Sources of Error in the Determination of Platelet Monoamine Oxidase: A Review of Methods

by C. David Wise, Steven G. Potkin, T. Peter Bridge, Bruce H. Phelps, H. Eleanor Cannon-Spoo, and Richard Jed Wyatt

Abstract

The methods used in the determination of platelet monoamine oxidase (MAO) activity in 26 studies of chronic schizophrenic patients and normal subjects (reviewed by Wyatt, Potkin, and Murphy 1979) are examined. The most commonly used substrate was tryptamine (64 percent) followed by tyramine, benzylamine, and β-phenylethylamine. When the relationship between the MAO activity and substrate concentration was examined, it was found that as many as 75 percent of the studies used suboptimal substrate concentrations. It appears that some chronic schizophrenics have a lower Michaelis constant (Km) and maximal velocity (Vmax). Thus, the use of suboptimal substrate concentrations could lead not only to a low estimate of the MAO activity, but also to erroneous comparisons between schizophrenic and normal groups. Because platelets are markedly heterogeneous in size and density, the differential centrifugation methods used for platelet preparation can produce up to an eight-fold error in the estimation of the MAO activity. We describe the use of a new method for the preparation of platelets that avoids many of the problems of the differential centrifugation methods. The new method shows substantially less intrasubject variance in MAO activity and is significantly different from the differential centrifugation procedures (by χ² of pooled intraclass correlations, p < .005).

Figure 1. MAO activity in normal subjects in studies using tryptamine as a substrate

Platelet monoamine oxidase (MAO) activity has been extensively investigated in normal and psychiatrically ill patients. A recent literature review (Wyatt, Potkin, and Murphy 1979) from our laboratory pointed out that, despite continuous efforts, consensus is still lacking concerning the normal range of platelet MAO activity. Figure 1 shows a greater than four-fold range in mean platelet MAO activity for normal groups in the studies using tryptamine as sub-

1 From table 3 in Wyatt, Potkin, and Murphy (1979).

Reprint requests should be sent to Dr. Wise at Laboratory of Clinical Psychopharmacology, Division of Special Mental Health Research, Intramural Research Program, National Institute of Mental Health, WAW Building, St. Elizabths Hospital, Washington, DC 20032.
strate. This wide range for normals is probably not primarily due to choosing different groups of normal individuals but rather to differences in platelet preparation and MAO assay procedures. In an attempt to understand better the reasons for the discrepant values in the literature, we scrutinized the methods used in 26 studies of normal subjects and chronic schizophrenic patients (reviewed by Wyatt, Potkin, and Murphy 1979). We will also report on the use of a new method (Corash, Shafer, and Perlow 1979) for the preparation of the entire platelet population, which decreases the degree of intrasubject variation over time.

Factors That Can Alter Platelet MAO Activity

Many factors are claimed to affect platelet MAO activity, e.g., acute versus chronic schizophrenic patients, selection of control groups, age, hormones, and diet (for review, see Wyatt, Potkin, and Murphy 1979). Most of these factors can be controlled for by the careful selection and matching of the patient group with appropriate individuals in the control group. There are, however, two additional factors which, in our opinion, require more attention: the methods used in the preparation of the platelets and the assay procedures employed. We feel that these two factors account for much of the intrasubject variance of MAO activity and perhaps the variance among studies reported in the literature.

MAO Assay Procedure

One parameter of the platelet MAO assay that has received relatively little attention is the relationship of substrate concentration to enzyme activity. As the theoretical curves in figure 2 illustrate, the enzyme activity in group II is less at substrate concentration C, no different at concentration B, and greater at concentration A if both the Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) are smaller in group II. Of course, other theoretical curves can be drawn, but there is evidence to suggest that the curves in figure 2 may represent the situation with platelet MAO in some schizophrenic patients. That is, the majority of studies (Wyatt, Potkin, and Murphy 1979) show that the platelet MAO activity in chronic schizophrenics is lower, and we (Kobes et al. 1979) and others (Berrettini, Prozialeck, and Vogel 1978; Berrettini, Vogel, and Clouse 1977) have reported that the $K_m$ in this group is also lower. Thus, when measuring platelet MAO activity in normal controls and patients at only one substrate concentration, it is essential to

