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Analysis of citrulline in rat brain tissue after perfusion with haloperidol by liquid chromatography–mass spectrometry

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Abstract

We have investigated the potential of high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) to determine enrichments of citrulline as a marker for in vivo nitric oxide (NO) production in brain tissue. The analysis of citrulline as the butyl ester derivative was evaluated using two types of ionization: electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI). APCI-MS appeared to be more suitable for determination of citrulline than ESI-MS, because the ion intensity of the protonated molecule ion $[M+H]^+$, m/z 232, of citrulline in the former was about twelve times higher than in the latter. The chromatography was carried out on a reversed C_8 column with the mobile phase consisting of 15% acetonitrile: 85% H_2O: 0.2% acetic acid (v/v). The calibration curve had good linearity within the concentration range investigated (5 ng to 500 ng/ml). The limit of determination was estimated to be ca. 1 ng/ml of standard solution. The method was applied to the analysis of citrulline in the brain dialysate obtained from rat after perfusion of the striatum with haloperidol (HP, 0.1 m*M*). It is concluded that APCI-MS in combination with HPLC can be successfully applied to determination of citrulline in brain tissue, thus providing a useful tool for assessment of in vivo NO production.
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Keywords: Citrulline; Haloperidol

1. Introduction

Nitric oxide (NO) is a unique biological messenger molecule in the central nervous system [1,2]. It is an unusual transmitter since it is a free radical gas which is not stored in synaptic vesicles. NO is formed by the conversion of arginine to citrulline in a reaction catalyzed by calcium-activated nitric oxide synthase (NOS) (Fig. 1) [3,4]. The physiological action of NO has not yet been well clarified, especially in the central nervous system. However, it can exert neurotoxicity, possibly through its ability to combine with superoxide [5]. This reaction forms peroxynitrite, which is itself toxic and can also decompose to produce the highly reactive, cytotoxic hydroxyl radical [6,7]. Thus, the increase of NO

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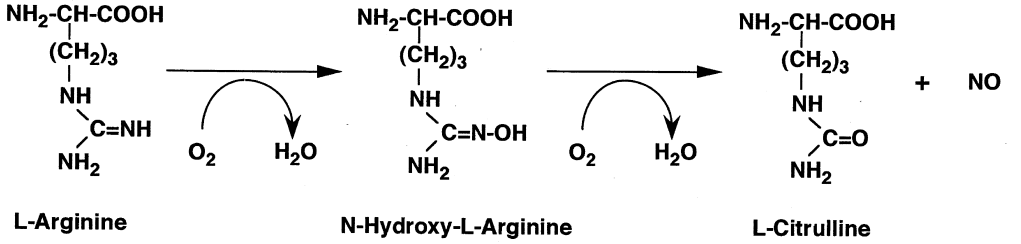

Fig. 1. NO-synthase catalyzed conversion of arginine.

production in neurons may lead to cell death. Recent reports suggest that the toxic effects of the Parkinsonism-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **1**) (Fig. 2) are mediated through an excessive production of NO [8–11].

Haloperidol, 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol, (HP, **3**) (Fig. 2) is a widely used antipsychotic agent that causes often extrapyramidal side effects (motor disturbances) including Parkinsonism and, following chronic treatment, tardive dyskinesia [12]. HP has structural features similar to those of MPTP and has been reported to be metabolized to a pyridinium compound, 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium ion (HPP^+, **4**) (Fig. 2), in rodents [13–15] and humans [16–18] which is a structural analog of the neurotoxin 1-methyl-4-phenylpyridinium ion (MPP^+, **2**) (Fig. 2), derived from MPTP via monoamine oxidase B (MAO-B). Intracerebral microdialysis [19,20] and neuronal cell culture [21,22] studies have shown that HPP^+ has neurotoxic properties resembling those of MPP^+.

