[Chem. Pharm. Bull. 26(11)3471—3476(1978)]

UDC 577.158.02.08 : 543.426.062

A Sensitive Fluorometric Assay of Human Platelet Monoamine Oxidase and Its Application to Assessment of Monoamine Oxidase Inhibitor

Kiyoshi Zaitsu, Hideaki Nagai, Kenji Ohtsubo, Seiji Eto, Kazuya Kohashi,[1] and Yosuke Ohkura[1a]

Faculty of Pharmaceutical Sciences, Kyushu University, 62[1]

(Received June 1, 1978)

An assay method of human platelet monoamine oxidase is presented based on the fluorometric determination of benzaldehyde formed from the substrate benzylamine under the optimal enzyme reaction conditions by the 1,2-diaminonaphthalene method. This method is very sensitive and permits the assay even with 50 μg of platelet protein.

The method was successfully applied to the assessment of inhibitory effect of monoamine oxidase inhibitors (tranylcypromine, pargyline, iproniazid, nialamide, isoniazid and cuprizone).

Keywords——fluorometry; human platelet monoamine oxidase; benzylamine as substrate; benzaldehyde determination; 1,2-diaminonaphthalene sulfate; monoamine oxidase inhibitor

Inhibitors of monoamine oxidase (MAO) have been used for the therapy of hypertension, angina, affective disorders and narcolepsy. Many investigators are searching for new MAO-inhibitors which do not inhibit the destruction of dietary tyramine, because MAO-inhibiting drugs occasionally interact with the amine to cause a hypertensive crisis.[2] Thus convenient method of monitoring the enzyme in man have been required.

Use of platelet MAO assay in the direct assessment of the MAO-inhibitory potency of drugs in man was first suggested by Robinson *et al.*[3] Subsequently, various factors influencing platelet MAO activity and the associations between the enzyme activity and clinical psychiatric disorders have been reported.[4–7]

For the assay of platelet MAO, radiometric methods have been mainly used. In the methods, ^{14}C-labelled substrates for platelet MAO such as benzylamine,[3–5,8–10] tyramine,[3,5,8–11] tryptamine,[3,4,10–12] serotonine,[3,9,12,13] phenylethylamine[4,12,13] and dopamine[10,12,13] were used, the radioactive products formed in the enzyme reaction were isolated and measured their radioactivities. Fluorometric methods have been also utilized for platelet MAO assays which included measurements of 5-hydroxyindoleacetic acid formed from substrate serotonin,[14]

1) Location: *Maidashi, Higashi-ku, Fukuoka, 812, Japan*; *a*) To whom correspondence should be addressed.
2) R.J. Wyatt, "Neuropsychopharmacology of Monoamines and Their Regulatory Enzymes," ed. by E. Usdin, Raven Press, New York, 1974, pp. 1—2.
3) D.S. Robinson, W. Lovenberg, H. Keiser, and A. Sjoerdsma, *Biochem. Pharmacol.*, **17**, 109 (1968).
4) D.J. Edwards and M.O. Burns, *Life Sci.*, **15**, 2045 (1974).
5) V. Gentle, M.H. Greenwood, and M.H. Lader, *Psycopharmacologia* (Berl.), **44**, 187 (1975).
6) D.L. Murphy, "Monoamine Oxidase and its Inhibition," Ciba Foundation Symposium 39, Elsevier, Amsterdam, 1976, pp. 341—351.
7) I. Brockington, T.J. Crow, E.C. Johnstone, and F. Owen, "Monoamine Oxidase and its Inhibition," Ciba Foundation Symposium 39, Elsevier, Amsterdam, 1976, pp. 353—362.
8) R.J. Wurtman and J. Axelrod, *Biochem. Pharmacol.*, **12**, 1439 (1963).
9) P.A. Bond and R.L. Cundall, *Clin. Chim. Acta*, **80**, 317 (1977).
10) G.G.S. Collins and M. Sandler, *Biochem. Pharmacol.*, **20**, 289 (1971).
11) M. Jain, *Clin. Chim. Acta*, **47**, 107 (1973).
12) L. Demisch, H.J. Bochnik, and N. Seiler, *Clin. Chim. Acta*, **70**, 357 (1976).
13) M.B.H. Youdim, D.G. Grahame-Smith, and H.F. Woods, *Clin. Sci. Mol. Med.*, **50**, 479 (1976).
14) S. Takahashi and T. Karasawa, *Clin. Chim. Acta*, **62**, 393 (1975).

