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An enzymatic assay for the MAO-B inhibitor selegiline in plasma

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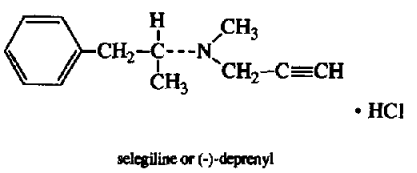
Abstract: A sensitive fluorimetric assay based on inhibition of rat brain monoamine oxidase-B (MAO-B) *in vitro* has been described. The procedure measures the inhibition of MAO activity produced by the addition of selegiline extracted from human plasma. This method uses the substrate kynuramine which is converted by MAO to the product 4-hydroxyquinoline which fluoresces in alkaline solution. Human plasma (500 μl) containing different concentrations of selegiline was deproteinized and extracted with ethyl acetate–butyl chloride. After reconstitution with 200 μl phosphate buffer, 50 μl of rat brain homogenate was added to study the MAO-B inhibition. Selegiline metabolites, amphetamine and methamphetamine (50 ng ml^{-1}), and desmethylselegiline (20 ng ml^{-1}), showed no inhibitory effect on MAO-B inhibition. Selegiline concentrations as low as 0.25 ng ml^{-1} can be detected. The standard curve was linear from 125 pg (0.25 ng ml^{-1}) to 4000 pg (8.0 ng ml^{-1}) in the incubation tube. This method should be helpful to determine pharmacokinetic parameters of selegiline after i.v. or oral dosing.

Keywords: *Selegiline: MAO-B; selegiline metabolites; enzymatic assay; fluorescence detection.*

Introduction

Selegiline or (−)-deprenyl (phenylisopropyl-*N*-methylpropinylamine; Fig. 1) was discovered by Knoll and Ecseri in 1965 [1] and is classified as a selective, irreversible monoamine oxidase-B inhibitor. Monoamine oxidase (MAO) protects the intestines and liver against the effects of exogenous biogenic amines, whereas in other tissues, such as the brain, it breaks down mediator substances and thus controls their concentrations [2]. Two subtypes of MAO, A and B, have been recognized [3]. In the human brain, most of the dopamine is metabolized by MAO-B. Two mechanisms of selegiline action in the human brain have been proposed by Knoll [3, 4]: by inhibiting the metabolism of dopamine, selegiline improves the duration of action of levodopa, and selegiline inhibits re-uptake of dopamine and increases the synthesis rate of dopamine by blocking the presynaptic dopamine autoreceptors that regulate the synthesis of dopamine.



selegiline or (-)-deprenyl

Figure 1
The structure of selegiline.

After oral administration, selegiline is metabolized in the liver to (−)-desmethylselegiline and (−)-methamphetamine, which is further metabolized to amphetamine. The metabolites of selegiline are in low concentration (ng ml^{-1}) and are the levorotatory forms [5]. The MAO-B inhibitory property of amphetamine (A) is about 500 times weaker and that of methamphetamine (MA) is 1000 times weaker than of selegiline. Desmethylselegiline inhibits MAO-B to a certain extent, though 30 times weaker than selegiline when measured *in vitro* by platelet MAO inhibition [6].

Currently, no pharmacokinetic data are available in the literature for selegiline, mainly due to lack of analytical methods which can measure <10 ng ml^{-1} in plasma. In the past, attempts to quantify selegiline have not met with success. The drawback of the method developed by Juvancz *et al.* [7] was the large plasma volume (3–5 ml) and the application of steam distillation for sample preparation, which was laborious and time consuming.

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Patrick *et al.* [8] have recently described a gas chromatographic-mass spectrometric method for the determination of plasma selegiline. The method requires 1 ml plasma and provides a limit of quantitation of 1 ng ml^{-1}. A simple, sensitive and specific enzymatic method is presented here which has been validated for the quantitation of selegiline in plasma (0.25–8.0 ng ml^{-1}) for a pharmacokinetic study in man or animals. MAO-B from rat brain homogenate was assayed as described by Morinan and Garrat [9]. This fluorimetric assay uses kynuramine as the substrate for conversion by MAO-B to 4-hydroxyquinoline, which fluoresces in alkaline media. The method was designed to measure selegiline at concentrations <10 ng ml^{-1} in plasma and was validated to determine sensitivity, linearity, precision, accuracy and ruggedness.

Materials and Methods

Preparation of rat brain supernatant

Brains from male Sprague–Dawley rats (200–250 g) were removed and homogenized in 10 vol (w/v) of 10 mM potassium phosphate buffer (pH 7.2) using a Potter-Elvejhem homogenizer with a Teflon pestle. The homogenate was centrifuged for 10 min at 2700 rpm to remove cell debris and nuclei. The supernatant was further centrifuged for 10 min. The supernatant after the second centrifugation was transferred to an amber coloured bottle, sonicated for 30 s, and stored frozen at −20°C. The total protein concentration in the rat brain homogenate was measured by the biuret method (Procedure No. 541, Sigma Chemical Co., St Louis, MO). For the enzymatic assay, protein concentrations were adjusted to 5 mg ml^{-1} in the homogenate.