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Figure 2. Theoretical curves of enzyme activity as a function of substrate concentration

![Diagram showing theoretical curves of enzyme activity](image-url)
determine, at least on subgroups of patients and normals, the enzyme activity over a wide range of substrate concentrations. This information allows one to establish, for the particular substrate, the optimal conditions for screening the remaining samples. One should use a substrate concentration that is on the plateau region of the saturation curve and not on either the ascending or descending (substrate inhibition) part of the curve. Also, because of variations in assay procedures, each laboratory should establish its own optimal substrate concentration. The necessity of determining this parameter is obvious from the wide range of apparent $K_m$ values reported in the literature for tryptamine from 7.4 to 35 $\mu$M (Collins and Sandler 1971; Donnelly and Murphy 1977; Edwards and Chang 1975; Hornecker et al. 1976; Robinson et al. 1968).

The results of our literature analysis show that tryptamine has been the most frequently used substrate followed by tyramine, benzylamine, and $\beta$-phenylethylamine (table 1). The data in figure 3 show the tryptamine concentrations used in the 18 studies of table 1. Also shown in figure 3 is a saturation curve for tryptamine determined in our laboratory. It is clear that substrate saturation occurs at a concentration greater than 100 $\mu$M. Two-hundred micromolar or greater would be a safe working range of substrate concentrations to be assured of measuring enzyme activity under saturating conditions. One-hundred micromolar or less would be in a suboptimal substrate concentration range. However, 72 percent of the studies cited in table 1 were probably run in the suboptimal range (table 2).

For tyramine (figure 4) all of the studies were run in a suboptimal range. In the case of $\beta$-phenylethylamine (figure 5), we and Hornecker et al. (1976) find a very narrow range of substrate concentrations from 8 to 10 $\mu$M that yield maximal enzyme activity. At concentrations greater than 10 $\mu$M, $\beta$-phenylethylamine is a potent MAO inhibitor. Again, most studies have used substrate concentrations outside the optimal range. The data summarized in table 2 indicate that 75 percent of the 26 studies may have used suboptimal substrate concentrations. In some cases, at least, the lower MAO values shown in figure 1 can be explained by the use of suboptimal (nonsaturating) substrate concentrations.

**Methods Employed in the Preparation of Platelets**

The other major factor that can greatly affect platelet MAO activity is the method for platelet pellet preparation. Studies have shown that platelets are markedly heterogene-

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**Table 1. Most frequently used substrates in the assay of platelet MAO**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Number of studies</th>
<th>Percent of all studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptamine</td>
<td>18</td>
<td>64.2</td>
</tr>
<tr>
<td>Tyramine</td>
<td>5</td>
<td>17.9</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>4</td>
<td>14.2</td>
</tr>
<tr>
<td>$\beta$-Phenylethylamine</td>
<td>1</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**Figure 3. Platelet MAO activity with tryptamine as substrate**

Note.—Data on left show concentration of tryptamine in the 18 studies that used this substrate (see table 1 in Wyatt, Potkin, and Murphy 1979). Graph on the right shows a typical curve of normal MAO activity as a function of tryptamine concentration. Average $K_m$ data for a group of four subjects are indicated.
Table 2. Substrate concentrations (optimal/suboptimal) used in 26 studies

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Number of studies</th>
<th>Substrate concentrations</th>
<th>Suboptimal percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptamine</td>
<td>18</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Tyramine</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>β-Phenyl-ethylamine</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>7</td>
<td>21</td>
</tr>
</tbody>
</table>

Figure 4. Platelet MAO activity with tyramine as a substrate

Note.—Data on left are from studies reviewed by Wyatt, Potwin, and Murphy (1979). Graph on right shows a typical curve of normal MAO activity as a function of tyramine concentration. Average $k_m$ data taken from Kobes et al. (1979).