NII-Electronic Library Service

4-hydroxyquinoline from substrate kynuramine[10,13] and hydrogen peroxide from various substrates (*m*-iodobenzylamine, tyramine, tryptamine and octopamine)[15] in the enzyme reaction.

In the previous paper a sensitive and rapid fluorometric assay of human serum MAO activity was reported,[16] based on the measurement of benzaldehyde formed from substrate benzylamine in the enzyme reaction as a fluorophore 2-phenylnaphtho[1,2-*d*]imidazole by means of the determination method of the aldehyde with 1,2-diaminonaphthalene.[17,18]

This paper extends the study to the assay method of platelet MAO activity under optimal conditions of the enzyme reaction and describes an application of the method for the assessment of MAO-inhibitory potency of MAO inhibitors.

Experimental[19]

Preparation of Human Blood Platelet——Twenty ml portions of human platelet concentrate (PC) were obtained from 200 ml portions of venous blood collected in 30 ml of portions of ACD anticoagulant solution at Japan Red Cross (Fukuoka) and treated between 6—10 hr after the preparation by the methods of Takahashi *et al.*[14] and Shulman *et al.*[20] with some modifications as follows.

Transfer 5 ml of PC into a polycarbonate centrifuge tube and centrifuge at $130 \times g$ for 5 min to sediment erythrocytes and leucocytes. Using a plastic pipet, transfer the supernatant, platelet-rich plasma, into a polycarbonate centrifuge tube containing 2.0 ml of 0.5% Na_2-EDTA in 0.9% NaCl (pH 7.4), and centrifuge in the same way. Transfer the resulting supernatant into a polycarbonate centrifuge tube and centrifuge at $1500 \times g$ for 10 min. Suspend the precipitated white platelet pellet in 4.0 ml of the EDTA solution and centrifuge in the same way. Again suspend the pellet in 4.0 ml of 0.9% NaCl and recentrifuge at $1500 \times g$ for 10 min. Carry out the entire procedure at 0—4°. Resuspend the platelet pellet in 4.0 ml of the saline solution and sonicate[21] gently for 30 sec. Take a part of the platelet suspension for protein determination and store the remainder at −18° until assay.

Reagent Solutions——Phosphate buffer (0.2 M): Prepare by mixing 0.2 M Na_2HPO_4 and KH_2PO_4 in the usual manner (pH 8.4 at 37°). Benzylamine·HCl solution (2.0 mM): Dissolve 28.72 mg of benzylamine·HCl[22] in 100 ml of the phosphate buffer, store in a refrigerator and use within 2 days. 1,2-Diaminonaphthalene monosulfate (1,2-DNS) solution (45 μg/ml): Dissolve 9.0 mg of pure 1,2-DNS[17] in 3.0 ml of concentrated H_2SO_4 (concentration, above 95%) under mixing on a Vortex-type mixer. To the resulting mixture, add about 30 ml of H_2O in small portions, cool rapidly to room temperature and dilute with H_2O to 200 ml. This is usable for a week when kept frozen at −18° and for a day in a refrigerator at 5°. Benzaldehyde standard solutions: Prepare 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 nmol/0.4 ml benzaldehyde solutions by dissolving benzaldehyde[23] in the phosphate buffer. Use within a day. MAO inhibitor solutions: Dissolve each MAO inhibitor[24] in the phosphate buffer to desired concentrations.