Assay of MAO activity in rat brain supernatant

To 200 μl of 10 mM potassium phosphate buffer (pH 7.2), 50 μl of supernatant was added. The reaction was started by the addition of 10 μl of 3.07 mM kynuramine (Sigma Chemical Co., St Louis, MO). After 15 min of incubation at 37°C, the reaction was terminated by the addition of 100 μl of 0.6 M perchloric acid. The mixture was centrifuged for 10 min (1000 rpm) and 0.30 ml aliquots of the supernatant were transferred to test tubes containing 2.0 ml of 1 N sodium hydroxide. The fluorescence intensity as 4-hydroxyquinoline was measured at an excitation wavelength of 315 nm and an emission wavelength of 380 nm using a fluorescence spectrofluorimeter (Perkin–Elmer LS 50, Buckinghamshire, England).

The concentration of product was calculated from a standard curve of 4-hydroxyquinoline. The standard curve was prepared by adding 4-hydroxyquinoline (0–10 nmol) to 50 μl enzyme and 10 mM potassium phosphate buffer (pH 7.2) in a total volume of 260 μl, and the fluorescence was measured as described previously.

Different concentrations (2.5–80.0 ng ml^{-1}) of selegiline were prepared in buffer to determine MAO-B inhibition. One hundred microlitres of different concentrations of selegiline mixed with 100 μl buffer was incubated for 20 min at 37°C and then with 10 μl of 3.07 mM kynuramine for 15 min. The assay was then carried out as described previously.

Enzymatic activity was expressed as nanomoles converted in 15 min in the control (uninhibited) samples (V_o) divided by nanomoles converted in 15 min in the inhibited samples (V_i). If the enzyme and substrate concentrations are constant, the plot of the ratio (V_o/V_i) against concentration gives a straight line [10]. This concept can be explained by the following equations. The Michaelis–Menten equation in the absence of inhibitor is given by:

$$V_o = \frac{V_{max}[S]}{[S] + Km} . \quad (1)$$

In the presence of a non-competitive inhibitor, the Michaelis–Menten equation can be written as

$$V_i = \frac{V_{max}[S]}{([S] + Km)(1 + [I_o]/k_i)} , \quad (2)$$

where; I_o is the inhibitor concentration and K_i is the inhibitor constant.

From equations (1) and (2) the ratio of uninhibited to inhibited rates V_o/V_i may be described as

$$\frac{V_o}{V_i} = 1 + \frac{[I_o]}{K_i} . \quad (3)$$

Each standard curve included six concentrations of selegiline in duplicate and a linear relation was fitted to the data using least-squares regression analysis.

Extraction of selegiline from plasma

Human plasma was mixed with selegiline to prepare selegiline concentrations of 0.25–8.0 ng ml^{-1}. The samples were prepared 24 h before the extraction and stored at −20°C. Five hundred microlitres of plasma was deproteinized by the addition of 500 μl of 0.6 M perchloric acid. The mixture was centrifuged for 10 min and 750 μl of the supernatant was transferred to a tube containing 1.0 ml of 1 N sodium hydroxide. The supernatant was extracted with 3.0 ml ethyl acetate–butyl chloride (1:3, v/v) mixture. The samples were slowly vortexed for 10 min so as to avoid formation of an emulsion, and then centrifuged for 5 min (2700 rpm). The tubes were then cooled in an acetone–dry ice bath. The whole organic layer was transferred to a tube containing 15 μl phosphate buffer (pH 6.2). The samples were dried under nitrogen and reconstituted with 200 μl phosphate buffer (pH 7.2) and the enzymatic assay was carried out as described previously.

Besides analysis in 500 μl human plasma, selegiline concentrations in 500 μl dog plasma and 25 and 100 μl human plasma were also analysed. The concentration of selegiline in dog plasma ranged from 0.25 to 8.0 ng ml^{-1}, whereas the concentration in 25 and 100 μl human plasma ranged from 25 to 150 and 1.25 to 25 ng ml^{-1}, respectively. The procedure for selegiline extraction from dog plasma was the same as described with 500 μl human plasma. When 25 or 100 μl human plasma was used, the volumes of perchloric acid and sodium hydroxide were reduced to 100 and 200 μl, respectively, and 1 ml organic solvent was used to extract the drug.

Recovery of selegiline from plasma

The recovery of selegiline from plasma was determined by comparing the selegiline ratio of uninhibited to inhibited rate from the standard curves prepared in buffer with the standard curves from the spiked plasma samples prepared for linearity, precision and accuracy studies. Each concentration on the standard curve was compared against the mean value of the standard curve made in buffer.

