other HPLC systems for the determination of substances in the brain such as biogenic amines and amino acids, in which laborious

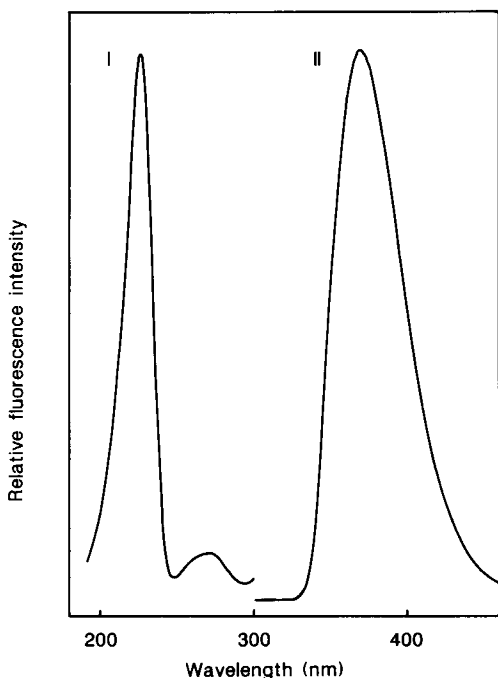

Fig. 1. Excitation and emission spectra of $NMIQ^+$. The fluorescence spectra of $NMIQ^+$ (10 μM) were measured at room temperature. (I) Excitation spectrum of $NMIQ^+$, measured at 375 nm emission wavelength; (II) emission spectrum of $NMIQ^+$, measured by excitation at 225 nm.

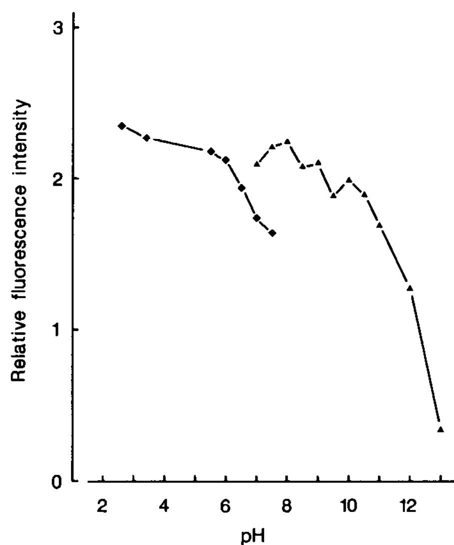

Fig. 2. Relationship between fluorescence intensity of $NMIQ^+$ and pH. The fluorescence intensity at 375 nm with excitation at 225 nm was measured in 0.1 *M* buffer. Sodium phosphate buffer was used from pH 2.6 to 7.7 and boric acid–sodium borate buffer from pH 7 to 13.

extraction and/or purification steps are essential before HPLC, the present study clearly shows that such pretreatment can be omitted with these chromatographic condition for $NMIQ^+$. The recovery of $NMIQ^+$ added to rat brain homogenate was nearly 100%. The uptake was dependent on the amount of striatal slices (Fig. 5) and the reaction time. The uptake was linear up to 20 min at 37°C. The effect of reaction temperature was also examined. As shown in Fig. 6, the uptake increased in a temperature-dependent manner and reached the maximum at 27–37°C.

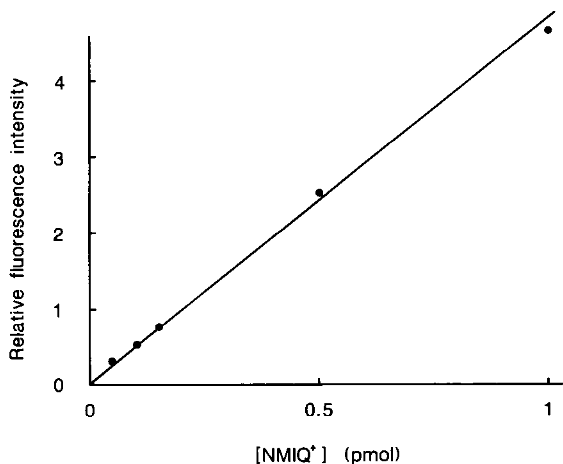

Fig. 3. Relationship between amount of $NMIQ^+$ and fluorescence intensity. The amounts of $NMIQ^+$ subjected to HPLC were plotted against the relative fluorescence intensity at 375 nm with excitation at 225 nm.

