Method Title: Recombinant Human MAO-B Inhibition Assay

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Summary

This document describes an in vitro method for determining potential inhibition of the human monoamine oxidase isoform B (MAO-B) by test drugs. The assay uses membrane preparations in which human MAO-B is heterologously expressed in baculovirus-infected BTl insect embryonic cells. Recombinant human MAO-B (rhMAO-B) preparations provide a relatively pure source of monoamine oxidase activity with very low levels of interfering activities, and without interference by the A isoform of MAO, which may be present in tissue preparations. The substrate used to measure enzymatic activities is an aminopropylether analog of methyl ester luciferin, which is converted to D-luciferin by MAO-B. The amounts ofD-luciferin formed are subsequently determined using the enzyme luciferase, which produces light that can be measured in a plate luminometer. A selective inhibitor ofMAO-B, selegiline (Deprenyl), is used as a positive control for inhibition. The use of this assay to measure recombinant and native MAO-B activities has been reported (1).

Standard reaction conditions for the recombinant human MAO-B assay are:

- Substrate concentration: 4.0 flM
- Protein concentration: 0.2 mg/mL (10 flg/well)
- Format: 96-well black opaque plate
- Incubation time and temperature: 1 hour at ambient temperature

Validation experiments used to select these reaction conditions are presented in Appendix 1.

Reagents and Materials

Equivalents may be used

1. MAO-Glo assay kit (Promega #Vl401, enough for 200 reactions), which consists of:
   a. MAO Substrate (arninopropylether analog of methyl ester luciferin)
   b. MAO-B Reaction Buffer- 100 mM HEPES, pH 7.5; 5% glycerol; 10% DMSO
   c. Reconstitution Buffer with Esterase (converts luciferin methyl ester precursor to D-luciferin) – make sure this reagent contains esterase
   d. Luciferin Detection Reagent (contains ATP and luciferase)
   e. rhMAO-B, 5 mg protein/mL (BD Biosciences #456284)
2. Control membranes, 5 mg protein/mL (BD Biosciences #456201)
3. Selegiline hydrochloride (Sigma M003-250MG)
4. 96-well black plates
5. Plate luminometer capable of reading a 96-well plate (Perkin-Elmer TopCount)

Assay Procedures

Preparation of Reagents

Volumes of reagents may be adjusted by using proportional weights and volumes.

1. **MAO-B Reaction Buffer** (100 mM HEPES, pH 7.5, 5% glycerol, 10% DMSO):
   a. To make 250 mL of buffer, dissolve 5.96 g HEPES (MW 238.3 g/mol) in about 200 mL of water; adjust pH to 7.5.
   b. Add 12.5 mL of glycerol and 25 mL ofDMSO. Bring the volume to 250 mL with water. And mix by inversion. Store refrigerated for up to 1 year.

2. 4x **Substrate Stock Solution** (16 J..lM substrate in MAO-B Reaction Buffer):
   a. Dissolve the dried MAO Substrate in 105 J..lL ofDMSO for the 200 reaction kit or 525 fl.L ofDMSO for the 1000 reaction kit. This will produce a 4 mM substrate stock solution.
   b. Dilute the DMSO substrate stock with MAO-B Reaction Buffer to a concentration of 16 J..lM (250x dilution). For example, for a 96-well plate of reactions, use 5.0 flL of MAO substrate stock diluted to a final volume of 1250 J..lL.

3. **Lucifer-in Detection Reagent:**
   a. Equilibrate the Reconstitution Buffer with Esterase and lyophilized Luciferin Detection Reagent to room temperature.
   b. Transfer the entire contents of the bottle of Reconstitution Buffer with Esterase to the amber bottle of lyophilized Luciferin Detection Reagent. Mix by swirling or inverting several times to obtain a homogeneous solution. Do not vortex or shake.