Fluorescence Spectra and Intensities——They were measured with a Hitachi MPF-4 spectrofluorometer equipped with a Ushio 150 W xenon arc-lamp UXL-150DS, a Hitachi 056 recorder and a quartz cell of 1×1 cm optical path lengths. The slit-widths in the exciter and the analyser in terms of wavelengths were set at 2 and 10 nm, respectively. A daily check of sensitivity of the fluorometer was made by measuring the fluorescence intensity of a 1.0 μg/ml quinine solution in 0.1 N H_2SO_4 at an emission wavelength of 450 nm with excitation at 350 nm. The fluorescence excitation and emission maxima described in this paper are uncorrected.

Assay Procedure of MAO Activity (Procedure A)——Place 0.40 ml of the phosphate buffer in a glass-stoppered test tube, add 0.10 ml of platelet saline suspension (about 50—100 μg protein) and preincubate at 37° for 5 min. Add 0.50 ml of benzylamine·HCl solution and incubate at 37° for 40 min. At the end

15) H.Y. Meltzer and S.M. Stahl, *Res. Commun. Chem. Pathol. Pharmacol.*, 7, 419 (1974).
16) K. Zaitsu, H. Nagai, K. Kohashi, and Y. Ohkura, *Chem. Pharm. Bull.* (Tokyo), 25, 918 (1977).
17) Y. Ohkura and K. Zaitsu, *Talanta*, 21, 547 (1974).
18) K. Zaitsu and Y. Ohkura, *Chem. Pharm. Bull.* (Tokyo), 23, 1057 (1975).
19) H_2O distilled after deionization was used throughout the work. All chemicals used were of reagent grade unless otherwise noted.
20) N.R. Shulman, V.J. Marder, M.C. Hiller, and E.M. Collier, *Prog. Hematol.*, 4, 222 (1964).
21) Branson ultrasonic cleaner (Model 12, 80 Watts) was used.
22) Freshly distilled benzylamine was converted to its hydrochloride in the usual way, which was recrystallized at least twice from EtOH.
23) Benzaldehyde freshly distilled *in vacuo* in a stream of N_2 was used.
24) Iproniazid·H_3PO_4 and isoniazid were obtained from Wako Pure Chem. Ind., nialamide, tranylcypromine·HCl and pargyline·HCl from Sigma Chem. Co. and cuprizone from Tokyo Chem. Ind.

NII-Electronic Library Service

of the incubation, add 2.0 ml of 1,2-DNS solution and heat in a boiling water-bath for 20 min to develop the fluorescence. Cool in ice-water bath for about 5 min, add 2.0 ml of 10% NaOH solution. Prepare a blank in the same way, but add the substrate after adding 1,2-DNS solution. Allow to stand the resulting mixtures at room temperature for about 10 min after adding the NaOH solution, measure the fluorescence intensities of the test and the blank at 390 nm with the excitation at 356 nm. Read the nmol of benzaldehyde on the calibration curve described below. The enzyme activity was expressed as nmol of benzaldehyde formed per mg of platelet protein per hr at 37°.

Calibration Curve——Place 0.10 ml of the phosphate buffer in a glass-stoppered test tube, add successively 0.40 ml of benzaldehyde standard solution (and of the phosphate buffer for blank), 2.0 ml of 1,2-DNS solution and 0.50 ml of benzylamine·HCl solution and then treat in the same way as described in the Procedure A to develop the fluorescence. A linear relationship was observed between the measured fluorescence intensities and the amounts of benzaldehyde (0.5—6.0 nmol).

Protein Determination——Determine the content of protein in platelet suspension by the method of Lowry[25] using bovine plasma albumin[26] as a reference standard.

