Application of Assay of Platelet Monoamine Oxidase Type-B (MAO-B) Activity to the Discrimination of Selegiline Use from Methamphetamine Abuse

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An assay of platelet monoamine oxidase type-B (MAO-B) activity has been explored in the search for a reliable method of discriminating selegiline (SG) use from methamphetamine (MA) abuse. MAO-B activity was measured by fluorimetry of 4-hydroxyquinoline (4HOQ) produced by oxidative deamination of kynuramine, the substrate for MAO-B. MA and most of its related compounds including its precursors produced no platelet MAO-B inhibition *in vitro* even at their lethal levels, though SG, its specific metabolites selegiline *N*-oxide (SGO) and *N*-desmethylselegiline (DM-SG), as well as its enantiomer *d*-deprenyl exhibited high platelet MAO-B inhibitory potency. *In vivo*, remarkable inhibition of MAO-B occurred even within an hour after drug administration, and the inhibition apparently lasted approximately 6–8 days after both 2.5 and 7.5 mg oral doses of SG hydrochloride. The present study suggests that the decrease of platelet MAO-B activity would be a significant marker to discriminate SG use from MA abuse, even a week after drug use.

Key words —— platelet monoamine oxidase type-B, selegiline, methamphetamine

INTRODUCTION

Selegiline [(*R*)-(–)-*N*,α-dimethyl-*N*-2-propynylphenethylamine] (SG) is a potent, irreversible and selective inhibitor of monoamine oxidase type-B (MAO-B).[1)] It has been widely prescribed alone or in combination with levodopa in the treatment of Parkinson's disease,[2)] and has also been sold as a prescription medicine in Japan since 1998 under the trade name FP. In 1996, the Japanese authorities classified SG as a "precursor" of the illicit stimulant methamphetamine (MA), and began to control its possession and use.

It is well established that SG is predominantly metabolized into MA and amphetamine (AP), which are among the most prevalent illicit drugs throughout the world.[3,4)] Therefore, the clear discrimination of legitimate therapeutic use of SG from illicit use of MA is essensial in drug enforcement. The high metabolism of SG, however, usually makes the detection of unchanged SG in urine difficult.[4–6)] Thus, much attention has been given to the development of a urinalysis procedure that can detect more specific metabolites to clearly distinguish SG use from MA use.

The discrimination of SG use from its counterpart has already been attempted by the enantiomeric determination of MA and AP based on the difference in their optical natures: the predominantly used illicit MA has been (+)-enantiomers or racemic isomers,[7)] whereas the metabolites of SG are (–)-enantiomers.[5,6)] However, (–)-MA has often been seized in Japan since 1998.[8,9)] This makes the discrimination of SG use by such an enantiomeric analysis problematic.

The detection of desmethyl selegiline (DM-SG) and selegiline *N*-oxide (SGO), specific metabolites of SG, have also been reported to be indicators of SG use.[4,6,10)] However, DM-SG and SGO were only detectable (> 0.2 ng/ml) in urine for not more than 60 hr after a single oral administration of 2.5–10 mg SG.[4,6)] The presence of DM-SG and/or SGO can reportedly be employed as the indicator only for urine

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samples collected within a limited period after intake. Thus, an accurate and reliable method for discriminating SG use from MA abuse for a longer period after intake is strongly required.

As mentioned above, SG has been known to potently and selectively inhibit MAO-B for several days,[11] and this specific property of SG is considered promising in the discrimination because MA was reported to exhibit no MAO-B-inhibiting property.[12] Platelets are also a convenient source for assessing MAO-B activity because more than 95% of the platelet MAO is of type B. Also, MAO in platelet and in the human brain are essentially the same.[13,14] In the present study, the MAO-B-inhibiting properties of MA and its related compounds including SG were evaluated *in vitro* using human platelets. The platelet MAO-B activity for SG users was also monitored for 15 days, and assay of the platelet MAO-B activity was evaluated for the discrimination of SG use from MA abuse.

