Alteration in the plasma concentration of a DAAO inhibitor, 3-methylpyrazole-5-carboxylic acid, in the ketamine-treated rats and the influence on the pharmacokinetics of plasma D-tryptophan

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Abstract: A determination method for 3-methylpyrazole-5-carboxylic acid (MPC), an inhibitor of D-amino acid oxidase (DAAO), in rat plasma was developed by using high-performance liquid chromatography-mass spectrometry (LC-MS). The structural isomer of MPC, 3-methylpyrazole-4-carboxylic acid, was used as an internal standard, and the intra- and inter-day accuracies and precisions were satisfactory for the determination of plasma MPC.

Next, the LC-MS method was applied to determine the plasma MPC concentration in ketamine (Ket)-treated rats after intraperitoneal administration of MPC (5.0 or 50 mg·kg$^{-1}$). The $C_{\text{max}}$ value of plasma MPC concentration in the Ket-treated rats was significantly higher than that in the control group when a high dose of MPC (50 mg·kg$^{-1}$) was administered. In addition, it was found that plasma D-tryptophan (D-Trp) concentration in Ket-treated rats administered D-Trp was not significantly increased by MPC, suggesting that the DAAO-inhibitory effect of MPC is attenuated in Ket-treated rats.

Keywords: 3-methylpyrazole-5-carboxylic acid, D-amino acid oxidase, inhibitor, rat, ketamine, D-tryptophan

Introduction

Schizophrenia, a serious neuropsychiatric disease, affects 1% of the general population and consists of positive symptoms, negative symptoms, and cognitive impairments. The glutamate hypothesis of schizophrenia states that the etiology of schizophrenia is associated with hypofunction of NMDA receptor. Phencyclidine [1-(1-phenylcyclohexyl)piperidine, PCP], an illicit drug, can act as non-competitive antagonist for an ionotropic glutamate receptor, the $N$-methyl-$D$-aspartate (NMDA) receptor, and an acute administration of PCP caused schizophrenia-like symptoms in humans. This finding suggested that blockage of glutamatergic neurotransmission via NMDA receptor was related with the onset of symptoms in schizophrenia. With regard to current therapeutic drug, it has been reported that available antipsychotics could improve only positive symptoms of schizophrenia. On the other hand, D-serine acts as an endogenous co-agonist at the glycine binding site of the NMDA receptor, and D-serine administration is considered as an effective therapeutic treatment for schizophrenia. In fact, Tsai et al. reported that co-administration of D-serine with the antipsychotics was effective for treating positive symptoms, negative symptoms, and cognitive impairments in patients with schizophrenia. From these reports, a drug-induced facilitation of NMDA receptor may take effect for the treatment of schizophrenia.

In 1994, it was reported that the intake of the anesthetic drug ketamine (Ket), which acts as a noncompetitive antagonist of the NMDA receptor, induced schizophrenia-like symptoms in humans. This finding suggests that Ket can be used to produce an experimental model of schizophrenia,
similar to that produced by PCP. In 2003, Becker et al. reported that sub-chronic administration of a sub-anesthetic dose of Ket (30 mg·kg⁻¹) to Sprague-Dawley (SD) rats exhibited some changes in rat behaviors, such as disruption of latent inhibition and decrease in non-aggressive behavior. In addition, an increase in hippocampal D₂ receptor binding was observed in the Ket-treated rat. These alterations indicated that the Ket-treated rat could be used, in part, for an animal model of schizophrenia. Recently, Watanabe et al. also reported that Ket-treated rats exhibited abnormal behaviors, including turning, weaving, and head-bobbing.

Recently, it has been reported that a single administration of Ket caused a dose-dependent and transient elevation in the levels of serine racemase (SR) and D-amino acid oxidase (DAAO) mRNA in all areas within the SD rat brain. In contrast, sub-chronic administration of Ket (50 mg·kg⁻¹ daily for 14 days) decreased the mRNA expression of SR in rat brain. These reports suggest that there are some links between gene expression of SR or DAAO mRNA and the blockade of NMDA receptors by ketamine treatment. It has been found that brain DAAO activity in schizophrenia patients was higher than that in a control group. DAAO plays a crucial role in the oxidative decomposition of D-serine, and therefore, an inhibition of DAAO by a drug may increase D-serine concentration in the brain tissue to induce facilitation of NMDA receptor.