Inhibitory effect of amphetamine, methamphetamine and desmethylselegiline

The inhibitory effect of amphetamine, methamphetamine and desmethylselegiline individually and in the presence of selegiline was determined. Desmethylselegiline was mixed with plasma at concentrations of 20, 40 and 50 ng ml^{-1}, while amphetamine and methamphetamine were investigated at concentrations of 50 and 100 ng ml^{-1}. The inhibitory effect of these metabolites on MAO-B was determined using the enzymatic assay described earlier in this report. Selegiline was mixed with plasma containing 50 ng ml^{-1} amphetamine, methamphetamine, or 20 ng ml^{-1} desmethylselegiline, to achieve concentrations of 0.375, 3.75 or 7.5 ng ml^{-1}. Five hundred microlitres of plasma containing selegiline or selegiline with metabolites were examined as described previously to measure the inhibitory effect.

Results and Discussion

Standard curve of hydroxyquinoline

A plot of the fluorescence intensity of 4-hydroxyquinoline as a function of the amount of 4-hydroxyquinoline reveals a linear relation, $y = -3.05 + 94.7x$ with a correlation coefficient of 0.999, which describes the standard curve.

Enzymatic activity of MAO-B in rat brain homogenate

MAO-B activity in the rat brain homogenate was 1.25 ± 0.07 nmol hydroxyquinoline per min per mg protein ($n = 3$). During a 15 min incubation, about 17% of the substrate was utilized. The activity of the enzyme preparation was linear from 5 to 30 min and with enzyme protein concentrations up to 0.5 mg.

Inhibition of MAO-B by selegiline

Results from this study show that selegiline can inhibit MAO-B activity at a concentration as low as 0.25 ng ml^{-1}. Six standard curves were prepared in human and dog plasma, and linear relations, as described in equation (3), were found. The result in human plasma has been summarized in Table 1. The standard curve of selegiline in dog plasma was linear from 0.25 to 8.0 ng ml^{-1} with a mean correlation coefficient and standard deviation of 1.0 ± 0. The results have been summarized in Table 2. No substantial difference was found in the standard curves of selegiline prepared in human or dog plasma.

The standard curve of selegiline using 100 μl human plasma was linear from 1.25 to 25.0 ng ml^{-1}. The mean intercept, slope and corre-

Table 1
Regression statistics of calibration curves of selegiline in 500 μl human plasma

Parameter	Mean	SD	RSD (%)
Slope	0.264	0.00784	2.97
Intercept	0.986	0.01428	1.45
R	1.00	0.00	0.00
0.25 ng ml^{-1}			
% Inhibition	5.94	1.16	19.5
Ratio	1.06	0.02	1.42
0.50 ng ml^{-1}			
% Inhibition	11.1	0.60	5.43
Ratio	1.13	0.01	0.74
1.00 ng ml^{-1}			
% Inhibition	18.9	1.26	6.65
Ratio	1.23	0.02	1.58
2.00 ng ml^{-1}			
% Inhibition	34.3	1.58	4.61
Ratio	1.52	0.04	2.51
4.00 ng ml^{-1}			
% Inhibition	50.8	1.75	3.44
Ratio	2.04	0.07	3.42
8.00 ng ml^{-1}			
% Inhibition	67.8	0.64	0.94
Ratio	3.11	0.06	2.06

Table 2
Regression statistics of calibration curves of selegiline in 500 μl dog plasma

Parameter	Mean	SD	RSD (%)
Slope	0.24	0.001	3.79
Intercept	1.00	0.03	2.55
R	1.00	0.00	0.50
0.25 ng ml^{-1}			
% Inhibition	5.53	0.78	14.1
Ratio	1.06	0.02	1.96
0.50 ng ml^{-1}			
% Inhibition	11.3	1.73	15.3
Ratio	1.13	0.01	0.51
1.00 ng ml^{-1}			
% Inhibition	17.0	1.92	11.3
Ratio	1.24	0.02	1.40
2.00 ng ml^{-1}			
% Inhibition	32.7	2.05	6.26
Ratio	1.52	0.05	3.48
4.00 ng ml^{-1}			
% Inhibition	50.5	3.04	6.01
Ratio	2.08	0.04	1.95
8.00 ng ml^{-1}			
% Inhibition	65.5	0.70	1.06
Ratio	3.14	0.08	2.53

lation coefficient of the two batches were 1.06, 0.051 and 0.998, respectively. When 25 μl human plasma was used, the standard curve of selegiline was linear from 25.0 to 150.0 ng ml^{-1}. The mean intercept, slope and correlation coefficient of the two batches were 1.23, 1.09 and 0.995, respectively.