MATERIALS AND METHODS

Chemicals —— SG (*l*-deprenyl) hydrochloride and DM-SG hydrochloride were generously provided by Fujimoto Pharmaceuticals (Osaka, Japan). *d*-MA hydrochloride and *l*-dimethylamphetamine hydrochloride were obtained from Dainippon Pharmaceuticals (Osaka, Japan). *dl*-Pseudoephedrine hydrochloride and *d*- and *l*-ephedrine hydrochloride were obtained from Alps Pharmaceutical (Gifu, Japan), and *dl*-methylephedrine hydrochloride was purchased from Kansai Yakuhin (Osaka, Japan). *dl*-Methoxyphenamine hydrochloride was obtained from Nippon Shinyaku (Kyoto, Japan). *dl*-Norephedrine hydrochloride and *dl*-β-phenethylamine were purchased from Tokyo Kasei Organic Chemicals (Tokyo, Japan), and *dl*-benzylamine from Wako Pure Chemical Industries (Osaka, Japan).

l-MA hydrochloride was extracted from a Vicks inhaler and purified as hydrochloride salt in our laboratory. SGO was prepared by oxidation of SG with m-chloroperbenzoic acid in our laboratory.[10] *d*-Dimethylamphetamine (DMA) hydrochloride and *d*-benzphetamine (BZP) hydrochloride were prepared by methylation and benzylation of *d*-MA, respectively. *dl*-Methylenedioxyamphetamine (MDA), *dl*-methylenedioxymethamphetamine (MDMA), *dl*-*p*-methoxyamphetamine (PMA), *dl*-*p*-methoxymethamphetamine (PMMA) and *dl*-ethylamphetamine (EAP) were synthesized by reductive amination of appropriate ketones and amines with sodium cyanoborohydride, and purified as their hydrochlorides. *d*- and *l*-AP hydrochloride were prepared by oxidative *N*-demethylation of *d*- and *l*-methamphetamine with alkaline ferricyanide, respectively.[15] *d*-Deprenyl was synthesized by propargylation of *d*-MA, and purified as its hydrochloride. *dl*-*p*-Hydroxyamphetamine hydrochloride and *dl*-*p*-hydroxymethamphetamine hydrochloride were synthesized according to the procedure described in a previous paper.[16] *dl*-Phentermine hydrochloride was one of the extensive authentic standards being stored in our laboratory.

Kynuramine dihydrobromide purchased from Sigma (St. Louis, MO, U.S.A.) was prepared in phosphate buffer (62.5 mM, pH 7.4) at the concentration of 2 mg/ml as the stock solution, and stored at –20°C until use. The stock solution was diluted to 0.33 mg/ml with the phosphate buffer immediately prior to use, and was used as the substrate for MAO-B. 4-Hydroxyquinoline (4HOQ) was prepared in 1 N NaOH at the concentration of 145 μg/ml as the standard stock solution, and stored at –20°C until use. The stock solution was diluted to 1.45 μg/ml with 1 N NaOH immediately prior to measurement as the standard for fluorimetric sensitivity. Other chemicals used were of analytical grade.

Sample Preparation —— Human venous blood (drug-free human blood or SG user's blood), to which 3.8%(w/v) sodium citrate aqueous solution was added, was centrifuged at 170 × *g* for 10 min, and the supernatant was separated as platelet-rich plasma (PRP). The residual layer was further centrifuged at 1700 × *g* for 10 min, and the supernatant was separated as platelet-poor plasma (PPP). The PRP and PPP were stored frozen at –20°C until use after measuring the number of platelets.

The PRP and PPP were defrosted immediately prior to use, adjusted to 300000 cells/μl with 62.5 mM phosphate buffer, and dispersed in suspension by ultrasonication. The suspensions of PRP and PPP were employed as an enzyme solution and a blank enzyme solution in measuring MAO-B activity, respectively.

