Biochemical Pharmacology, Vol. 28, pp. 2345–2347.

0006-2952/79/0801-2345 $02.00/0

DETERMINATION OF PLATELET MONOAMINE OXIDASE ACTIVITY IN HUMAN PLATELET-RICH PLASMA—

A NEW MICROFLUORESCENT ASSAY UTILIZING KYNURAMINE AS SUBSTRATE

JOHN E. MCENTIRE, STEPHEN J. BUCHOK and BEN W. PAPERMASTER

Cancer Research Center, 115 Business Loop 70 and Garth, Columbia, MO 65201, U.S.A.

(*Received* 3 *July* 1978; *accepted* 8 *January* 1979)

Abstract—A simple assay system for the measurement of human platelet monoamine oxidase is described. The assay is based on the fluorometric measurement of 4-hydroxyquinoline produced by enzymatic oxidative deamination of kynuramine. The results obtained on 21 individuals correlate ($r^2 = 0.97$) with data obtained in parallel with a [^{14}C]benzylamine/pargyline assay. This assay system appears to be technically and economically favorable as a method for screening large numbers of individuals in order to establish distributions of monoamine oxidase activities for normal and affected populations.

Platelet monoamine oxidase (MAO) has been shown recently to be of potential importance as a marker enzyme in schizophrenia [1–3] and as a means of predicting lithium responsiveness in bipolar affective disorders [4, 5]. These differences in MAO levels are only observed when assayed with certain substrates. Radiolabeled substrates, such as [^{14}C]benzylamine, have been traditionally used in order to obtain the sensitivity necessary to perform replicate assays from small sample volumes. Since these assay systems involve organic extraction and the handling of radioactive material, they are not readily adaptable for use in large scale screening.

In this report we describe a simple, fast, and accurate fluorescent assay which can be used to determine MAO activity of platelet-rich plasma (PRP) samples. The assay is based on the appearance of 4-hydroxyquinoline (4-HOQ), a fluorescent product of the oxidative deamination of kynuramine [6]. The results correlate highly with those obtained from parallel assays using [^{14}C]benzylamine as substrate.

MATERIALS AND METHODS

Collection of blood

Fresh venous blood was collected in glass tubes containing 14% (v/v) anticoagulant (citrate phosphate dextrose). PRP was obtained by pooling the plasma from sequential centrifugations of the sample at 175 *g* for 10 min and 300 *g* for 10 min. The platelet concentration of the PRP was determined using a TOA platelet counter (Scimetrics, Inc., Houston, TX.). Duplicate dilutions (1:5000) of the platelet suspensions were counted at least 5 times each. PRP samples having less than 1×10^8 platelets/ml were not used. Visual inspection of the PRP samples indicated less than 0.1 per cent contamination by other cellular components. Platelet counting and distribution of aliquots for assay were performed immediately after PRP preparation. Assays were carried out within 4 hr of blood collection.

Enzyme assay

Assay of MAO activity was performed using both [^{14}C]benzylamine and kynuramine as substrates.

[^{14}C]*benzylamine.* The [^{14}C]benzylamine assay was performed as described by Murphy *et al.* [7]. [Methylene-^{14}C]benzylamine HCl, 12.5 mCi/nmole (ICN Pharmaceuticals, Irvine, CA) was combined with unlabeled benzylamine (Sigma Chemical Co., St. Louis, MO) in 0.01 N HCl to obtain a solution of 2 mCi/mmole. The substrate was washed with 15–20-ml aliquots of toluene until counts in the organic phase stabilized. Six aliquots of 0.25 ml PRP were prepared; to three of these samples 10 μl of 10^{-4} M pargyline in 0.01 N HCl was added and they were incubated for 15 min at 37°. After incubation, 0.25 ml of a buffer–substrate mixture containing 0.2 M Tris, pH 9.1, and 4×10^{-4} M benzylamine (solution prepared above) was added to each of the six tubes. After incubation for 30 min at 37°, the reaction was terminated by the addition of 50 μl of 3 N HCl. After 15 min, 3.0 ml heptane (Sigma Chemical Co.) was added; the tubes were shaken for 10 min, centrifuged at 200 *g* for 10 min, and frozen in an alcohol–dry ice bath. The unfrozen heptane layer of each tube was decanted into a scintillation vial containing 10.0 ml of aqueous counting scintillant (Amersham-Searle, Arlington Heights, IL) and counted. Platelet MAO activity was determined by calculating the difference in pargyline-treated and non-treated samples and is expressed as nmoles benzylamine hydrolyzed/10^8 platelets/hr.

