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Determination of platelet monoamine oxidase activity by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A procedure for determining human platelet monoamine oxidase (MAO) with dopamine (DA) as substrate is described. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) was used to separate and detect components of the reaction mixture. The method for platelet preparation was also improved and only 2 ml of blood were required. Following a 10-min incubation of the platelet preparation with DA in 0.1 *M* Tris buffer (pH 9.0), excess DA substrate was removed by adsorption on a cation-exchange resin. The reaction product, 3,4-dihydroxyphenylacetaldehyde, was adsorbed on acid-washed alumina, eluted with 0.1 *M* perchloric acid and analyzed by HPLC. Simple, clean chromatograms were obtained with good reproducibility using 3,4-dihydroxybenzylamine as an internal standard. The within-sample, between-samples and between-day relative standard deviations were 0.9, 3.7 and 6.1%, respectively. The apparent Michaelis constant and maximum velocity were 0.10 m*M* and 0.37 nmol/min · mg protein, respectively. This HPLC–ED method offers a good alternative to methods using radioactivity.

INTRODUCTION

Catecholamine therapy has become one of the cornerstones of critical care medicine. Patients experiencing major trauma, sepsis, myocardial infarction and shock routinely receive doses of dopamine, epinephrine and norepinephrine which results in plasma hormone concentrations that exceed by 100- to 10 000 fold the concentrations that occur endogenously even in the presence of stress or exercise [1]. The physiological response to these agents is highly variable. In order to understand the biological basis for this inter-individual variation in patient response, both the pharmacokinetics and the pharmacodynamics of exogenously administered catecholamines must be evaluated.

As inter-individual differences in drug responsiveness are most often related to inter-individual differences in drug metabolism [2], a thorough evaluation of the metabolism of exogenous catecholamines must be undertaken. This requires the assessment of the activities of each of the enzymes involved in their metabolism in a tissue that is both readily obtainable from patients and able to be sampled repeatedly. Clearly, the blood elements best fit this description and, among these, white blood cells and platelets are the most logical choices as they are seldom transfused.

There are three ways in which catecholamines may be metabolically inactivated: 3-O-methylation via catechol-O-methyltransferase, deamination by monoamine oxidase and conjugation with sulfur via phenolsulfotransferase [3]. In this

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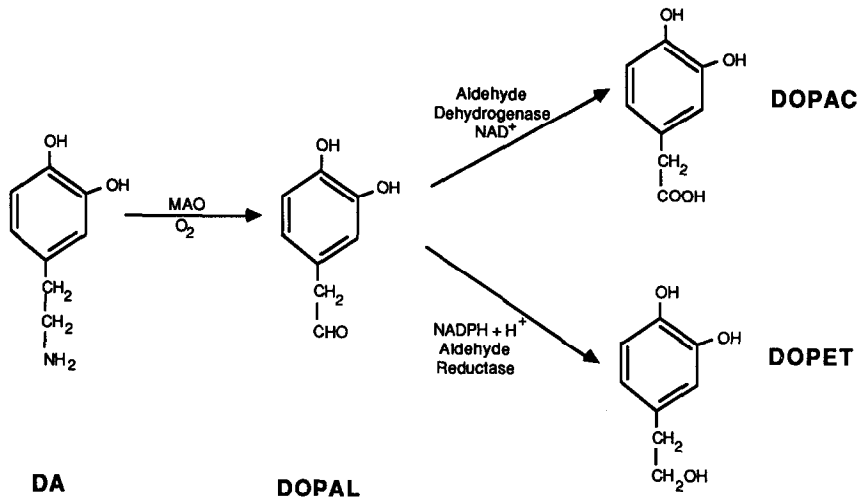

Fig. 1. Metabolism of dopamine (DA) by monoamine oxidase (MAO). DOPAL = 3,4-dihydroxyphenylacetaldehyde; DOPAC = 3,4-dihydroxyphenylacetic acid; DOPET = 3,4-dihydroxyphenylethanol.

study we focused on the metabolism of dopamine (DA) by monamine oxidase (MAO) in human platelets.

Monoamine oxidase (monoamine O_2 oxidoreductase, EC 1.4.3.4) catalyzes the oxidative deamination of most naturally occurring biologically active monoamines, including the neurotransmitter DA. It is usually localized in mitochondria. On the basis of studies with substrate-selective inhibitors, MAO has been classified into two types, MAO-A and MAO-B [4]. Unlike human brain and liver, which contain both types, only MAO-B appears to be present in human platelets [5,6]. This human platelet MAO preferentially deaminates DA [7], benzylamine and phenylethylamine, in contrast to its lower activity with 5-hydroxytryptamine, an MAO-A substrate [4].