Assay Procedure of MAO Activity in the Presence of MAO Inhibitor (Procedure B)——Place 0.20 ml of the phosphate buffer in a glass-stopered test tube, add 0.10 ml of platelet saline suspension and warm at 37° for 5 min. Add 0.20 ml of one of the inhibitor solutions and preincubate at 37° for 15 min. At the end of the pre-incubation, add 0.50 ml of benzylamine·HCl solution and incubate at 37° for 40 min. Immediately after the incubation, add 2.0 ml of 1,2-DNS solution and 0.20 ml of the phosphate buffer, and then treat in the same way as in the Procedure A to develop the fluorescence. Prepare a blank in the same way, but add the substrate after adding 1,2-DNS solution. Prepare a control, corresponded to 0% inhibition, in the same way, but add 0.20 ml of the phosphate buffer in place of the inhibitor solution and add 0.20 ml of the inhibitor solution after adding 1,2-DNS solution. Prepare a blank of the control in the same way, but add benzylamine·HCl solution after adding 1,2-DNS solution.

Results

The fluorescence spectra of the final reaction mixture in the Procedure A or B had the excitation maximum at 356 nm and the emission maximum at 390 nm, which were identical with those of the reaction mixture of benzaldehyde with 1,2-DNS.[17]

The enzyme reaction conditions were investigated so as to be optimal. As shown in Fig. 1, the optimum pH was found at pH 8.4.

Figure 2 showed that the reaction rate increased with increasing phosphate concentration in the range of 0.01—0.4 M and a 0.4 M phosphate gave maximum rate. Phosphate

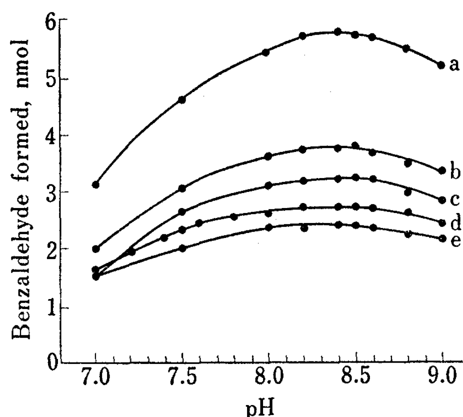
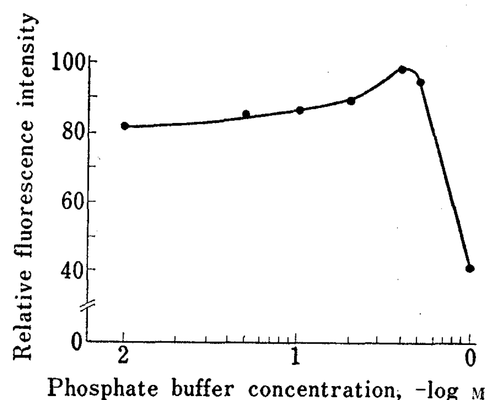


Fig. 1. Dependence of pH on Platelet MAO Activity

Platelet saline suspensions (100 μl) from different five human bloods were treated as in the Procedure A at various pH.

a, 151.3; b, 118.8; c, 77.5; d, 98.4; e, 142.5 μg platelet protein.



Feg. 2. Effect of phosphate Concentration in the Buffer on Plarelet MAO Activity

Aliquots (100 μl) of platelet suspensions (132.5 μg platelet protein/100 μl) were treaten as in the Procedure A with variously concentrated phosphate buffer solutions (pH 8.4).

25) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
26) Bovine albumin powder (Fraction V, Armour Pharmaceutical Co.) was used.

NII-Electronic Library Service

concentrations more than 0.4 M caused the reduction of the enzyme activity and the elevation of the blank fluorescence. A 0.2 M phosphate buffer of pH 8.4 was adopted in the present method.

Heat stability of the platelet MAO was investigated (Fig. 3). The MAO activity decreased markedly when platelet suspension was heated at 50°, though no loss of activity was observed at 37° for at least 45 min.