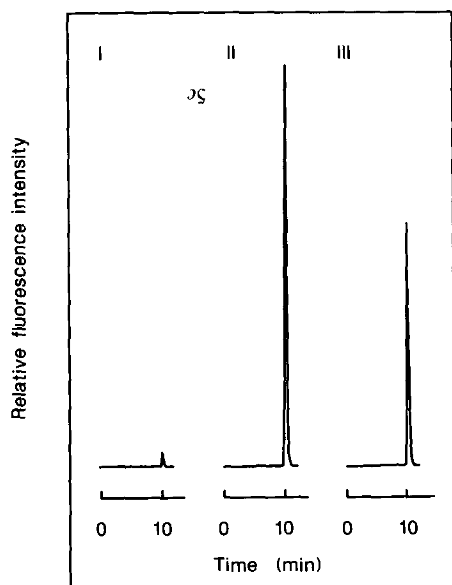

Fig. 4. High-performance liquid chromatograms of $NMIQ^+$. (I) Rat striatal slices (0.9 mg protein) were incubated with $NMIQ^+$ (5 nmol) for 1 h at 0°C; (II) rat striatal slices (1.0 mg protein) were incubated with $NMIQ^+$ (5 nmol) for 1 h at 37°C, then analysed as described under Experimental; (III) $NMIQ^+$ (5.3 pmol).

As reported previously, $NMIQ^+$ inhibited tyrosine hydroxylation, the rate-limiting step of dopamine biosynthesis, in rat striatal slices at 10^{-6}–10^{-3} M[8]. We determined the concentration of $NMIQ^+$ that was taken up into striatal slices during the incubation under the same conditions. When striatal slices were incubated in the Krebs–Ringer hydrogencarbonate medium containing 10^{-6}–10^{-3} M $NMIQ^+$ for

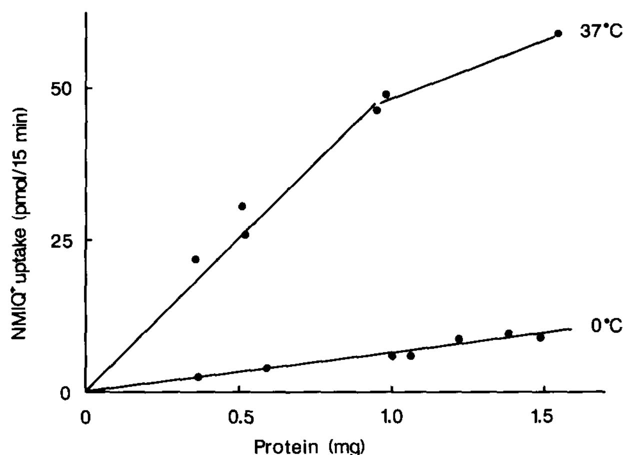

Fig. 5. Uptake of $NMIQ^+$ in rat striatal sices. Slices were incubated for 15 min at 0 or 37°C in a Krebs–Ringer hydrogencarbonate medium containing 1 μM $NMIQ^+$. The protein concentration in the slices is shown on the abscissa.

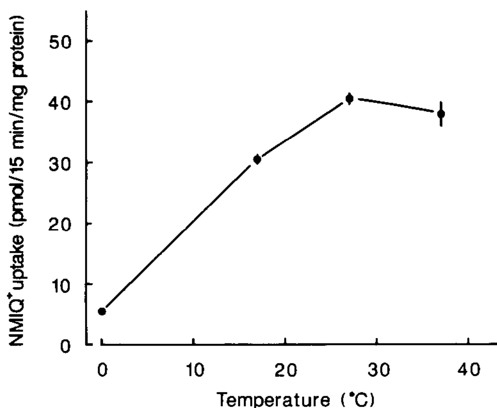

Fig. 6. Effect of temperature on $NMIQ^+$ uptake in rat striatal slices. Slices were incubated for 15 min at 0, 17, 27 or 37°C in a Krebs–Ringer hydrogencarbonate medium containing 1 μM $NMIQ^+$. Each value is the mean ± standard error of four experiments.