Fluorimetric Assay of MAO-B Activity —— MAO-B activity was conveniently assayed by the method of Krajl:[17] one can readily estimate MAO-B activity by monitoring fluorimetrically the appearance of 4HOQ arising from the spontaneous and complete cyclization of the intermediate aldehyde

produced by the oxidative deamination of kynuramine, for which MAO-B is responsible, because 4HOQ production depends only on MAO-B activity.

The incubations were carried out as follows: to 880 μl of the 62.5 mM phosphate buffer, 120 μl of the drug solutions adjusted to various concentrations was added (the final concentration being 1×10^{-4}–1×10^{-9} M), and preincubation followed for 5 min at 37°C. To the preincubated drug solution, 80 μl of the PRP or PPP obtained from drug-free human blood was added, and the solution was further preincubated 30 min at 37°C before 120 μl of the substrate kynuramine (0.33 mg/ml) solution was added. The incubation was continued for 120 min at 37°C after addition of the substrate.

In the measurement of MAO-B activity for the SG user, 120 μl of 62.5 mM phosphate buffer and 80 μl of the PRP or PPP obtained from the SG user's blood were employed instead of 120 μl of the drug solutions and 80 μl of the PRP or PPP obtained from drug-free human blood, respectively. The incubation was performed using the same procedure.

After the incubation, the reaction was terminated by adding 800 μl of 10%(w/v) trichloroacetic acid. The precipitated protein was spun down at $1700 \times g$ for 10 min and a 1.0 ml aliquot of supernatant was added to 2.0 ml of 1 N NaOH in a quartz cuvette. The solution was immediately submitted to fluorimetry on an FP-6200DS spectrophotofluorometer (JASCO, Tokyo, Japan): the solution was activated at 315 nm and the fluorescence was measured at 380 nm.

For the standard of fluorimetric sensitivity, 1.45 μg/ml of 4HOQ solution in 1 N NaOH was also submitted to measurement under the same conditions.

MAO-B activity was calculated as nmoles 4HOQ formed per 1×10^7 platelets per hour at 37°C (nmol 4HOQ/1×10^7 platelets/hr).

Drug Administration and Sample Collection —— After informed consent, six healthy male volunteers (V1: 27 years old, 64 kg body wt, V2: 36 years, 66 kg body wt; V3: 54 years, 61 kg body wt, V4: 39 years, 60 kg body wt; V5: 43 years, 56 kg body wt; V6: 46 years, 51 kg body wt) received a single oral dose of 2.5 mg (V1, V2, V3) or 7.5 mg (V4, V5, V6) of SG hydrochloride (FP Tablet 2.5®, Fujimoto Pharmaceuticals).

The blood samples were taken 0 hr, 1 hr, 6 hr, 1 day, 3 days, 6 days, 8 days, 10 days, 13 days, and 15 days after drug administration: Venous blood (10 ml) was collected in a plastic tube containing 1 ml of 3.8%(w/v) sodium citrate solution, and samples were subjected to preparation of PRP and PPP as soon as possible.

In order to examine variation in the baseline level of MAO-B activity, the venous blood samples were also taken from one volunteer (V4) on three different days after drug administration with at least a 4-week washout period.

RESULTS

Precision of Platelet MAO-B Activity Assay

To evaluate the precision of the proposed assay of platelet MAO-B activity, the relative standard deviations (RSDs) in the intra- and inter-assay were calculated using, as a sample, PRP and PPP obtained from V4. The RSDs in the intra- and inter-assay were estimated to be 2.9 ($n = 5$) and 5.3% ($n = 6$), respectively, demonstrating that the present assay is reliable for the estimation of platelet MAO-B activity.

