Sensitive, Specific Detection of MAO Activity

The MAO-Glo™ Assay: A Bioluminescent-Coupled Assay for Monoamine Oxidase Activity

Michael P. Valley, Ph.D.[1], Erika M. Hawkins, M.S.[1], John Shultz, Ph.D.[1], Terry L. Riss, Ph.D.[1], Keith V. Wood, Ph.D.[1], Wenhui Zhou, Ph.D.[2], Laurent Bernad, Ph.D.[2], Troy Good, Ph.D.[2], Dave Good, B.S.[2], and Dieter H. Klaubert, Ph.D.[2] [1]Promega Corporation, [2] Promega Biosciences, Inc.

Introduction

Although cytochrome P450 enzymes are responsible for the majority of drug metabolism reactions, the monoamine oxidases (MAOs) are arguably the second-most important phase I biotransformation enzymes. MAOs catalyze the oxidative deamination of a number of biogenic and xenobiotic amines to the corresponding aldehydes with consumption of O_2 and production of H_2O_2 (1). These flavoenzymes are located in the outer membrane of mitochondria and exist in two forms, MAO A and MAO B (2). In humans, these isozymes are 70% identical and exhibit different yet overlapping tissue expression and substrate specificity (3). Since MAOs can oxidize neurotransmitters and produce toxic H_2O_2, they have been associated with a variety of behavioral conditions including smoking, alcoholism, aggression, and certain neurodegenerative disorders (4).

The MAO-Glo™ Assay can detect MAO activity over a 10,000-fold range of enzyme concentration with limits of detection of 1 and 6ng for MAO A and B, respectively.

The ability to measure MAO activity and the effects of test compounds on that activity is critical for selecting specific MAO inhibitors, discovering potential drug-drug interactions and assessing the MAO-catalyzed detoxification or bioactivation of various target compounds (5–7). However, there are several disadvantages to current methods for measuring MAO activity. HPLC and radiochemical methods are common but are typically time-consuming and expensive. Fluorescent assays can be rapid and sensitive, but they are often susceptible to interference. Therefore, we have developed an assay that couples the activity of MAO to the production of light by luciferase. The MAO-Glo™ Assay[a,b,c] is a simple, homogeneous and robust two-step bioluminescent-coupled assay for the rapid and sensitive detection of MAO activity.

Assay Design and Chemistry

In the MAO-Glo™ Assay, the substrate for the MAO reaction is an aminopropylether analog of luciferin methyl ester (Figure 1). The MAO A and MAO B enzymes used in this study were purchased from Sigma-Aldrich (Cat.# M7316 and M7441) in the form of

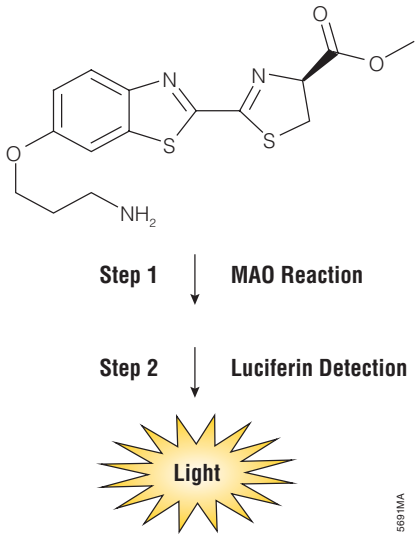

Figure 1. The MAO-Glo™ Assay reaction.

microsomal protein prepared from insect cells infected with recombinant baculovirus containing cDNA inserts for the human MAO genes. In the first assay step, the MAO enzyme oxidizes the amine of the substrate to an imine, which is subsequently hydrolyzed to the corresponding aldehyde. The aldehyde then spontaneously undergoes a β-elimination reaction to form luciferin methyl ester. In the second assay step, the Luciferin Detection Reagent inactivates the MAO enzyme, and the esterase and luciferase enzymes in the reagent sequentially hydrolyze the methyl ester and oxidize luciferin to produce light. Although a number of reactions are required to generate the luminescent signal, most of these reactions are rapid, and the amount of light produced in the second step is proportional to the activity of MAO in the first step.

The substrate exhibits low K_m values (4–40µM) with both isozymes, and the maximal activity is only about tenfold different, favoring MAO A over MAO B. The amount of MAO used and the reaction time in Step 1 will affect the level of luminescence (Figure 2, Panel A). Reactions with 0.2µg, 1µg or 5µg of microsomal protein incubated for three hours, 40 minutes or 10 minutes, respectively, all produce a similar luminescent signal. The proprietary formulation of the Luciferin Detection Reagent eliminates the need for strictly timed luminescent detection and generates a stable luminescent signal (Figure 2, Panel B).