Kynuramine. MAO activity using kynuramine as substrate was assayed by a modification of the fluorescent technique reported by Kraml [6]. Six replicate tubes were prepared, three of which served as controls. The enzyme reaction mixture contained 1.6 ml 0.1 M borate, pH 8.2, and 0.2 ml PRP. Control tubes were incubated in a 100° bath for 45 sec. After equilibration at 37°, the reaction was initiated by the addition of 0.2 ml 1.0 mM kynuramine diHBr (Sigma Chemical Co.). After incubation at 37° for 1 hr, the reaction was

terminated by incubation at 100° for 45 sec. One ml of the reaction mixture was mixed with 2.0 ml of 1 N NaOH, and the fluorescent intensity at 315 nm excitation and 380 nm emission was read on an Aminco Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, MD.). Activity is calculated from a standard curve of 4-HOQ vs. fluorescence. Specific activity is expressed as nmoles 4-HOQ produced/10^8 platelets/hr. In preliminary studies plasma was clarified of platelets by centrifugation and assayed similarly.

Data analysis

Data were analyzed using a Tektronics 4051 Graphics minicomputer and related software (Tektronics, Inc., Beaverton, OR).

RESULTS

[^{14}C]*Benzylamine assay.* Results from the assay of 21 samples (Table 1) gave findings consistent with those reported by Murphy *et al.* [7]. The mean specific activity of this group was 10.93 nmoles/10^8 platelets/hr and its range 5.91–22.98. One-way analysis of variance, utilizing values of three individual replicates per sample, gave a variance ratio of F = 463 (Table 1).

Kynuramine assay. Assay of MAO with kynuramine as substrate gave activities shown in Table 1. Blank values ranged from 0.6 to 0.75 relative fluorescent units. Studies on pooled platelet-rich plasma samples indicated that this representative sample had a K_m of

Table 1. Specific activities of platelet MAO using [^{14}C]benzylamine and kynuramine as substrates*

Sample No.	Specific Activity (mean ± S.E.) (nmoles Benzylamine/10^8 platelets/hr)	(nmoles 4-HOQ/10^8 platelets/hr)
1	6.68 ± 0.10	6.87 ± 0.03
2	12.94 ± 0.40	13.20 ± 0.13
3	6.80 ± 0.17	6.38 ± 0.05
4	21.99 ± 0.59	19.90 ± 0.14
5	5.91 ± 0.11	5.67 ± 0.03
6	22.98 ± 0.08	23.35 ± 0.21
7	11.38 ± 0.11	12.25 ± 0.07
8	9.79 ± 0.14	10.48 ± 0.16
9	10.36 ± 0.15	10.62 ± 0.16
10	10.71 ± 0.17	10.19 ± 0.04
11	12.20 ± 0.16	11.11 ± 0.04
12	9.59 ± 0.11	9.91 ± 0.03
13	6.18 ± 0.21	6.60 ± 0.05
14	8.81 ± 0.06	8.47 ± 0.02
15	11.19 ± 0.25	9.99 ± 0.05
16	13.98 ± 0.05	14.88 ± 0.05
17	11.09 ± 0.10	10.30 ± 0.12
18	11.39 ± 0.14	10.42 ± 0.03
19	8.51 ± 0.02	8.78 ± 0.04
20	9.54 ± 0.09	9.65 ± 0.38
21	7.71 ± 0.32	7.84 ± 0.06

* The numbers shown in each column are the means of three replicates from each sample. Variation in measurement was analyzed by one-way analysis of variance (ANOVA) on a Tektronics 4051 Minicomputer. The overall coefficient of variation (CV) for the benzylamine assay was 4 per cent, and for the fluorescent assay was 2.2 per cent, suggesting less variation among replicates for the fluorescent assay, although both assays can be performed well within tolerance limits of < 5 per cent CV.