Based on these considerations, it has been proposed that a specific inhibitor of DAAO may be therapeutic for the treatment of schizophrenia. In 2008, Adage et al. reported the pharmacological profiles of 3-methylpyrazole-5-carboxylic acid (MPC) (Fig. 1) as an inhibitor of DAAO and that MPC was able to increase brain D-serine levels and ameliorate PCP-induced abnormal behavior in SD rats, suggesting that MPC can inhibit DAAO activity in vivo and is a potential therapeutic drug for the treatment of schizophrenia. Our recent studies also indicated that pre-administration or infusion with MPC inhibited the metabolism of D-tryptophan (D-Trp) or D-kynurenine by DAAO in SD rats.

Until now, there has been little information on the pharmacokinetics of MPC in the animal model of schizophrenia. In the present study, we investigated time-course profiles of MPC concentration in the plasma of Ket-treated rats after administration of MPC by using HPLC with mass spectrometric detection (MS). The pharmacokinetic parameters were determined and compared between control and Ket-treated rats. In addition, the effect on inhibition of DAAO activity by MPC in Ket-treated rats was also examined. As a representative substrate for DAAO, D-Trp (Kₘ value, 845 µM) was administered to control and Ket-treated rats that were pretreated with MPC, and the pharmacokinetic data on plasma D-Trp concentration were compared between the groups.

**Materials and methods**

**Chemicals.** MPC, 3-methylpyrazole-4-carboxylic acid, used as an internal standard (I.S.), and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol 200 (PEG) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Methanol (MeOH) and acetonitrile (CH₃CN) were obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan). Water was used after purification by a WR600G (Nihon Millipore K.K., Tokyo, Japan).

**Animal experiments.** All animal experiments were approved (No. 11-55-17) by the Committee of Animal Care, Toho University. Male SD rats were purchased from Charles River Japan (Kanagawa, Japan) and were housed in an environmentally controlled room for at least 1 week before use.

Administration of Ket to rats was carried out as previously reported. Ketamine hydrochloride (Wako Pure Chemicals Co., Ltd., Osaka, Japan) was dissolved in physiological saline (1.0 mg·mL⁻¹) and then intraperitoneally (i.p.) administered (30 mg·kg⁻¹) to 7-week-old rats daily for 5 consecutive days. The control animals received only saline (1 mL·kg⁻¹·day⁻¹). The rats were used for experiments 4 weeks after cessation of the ketamine or saline treatment.

![Fig. 1. Chemical structures of 3-methylpyrazole-5-carboxylic acid (MPC) and 3-methylpyrazole-4-carboxylic acid (I.S.).](image-url)
Administration of MPC. A solution of MPC (5.0 or 50 mg·kg⁻¹·3 mL⁻¹) was prepared in PBS/ethanol/PEG (2.8/0.1/0.1) and was i.p. administered to rats. A heparinized syringe with a 25-gauge needle was used to draw blood (approximately 0.20 mL) from the left jugular vein at 0.083, 0.25, 0.5, 1, 2, and 3 h after administration of MPC (5.0 mg·kg⁻¹, n = 4), or at 0.083, 0.25, 0.5, 1, and 3 h after administration of MPC (50 mg·kg⁻¹, n = 5–6). Before administration of either dose of MPC, blood (approximately 0.20 mL) was obtained from each rat and used as the control. The blood was centrifuged at 3,000 g for 10 min at 4°C to obtain plasma. The plasma was transferred to another tube and stored at −80°C until it was analyzed.

Sample pretreatment. For determining the plasma concentration of MPC, 2 calibration curves (31.25–250 µM and 200–800 µM) were constructed. Ten microliters of PBS or rat blank plasma was spiked with 10 µL of 31.25–200 µM I.S. dissolved in CH₃CN and 10 µL of 200 µM I.S. dissolved in CH₃CN, and vigorously mixed with 70 µL of CH₃CN previously published HPLC method. Before administration of either dose of MPC, blood (approximately 0.20 mL) was obtained from each rat and used as the control. The blood was centrifuged at 3,000 g for 10 min at 4°C to obtain plasma. The plasma was transferred to another tube and stored at −80°C until it was analyzed.

After centrifugation at 2,500 rpm for 5 min, 50 µL of the supernatant was sampled and evaporated in vacuo. The obtained residue was dissolved in 100 µL of the mobile phase, 60 µL (31.25–250 µM) or 20 µL (200–800 µM) of the final solution after filtration with Durapore (0.2 µm membrane, Millipore K.K., Tokyo, Japan), and was injected into the LC-MS system.

Administration and determination of D-Trp concentration. D-Trp was dissolved in PBS (10 mg·mL⁻¹) and administered i.p. to control or Ket-treated rats (100 mg·kg⁻¹; n = 3–5) at 30 min after i.p. administration of MPC (50 mg·kg⁻¹). A heparinized syringe with a 25-gauge needle was used to draw blood (approximately 0.20 mL) from the left jugular vein 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h after administration of D-Trp. The blood was treated in a similar manner as described in previous paper. Plasma D-Trp concentration was determined by our previously published HPLC method.