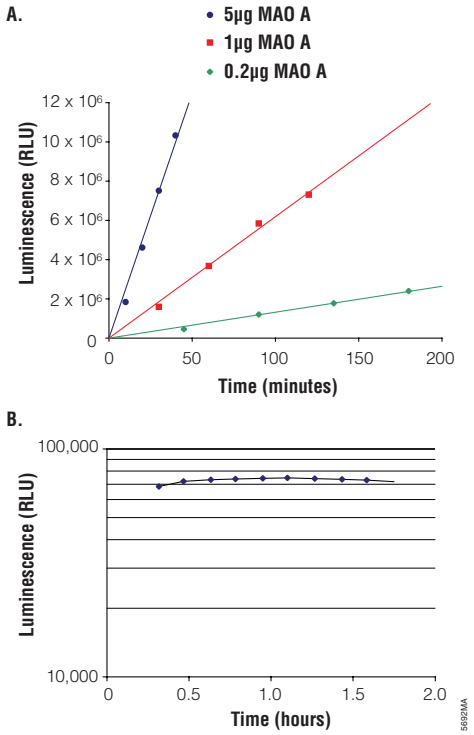

Figure 2. The kinetics of the MAO-Glo™ Assay. Panel A. In a volume of 50µl, 5µg (circles), 1µg (squares) or 0.2µg (diamonds) of microsomes containing MAO A were added to 40µM MAO Substrate in MAO Reaction Buffer at 25°C. After various incubation times, 50µl of reconstituted Luciferin Detection Reagent was added, and the luminescent signal was measured after 20 minutes with a GloMax™ 96 Microplate Luminometer (Cat.# E6501). **Panel B.** The same reaction as in Panel A was performed with 2µg of microsomes containing MAO A. After an incubation time of 45 minutes, 50µl of reconstituted Luciferin Detection Reagent was added, and the luminescent signal was measured at various times.

Take Advantage of the Luminescent Format

The MAO-Glo™ Assay has several advantages over comparable fluorescent assays. Fluorescent detection methods can be confounded by the absorbance or fluorescence properties of the compounds being tested, while bioluminescent assays are much less sensitive to these effects. For the detection of MAO activity, one particular fluorescent assay couples the production of H_2O_2 by MAO to the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine by horseradish peroxidase (HRP; 8). This fluorescent assay is an indirect and nonspecific measure of MAO activity since H_2O_2 levels can be affected by a number of enzymes and cellular processes. Any compounds that can alter the concentration of H_2O_2 will elicit a change in the fluorescent signal regardless of MAO enzyme activity. The requirement of HRP by fluorescent assays is also unfavorable because numerous compounds can act as oxidizable substrates for HRP, including the resorufin product of the fluorescent assay (9). Such compounds will hinder the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine and uncouple the fluorescent signal from the activity of MAO. In addition, the stability of 10-acetyl-3,7-dihydroxyphenoxazine is a concern because it is sensitive to air, light, changes in pH and the presence of thiols. In contrast, the bioluminescent assay is specific for MAO, since only compounds that interfere with the ability of MAO to use the pro-luminescent substrate will cause changes in the resulting luminescent signal.

The quality of the MAO-Glo™ Assay was assessed by performing a Z´-factor analysis in a 384-well plate. Z´-factor values between 0.5–1.0 are indicative of high-quality assays (10); the Z´-factor value of 0.95 measured for both MAO A and MAO B suggests that the MAO-Glo™ Assay is an excellent method for the detection of MAO activity. The sensitivity of the MAO-Glo™ Assay was determined by measuring the limit of detection, defined as the amount of enzyme necessary to give a net luminescent signal equal to three times the noise of the signal in the absence of MAO. The MAO-Glo™ Assay can detect MAO activity over a 10,000-fold range of enzyme concentration with limits of detection of 1 and 6ng per reaction for MAO A and MAO B, respectively (Figure 3). By comparison, the fluorescent assay described above was much less sensitive. Using either of the two recommended substrates at concentrations equal to their K_m values, the limits of detection of the fluorescent assay were between 170–190ng (Figure 3).