4. 4x **Selegiline (SEL) Stock Solutions:** Selegiline is used as positive control inhibitor of MAO-B.
   a. Prepare 40 mM SEL stock (400x):- Dissolve 17.9 mg SEL HCl (MW 223.7 g/mol) in 2.0 mL ofDMSO.
   b. Prepare 4x SEL solutions in MAO-B Reaction Buffer.

Final cone. (J..I.M)	4x **Stock (J..I.M)**	**J..I.L of stock**	**IlL MAO-B Buffer**
100	400	10 J..lLof40 mM	990 f.lL
10	40	50 f..lL of 400 J..lM	450J..lL
1	4	50 flL of40 J..lM	450 f..lL
0.1	0.4	50 J..lLof4 J..lM	450 f..lL
0.01	0.04	50 J..lL of 0.4 J..lM	450 J..lL
0 (vehicle control)	0	0	500 f..lL

5. 4x **Test Drug Stock Solutions:** It is recommended that test drug solubility in DMSO and assay buffer be tested before preparing incubation mixes. The top concentrations of drug may need to be adjusted or omitted if solubility is limited.
   a. Prepare a 400x stock of each test drug in DMSO.
   b. Prepare 4x stocks (as above for SEL) to give final assay concentrations ofO, 0.01, 0.1, 1.0 10, and 100 J.!M.

6. 2x **rhMAO-B Enzyme Preparation:**
   a. Transfer 80 J.!L of 5 mg/rnL rhMAO-B stock to 920 L MAO-B Reaction Buffer. Keep suspensiÖns on Ice.

Incubation Procedures

1. Prepare incubation mixes:
   a. Test drug incubations: Pipette the following into wells:
      - 12.5 J.!L of 4x (16 J.!M) substrate solution
      - 12.5 J.J.L of 4x test article solutions
   b. Positive SEL controls: Pipette the following into wells:
      - 12.51-!L of4x (16 J.J.M) substrate solution
      - 12.5 11L of 4x SEL solutions
   c. Negative membrane controls (optional):
      - 12.5 L of 4x (16 11M) substrate solution
      - 12.5 11L ofMAO-B Reaction Buffer

2. Incubate samples: Start reactions by adding 25 lL of2x MAO-B enzyme preparation or control membranes to each well; mix. Incubate 60 minutes with shaking at ambient ternperature.
3. Process and analyze samples:
   a. Add 50 J.!L of reconstituted Luciferin Detection Reagent to each well of the plate and mix briefly.
   b. Incubate the plate at room temperature for 20 minutes and measure the luminescence of the wells.
4. Calculations:
   a. Raw values for relative luminescence units (RLU) are corrected by subtracting mean negative membrane control values from test drug and positive control values. Means and standard deviations are then determined for vehicle controls (0 11M).
   b. Calculate percent of vehicle control values by dividing sample and positive control RLU values by the mean RLU value of the corresponding vehicle control.

c. Determine the means and standard deviations of the percent of vehicle control values for test drug and positive control treatments.

References

1. Valley, M. P., Zhou, W., Hawkins, E. M., Shultz, J., Cali, J. J., Worzella, T., Bemad, L., Good, T., Good, D., Riss, T.L., Klaubert, D. H., and Wood, K. V. (2006). A bioluminescent assay for monoamine oxidase activity. *Analyt. Biochem*. 359, 238-46.