Benzylamine and Murphy et al. (1978) separated platelets according to different densities and have shown that there is a 2- to 15-fold difference in MAO activity between their lightest and heaviest subfractions, i.e., the heaviest platelets contained platelet MAO with the highest specific activity.

In the differential centrifugation method, the $g$-force multiplied by the time of the initial centrifugation step ($g$-minutes) is the main factor that determines the type or density of platelet obtained in the final pellet, and thus, the specific activity of the MAO in the platelet pellet. In our review of the literature, there was a 10-fold range of values for the $g$-minute factor (figure 6). Therefore, it is not surprising to find such a wide range of values for normal platelet MAO (figure 1).

There are at least four other factors that interact with the differential centrifugation method to give erroneous or variant results. Platelets prepared in the cold at 4°C yield 12 percent lower MAO activity than those prepared at room temperature, apparently because the cold causes dense platelets to settle in the buffy layer with the white cells. A second factor is the contamination of the pellet with plasma proteins, which results in erroneous platelet protein values. Most methods that we reviewed merely rinse the intact pellet at the bottom of the tube leaving behind large amounts of trapped plasma proteins. Even in those methods that resuspend the pellet, a large percentage of the platelets remain clumped together and trap the plasma proteins. The third factor is red and white cell contamination of platelet preparations. This could be a serious problem, especially since it has been shown that lymphocytes contain MAO (Ibragimosa and Glasov 1976; Sullivan et al. 1978). At low $g$-forces, there will always be a trade-off between the recovery of the more dense platelets and contamination with lymphocytes.
Figure 5. Platelet MAO activity with phenylethylamine (PEA) as substrate

In addition to the one study in the review of Wyatt, Potkin, and Murphy (1979) that used PEA (Demisch et al. 1976), the data for three other studies are included (Yang and Neff 1973; Edwards and Chang 1975; Donnelly and Murphy 1977) including one where MAO activity was measured in rat brain. Graph on right shows a typical curve of normal MAO activity as a function of PEA concentration. Note the substantial inhibition of enzyme activity at PEA concentrations of greater than 10μM.

The final factor is that the distribution and modal platelet density are not uniform among all individuals. Therefore, in the differential centrifugation method, the recovery of the more dense platelets will vary among individuals. We have found that this factor alone can lead to an eight-fold error in the platelet MAO activity determination.

Many of the above problems also apply to the preparation of platelet-rich plasma (PRP) used for the determination of platelet MAO. In addition, there are problems associated with obtaining accurate platelet counts that are used to express the MAO activity. Finally, platelet MAO activity measured in the presence of plasma is, in some cases at least, subjected to the effects of activators (Wise et al. 1979; Yu and Boulton 1979) or inhibitors (Berrettini and Vogel 1978), which could lead to altered results.

The Corash Method for the Preparation of Platelets

A new method for the preparation of platelet pellets has been developed by Corash and Shafer (see Corash, Shafer, and Pertlow 1979) that avoids many of the above problems. This procedure gives 98 percent recovery of the platelet population with no plasma protein contamination and with virtually no red blood cell or leukocyte contamination. The following is a description of the modified procedure used routinely to prepare platelets for MAO assay. In brief, 4½ ml of venous blood is drawn through a 19-gauge needle and placed in a polypropylene tube that contains .5 ml of citrate (.132 M)-EDTA (disodium,.01 M). The contents are gently mixed and kept at room temperature. Two ml of an isotonic buffer (.145 M sodium chloride,.01 N monosodium phosphate and 3.14 mM disodium EDTA, pH 6.5, "wash-out buffer") is added and the contents gently mixed and then centrifuged at 750g (or 2,100 rpm) for 3 minutes in a GLC-2 tabletop cen-
the wash-out buffer; the suspension with a glass pasteur pipette. Each of the two ml aliquots are then combined and centrifuged at 700g for 30 minutes. The pellet is again resuspended in 1 ml of wash-out buffer; an additional 1 ml of buffer is added, mixed, and the tube is centrifuged at 1500g for 20 minutes to obtain the final pellet. The entire procedure is carried out at room temperature. After the supernatant is removed, the pellets are stored at −50°C. Up to 16 samples can be processed in 4 to 6 hours. Every aspect of this procedure has been carefully designed so that the platelets remain physiologically intact and do not clump and trap plasma proteins (Corash, Shafer, and Perlow 1979).