Deamination of amines with MAO leads to the formation of an aldehyde (Fig. 1). In the presence of crude tissue preparations, this aldehyde may undergo further oxidation to the corresponding acid or reduction to the corresponding alcohol [3,8] (Fig. 1). Consequently, the ideal MAO assay should minimize further metabolism of the aldehyde and separate it from any acid and alcohol metabolites formed. A number of different methods have been used for the assay of MAO [9]. The most commonly used methods are radiometric with the use of a ^{14}C-labelled substrate [8–10]; however, in these procedures all radiolabelled metabolites formed are counted.

High-performance liquid chromatography with electrochemical detection (HPLC–ED) provides the option of separating the three DA metabolites and avoiding the expense of radioactive materials. To our knowledge, there is no specific HPLC–ED method for determining MAO platelet activity with a native substrate, although some investigators have examined the metabolism of DA and its metabolites using HPLC–ED [11,12]. In this study, a relatively rapid procedure was characterized for analyzing MAO in human platelets. DA, which is widely used for the treatment of shock and hypotension and as an endogenous neurotransmitter, was used as substrate and the products were identified by HPLC–ED.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical-reagent or HPLC grade. 3-Hydroxytyramine (DA), 3,4-dihydroxybenzylamine (DHBA), 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine, ethylenediaminetetraacetic acid, disodi-

um salt (Na_2EDTA), L-ascorbic acid, tris-(hydroxymethyl)aminomethane (Tris base), tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), N-methyl-N-propargylbenzylamine hydrochloride (pargyline), Amberlite CG-50 (hydrogen form, wet mesh 100–200), 2,4-dinitrophenylhydrazine and MAO from bovine plasma were purchased from Sigma (St. Louis, MO, USA). Amberlite was prepared according to the method of Pisano [13].

Sodium chloride (NaCl), sodium hydroxide (NaOH) pellets, hydrochloric acid (HCl), perchloric acid ($HClO_4$), sodium phosphate, monobasic (NaH_2PO_4), 85% phosphoric acid (H_3PO_4) and glacial acetic acid were purchased from Fischer Scientific (Pittsburgh, PA, USA).

HPLC-grade acetonitrile and ethyl acetate were purchased from Burdick and Jackson Labs. (Muskegon, MI, USA), sodium octylsulfate from Eastmann Kodak (Rochester, NY, USA), acid-washed alumina from Bioanalytical Systems (West Lafayette, IN, USA) and phenobarbital sodium from Mallinckrodt (Paris, KY, USA).

3,4-Dihydroxyphenylacetaldehyde (DOPAL) was synthesized from epinephrine by the method of Fellman [14].

HPLC equipment and conditions

Analyses were performed using a Varian (Palo Alto, CA, USA) Model 5020 liquid chromatograph equipped with an automated Valco Model C6U column switching valve with a 100-μl loop. Chromatography was performed on a 250 mm × 4.6 mm I.D. Biophase ODS reversed-phase column, 5 μm particle size (Bioanalytical Systems), at a flow-rate of 1.5 ml/min. Peaks were detected with an LC-4B amperometric detector (Bioanalytical Systems) fitted with a glassy carbon working electrode maintained at +0.65 V *vs.* Ag/AgCl. Peak areas were integrated on a DS 604 computer (Varian) and printed on a Hewlett-Packard Think Jet printer. The detector range was usually set at 5 nA.

The mobile phase was prepared as follows: 32.1 g of monochloroacetic acid, 9.35 g of NaOH pellets and 1.5 g of Na_2EDTA were added to 1800 ml of deionized water and adjusted to pH 3.1 with 5 *M* NaOH. Then 300 mg of sodium octylsulfate were added and the volume was adjusted to 2 l. After filtration through a 0.2-μm filter, 60 ml of this solution were replaced with the same volume of filtered acetonitrile to make a 3% (v/v) acetonitrile solution. This mobile phase was then degassed with nitrogen for 20 min and could be recycled continuously for up to one month. As the column aged and peaks eluted more quickly, the acetonitrile concentration was decreased to maintain baseline resolution between peaks.