Appendix 1: Assay Validation Results

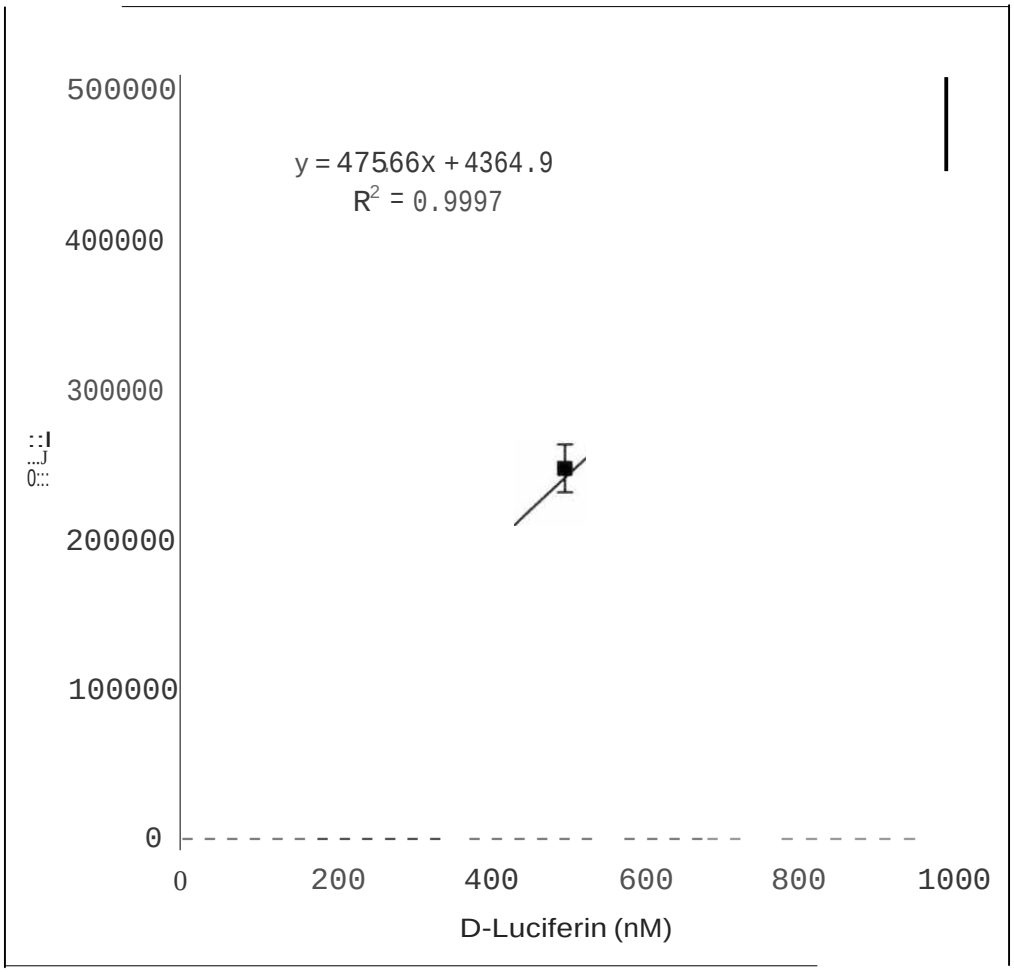

Figure 1: Linearity of luminescent response for a D-luciferin standard curve. The assay gives a linear response up to about 5×10^5 relative light units (RLU).