Recovery of selegiline from plasma

The recovery of selegiline from 500 μl human plasma ranged from 64 to 94% over the concentration range of 0.25–4.0 ng ml^{-1}. In dog plasma, the recovery ranged from 63 to 93% over the same concentration range. The recovery study was found to be satisfactory as indicated by the low values of the relative standard deviation. Since data for 8.0 ng selegiline in buffer was not collinear with the standard curve, the comparison of recovery from buffer was only possible from 0.25 to 4.0 ng ml^{-1} of selegiline in plasma.

Table 3
Inhibitory effect of desmethylselegiline, amphetamine and methamphetamine on MAO-B

ng ml^{-1}	Inhibition (%)
Desmethylselegiline	
20.00	2.07
40.00	4.30
50.00	7.20
Methamphetamine	
50.00	0.00
100.00	0.00
Amphetamine	
50.00	0.00
100.00	0.00

Inhibitory effect of desmethylselegiline, amphetamine and methamphetamine on MAO-B

It was found that neither amphetamine nor methamphetamine has any measurable inhibitory effect on MAO-B at 50 ng ml^{-1}, whereas desmethylselegiline did not show any substantial inhibitory effect at 20 ng ml^{-1} (Table 3). Furthermore, desmethylselegiline (20 ng ml^{-1}) in the presence of different concentrations of selegiline did not show any additional inhibitory effect when compared with the inhibitory effect of selegiline alone (Table 4).

Method validation

The limit of detection (LOD) and limit of quantitation (LOQ) of the assay method, in human plasma, were 0.25 ng ml^{-1}. The within batch precision (RSD) of the validation pool ranged from 1.41 to 16.7%. Batch-to-batch precision (RSD) of the validation pool ranged from 6.9 to 14.4%. The within batch accuracy of the validation pool ranged from 90.1 to 111.5%, whereas batch-to-batch accuracy ranged from 98.3 to 104%. In the presence of metabolites at the concentration described earlier, the within batch precision (RSD) of the validation pool ranged from 2.65 to 16.22%.

Table 4
Accuracy and precision of selegiline assay

Concentration (ng ml^{-1})	Selegiline	Accuracy (%)	Selegiline + Metabolite	Accuracy (%)
0.375	0.39 ± 0.06	104.00	0.41 ± 0.06	109.33
3.75	3.82 ± 0.37	101.87	3.84 ± 0.31	102.40
7.50	7.37 ± 0.51	98.27	7.49 ± 0.37	99.56

$n = 30$.

Batch-to-batch precision (RSD) of the validation pool ranged from 5.0 to 14.19%. The within batch accuracy (RSD) of the validation pool ranged from 99.0 to 119.0%, whereas batch-to-batch accuracy (RSD) ranged from 99.9 to 109%. A paired *t*-test was used to determine the difference between the mean concentration values determined for selegiline (concentrations 0.375, 3.75 and 7.5 ng ml^{-1}) alone and in the presence of metabolites. At the 0.05 level, no statistical differences were detected between the two groups.

In dog plasma, the within batch precision (RSD) of the validation pool ranged from 3.95 to 16.35%; batch-to-batch precision (RSD) of the validation pool ranged from 4.92 to 15.39%. The within batch accuracy of the validation pool ranged from 98.66 to 112.53%, whereas batch-to-batch accuracy of the validation pool ranged from 101.33 to 101.47%.

The LOD and LOQ of the assay method, using 100 µl human plasma was 1.25 ng ml^{-1}. The within batch precision (RSD) of the validation pool ranged from 3.78 to 9.60%. Batch-to-batch precision (RSD) of the validation pool ranged from 3.66 to 8.55%. The within batch accuracy of the validation pool ranged from 96.8 to 105.47%; whereas batch-to-batch accuracy of the validation pool ranged from 98.00 to 104%. The LOD and LOQ of the assay method, using 25 µl human plasma was 25 ng ml^{-1}. The within batch precision (RSD) of the validation pool ranged from 2.90 to 11.78%. Batch-to-batch precision (RSD) of the validation pool ranged from 6.51 to 11.60%. The within batch accuracy of the validation pool ranged from 91.94 to 108.60%, whereas batch-to-batch accuracy of the validation pool ranged from 97.29 to 104.33%.

The ruggedness of the method was evaluated from the inter-day accuracy of the data. Samples prepared and analysed on day 2 or 3 were quantified using the calibration curves prepared on day 1. No significant difference was observed in inter-day accuracy.

Conclusions

The described method resulted in precise and accurate quantification of selegiline in human plasma from 0.25 to 8.0 ng ml^{-1}. This simple and rapid method may be useful for the assay of selegiline in blood, urine and possibly tissues for pharmacokinetic studies in animals, healthy volunteers, or Parkinsonian patients. This method has also been validated in dog plasma and a pharmacokinetic study of selegiline in the dog has been completed.

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