LC-MS conditions. The LC-MS apparatus comprised an Agilent 1200 series HPLC (Agilent Technologies, CA, U.S.A.) and a time of flight (TOF)-MS, JMS-T100 LP AccuTOF LC-Plus, equipped with an electrospray ionization (ESI) source (JEOL Co. Ltd., Tokyo, Japan). The separation column used in this study was TSKgel ODS-80Ts QA (150 mm × 2.0 mm; i.d., 5 µm) (Tosoh Corporation, Tokyo, Japan). For the mobile phase, 0.1% HCO₂H in H₂O/CH₃CN (95/5) was eluted isocratically at 0.16 mL·min⁻¹. Column temperature was maintained at 40°C. The conditions for ESI-MS detection were as follows: positive ion mode; needle voltage was set at 2,000 V; and the ring lens and orifice 1 and 2 voltages were set at 8, 49, and 4 V, respectively. Nitrogen was used as the nebulizing and desolvating gas, and the pressure was constant at 0.617 MPa. The desolvation chamber and orifice 1 temperatures were 250°C and 80°C, respectively. The data were obtained using Mass Center software, MS-56010MP (JEOL).

Precision and accuracy. The precision was expressed as relative standard deviation (RSD, %), and the accuracy was expressed as relative mean error (RME, %). RSD and RME were calculated by following equations [1] and [2], respectively.

\[
\text{RSD} (\%) = \left( \frac{\text{standard deviation}}{\text{mean}} \right) \times 100 \quad [1]
\]

\[
\text{RME} (\%) = \left( \frac{|\text{measured concentration} - \text{added (theoretical) concentration}|}{\text{added (theoretical) concentration}} \right) \times 100 \quad [2]
\]

Pharmacokinetic parameters and statistical analysis. Pharmacokinetic parameters obtained in the present study were determined by a simplex method analysis or moment analysis. Statistical analyses were performed using a one-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison test. A p value below 0.05 was judged as a significant difference.

Results and discussion

Plasma MPC concentration. In the present study, LC-TOF/MS was employed for determination of plasma MPC concentration in rats after its i.p. administration. Figure 2(a) shows a representative MS spectrum of a standard of MPC (0.4 mM), showing the m/z at 127.05. For determining MPC concentration, a structural isomer of MPC, 3-methylpyrazole-4-carboxylic acid (Fig. 1), was used as the I.S. Under the present HPLC conditions, in which the mobile phase was 0.1% HCO₂H in H₂O/CH₃CN (95/5) with an ODS column (150 × 2.0 mm; i.d., 5 µm), the I.S. and MPC peaks were found to be separated and eluted at close retention times, i.e., 8.3 and 12.5 min, respectively. Therefore, selected ion monitoring (SIM) at m/z 127.05 showed that 2 clear peaks were simultaneously detected (Fig. 2(b)).
intra- and inter-day accuracies (RME) and precisions (RSD) were in the range of −10.49% to 4.19% and −8.86% to 4.36% (n = 4), and 0.61% to 2.85% and 0.55% to 2.91% (n = 4), respectively (Table 1).

Figure 2(c) shows the SIM at m/z 127.05 of the blank plasma sample spiked with 10 µL of MPC (400 µM) and I.S. (200 µM), obtained by the proposed LC-MS. Both MPC and I.S. peaks were clearly detected, and the detection limit for MPC was approximately 3.0 pmol (100 nM in plasma) on the column (signal to noise ratio, 3). The 2 calibration curves, which covered lower (31.25–250 µM) and higher concentration ranges (200–800 µM), showed good linearity (r² = 0.9982 and 0.9996, respectively). These validation data indicated that the present LC-MS method, including the pretreatment procedure, was sufficient for the determination of plasma MPC concentration. Using the LC-MS, time-course profiles of plasma MPC concentration in control and Ket-treated rats, which are in part an animal model of schizophrenia,9) were investigated after i.p. administration of MPC (5.0 or 50 mg·kg⁻¹). As shown in Fig. 2(d), the MPC peak in rat plasma was clearly detected at 5.0 min after its administration (5.0 mg·kg⁻¹).