Preparation of human platelets

Platelets were isolated from blood by a modification of the procedures of Wise *et al.* [15] and Corash *et al.* [16]. Briefly, 2 ml of venous blood were added to 7.5 mg of Na_2EDTA in a 5-ml polypropylene tube and gently mixed at room temperature. A 1-ml volume of an isotonic phosphate buffer (IPB: 0.145 *M* NaCl–0.01 *M* NaH_2PO_4–3.14 m*M* Na_2EDTA, pH 7.4) was added and the sample mixed gently and centrifuged at 700 *g* for 3 min at 20°C in a Beckman (Palo Alto, CA, USA) Model TJ-6 centrifuge. The supernatant containing the platelet-rich plasma was transferred with a plastic pipet (without disturbing the interface) to another polypropylene tube. The red cell pellet was washed three more times with 1 ml of IPB and the four combined supernatant solutions were centrifuged at 700 *g* for 20 min at 4°C. This supernatant was discarded. The platelet pellet was overlaid with 0.4 ml of IPB, shaken gently to disperse any visible clumps of platelets and centrifuged at 700 *g* for 20 min at 4°C. The pellet was finally resuspended in 0.35 ml of 0.1 *M* Tris–HCl buffer (pH 9.0) containing 0.1 m*M* ascorbic acid and 0.1 m*M* Na_2EDTA to limit non-enzymatic oxidation of DA [5]. This platelet suspension was used immediately for assay at pH 9 or stored at −70°C for later assay. MAO activity was stable for at least four weeks at −70°C.

If MAO was to be determined at pH 7.4, the pellet was resuspended in 0.1 *M* sodium phosphate (pH 7.4) containing 0.1 m*M* ascorbic acid

and 0.1 m*M* Na_2EDTA and used on the same day.

Determination of MAO activity, standard procedure

The platelet suspension for assay at pH 9 was sonicated at 70 W for 15 s with a sonifier cell disrupter (Heat Systems–Ultrasonics, Plainview, NY, USA) in an ice-bath.

For all assays, protein concentration was determined on an aliquot of sonicated suspension by the method of Lowry *et al.* [17] using bovine serum albumin as a standard.

To measure the conversion of DA to DOPAL, 430 μl of buffer and 50 μl of the sonicated platelet suspension (containing typically 0.15 mg of protein) were combined in a 5-ml glass tube and preincubated for 10 min at 37°C. A blank in which buffer was substituted for the sonicated platelet suspension was run with every set of assays (see results regarding choice of blank). The reaction was initiated by adding 20 μl of 50 μM DA in 0.1 *M* $HClO_4$. After 10 min the reaction was stopped by transferring the tube to an ice–salt bath. [Assays at pH 7.4 contained 380 μl of freshly made 0.1 *M* sodium phophate (pH 7.4) and 100 μl of a fresh, unsonicated platelet suspension and were incubated for 20 min.]

DA was separated from the reaction products by adsorption on 3 ml of Amberlite cation-exchange resin in a 10-ml polypropylene chromatographic column (Bio-Rad Labs., Richmond, CA, USA). The reaction mixture, a 0.5-ml deionized water rinse of the assay tube and 2 × 2 ml of water were sequentially eluted through the column and collected in a 10-ml polypropylene tube in an ice-bath. An internal standard, 100 μl of 2 μM DHBA in 0.1 *M* $HClO_4$, was added to the eluate.

The catechols were purified by selective binding to alumina. To the Amberlite eluate was added 1 ml of 1 *M* Tris–HCl (pH 8.65) containing 2% Na_2EDTA and 100 mg of alumina. The solution was mixed on a rotary mixer at high speed, setting 7 (Roto-Torque, Cole-Parmer Instrument, Chicago, IL, USA) for 10 min, centrifuged for 30 s at *ca.* 5100 *g* (setting 7) in an International Clinical Centrifuge, Model CL (IC centrifuge, International Equipment, Needham, MA, USA) and the supernatant discarded. The alumina was mixed three times with 10 ml of deionized water, allowed to settle in an ice-bath and the supernatant discarded. Finally, 1.5 ml of deionized water were added, the alumina mixture was transferred to an MF-1 microfilter (Bioanalytical Systems), the microfilter centrifuged for 3 min at *ca.* 3600 *g* (setting 5) and the eluate discarded.

To elute the catechols from the alumina, 400 μl of 0.1 *M* $HClO_4$were added to the microfilter and the assembly was vortex-mixed fully. After the alumina had settled for 5 min it was centrifuged for 3 min at *ca.* 1500 *g* (setting 2). The eluate was analyzed within 30 min or stored at −70°C until analysis. DOPAL and DHBA were stable for at least one month in 0.1 *M* $HClO_4$ at −70°C.

Determination and calculation of the DOPAL produced in the assay

DOPAL was synthesized by the method of Fellman [14] starting with 100 mg of epinephrine and heating for 50 s in a 125°C oil-bath. The product was extracted into one volume of ethyl acetate and dried under vacuum. The residue was dissolved in 0.5 ml of 0.1 *M* perchloric acid and 100 μl were injected onto the HPLC column in a mobile phase of 0.01 *M* ammonium formate (pH 3.1). The DOPAL peak was collected and the concentration determined using the absorbance of its 2,4-dinitrophenylhydrazine derivative and the published absorptivity for aldehyde derivatives ($A_{max} = 2.72 \cdot 10^4$ at 480 nm) [18]. Absorbances were determined using a Response UV–VIS scanning spectrometer (Response, Ciba Corning Diagnostics, Oberlin, OH, USA).