MAO-B Inhibiting Property of MA and Its Related Compounds *in Vitro*

MAO-B activity with various concentrations of MA and its related compounds was measured *in vitro*, and the inhibitory potency of each on MAO-B was expressed as the concentration that gave 50% MAO-B inhibition, IC_{50}. As summarized in Table 1, extremely high inhibitory potency of SG on MAO-B was confirmed. It was also shown that the specific SG metabolites, SGO and DM-SG, and the enantiomer of SG, *d*-deprenyl exhibited relatively high inhibitory potencies. These findings suggest that the propargyl moiety and its optical disposition would be responsible for inhibition of MAO-B activity.

In addition, MA and its related compounds, except for SG, SGO, DM-SG and *d*-deprenyl, were found to produce no or very low inhibition of MAO-B. Especially, not only MA and AP but also their precursors DMA and BZP showed no inhibition of MAO-B even at the lethal level of MA in blood, i.e.,(1×10^{-4}–1×10^{-5} M). This suggests that the platelet MAO-B activity would be promising as an indicator for discriminative screening of SG use from MA abuse.

Table 1. Inhibitory Potency of Methamphetamine and Its Related Compounds on Platelet MAO-B

Compound	IC_{50}[a)] (M)
Selegiline	3.6×10^{-9}
Desmethylselegiline	3.0×10^{-7}
Selegiline *N*-oxide	2.9×10^{-8}
d-Deprenyl	9.1×10^{-8}
d-Methamphetamine	$> 1 \times 10^{-4}$
l-Methamphetamine	$> 1 \times 10^{-4}$
d-Amphetamine	$> 1 \times 10^{-4}$
l-Amphetamine	$> 1 \times 10^{-4}$
d-Dimethylamphetamine	$> 1 \times 10^{-4}$
l-Dimethylamphetamine	$> 1 \times 10^{-4}$
d-Benzphetamine	$> 1 \times 10^{-4}$
dl-p-OH Methamphetamine	$> 1 \times 10^{-4}$
dl-p-OH Amphetamine	$> 1 \times 10^{-4}$
dl-Ethylamphetamine	$> 1 \times 10^{-4}$
dl-MDA	$> 1 \times 10^{-4}$
dl- MDMA	$> 1 \times 10^{-4}$
dl-PMA	5.1×10^{-5}
dl-PMMA	6.2×10^{-5}
dl-Methoxyphenamine	$> 1 \times 10^{-4}$
dl-Phentermine	$> 1 \times 10^{-4}$
dl-Methylephedrine	$> 1 \times 10^{-4}$
l-Ephedrine	5.8×10^{-5}
d-Ephedrine	$> 1 \times 10^{-4}$
dl-Norephedrine	$> 1 \times 10^{-4}$
dl- Pseudoephedrine	$> 1 \times 10^{-4}$
dl-Benzylamine	1.0×10^{-4}
dl-β-Phenethylamine	2.6×10^{-6}

a) IC_{50}: Concentration giving 50% MAO-B inhibition.

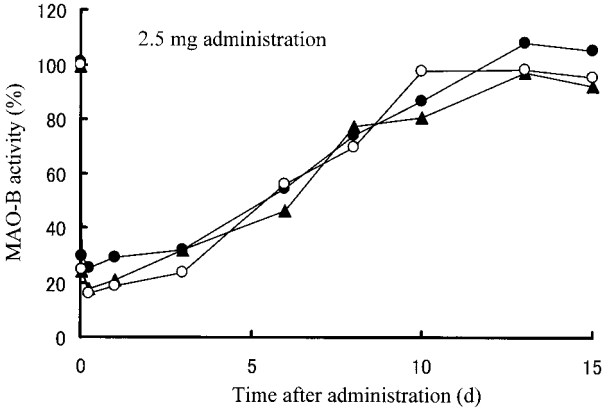

Fig. 1. Time-Course of MAO-B Activity after a Single Oral Dose of 2.5 mg of Selegiline Hydrochloride
Symbols: ●, Volunteer 1; ○, Volunteer 2; △, Volunteer 3.