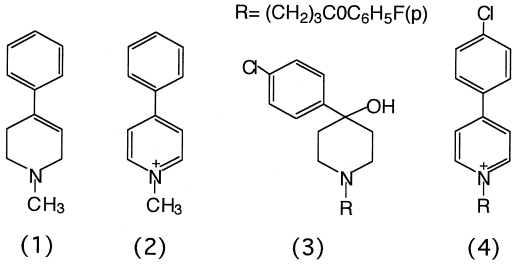

Fig. 2. Chemical structures discussed in this report. (1) MPTP, (2) MPP^+, (3) HP, (4) HPP^+.

We have been interested in the possible role of NO in the extrapyramidal side effects derived from HP as the results of MPTP. In the present study, to examine whether the NO production in the brain increases in the drug-induced Parkinsonism model animals, we determined the citrulline contents as a marker for in vivo NO production in rat brain treated with the antipsychotic agent HP using the more sensitive and selective LC–MS methodology. Several methods to determine NO production have already been reported: the colorimetric assay of nitric azo-coupling reaction (Griess reaction) [23], the chemiluminescence assay [24,25], the oxyhemoglobin (HbO_2) oxidation method [26], the GC–MS detection assay [27], etc. However, these methods are not specific for NO and are technically complex. Moreover, in order to do the quantitative analysis on NO production in the brain, these methods do not satisfy our requirements for a direct, nonradiometric assay. On the other hand, as the conversion of arginine to citrulline is stoichiometric with the enzymatic formation of NO [28], the determination of citrulline as a marker of NO production is more suitable than those described above.

HPLC is generally used for the determination of amino acids and amines following precolumn derivatization using *o*-phthaldialdehyde (OPA) [29]. But this method requires a long time (about 60 min) for the separation and determination of the many derivatized amino acids in the samples. Thus, we developed a highly sensitive and selective assay for the determination of citrulline in brain using APCI-MS in combination with HPLC and applied it to the dialysate samples from rats perfused in the striatum with HP.

2. Experimental

2.1. Chemicals and reagents

Haloperidol, L-arginine and L-citrulline were purchased from Sigma Chemical (St. Louis, MO, USA). Citrulline propyl ester (internal standard) was synthesized in our laboratory. Tween-20, butanolic HCl (10%), HPLC-grade acetonitrile and acetic acid were obtained from Nacalai Tesque (Kyoto, Japan). Sodium pentobarbital solution (50 mg/ml) was obtained from Dainippon Pharmaceutical (Osaka, Japan). All other chemicals and reagents were of analytical grade from commercial sources.

2.2. Sample collection

Anesthetized (50 mg/kg, intraperitoneally, sodium pentobarbital) male Wistar rats (250–300 g, SLC, Shizuoka, Japan) were stereotaxically implanted with 22-gauge cannulae in the left striata at anteroposterior +0.4 mm, lateral +3.0 mm from the bregma, and −3.5 mm from the skull, according to the stereotaxic atlas of Paxinos and Watson [30]. Dummy probes were then placed inside the cannulae. The rats were housed in plastic cages (35×35×40 cm) with free access to food and water, and a 20 h recovery period was allotted. The microdialysis probes with dialysis area of 3 mm length were of the I-shaped type reported previously [31]. The dialysis tube (0.2 mm I.D., 0.31 mm O.D.) was prepared from a polyacrylonitrile/sodium methylsulfonate membrane (Hospal, Bologna) with a molecular weight cut-off of 1100 Da. After insertion through the guide cannulae, the probe was connected to a microinfusion pump and perfused with Ringer's solution at a flow-rate of 2 μl/min for 180 min. Then, HP (0.1 m*M*) in Ringer's solution was perfused for 10 min followed by sequential perfusion with Ringer's solution for 240 min. The dialysate was collected every 30 min during the perfusion. As a control experiment, 0.1 m*M* sucrose in Ringer's solution instead of HP was perfused for 10 min.