Figure 3 shows time-course profiles of plasma MPC concentration after its i.p. administration (5.0 or 50 mg·kg⁻¹). In the rat groups administered the low dose (5.0 mg·kg⁻¹) of MPC, the maximum concentration (Cmax) of plasma MPC was reached at 0.5 h in both control and Ket-treated rats. Subsequently, similar declines were observed until 2 h after administration. Between the control and Ket-treated rats, no remarkable differences were observed in PK parameters, such as Cmax, half-life (t1/2), or area under the curve (AUC).

Previously, the PK parameters of plasma MPC concentration in SD rats (p.o. and i.v. administration, 10 mg·kg⁻¹) were reported by Adage et al.16) However, their previous data, especially the t1/2 (5.59 h) and clearance (CL) (0.22 L·h⁻¹·kg⁻¹), were different from our present data on the control rats (t1/2, 50.8 min and CL, 18.3 L·h⁻¹·kg⁻¹). These differences might be attributed to differences in the
administration route, analytical method, or the calculation method for the PK parameters.

When a high dose (50 mg·kg$^{-1}$) of MPC was administered, plasma MPC concentrations were significantly increased than those in the case of a low dose (5.0 mg·kg$^{-1}$) of MPC ($p < 0.01$). In addition, an interesting phenomenon occurred. In the rat groups that were administered the high dose, plasma MPC concentrations at 5.0 and 15 min in the Ket-treated rats were significantly higher than those in the control groups ($p < 0.01$ and $p < 0.05$, respectively; Fig. 3). After 1 h, the MPC concentrations declined similarly in both control and Ket-treated rats.

We therefore considered that plasma MPC concentration might differ between the control and Ket-treated rats when a high dose of MPC is administered. In the present study, samples from Ket-treated rats were taken 4 weeks after cessation of the repeated ketamine treatment. Therefore, we assumed that ketamine and its major metabolite, norketamine, were completely eliminated from the rat body, because their half-lives in plasma are approximately 0.5–0.6 h and 0.6–0.8 h, respectively.$^{22,23}$

These findings suggest that the repeated Ket treatment alters the expression of some proteins responsible for the absorption, distribution, metabolism, or elimination (ADME) of MPC. Based on the significant difference in the $C_{\text{max}}$ values that were observed with the high dose of MPC, proteins involved in the ADME of MPC, such as transporters or metabolic enzymes, might be saturated by the high dose of MPC. This suggests that the expression of these proteins was reduced in the Ket-treated rats. Thus, it is likely that deficiency of transporters, by which MPC is incorporated into cells, caused the observed increase in plasma MPC concentration. The lack or attenuated expression of an enzyme responsible for metabolizing MPC in cells might increase the plasma MPC concentration. These findings indicate that DAAO-inhibitory effects of MPC might be altered in the Ket-treated rats.

### Table 1. Validation data on the determination of plasma MPC concentration in the ranges of 31.25–250 µM and 200–800 µM by the proposed LC-MS method ($n = 4$, mean ± S.D.)

| Concentration spiked in plasma (µM) | 31.25  | 62.5 | 125  | 250  |
|-------------------------------------|--------|------|------|------|
| **Intra-day**                        |        |      |      |      |
| mean ± S.D.                          | 27.97 ± 0.80 | 64.65 ± 1.57 | 130 ± 1.96 | 248 ± 3.35 |
| RSD (%)                              | 2.85   | 2.42 | 1.50 | 1.35 |
| RME (%)                              | -10.49 | 3.44 | 4.19 | -0.92 |
| **Inter-day**                        |        |      |      |      |
| mean ± S.D.                          | 28.48 ± 0.81 | 62.68 ± 0.91 | 130 ± 3.79 | 244 ± 6.63 |
| RSD (%)                              | 2.86   | 1.45 | 2.91 | 2.72 |
| RME (%)                              | -8.86  | 0.29 | 4.36 | -2.41 |

| Concentration spiked in plasma (µM) | 200  | 400 | 600 | 800  |
|-------------------------------------|------|-----|-----|------|
| **Intra-day**                        |      |     |     |      |
| mean ± S.D.                          | 191 ± 1.17 | 406 ± 5.21 | 614 ± 15.2 | 787 ± 7.40 |
| RSD (%)                              | 0.61  | 1.28 | 2.47 | 0.94 |
| RME (%)                              | -4.59 | 1.43 | 2.33 | -1.58 |
| **Inter-day**                        |      |     |     |      |
| mean ± S.D.                          | 196 ± 5.18 | 403 ± 8.91 | 602 ± 5.76 | 786 ± 4.33 |
| RSD (%)                              | 2.64  | 2.21 | 0.96 | 0.55 |
| RME (%)                              | -1.79 | 0.78 | 0.26 | -1.70 |