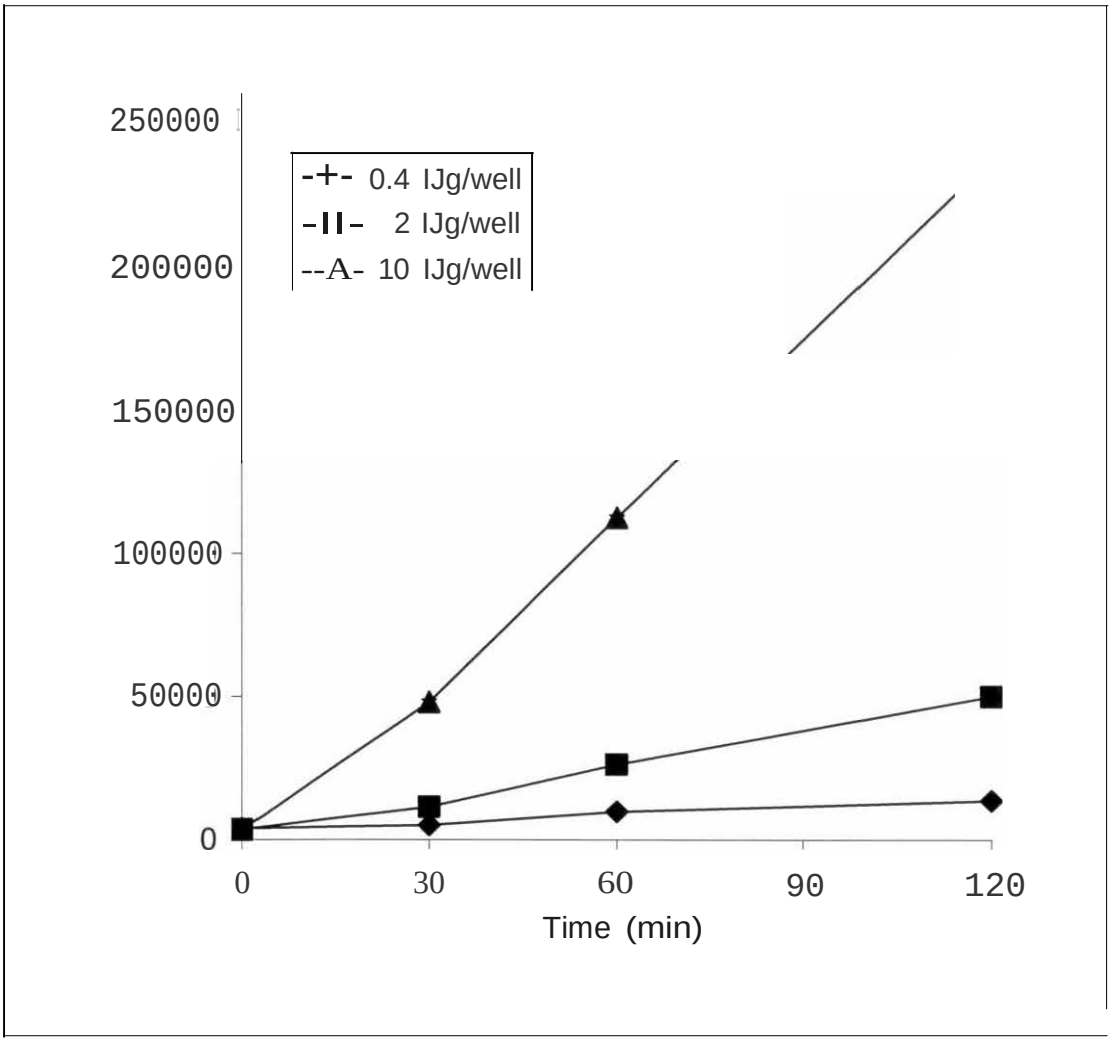

Figure 2: Time-dependence of assay response. The linear dependence of the reaction rate on time indicates that initial rate conditions have been satisfied for the assay, which is required for determination of the Michaelis-Menten constant, Km.