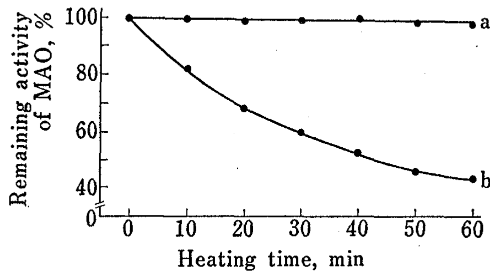


Fig. 3. Effect of Heating Time and Temperature in the Pre-incubation on Platelet MAO Activity

Aliquots (100 μl) of platelet suspensions (78.0 μg platelet protein/100 μl) were added to 0.4 ml of the phosphate buffer (0.2 M, pH 8.4) and heated at 37° (a) and 50° (b) for periods, and remaining MAO activities were measured by the Procedure A.

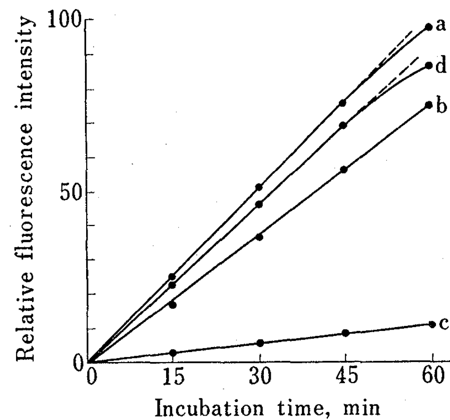


Fig. 4. Effect of Incubation Time and Temperauture on the Measured Fluorescence Intensity

Aliquots (100 μl) of platelet suspensions (83.3 μg platelet protein/100 μl) were treated as in the Procedure A for periods of incubation.

a: 37°, b: 30° and c: 25° without shaking the incubation mixtures. d: 37° with shaking at 120 cycles/min.

As shown in Fig. 4, a higher incubation temperature below 37° gave a higher activity of the enzyme, and the amount of benzaldehyde formed increased in proportional to the incubation time for at least 45 min. Shaking the mixture during the incubation at 37° caused some loss of MAO activity. Thus the incubation of 40 min at 37° without shaking was employed in the procedure.

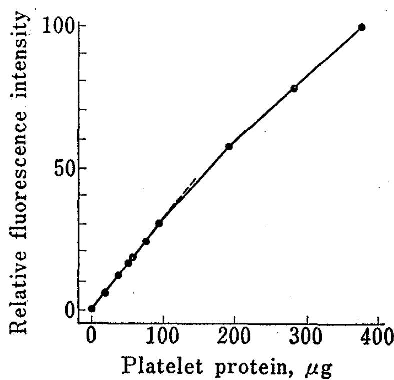


Fig. 5. Relationship between the Amount of Platelet Protein and the Measured Fluorescence Intensity

Aliquots (100 μl) of platelet suspensions were treated under the conditions of the Procedure A.

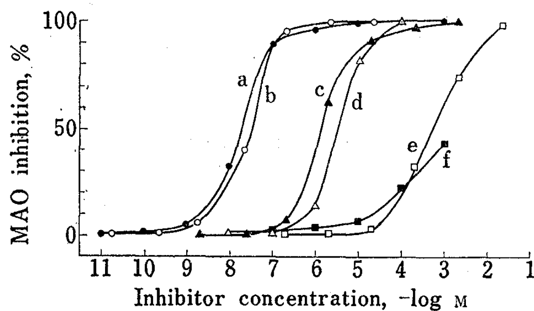


Fig. 6. Inhibition Curves of Various Inhibitors against Human Platelet MAO

Each inhibitor was treated under the conditions of the Procedure B with three platelet samples from different bloods.

a, tranylcypromine; b, pargyline; c, iproniazid; d, nialamide; e, isoniazid; f, cuprizone.

NII-Electronic Library Service

Michaelis constant (Km) for benzylamine was obtained with 14 platelet samples from different bloods as 36.7 ± 10.1 μM (mean±SD), in which the minimum and maximum values were 22.7 and 64.5 μM, respectively. Thus, a concentration of the amine, 1.0 mM, in the incubation mixture was prescribed in the procedures as a saturated amount. The fluorescence intensity was proportional to the amount of platelet protein up to about 100 μg (Fig. 5).