1 h at 37°C, $NMIQ^+$ was taken up into striatal tissues as shown in Table I. The molarity in the tissue slices was also determined (Table I). The results suggest that $NMIQ^+$ was concentrated in striatal slices to an extent 3–18 times higher than the concentration in the medium.

The effect of nomifensine, a selective inhibitor of dopamine uptake, was examined. The uptake of $NMIQ^+$ from a 5 μM solution was reduced in the presence of nomifensine (5 μM). The $NMIQ^+$ uptake (pmol/h · mg protein) was 240.0 ± 4.7, which was much lower than the control value of 502.1 ± 9.5 in the absence of nomifensine. The MPP^+ uptake had been found to be mediated by the dopamine uptake system using mouse brain synaptosomes[5]. The present result also suggests that

TABLE I

EFFECT OF $NMIQ^+$ CONCENTRATION IN THE INCUBATION MEDIUM ON DOPA FORMATION AND ON ITS UPTAKE INTO RAT STRIATAL SLICES

The slices were incubated for 1 h at 37°C.

$NMIQ^+$ (M)	*DOPA formation[a] (% of control)*	*$NMIQ^+$ taken up*	
		nmol/mg protein	*μM*
0	100 ± 2	0	
10^{-6}	99 ± 7	0.16 ± 0.01	18
10^{-5}	62 ± 3[c]	0.19 ± 0.07	135
10^{-4}	24 ± 1[c]	4.70 ± 0.19	533
10^{-3}	21 ± 5[c]	25.57 ± 0.34	2905

[a] Each value is the mean ± standard error of four experiments; the control (100%) value was 778.4 ± 18.9 pmol DOPA formed/h · mg protein.

[b] The molarity in the tissue slices was determined based on the assumption that 1 mg wet tissue weight was equivalent to 0.114 mg protein measured by the Bradford method using bovine serum albumin as a standard.

[c] $P < 0.01$ for the difference from the control.

NMIQ$^+$ is taken up into striatal slices by the dopamine uptake system. Furthermore, nomifensine at 5 μM also prevented the inhibition of tyrosine hydroxylation caused by 5 μM NMIQ$^+$. These results suggest that NMIQ$^+$ in addition to MPP$^+$ is taken up into striatal dopamine neurons and then inhibits dopamine synthesis.

MPP$^+$ inhibited tyrosine hydroxylation in rat striatal slices, as reported previously[7]. The concentration of MPP$^+$ producing significant inhibition was lower than that of NMIQ$^+$, and the maximum inhibition produced by MPP$^+$ was greater than that caused by NMIQ$^+$. This could be explained by the accumulation of NMIQ$^+$ being less pronounced than that of MPP$^+$. Thus, the previously related data on uptake systems were generated from the MPP$^+$ neurotoxin using striatal synaptosomal preparations at nanomolar to micromolar concentrations[5,15] whereas the concentrations used in this work were from micromolar to millimolar. MPTP is now the most potent neurotoxin to induce Parkinson's disease but the only known source of MPTP is artificial. The fact that NMIQ$^+$ is taken up into dopaminergic neurons and inhibits dopamine synthesis supports the view that they may be naturally occurring neurotoxins involving the pathogenesis of parkinsonim and other neurodegenerative diseases of the central nervous system. Although NMIQ$^+$ is a relative weak neurotoxin compared with MPP$^+$, damage of dopaminergic neurons could be produced by a cumulative effect of exposure to minute quantities over many years.

The method reported here can be used for the determination of NMIQ$^+$ in a small amount of crude sample. This procedure should have broad applications in biochemical and pharmacological studies on NMIQ$^+$ toxicity.

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