**Platelet MAO Activity Prepared by the Corash Method**

In order to evaluate this new method, we have determined the intrasubject variation of platelet MAO activity over time by the Corash method and compared it to that of the other commonly used procedures. Two separate blood drawings were obtained from a group of normal subjects and chronic schizophrenic patients. With each blood drawing, the platelets were prepared by three different methods, i.e., the Corash method to give a platelet pellet, a differential centrifugation method to give either a platelet pellet (done at 4°C), or PRP (done at 22°C to 26°C). Furthermore, two different substrates, tyramine and benzylamine, were investigated with different preparations.

Thirteen chronic schizophrenic patients, hospitalized at Saint Elizabeths Hospital or currently living in halfway house facilities, who received a diagnosis of schizophrenia according to DSM-II (American Psychiatric Association 1968) and by Research Diagnostic Criteria (RDC; Spitzer, Endicott, and Robins 1975) were studied. The patients who satisfied these criteria had a chronic illness for at least 6 months without return to premorbid level of functioning. Normal control samples were obtained mostly from paid volunteers (n = 11) and a few (n = 4) from staff of the psychiatric unit.

The enzyme assay was basically that of Wurtman and Axelrod (1963) with slight modifications. When tyramine-14C was used as substrate (obtained from New England Nuclear), a final concentration of 2 mM was used, well within the optimal range for saturating the enzyme (see above discussion and figure 4). The platelet pellets or PRP were frozen and thawed three times to obtain maximum enzyme activity. After the addition of 1.5 ml of 0.067M potassium phosphate buffer, the pellet was loosened from the bottom and then blended for 5 seconds at maximum speed with a polytron (Tekmar, Model STT). A 250 µl aliquot of enzyme (that contained .05 to .175 mg protein) was mixed with 200 µl buffer and the reaction initiated by adding 50 µl of substrate. The reaction mixture was incubated for 30 minutes and the reaction

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2 The data presented in this article are part of a repeated measures design that has not been completed. For that study, a smaller group (n = 10) of normals and patients will be carefully matched (see above discussion). However, for the purposes of this article, we are interested in possible differences in the intraclass correlations among the different preparations in normal and patient populations.

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stopped with 50 μl of 3 N HCl. The product was extracted into toluene and the amount of radioactivity determined in a Beckman LS-9000. The amount of product obtained by a single extraction was 62.3 percent (the values reported here are corrected values). MAO activity was found to be linear with respect to protein concentration and time of incubation. When benzylamine-14C (obtained from Amersham) was used as a substrate, the procedure of Murphy et al. (1976b) was followed. For the Corash procedure, the in-

Table 3. Intraclass correlation (ICC) of MAO activity in platelets from normal and chronic schizophrenic patients obtained on two separate occasions by three different methods of platelet preparation

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature</th>
<th>Substrate</th>
<th>Normals (n = 15)</th>
<th>Patients (n = 13)</th>
<th>Combined (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corash, Shafer, and Perlow (1979)</td>
<td>22–26°C</td>
<td>Tyramine3</td>
<td>.92</td>
<td>.79</td>
<td>.936</td>
</tr>
<tr>
<td>Differential centrifugation1</td>
<td>4°C</td>
<td>Tyramine3</td>
<td>.436</td>
<td>.38</td>
<td>.59</td>
</tr>
<tr>
<td>Differential centrifugation2</td>
<td>22–26°C</td>
<td>Tyramine3</td>
<td>.73</td>
<td>.29</td>
<td>.65</td>
</tr>
<tr>
<td>Differential centrifugation2</td>
<td>22–26°C</td>
<td>Benzylamine4</td>
<td>.75</td>
<td>.45</td>
<td>.77</td>
</tr>
</tbody>
</table>