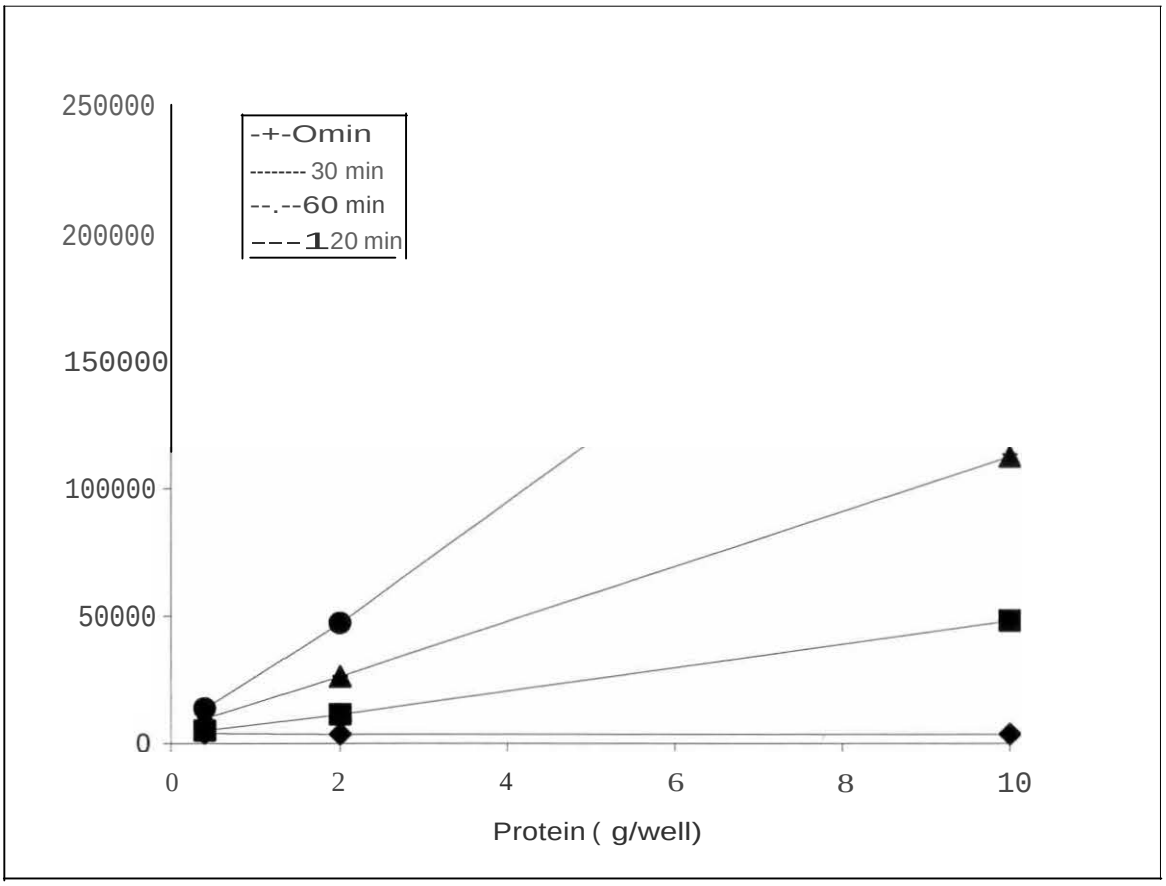

Figure 3: Protein-dependence of assay response. The linear dependence of the reaction rate on protein concentration also indicates that initial rate conditions have been satisfied for the assay. Based on these results, assay conditions of I0 *J.g* protein/well and one hour incubation time were chosen.