2.3. Sample preparation for LC–MS

Brain dialysate samples obtained from rats after perfusion with HP solution were evaporated to dryness in vacuo below 40°C. The residue was derivatized with 100 μl of 10% butanolic HCl by heating at 80°C for 60 min. After removing the excess butanolic HCl, the derivatized residue was dissolved in 200 μl of internal standard solution (I.S., citrulline propyl ester, 50 ng/ml in the mobile phase solution). After passing through a 0.45 μm filter, aliquots of 100 μl of the samples were subjected to APCI-LC–MS system.

2.4. APCI-LC–MS

APCI-LC–MS was performed using an ion trap mass spectrometer (LCQ, Thermoquest, USA) equipped with a reversed-phase C_8 column (4.6 mm, I.D.×150 mm, 5 μm particle diameter, Nacalai Tesque, Japan). A mobile phase consisting of 15% acetonitrile: 85% H_2O: 0.2% acetic acid (v/v) was delivered at a flow-rate of 1.0 ml/min at ambient temperature. The conditions for APCI-LC–MS were as follows; electric field 5.0 kV, nitrogen sheath gas 80 μl, vaporizer temperature 450°C, capillary temperature 200°C, discharge current 5.0 μA.

3. Results and discussion

At first, we compared the usefulness of the two ionization modes, APCI and ESI, for the determination of the citrulline contents in brain dialysates using LC–MS. The APCI and ESI mass spectra of citrulline as the butyl ester derivative are shown in Fig. 3. Citrulline showed a protonated molecule ion $(M+H)^+$ of m/z 232. The ion intensities of APCI and ESI were measured using this m/z. As shown in Fig. 4, the ion intensity in the full scan mode of the m/z 232 of citrulline butyl ester (2.0–20.0 ng) in the APCI mode was about twelve times higher than that in ESI. Based on this finding, APCI ionization was chosen for the determination of citrulline using LC–MS.

The mass chromatograms in APCI-LC–MS of standard solutions are shown in Fig. 5. As the derivatized arginine has an isotope ion, $[M+2+H]^+$ at m/z 232, the peaks of derivatized citrulline and arginine have to be well separated in the mass