1 Method of Murphy et al. (1976a) was used to obtain platelet pellet.
2 Platelet-rich plasma was obtained by method of Murphy et al. (1976a).
3 Final concentration of tyramine-14C was 2 mM.
4 The assay of Murphy et al. (1976a) was used. Normal group n = 10.
5 Different from the Corash method by χ2 of pooled ICC, p < .05.
6 Different from each of the other methods by χ2 of pooled ICC, p < .005.

Table 4. Mean platelet MAO activity in normal and chronic schizophrenic patients from blood drawn on two separate occasions

<table>
<thead>
<tr>
<th>Method of platelet preparation1</th>
<th>Mean MAO activity and t-test comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First blood drawing</td>
</tr>
<tr>
<td></td>
<td>Normals (n = 15)</td>
</tr>
<tr>
<td></td>
<td>Normals (n = 15)</td>
</tr>
<tr>
<td>Corash et al. (1979)2</td>
<td>38.2 ± 1.28</td>
</tr>
<tr>
<td></td>
<td>t = 4.43</td>
</tr>
<tr>
<td></td>
<td>p &lt; .0003</td>
</tr>
<tr>
<td>Differential centrifugation2</td>
<td>27.9 ± 1.88</td>
</tr>
<tr>
<td></td>
<td>t = 3.39</td>
</tr>
<tr>
<td></td>
<td>p &lt; .003</td>
</tr>
<tr>
<td>Differential centrifugation3</td>
<td>61.0 ± 2.22</td>
</tr>
<tr>
<td></td>
<td>t = 3.35</td>
</tr>
<tr>
<td></td>
<td>p &lt; .003</td>
</tr>
<tr>
<td>Differential centrifugation4</td>
<td>13.6 ± 0.73</td>
</tr>
<tr>
<td></td>
<td>t = 4.45</td>
</tr>
<tr>
<td></td>
<td>p &lt; .004</td>
</tr>
</tbody>
</table>

1 See table 3 for temperature and substrate parameters and references for methods of preparation.
2 MAO activity as nmoles product/hr/mg protein.
3 MAO activity as nmoles product/hr/10^8 platelets.
4 MAO activity as nmoles product/hr/10^8 platelets, n = 10 for normal group.
traclass correlation (ICC) for split samples run on the same day was .99 (n = 14) and the ICC for split samples run on different days was .94 (n = 28). The data for the ICC of MAO activity for platelets obtained on two separate occasions are shown in table 3. The Corash method shows significantly less variance than the other currently employed methods. The data in table 3 also show that the ICC for patients is consistently lower for all four methods. This greater variance for patients may reflect something that is intrinsically different about the platelet MAO of chronic schizophrenic patients.

As discussed earlier, there are differences among individuals with regard to the distribution of platelet populations on density gradients (Murphy et al. 1978). We find that this factor interacts with the differential centrifugation method to give up to an 8- to 10-fold error in the determination of platelet MAO activity. For example, with the differential centrifugation method, we found one patient's MAO activity (nM/hr/mg) was 2.5 ± 0.13 (SEM) (n = 4). However, blood drawn at the same four times but processed by the Corash method was actually 21.4 ± 1.11. Also, the percent difference in activity for samples processed by these two methods is not constant and varies considerably from individual to individual. Despite the potential for artifact in the differential centrifugation method, the data indicate that all methods showed significant differences between the normal and patient groups (table 4). However, it is possible that some of the studies in the literature that failed to show significant differences may have resulted from the variance due to the method of platelet preparation and/or to the parameters chosen for the MAO assay procedure.

References


Sullivan, J.L.; Cavenar, J.O., Jr.; Stanfield, C.N.; and Hammett, E.B.


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