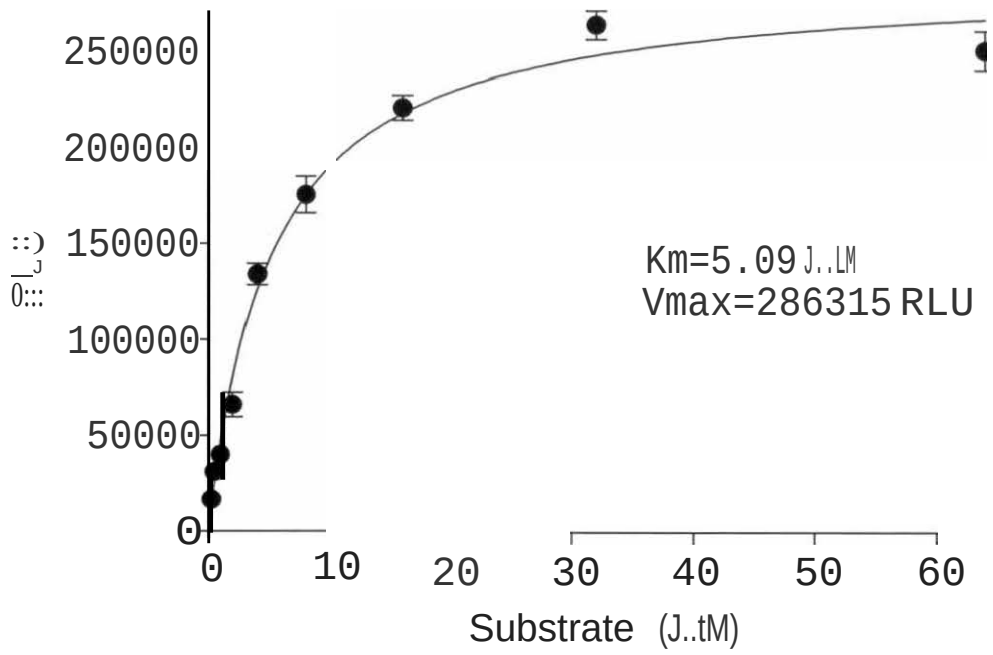

Figure 4: Substrate-velocity plot of rhMAO-B assay. Under standard reaction conditions the Michaelis-Menten constant, Km, was determined to be 5.09 j.!M. Therefore, a substrate concentration of 4.0 llM was chosen for the rhMAO-B inhibition assay.