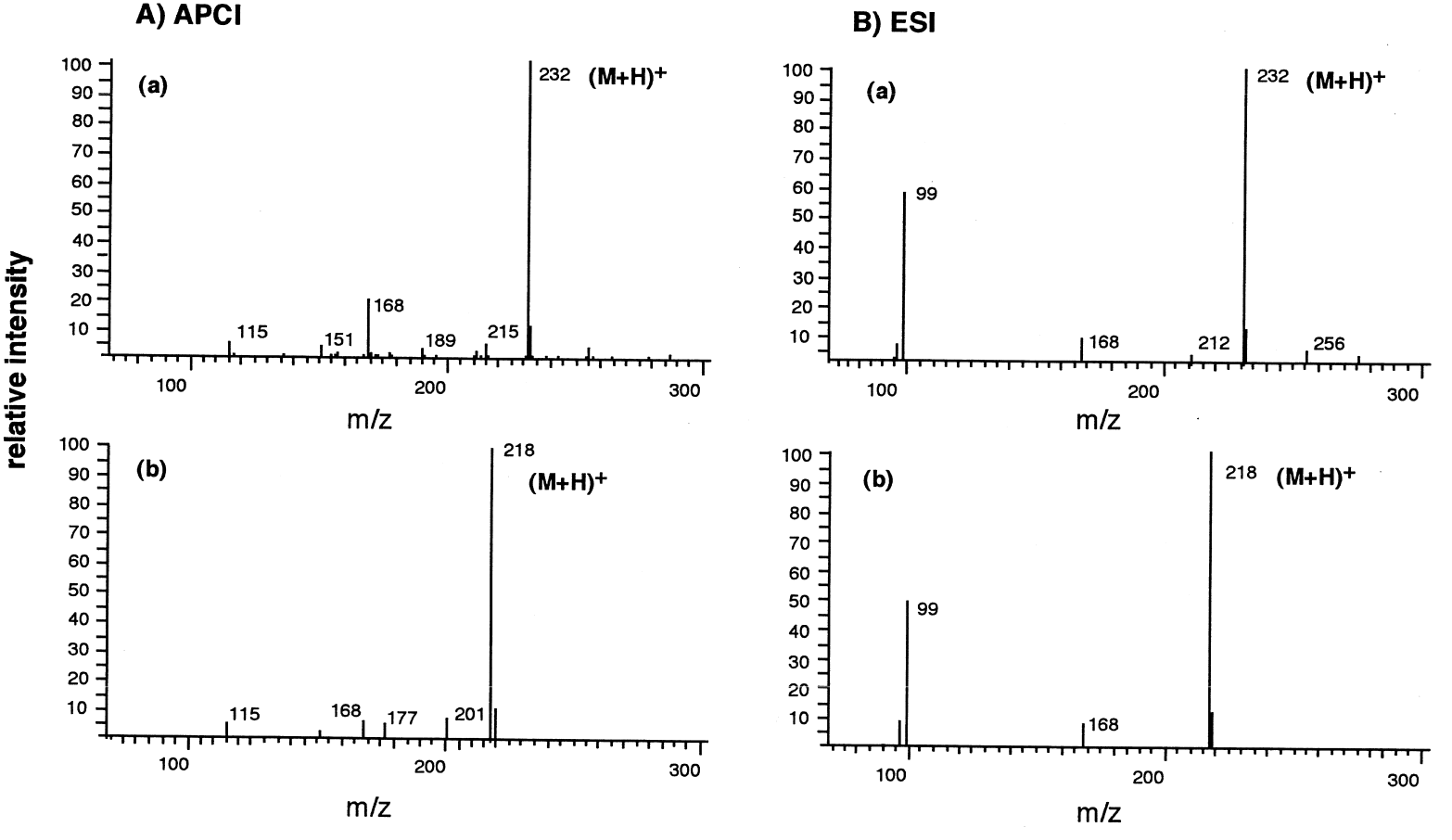

Fig. 3. APCI (A) and ESI (B) mass spectra of citrulline as butyl ester derivative (MW 231, a) and as propyl ester derivative (MW 217, b).

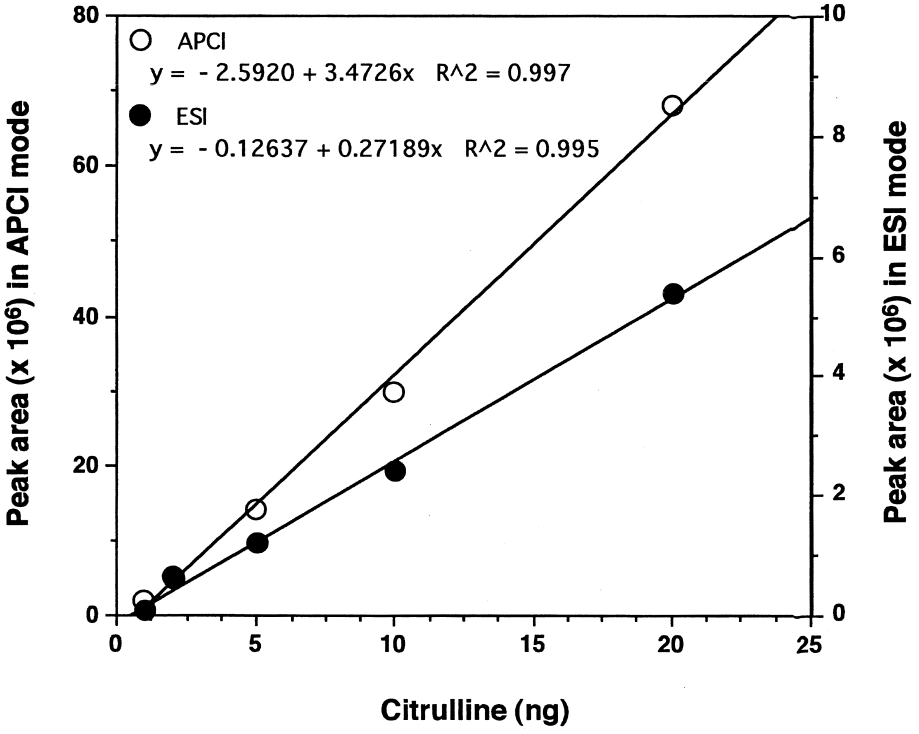

Fig. 4. Ion intensities of derivatized citrulline (m/z 232) in APCI-MS and ESI-MS.