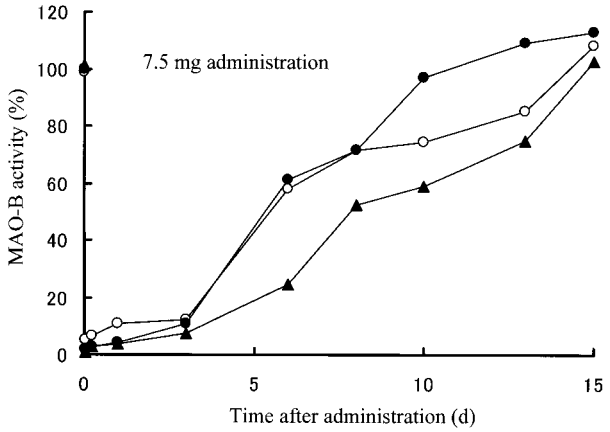

Fig. 2. Time-Course of MAO-B Activity after a Single Oral Dose of 7.5 mg of Selegiline Hydrochloride
Symbols: ○, Volunteer 4; ●, Volunteer 5; △, Volunteer 6.

Platelet MAO-B Activity for SG Users

Time–Dependent Intraindividual Variation of Platelet MAO-B Activity: The time-dependent interindividual variation of platelet MAO-B activity was examined by collecting blood samples from Volunteer 4 on 4 different days, and the MAO-B activities were measured. The mean value was 7.6 nmol 4HOQ/1 $\times 10^7$ platelets/hr, and the RSD was as low as 5.1%.

Time Course of Platelet MAO-B Activity for SG Users: The baseline MAO-B activity of samples collected from six subjects (V1–V6) immediately before drug administration (0 hr) was found to vary between individuals from 3.1 to 7.5 nmol 4HOQ/1 $\times 10^7$ platelets/hr. The time-course changes of MAO-B activity were, therefore, evaluated as the percentage of the activity against baseline activity obtained from each subject.

As depicted in Figs. 1 and 2, a higher dose led to greater MAO-B inhibition within the administered dose range, and 2.5 mg and 7.5 mg oral doses produced MAO-B inhibition values greater than 70% and 90%, respectively. These data are well consistent with those previously reported.[11)]

The inhibition of MAO-B occurred rapidly within even one hour after administration, and the maximal inhibition was reached 1–6 hr after intake. The inhibition lasted for approximately 10 days and 10–14 days after the oral doses of 2.5 mg and 7.5 mg SG hydrochloride, respectively. The 50% recovery took approximately 6–8 days after the oral dose. These demonstrate that SG use causes remarkable decrease of MAO-B activity for approximately a week after its ingestion. As mentioned in the previous section, not only *d*- and *l*-MA but also their precursors including DMA and BZP produced no MAO-

B inhibition *in vitro*, while SG and its specific metabolites produced great inhibition. These results suggest that the assay of platelet MAO-B activity would be applicable to the discriminative screening of SG use from MA abuse, even a week after drug use.

DISCUSSION

The rapid assay of platelet MAO-B activity by fluorimetrically measuring the appearance of 4HOQ produced by oxidative deamination of kynuramine, the substrate for MAO-B was applied to distinguish of SG use from MA abuse. MA and most of its related compounds including its precursors produced no MAO-B inhibition *in vitro* even at their lethal levels, though SG, its specific metabolites SGO and DM-SG, as well as its enantiomer *d*-deprenyl exhibited high MAO-B inhibitory potency. In addition, the remarkable inhibition of MAO-B occurred rapidly even within an hour after drug administration, and the inhibition appeared to last approximately 6–8 days after oral doses of either 2.5 mg or 7.5 mg of SG hydrochloride. The findings of this study suggest that the decrease of platelet MAO-B activity would be a marker for discriminative screening of SG use from MA abuse even a week after drug use. A combination of the present assay of platelet MAO-B activity, the analysis of the specific urinary metabolites SGO and/or DM-SG, and enantiomeric analysis of urinary MA will be a stronger and more reliable method to differentiate SG use from MA abuse.

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