The amount of DOPAL produced in the assay was determined by adding known amounts of DOPAL to separate reaction mixtures without platelets (platelets do not affect recovery; see Results). After a 10-min incubation the mixture was transferred to an Amberlite resin column and eluted with 4.5 ml of deionized water. Samples were then processed by the standard assay and a calibration graph of DOPAL/DHBA peak-area ratio *versus* the amount of DOPAL in the initial

reaction mixture was obtained. MAO activity is expressed as nanomoles of DOPAL formed per minute per milligram of protein at 37°C.

Calculations

All linear regressions were analyzed by the method of least squares. Significance was tested by a Student's *t*-test [19].

RESULTS

DA metabolism by platelets at physiological pH

Incubation of fresh, intact platelets with DA at pH 7.4 resulted in the generation of three presumptive metabolite peaks (peaks I, Ia and Ib) and peaks for the internal standard 3,4-dihydroxybenzylamine (DHBA, peak II) and variable amounts of residual DA substrate (peak III) (Fig. 2). It was expected that the major metabolite would reflect the oxidation of DA by MAO to the aldehyde DOPAL. The latter could then be metabolized further to 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylethanol (DOPET) via aldehyde dehydrogenase and aldehyde reductase respectively (Fig. 1) [11].

Peak I was identified as DOPAL by both enzyme and chemical synthesis. Thus, when bovine plasma MAO was substituted in the assay for the platelet preparation, the aldehyde oxidation product of DA appeared at the same HPLC retention time as peak I. The formation of this peak and its metabolites was completely inhibited by 1 m*M* pargyline, an MAO-B inhibitor (see Fig. 2). In addition, when DOPAL was synthesized from epinephrine, the major product eluted with a retention volume equal to that of peak I. Owing to the great instability of DOPAL to light and heat, it was not possible in our hands to dry the relatively pure ethyl acetate extract of DOPAL without generating new compounds that were easily detected by HPLC.

Peak Ia was identified presumptively as DOPET because its formation was inhibited to the largest extent by phenobarbital, an inhibitor of brain aldehyde reductase [20]. Production of

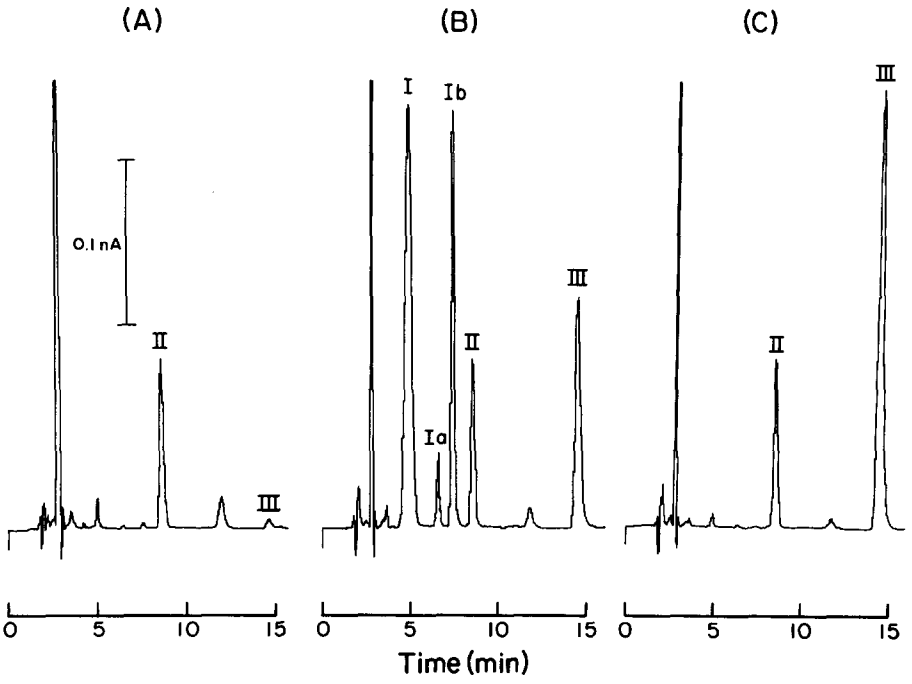

Fig. 2. HPLC–ED of MAO assayed in 0.1 *M* phosphate buffer (pH 7.4) for 20 min. (A) Blank without platelets; (B) platelet assay; (C) platelet assay with 1 m*M* pargyline. Peaks: I = DOPAL; Ia = DOPET; Ib = DOPAC; II = DHBA; III = DA.