Assay	SS	MS	F	P
Benzylamine	1175.5	58.8	463.4	0.001
Kynuramine	1097.3	54.9	1202.2	0.001

Both assays are capable of distinguishing among different samples with P = 0.001.

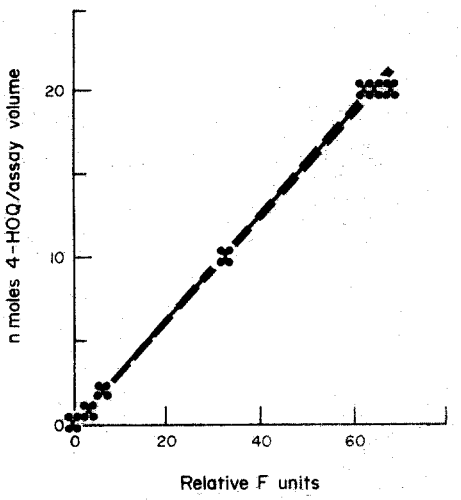

Fig. 1. Standard curve for 4-HOQ concentration. A standard solution of 4-HOQ was added at varying concentrations to a mixture of 1.6 ml of 0.1 M borate, pH 8.2, and water for a final volume of 2.0 ml. The 2.0 ml volume contained from 0.02 to 20 nmoles of 4-HOQ. One ml of this solution was removed and mixed with 2.0 ml of 1 N NaOH. Fluorescence was determined using an Aminco Bowman spectrophotofluorometer at 315 nm excitation and 380 nm emission. The standard curve was performed independently by three laboratory personnel. Broken lines are 95 per cent confidence limits.

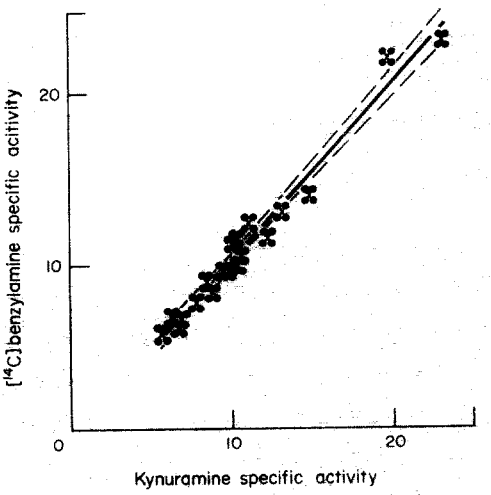

Fig. 2. Correlation of MAO specific activities using [^{14}C]benzylamine and kynuramine as substrates. Specific activities were determined as described in Materials and Methods. The values obtained were plotted and linear regression analyses performed. Broken lines indicate 95 per cent confidence intervals.

3×10^{-5} M, pH optima at 8.2, and was linear for at least 2 hr. The substrate concentration (1.5×10^{-4} M) for further studies was chosen so as to be 5 times the K_m. Under the conditions employed, a fluorescent reading of 32 relative units represented 4.9 per cent substrate utilization and was arbitrarily defined as the maximum acceptable reading. Plasma samples which had been centrifuged to remove platelets did not hydrolyze kynuramine under the assay conditions used; therefore, activity could be attributed entirely to platelet MAO. Analysis of variance performed on replicate samples varied hardly at all, and different samples were easily distinguishable one from the other ($F = 1202$). The overall variation in replicates was less in the fluorescent assay (legend to Table 1). The standard curves for 4-HOQ (Fig. 1), when performed independently by three laboratory personnel, were very similar ($r^2 = 0.99$) as were replicate assays of the same sample performed by two different personnel ($r^2 = 0.97$).