chromatogram. HPLC conditions were examined therefore for a good separation of these compounds. As a result, citrulline and arginine peaks in the mass chromatogram were clearly separated using a reversed-phase C_8 column and a mobile phase consisting of 15% acetonitrile:85% H_2O:0.2% acetic acid (v/v).

The calibration curve for citrulline was obtained by plotting the peak area ratio of derivatized citrulline (m/z 232) to I.S. (m/z 218) versus the amount of citrulline. A good linearity was observed over the concentration range examined (5–500 ng/ml for standard solution, $y=0.013\ x+0.034$, $r^2=0.9974$). The calibration curve showed little day-to-day variability in slopes and intercepts [coefficient of variation (C.V.), <8%]. The lower limit of detection was approximately 1 ng/ml ($S/N=4$) for standard solution. Experiments with spiked samples resulted in a recovery of 95.2±5.5% at a concentration of 20 ng/ml of brain dialysate (data not shown).

The citrulline contents in rat brain dialysates after perfusion with HP (0.1 mM) in Ringer's solution were determined by APCI-LC–MS. Fig. 6 shows the mass chromatograms of an extract obtained from rat brain dialysate. The citrulline and arginine peaks in brain dialysate were detected at retention times of 4.8 and 4.3 min, respectively. These peaks were well separated in the chromatogram, and the mass chromatogram was free of interference at the retention times of interest. The analysis time for the APCI-MS determination of each sample is less than 6 min, and was rapid compared with HPLC/fluorescence detection using OPA reagent as reported previously [32,33]. Moreover, MS/MS analysis of the peak on mass chromatogram of m/z 232 ion showed the product ion of m/z 70 which is a neutral loss of m/z 162 (data not shown). The citrulline contents in rat brain dialysates before and after HP perfusion are shown in Table 1. Whereas the citrulline content in brain dialysate was 91.4±15.2 ng/ml in preperfusion with Ringer's solution, that in brain dialysate collected at 0–1 h after HP perfusion was 160.8±20.4 ng/ml.

The significant increase of the citrulline content in

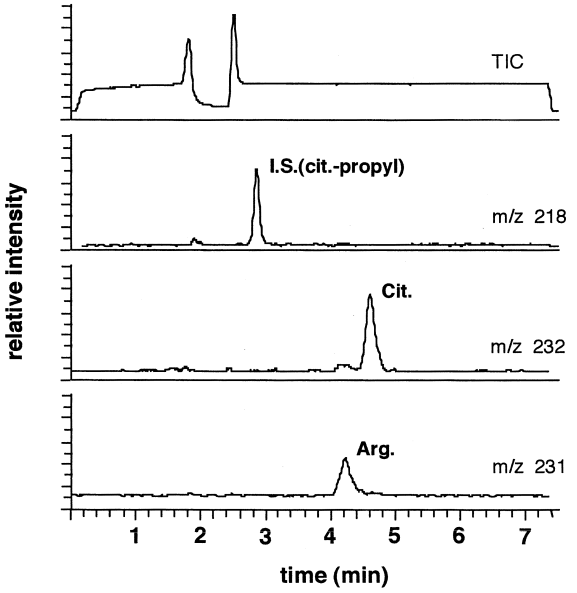

Fig. 5. APCI mass chromatograms of derivatized standard solutions. The standard solution used was the mixture of citrulline, arginine and I.S. (each 200 ng/ml). Chromatographic conditions: C_8 column, 150 m×4.6 mm I.D.; mobile phase, 15% acetonitrile: 85% H_2O: 0.2% acetic acid (v/v); flow-rate, 1.0 ml/min. I.S.: citrulline propyl ester; Cit.: citrulline; Arg.: arginine.