TABLE I

INHIBITION OF THE FORMATION OF DOPET AND DOPAC IN PLATELETS

The MAO assay was performed at pH 7.4 with freshly isolated platelets and DA in the presence of known inhibitors of aldehyde reductase. The yield of the three metabolites was determined using values for recovery and the electrochemical detector response. For DOPAL and DOPAC the values were obtained as described in the text and they were very similar for the two metabolites. The amount represented by the peak presumed to be DOPET was calculated by using the average of these known values for DOPAL and DOPAC.

Assay time (min)	Inhibitor	Proportion of total product (%)			Total (nmol per assay)
		DOPAL	DOPET	DOPAC	
90	None	65.0	5.0	30.0	2.67
	1 m*M* phenobarbital	77.8	2.6	19.6	2.76
	5 m*M* phenobarbital	91.6	0.4	8.1	2.72
60	None	62.4	4.2	33.4	2.02
	1 μM quercetin	62.3	2.9	34.8	2.08
	10 μM quercetin	75.5	2.4	22.1	2.26

DOPAC (peak Ib) was also inhibited by phenobarbital but to a lesser extent and MAO showed no inhibition as the total production of DOPAL, DOPET and DOPAC remained constant (Table I). Similar results were found for quercetin, a potent inhibitor of three human brain aldehyde reductases [21].

The time course of the appearance of the three metabolites is shown in Fig. 3. As might be expected, there was an initial lag in the production of the secondary metabolites DOPET and DOPAC. As extraneous peaks from DA appeared over time in the pH 7.4 phosphate-buffered assay, blanks without platelets were run at each time point.

Preliminary evidence suggested that the aldehyde dehydrogenase and the aldehyde reductase activities in platelets were fairly labile. Platelet sonication reduced the conversion of DOPAL to DOPAC and DOPET by 50% and freezing platelets at −70°C followed by thawing at 0°C virtually eliminated aldehyde metabolism. Thus, although it was possible to determine some aspects of MAO activity at physiological pH, analyses from clinical studies would be complicated by the variable further metabolism of the pri-

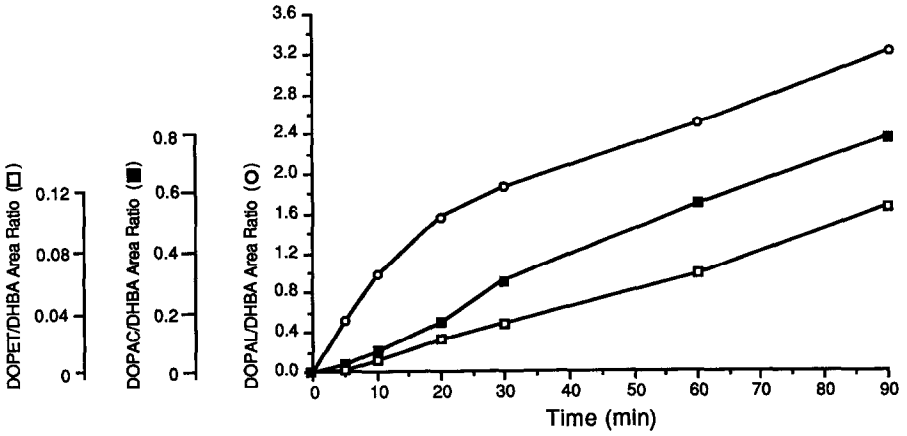

Fig. 3. Time course of the appearance of the three DA metabolites in 0.1 *M* phosphate buffer (pH 7.4) over 90 min.

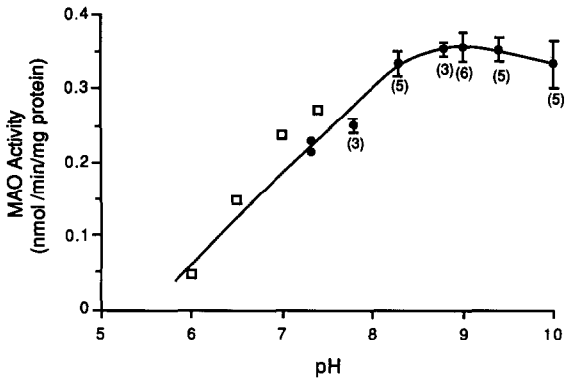

Fig. 4. Variation of platelet MAO activity with pH after 10-min incubation. (●) 0.1 *M* Tris between pH 7.3 and 10.0, mean ± S.D. (*n* in parentheses); (□) 0.1 *M* phosphate between pH 6.0 and 7.4.

mary metabolite DOPAL and by the precise method of sample collection and storage. Therefore, we developed a system that would isolate MAO activity and preclude further metabolism of the primary aldehyde metabolite.