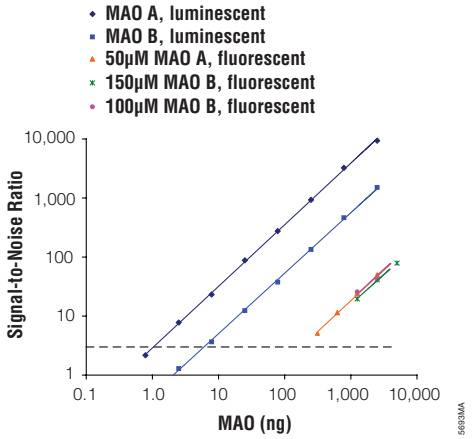

Figure 3. The sensitivity of the MAO-Glo™ Assay. In a volume of 50µl, various amounts of microsomes containing MAO A (diamonds) were added to 40µM MAO Substrate in MAO Reaction Buffer at 25°C. After a one-hour incubation, 50µl of reconstituted Luciferin Detection Reagent was added, and the luminescent signal was measured after 20 minutes with a GloMax™ 96 Microplate Luminometer (Cat.# E6501). The same experiment was performed with MAO B (squares) and 4µM MAO Substrate in MAO B Reaction Buffer. Fluorescent assays were performed on a Fluoroskan Ascent plate-reading fluorometer (Labsystems) as described by Molecular Probes, Inc., except the substrate concentrations were adjusted to match their reported K_m values of 50µM *p*-tyramine with MAO A (triangles), 150µM *p*-tyramine with MAO B (asterisks), or 100µM benzylamine with MAO B (circles). The dashed line indicates the limit of detection at a signal-to-noise ratio of 3.

Accurately Determine Effects of Test Compounds

The ability of the MAO-Glo™ Assay to detect changes in the activity of MAO was demonstrated by performing the assay in the presence of compounds known to be substrates and inhibitors of the MAO enzymes (Figure 4). Clorgyline and deprenyl are specific inhibitors for MAO A and MAO B, respectively, while phenylethylamine, serotonin, and dopamine are substrates with variable specificity for each isozyme. These compounds all caused a decrease in activity with the MAO substrate because both substrates and inhibitors interfere with the ability of MAO to oxidize the proluminescent substrate. The bioluminescent-coupled assay correctly predicted the specificity of these compounds and yielded K_m or K_i values that are comparable to previously published values. For calculated K_m or K_i values and the corresponding published values see Technical Bulletin #TB345.

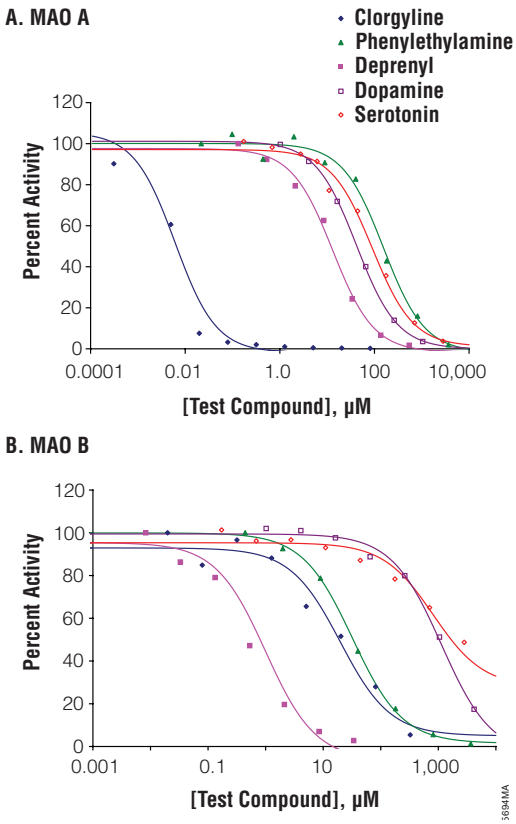

Figure 4. Measuring the affinity of test compounds for MAO with the MAO-Glo™ Assay. MAO A (**Panel A**) and MAO B (**Panel B**) were assayed as in Figure 3 in the presence of various concentrations of known substrates and inhibitors: clorgyline (closed diamonds), phenylethylamine (triangles), deprenyl (closed squares), dopamine (open squares), and serotonin (open diamonds). For the calculated K_m or K_i values and the corresponding published values, see Technical Bulletin #TB345.

The ability to detect the effects of test compounds was also demonstrated by screening LOPAC[1280], a collection of 1,280 pharmacologically active compounds available from Sigma-Aldrich (Figure 5). We defined a "hit" as any compound that caused a greater than 25% decrease in the luminescent signal relative to the no-test-compound control reactions. Using this criterion, 95 compounds were hits against MAO A, 60 compounds were hits against MAO B, and 58 compounds were hits against both enzymes. In order to determine if any of these compounds inhibit the Luciferin Detection Reagent, the subset of compounds that were hits with both enzymes was rescreened in the absence of MAO using 0.5µM luciferin methyl ester as substrate. Only 12 compounds were found to cause a greater than 25% decrease in the luminescent signal relative to the no-test-compound control reactions, indicating that the Luciferin Detection Reagent had only a 0.9% false-hit rate. This is consistent with the 0.8–1.3% false-hit rate reported for a similar bioluminescent-coupled protease assay (11). In contrast, analogous fluorescent-based protease assays have reported false-hit rates as high as 10% (12). With a more stringent cutoff of a greater than 80% decrease in luminescent signal to define a hit, the number of compounds that were hits with MAO A, MAO B or both enzymes decreases significantly (33, 5 and 8, respectively), but so does the number of false hits; only three of these compounds caused a greater than 80% decrease in the luminescent signal relative to the no-test-compound control, yielding an even lower false-hit rate of 0.2%.