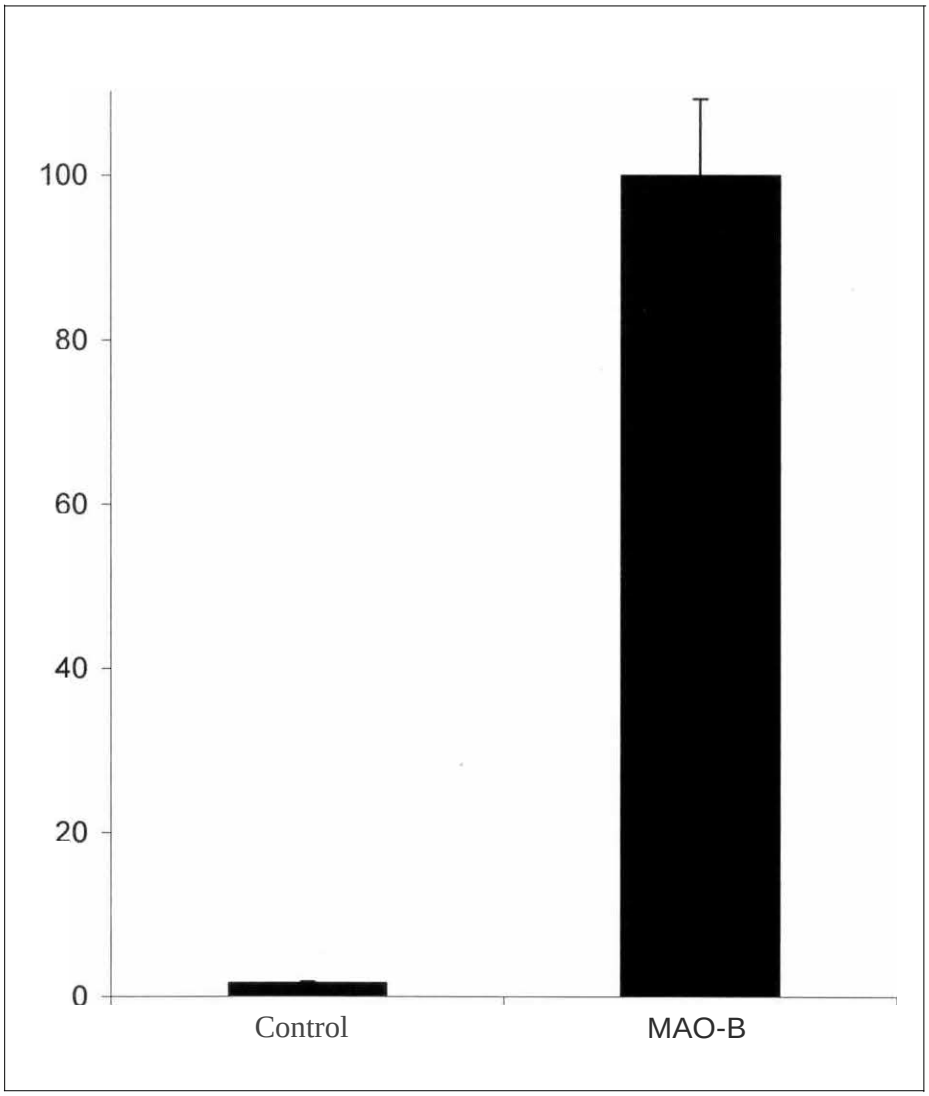

Figure 5: Determination of background MAO activity using control membranes. Control membranes from BTl insect cells transfected with the wild type viral vector without the MAO-B gene showed very low MAO activities relative to membranes from MAO-B transfected cells.

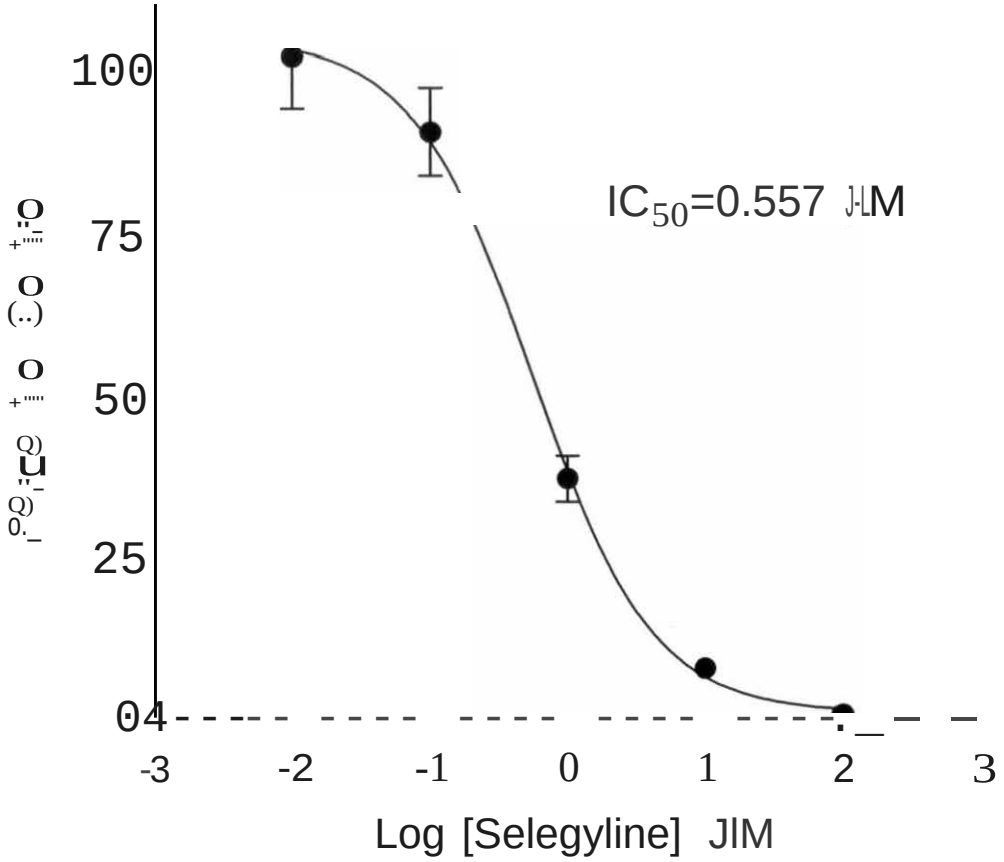

Figure 6: Selegiline inhibition of MAO-B. Selegiline, a selective inhibitor of MAO-B, produced a concentration-dependent inhibition of rhMAO-B with an IC_{50} of 0.557 JlM.