Selection of pH for MAO determination

pH profile. The pH profile for platelet MAO activity was determined in 0.1 *M* phosphate between pH 6 and 7.4 and 0.1 *M* Tris prepared at room temperature between pH 7.3 and 10.0 (Fig. 4). The mean measured pH values of the reaction mixtures at 37°C were 7.26, 7.56, 8.12, 8.54, 8.7, 8.97 and 9.17 from Tris prepared at pH 7.3, 7.8, 8.3, 8.8, 9.0, 9.4 and 10.0, respectively. A high pH range, 8.66–9.83, was tested using 0.1 *M* CHES. However, when the pH of the reaction mixture was >9.3 the mixture turned brown, presumably owing to the instability of DA. As shown in Fig. 4, maximum MAO activity occurred at pH 9.0 (the pH of the final reaction mixture at 37°C was 8.7); no other DA metabolites were detectable at this pH (Fig. 5) and the activity in platelets appeared to be stable when frozen at −70°C for at least one month. This pH and buffer were therefore chosen for further examination.

Assay blank. Chromatograms of the isolated DA metabolites often contained extra peaks. As

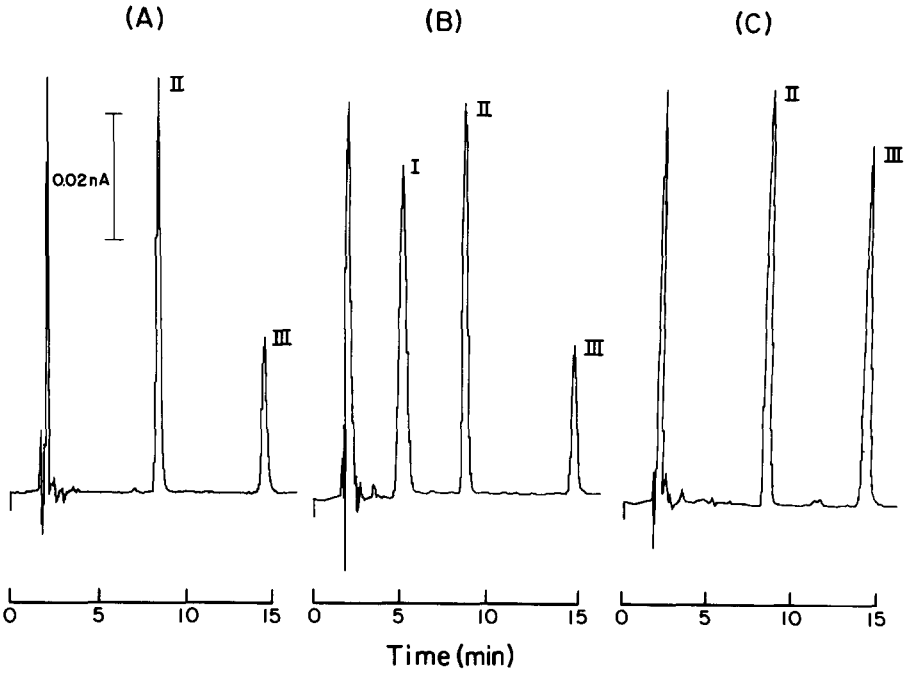

Fig. 5. HPLC–ED of MAO assayed in 0.1 *M* Tris buffer (pH 9) for 10 min. (A) Blank without platelets; (B) platelet assay; (C) platelet assay with 1 m*M* pargyline. Peaks: I = DOPAL; II = DHBA; III = DA.

these peaks sometimes interfered with the metabolite peaks, they should be present in the assay blank. The source of these peaks was identified by performing the assay at both pH 7.4 and 9 in reaction mixtures containing (a) boiled platelets, (b) no platelets or (c) no DA. Whereas there were no extra peaks in any Tris–HCl pH 9 assay, all pH 7.4 assays containing DA [*i.e.*, (a) and (b)] resulted in chromatograms with extra peaks. Even when DA was repurified [22], the extra peaks appeared. As platelets did not contribute extra peaks, they were omitted from the assay blank and DA was included. This also conserved the amount of blood initially required.

Validation and characterization of platelet MAO at pH 9

Recovery from alumina. Reaction mixtures incubated without platelets were transferred directly to an Amberlite column and eluted with 4.5 ml of deionized water. Known amounts of DOPAL, DHBA and DOPAC in 0.1 *M* $HClO_4$ were added to the eluate together with the usual Tris buffer and alumina. The buffering capacity of the Tris maintained the correct pH despite the addition of 0.1 *M* $HClO_4$. Samples were then processed by the standard method. The recoveries (mean ± S.D.) of DOPAL, DHBA and DOPAC were 36.4 ± 0.9, 87.9 ± 0.9 and 32.3 ± 2.2% (n = 5), respectively.