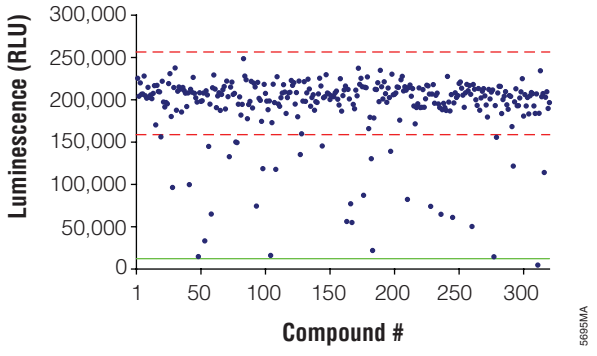

Figure 5. An example of screening LOPAC[1280] with the MAO-Glo™ Assay. Reactions with MAO A were performed in 384-well plates on a Freedom EVO® 200 (Tecan) with 0.5µg of microsomal protein and 10µM test compound in a volume of 30µl. Test compound was substituted with MAO Reaction Buffer and 5% glycerol) in the positive control reactions; MAO enzyme was substituted with buffer in the negative control reactions. After one hour, 30µl of Luciferin Detection Reagent was added, and the luminescent signal was measured after 20 minutes on a GENios Pro® detection instrument (Tecan). For clarity, reactions with only one fourth of the compounds of the LOPAC[1280] library are shown. The red dashed lines represent deviations of ±25% from the average of positive control reactions, and the green line represents the average of the negative control reactions.

The robustness of this bioluminescent-coupled assay is not only superior to fluorescent assays, but it also makes a provocative argument for screening compounds against MAO. Using the 25% cutoff, 201 of the compounds screened interact with the MAO A and/or MAO B enzymes. This suggests that, with a similar library of active compounds, there is a 16% chance that any one compound may interact with one or both of the MAO enzymes. In other words, the involvement of MAO in drug metabolism reactions may be more prevalent than is currently understood, warranting the inclusion of MAO activity assays in any drug metabolism studies.

Summary

The MAO-Glo™ Assay is a simple and homogeneous method for measuring MAO activity. This robust assay can easily be performed in less than 90 minutes, yet the luminescent signal is stable enough that it can be reliably measured over several hours. Compared to typical fluorescent assays, the MAO-Glo™ Assay is more sensitive, more specific for MAO and less susceptible to interference by test compounds. The MAO-Glo™ Assay accurately reflects the effects of test compounds and is amenable to automation. Furthermore, since a substantial percentage of library compounds appear to interact with MAO, the MAO-Glo™ Assay would be a beneficial component in future drug metabolism studies.

Acknowledgments

The authors would like to thank Brad Larson and Tracy Worzella for conducting the LOPAC^{1280} screen experiment.

References

1. Edmondson, D.E. *et al.* (2004) *Curr. Med. Chem.* **11**, 1983–93.
2. Bach, A.W. *et al.* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4934–8.
3. Benedetti, M.S. (2001) *Fundam. Clin. Pharmacol.* **15**, 75–84.
4. Shih, J.C., Chen, K. and Ridd, M.J. (1999) *Annu. Rev. Neurosci.* **22**, 197–217.
5. Kalgutkar, A.S. *et al.* (2002) *Curr. Drug Metab.* **3**, 379–424.
6. Kalgutkar, A.S. *et al.* (2001) *Chem. Res. Toxicol.* **14**, 1139–62.
7. Benedetti, M.S. and Dostert, P. (1994) *Drug Metab. Rev.* **26**, 507–35.
8. Zhou, M. and Panchuk-Voloshina, N.P. (1997) *Anal. Bioch.* **253**, 169–74.
9. Reszka, K.J. *et al.* (2005) *Anal. Bioch.* **342**, 327–37.
10. Zhang, J.H. *et al.* (1999) *J. Biomol. Screen.* **4**, 67–73.
11. O'Brien, M.A. *et al.* (2005) *J. Biomol. Screen.* **10**, 137–48.
12. Grant, S.K., Sklar, J.G. and Cummings, R.T. (2002) *J. Biomol. Screen.* **7**, 531–40.

Protocols

◆ *MAO-Glo™ Assay Technical Bulletin* #TB345, Promega Corporation.

Ordering Information

Product	Size	Cat.#
MAO-Glo™ Assay	200 assays	V1401
	1,000 assays	V1402

[a] Patent Pending.

[b] U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents pending.

[c] The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.