Recovery of 2.0 nmol of benzaldehyde added to the incubation mixture in the Procedure A was more than 99% in the presence of platelet protein below 90 μg and about 93% in the presence of the protein over the range of 134—233 μg.

The MAO assays on 33 platelet suspensions from different bloods revealed that the minimum activity was 20.4 units. Then platelet suspension containing about 50—100 μg protein/100 μl was to be subjected to the MAO assay for the reason described in the discussion.

The addition of an aldehyde dehydrogenase inhibitor, tetraethylthiuram disulfide, to the incubation medium in a concentration of 10 μM did not affect the MAO activity measured by the Procedure A.

The precision of the present method was studied by performing 15 determinations at the same time on 3 platelet samples with the mean activities of 23.6, 45.4 and 48.5 units (the amounts of platelet protein subjected to the assay were 76.3, 72.4 and 103 μg, respectively). The coefficients of variation were 3.4, 2.9 and 2.6%, respectively.

Estimation of Inhibitory Effect of MAO Inhibitors

Figure 6 showed the percentage inhibition of MAO activity in platelet against the concentration of MAO inhibitor. The values of I_{50} (concentration of inhibitor required to cause 50% inhibition of the MAO activity) were as follows (I_{50} was described in parenthesis): Tranylcypromine (20 nM), pargyline (30 nM), iproniazid (1 μM), nialamide (3 μM), isoniazid (600 μM) and cuprizone ($\simeq 2$ mM).

Discussion

The present method permits the MAO assay even with 50 μg of platelet protein and is almost comparable in sensitivity to the radiometric assay.[12] This sensitivity was achieved by use of benzylamine, one of the most suitable substrates for human platelet MAO (type B MAO)[27] and use of the sensitive determination method of benzaldehyde with 1,2-diaminonaphthalene. The minimum platelet MAO activity observed in this work was 20.4 units which corresponded to 0.68 nmol benzaldehyde formed under the conditions of the Procedure A when 50 μg of platelet protein was used. For routine assay, only 2 ml of whole blood may be required for the duplicate determinations for the test and blank, because 240—300 μg of platelet protein may be harvested from 2 ml of whole blood.[14]

The optimum pH for platelet MAO was revealed to be 8.4, although platelet MAO assays have been made generally at pH 7.4 which was rather optimal for serum MAO.[16]

The Km value for benzylamine obtained in this work, 36.7 μM, was in the same order of magnitude as those obtained under somewhat different conditions,[9] 70 and[10] 55 μM.

The mode of the heat stability of the enzyme (Fig. 4) was in a good agreement with that described by Collins *et al.*[10]

The data obtained in the examination of inhibitory effect of MAO inhibitors indicated that tranylcypromine and pargyline were potent inhibitors, iproniazid and nialamide moderately potent and isoniazid and cuprizone less potent for platelet MAO. Simple sigmoidal curves of percent-inhibition of platelet MAO against concentration of the inhibitors (Fig. 6)

27) D.L. Murphy and C.H. Donnelly, "Neuropsychopharmacology of Monoamines and Their Regulatory Enzymes," ed. by E. Usdin, Raven Press, New York, 1974, pp. 71—85.

NII-Electronic Library Service

demonstrated homogeneity of platelet MAO.[28] This simple mode of the inhibition indicates that the use of platelet MAO may be convenient for the easy screening of MAO-inhibitory potency of newly developed drugs.

Acknowledgement The authors express their gratitude to the staff of Central Clinical Laboratory of Kyushu University Hospital for the kind supply of platelet concentrate and to Mrs. M. Yamaguchi (née Tomita) for the capable technical assistance. A Grant-in-Aid for Scientific Research for the Ministry of Education, Science and Culture of Japan (277646) is gratefully acknowledged.

28) In enzyme sample possessing mixtures of both type A and B MAOs, stepwise and complicated inhibition was observed: J.P. Johnston, *Biochem. Pharmacol.*, **17**, 1285 (1968).

NII-Electronic Library Service