Recovery from Amberlite. The recovery of DOPAL from Amberlite in the absence of platelets was examined by the same procedure as above for alumina except that known amount of DOPAL were added to reaction mixtures after incubation but before transfer to Amberlite. The recovery (mean ± S.D.) of DOPAL was 37.8 ± 1.2% (n = 5). Because there was no difference between the recoveries from alumina and Amberlite, the recovery of DOPAL from Amberlite was 100%.

For recovery in the presence of platelets, it was necessary to prevent any reaction between DA and MAO. Therefore, duplicate reaction mixtures incubated without platelets were first placed in the ice–salt bath and then platelets and a known amount of DOPAL were added. The final recovery of DOPAL was 36.8% at 0.3 mg/ml protein (a typical assay concentration) and 34.4% at 0.6 mg/ml protein. Consequently, the recovery of DOPAL from Amberlite in the presence or absence of platelets was close to 100%. Similar experiments showed the recovery of DOPAC from Amberlite to be 100%.

Linearity and sensitivity. The electrochemical detector response was linear for 100-μl injections containing 2–200 pmol DHBA (r = 0.9999), 12.5–250 pmol DOPAC (r = 0.9999) and 3–327 pmol DOPAL (r = 0.9999).

To determine the linearity of the detector response for the amount of DOPAL generated in the assay, DOPAL between 0.05 and 5.57 nmol was tested as described under Experimental. There was good linearity (r = 0.9999) and the equation of the line was $y = 2.392x + 0.0139$. A similar procedure was used for DOPAC; the correlation coefficient was 0.9997 and the equation of the line was $y = 2.464x + 0.0193$.

MAO kinetics. The formation of DOPAL was linear with time up to 10 min (Fig. 6) and with platelet protein concentration (r = 0.991) up to at least 1.38 mg/ml in the final assay (Fig. 7).

To determine the Michaelis constants (K_M) for MAO, concentrations of DA between $5 \cdot 10^{-6}$ and $5 \cdot 10^{-3}$ *M* were chosen. The mean apparent K_M and maximum velocity (V_{max}) determined by Lineweaver–Burk analysis from four separate experiments were 0.103 m*M* [relative standard de-

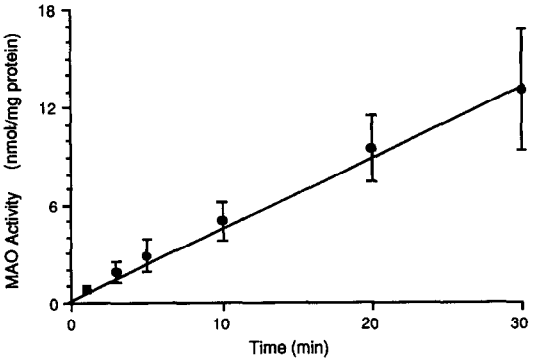

Fig. 6. Time course of platelet MAO activity with DA as substrate in 0.1 *M* Tris buffer (pH 9) over 30 min. The means of data from three separate platelet preparations ± S.D. are shown.

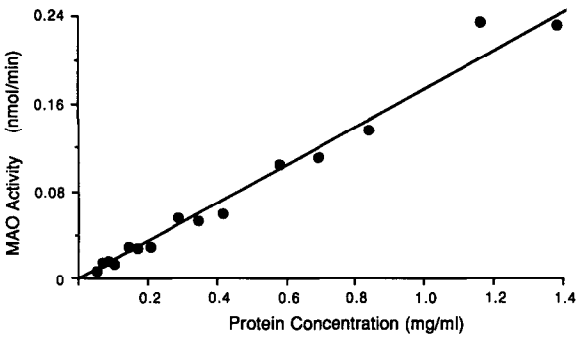

Fig. 7. Dependence of MAO activity on the concentration of platelet protein in the 10-min assay in 0.1 *M* Tris buffer (pH 9). Individual assays form three separate platelet preparations (r = 0.991 overall) are plotted.

viation (R.S.D.) = 35%] and 0.372 nmol/min · mg protein (R.S.D. = 7%), respectively (Fig. 8).

Reproducibility. Reproducibility was examined in four ways: (1) a single sample was injected multiple times on to the HPLC column; (2) samples from one platelet preparation were processed individually and analyzed within one day; (3) one platelet preparation was stored in aliquots at −70°C and analyzed on different days; and (4) different platelet preparations from one person were analyzed at different times. MAO activity showed excellent reproducibility