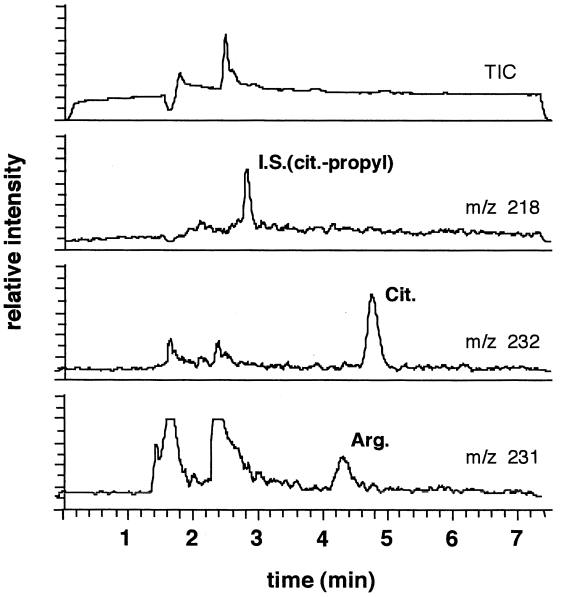

Fig. 6. APCI mass chromatograms of extracts obtained from brain dialysates after perfusion of the striatum with HP (0.1 m*M*). Chromatographic conditions are as Fig. 5.

the HP-perfused rat brains suggests that antipsychotic agent HP promotes the NO production in brain tissue. As HP increases dopamine turnover in humans and rats [34,35], it has been proposed that HP-induced oxidative stress arises from the generation of hydrogen peroxide as a consequence of catecholamine metabolism by monoamine oxidases [36,37]. Hydrogen peroxide promotes the release of glutamate from neuronal terminals and it induces the increase of intracellular Ca^{2+} level [38–40]. It has also been reported that the increase of Ca^{2+} level promotes the generation of NO free radical, which has been implicated in the toxic effects of glutamate. Therefore, it is assumed that chronic administration of HP induces oxidative stress. This hypothesis is partially supported by elevation of levels of lipid peroxidation on haloperidol treatment in rats [41] and in psychotic patients [42,43]. Furthermore, it is

Table 1
Citrulline concentration in striatal dialysates of rats following perfusion with haloperidol (0.1 m*M*)[a]

Collection time of dialysate (h)	Citrulline concentration (ng/ml)	Relative % (mean)
Control		
0–1	91.4±11.2	100.0
1–2	96.7±18.1	105.8
HP perfusion		
0–1	160.8±20.4	175.9
1–2	144.3±23.1	157.9

[a] Haloperidol (0.1 m*M*) in Ringer's solution was perfused for 10 min followed by sequential perfusion with Ringer's solution for 120 min. Data revealed mean±SD of three experiments.

known that coadministration of the antioxidant vitamin E with haloperidol results in some improvement in patients with tardive dyskinesia [44,45].

Microdialysis combined with LC–MS has been recently shown to be a powerful in vivo technique for physiological, pharmacological and toxicological studies [46–49]. This technique by which microdialysates collected in living animals can be injected onto the LC–MS system using either an off-line or an on-line mode has several advantages such as minimal sample preparation, real-time analysis and molecular identity of the analyte of interest. Additionally, a shortened sample preparation process is important when the metabolites to be analyzed are unstable. The present study demonstrated that this technique was very useful for an analysis of unstable NO in rat brain.

In conclusion, the citrulline content as a marker of in vivo NO production in brain tissue was determined by a combination of microdialysis in living rat brain with APCI-LC–MS. The citrulline contents in HP-perfused rat brains were increased significantly in comparison with that in preperfusion with Ringer's solution. This assay method has a short analysis time compared with HPLC/fluorescence detection using OPA reagent and provides a useful tool for assessment of in vivo NO production.

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