Fig. 3. Time-course profiles of plasma MPC concentration after i.p. administration of MPC to rats. Open triangles (control rats) and closed squares (Ket-treated rats) with dotted lines represent rats administered 5.0 mg·kg$^{-1}$ MPC ($n = 4$), and open (control rats) and closed circles (Ket-treated rats) with solid lines represent rats administered 50 mg·kg$^{-1}$ MPC ($n = 5$–$6$). * $p < 0.05$, ** $p < 0.01$, compared to control rats (50 mg·kg$^{-1}$). # $p < 0.01$, compared to control and Ket-treated rats (5.0 mg·kg$^{-1}$).
Plasma D-Trp concentration. To test this hypothesis, a representative D-amino acid, D-Trp, was administered i.p. (100 mg·kg⁻¹) to the Ket-treated rats after pre-administration of MPC (50 mg·kg⁻¹), and we determined if changes in the PK parameters of plasma D-Trp concentration occurred in Ket-treated rats. D-Trp was chosen for this experiment because it is easily metabolized by DAAO, with a reported $K_m$ value of 845 µM. After 30 min of pretreatment with MPC or the vehicle, D-Trp was administered to both control and Ket-treated rats (100 mg·kg⁻¹). As shown in Fig. 4A and B, the time-course profiles of plasma D-Trp concentration in the control and Ket-treated rats without MPC pretreatment (vehicle administration) were almost identical, suggesting that ADME, including the metabolism of D-Trp by DAAO, were not altered in Ket-treated rats. However, in the rats pretreated with MPC, a significant difference in plasma D-Trp concentration was observed between control and Ket-treated rats. As shown in Fig. 4C and D, the plasma D-Trp concentrations in Ket-treated rats pretreated with MPC were significantly lower than those in the control rats pretreated with MPC. Although no significant difference in plasma D-Trp concentration was observed between control and Ket-treated rats (Fig. 4A and B), the plasma D-Trp concentrations were significantly higher in the control rats pretreated with MPC (Fig. 4A and C), while there was no significant increase in the plasma D-Trp concentrations in Ket-treated rats pretreated with MPC (Fig. 4B and D).
plasma D-Trp concentration was significantly increased in normal rats pretreated with MPC, implying that MPC could inhibit D-Trp metabolism by DAAO in vivo in normal rats. However, in the Ket-treated rats, MPC did not inhibit D-Trp metabolism by DAAO. The PK parameters of plasma D-Trp concentration are summarized in Table 2. Among the measured PK parameters of plasma D-Trp concentration, $C_{\text{max}}$ and $AUC$ were significantly different between the MPC-pretreated control and Ket-treated rats. As shown in Table 2, MPC-pretreatment caused an increase in the $C_{\text{max}}$ and $AUC$, and a decrease in the $V_d$ in control rats, suggesting that the DAAO-inhibitory effect of MPC normally occurred in these rats. In contrast, there were no significant differences in the PK parameters in Ket-treated rats, with or without MPC pretreatment, indicating that the DAAO-inhibitory effect induced by MPC might be quite absent. This indicates that the DAAO-inhibitory effect of MPC was considerably attenuated in the Ket-treated rats.

These results suggest that the possibility of decreased MPC metabolism in Ket-treated rats should be ruled out, because the pharmacological action of MPC was attenuated. Therefore, we speculate that one of the reasons for the absence of the DAAO-inhibitory effect of MPC in the Ket-treated rats was that the MPC was not sufficiently incorporated into the liver or kidney cells, which possess relatively high DAAO activity, in Ket-treated rats. This explanation is based on the result that plasma MPC concentrations in Ket-treated rats were significantly higher than those in control rats (Fig. 3).

It seems likely that D-Trp metabolism by DAAO was not inhibited due to a decreased incorporation of MPC into cells. This could explain why the DAAO-inhibitory effect of MPC was not observed in the Ket-treated rats. Changes in expression of proteins that are involved in the incorporation of MPC into the liver or kidney will need to be examined in future studies. Indeed, it has been reported that repeated ketamine treatment could induce changes in the expression of some proteins in rats.

As Ket-treated rats are, in part, an animal model for schizophrenia, it is crucial to elucidate whether these changes occur in patients with schizophrenia.

**Conclusion**

The proposed LC-MS method was used to determine the concentration of a DAAO-inhibitory compound, MPC, in rat plasma after its administration. In Ket-treated rats, the $C_{\text{max}}$ value for plasma MPC was significantly higher than that in the control rats following i.p. administration (50 mg·kg$^{-1}$). This phenomenon was accompanied by attenuation of the DAAO-inhibitory effect of MPC in Ket-treated rats.

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