Correlation of the two assay systems. Identical samples assayed using both the benzylamine and kynuramine techniques showed extremely high correlation. Figure 2 illustrates linear regression of benzylamine-specific activity on kynuramine ($r^2 = 0.97$). Two-way analysis of variance indicated that the assays were of equal variability (sums of squares), and both substrates were capable of distinguishing differences in MAO-specific activity among platelet samples.

DISCUSSION

The data presented indicate that a fluorescent assay for platelet MAO using kynuramine as substrate gives results which are statistically nearly identical to those obtained by the standard [^{14}C]benzylamine assay. The use of the assay system we describe should enable MAO determinations to be carried out on large numbers of individuals with the same reproducibility, accuracy and sensitivity obtainable by the more complicated benzylamine assay. In addition to the advantage of technical simplicity which this system offers, there are also favorable comparisons as to assay time and cost. In our laboratory, the [^{14}C]benzylamine assay could be carried out in about 3 hr at a cost of $2.50/triplicate sample. Our fluorescent assay could be performed in only 70 min at a cost of $0.20/sample triplicate group. As mentioned previously, the kynuramine assay eliminates problems of handling and disposal of the hard beta emitter, ^{14}C.

The kynuramine assay does not allow one to estimate the plasma MAO activity as may be done with the benzylamine/pargyline assay. However, it should be noted that plasma MAO has not been shown to vary between normal and chronic paranoid schizophrenics [2].

The modifications which we have made to the kynuramine assay system of Kraml [6] include termination in a boiling water bath, optimization of pH and substrate concentration, and use of PRP as a source of enzyme. Termination of the reaction with trichloroacetic acid, as reported by Kraml, results in quenching of fluorescence and instability during the fluorescent reading. Exposure to boiling water for 45 sec completely inactivates the MAO present while causing neither kynuramine nor 4-HOQ to be broken down. The advantages of using PRP as a source of enzyme with specific activities based on platelet number instead of platelet protein are described by Murphy *et al.* [7]. Although our data on platelet MAO stability confirm those of Murphy *et al.*, we found that platelet counts became quite variable within 3–4 hr after preparation of PRP due to platelet aggregation. Therefore, all PRP samples were aliquoted and counted within 1 hr of preparation.

We feel that the modified kynuramine assay, which we report here, will enable a large data base of normal and schizophrenic MAO activities to be obtained more efficiently and economically than with currently used assay techniques.

Acknowledgements—This work was supported by the Multidisciplinary Research Program in Mental Health. The authors wish to thank Dr. Martine J. Young and Miss Jacquelyn R. Picard for technical assistance provided in these studies, Mr. Paul Sartwell (Scimetrics, Inc.) for use of the platelet counting equipment, and Mrs. Stephanie H. Sordahl for expert typing and editing of this manuscript.

REFERENCES

1. D. L. Murphy, R. Belmaker and R. J. Wyatt, *J. psychiat. Res.* **11**, 221 (1974).
2. S. G. Potkin, H. E. Cannon, D. L. Murphy and R. J. Wyatt, *New Engl. J. Med.* **298**, 61 (1978).
3. W. H. Berrittini, W. H. Vogel and R. Clouse, *Am. J. Psychiat.* **1341**, 805 (1977).
4. J. L. Sullivan, J. O. Cavenar, A. Maltbie and C. Stanfield, *Lancet* **2**, 1325 (1977).
5. J. Landowski, L. Wieslawa and S. Angielski, *Biochem. Med.* **14**, 347 (1975).
6. M. Kraml, *Biochem. Pharmac.* **14**, 1684 (1965).
7. D. L. Murphy, D. Wright, M. Buchsbaum, A. Nichols, J. I. Costa and R. J. Wyatt, *Biochem. Med.* **16**, 254 (1976).