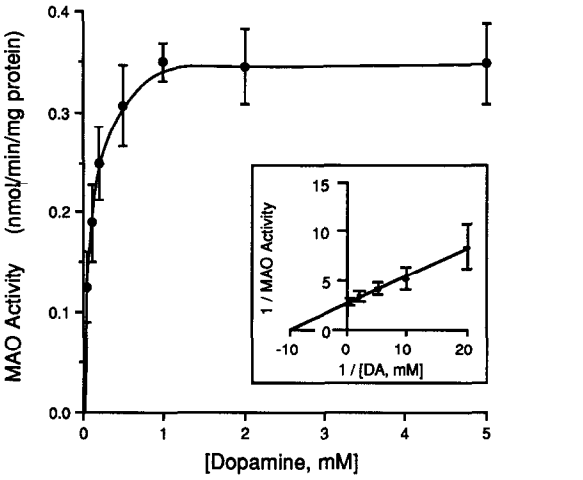

Fig. 8. Dependence of MAO activity on DA concentration for a 10-min assay in 0.1 *M* Tris buffer (pH 9) where the concentration was varied between 10^{-6} and 10^{-3} *M* DA. Inset: Lineweaver–Burk plot of the same data. The means of data from four separate experiments ± S.D. are shown.

TABLE II

INTER-INDIVIDUAL VARIATION IN HUMAN PLATELET MAO ASSAYS

Subject	MAO activity (nmol DOPAL/min · mg protein)	
	pH 7.4 assay	pH 9.0 assay
1	0.372	0.460
2	0.270	0.320
3	0.256	0.470
4	0.168	0.305
5	0.201	0.224
6	0.314	0.324
7	0.198	0.270
8	0.199	0.263
Mean	0.247	0.330
S.D.	0.070	0.090

on the same platelet preparation within sample (n = 5), between samples (n = 6) and between days (n = 5) with R.S.D.s of 0.9, 3.7 and 6.1%, respectively. Intra-individual MAO activity over time was more variable, with R.S.D. = 13.9% (n = 20). In all instances, the DOPAL peak-area variation was more than double the DHBA peak-area variation.

Inter-individual variation. The variation of MAO activity among individuals was analyzed in platelets from eight adults (four males and four females) at both pH 7.4 and pH 9. As shown in Table II, the activities in nmol/min · mg protein at pH 9 were higher than at pH 7.4. No statistically significant gender differences were noted in this limited sample.

DOPAC was also determined at pH 7.4 and it was produced at rates between 0.014 and 0.029 nmol/min · mg protein. In all instances DOPAC was $<12\%$ of the sum of DOPAC and DOPAL.

DISCUSSION

The assay for MAO activity presented here has the following advantages: (1) a minimum sample volume is employed, which permits repeated sampling from the same patient; (2) the procedure is relatively rapid and at least ten blood samples can be processed and analyzed within a

7-h workday; (3) by adjusting the pH either MAO activity alone or MAO plus aldehyde metabolism can be ascertained with each product quantitated separately; and (4) no radioactive materials are required.

This method requires only 2 ml of whole blood, which is less than that used by other investigators [7,10,12]. Cold centrifugation protects the platelets from heat denaturation. In addition, it incorporates the innovations of other investigators which ensure that variability from preparation to preparation is minimized [15] and that platelet pellets are virtually free from plasma proteins, erythrocytes and leukocytes [16].

The high pH optimum observed for MAO activity using DA is not unexpected. Roth [23] also found a high pH optimum for rat liver MAO using the substrates phenylethylamine and benzylamine under conditions which limited substrate inhibition.

In the present procedure with Tris–HCl (pH 9.0), DOPAL is the only detectable product (Fig. 5). This may have been partially due to direct inhibition by Tris, as nitrogen-containing buffers have been shown to inhibit aldehyde dehydrogenase [24], the enzyme that catalyzes the conversion of DOPAL to DOPAC. In contrast, in the commonly used phosphate buffer at pH 7.4, both DA and DOPAL become the source of additional products.

The recovery of DOPAL from alumina under these assay conditions is low but extremely reproducible (Table I). The recovery from Amberlite is 100%, but to maintain this recovery the protein concentration in the reaction mixture should remain below 1 mg/ml [8,25]. With the current procedure protein concentrations generally are $\leqslant 0.6$ mg/ml.

The Michaelis constant for human platelet MAO activity with DA as substrate cannot be compared precisely with the K_M values from other studies, as the latter employed a different pH and a radioactive substrate. Nevertheless, the apparent K_M of 0.22 mM and a V_{max} of 0.45 nmol/min · mg protein reported by Donnelly and Murphy [5] is similar to the K_M of 0.10 mM and V_{max} of 0.37 nmol/min · mg protein reported here. Glover *et al.* [7] reported a range of platelet MAO activity from 0.06 to 0.51 nmol/min · mg protein using a DA concentration of 0.15 mM. When individual subject MAO activities determined under optimum conditions at pH 9 are calculated for a DA concentration of 0.15 mM, they range from 0.14 to 0.29 nmol/min · mg protein (data not shown).

In conclusion, a sensitive and selective assay for the determination of MAO activity in human platelets using DA as substrate has been developed. The procedure is suitable for clinical studies.

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