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A RADIOISOTOPIC ASSAY FOR MONOAMINE OXIDASE DETERMINATIONS IN HUMAN PLASMA*

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Abstract—A method for monoamine oxidase is described which is based on the formation of a radioactive anisole-soluble end product from the substrate, ^{14}C-tyramine. After the enzyme incubation, the end product is extracted into anisole, the aqueous phase frozen, and the anisole containing both the radioactive end product and phosphor is poured into a counting vial for assay in a liquid scintillation spectrometer. This method can detect the metabolism of 1·7 ng of tyramine. With this assay, monoamine oxidase activity was found in human plasma. The data obtained from the use of specific amine oxidase inhibitors suggest that monoamine and diamine oxidase of human plasma are separate enzymes.

THE available methods for the assay of monoamine oxidase activity have been reviewed by Zeller.[1] They include the methods based on the disappearance of substrate, consumption of oxygen, production of ammonia, and production of peroxide. Monoamine oxidase has also been determined both *in vivo* and *in vitro* by the isotope dilution technique by measuring residual ^{14}C-tyramine or ^{14}C-tryptamine which was used as substrate.[2,3] More recently, fluorometric methods[4] such as the determination of residual tyramine or tryptamine or the production of 5-hydroxyindoleacetic acid from serotonin have been widely used for this purpose. Unfortunately, none of the existing methods was adaptable in our hands as a rapid micromethod for monoamine oxidase activity to accommodate a large number of samples.

Burstein and Dorfman[5] have recently devised a simple and ingenious isotopic method for the study of steroid sulfatase. This consisted of the extraction of a toluene-soluble reaction product formed from a toluene-insoluble isotopic substrate with the scintillation solution and the determination of radioactivity of the product in a liquid scintillation spectrometer after freezing and filtering the aqueous phase. Our laboratory has found this technique applicable for the determination of diamine oxidase.[6] We have now determined conditions required to apply this technique for a sensitive, rapid, and accurate determination of monoamine oxidase activity. The details are described in this paper.

During the course of this work, an assay similar to that presented here for monoamine oxidase has appeared, which employed ^{14}C-tryptamine as substrate.[7] However,

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detailed evidence supporting the validity of the method has not yet been presented. An abstract describing another micromethod for monoamine oxidase using ^{14}C-serotonin and ^{14}C-tyramine also appeared earlier,[8] but a subsequent report could not be found in the literature.

With the aid of the isotopic method described in this paper and amine oxidase inhibitors, evidence has been obtained for the occurrence of monoamine oxidase in human plasma.[1,9] The evidence supporting this claim is presented here.

MATERIALS AND METHODS

Radioactive materials

^{14}C-Tyramine, 4·6 mc/mmole, was purchased from California Corporation for Biochemical Research. This was diluted with nonisotopic tyramine to give a solution with an activity of 50,000 disintegrations per min (dpm) per 4 μg tyramine per 0·1 ml solution. ^{14}C putrescine was purchased from New England Nuclear Corporation and diluted with carrier to give a solution with an activity of 100,000 dpm/50 μg putrescine per 0·10 ml solution.

Enzyme inhibitors

All inhibitors were used in a final concentration of 10^{-4} M (calculated on the basis of the free base) unless otherwise stated. The inhibitors used were as follows: aminoguanidine sulfate (Distillation Products), 1-isopropyl-2-isonicotinylhydrazine or Marsilid (Hoffmann-La Roche, Inc.), β-phenylethylhydrazine or Nardil (Warner-Chilcott), *cis-trans*-phenylcyclopropylamine sulfate or Parnate sulfate (Smith, Kline & French), α-methylphenethylhydrazine or Catron (Lakeside), and isonicotinic acid hydrazide or INH (Distillation Products).

Enzyme preparation

Monoamine oxidase was prepared from hog kidney by the method of Satake.[10] Sixty-five grams of hog kidney acetone powder was extracted twice with 30 ml of 0·2 M phosphate buffer, pH 7·2. The residue was suspended in 20 ml water inside a Visking cellulose casing bag and dialyzed for 4 hr against tap water followed by 4 hr against distilled water This dialyzed suspension containing 1.12 mg N/ml had a specific activity of 44 μl O_2 uptake/hr per mg protein when tyramine was used as substrate.

Liquid scintillation counting method

A Packard Tri-Carb liquid scintillation spectrometer, model 314EX, was used in this study. The extraction solvent consisted of reagent-grade anisole containing 0·6 % 2,5-diphenyloxazole (PPO). This solution (anisole–PPO) gave 53 % counting efficiency after its use in extraction of end product(s). Toluene containing 0·4 % PPO (toluene–PPO) was also tested in preliminary studies as a possible useful solvent.

^{14}C-Tyramine was assayed at a counting efficiency of 50 % using a toluene–ethanol mixture (70:25 v/v) containing 0·37 % PPO and 0·01 %1, 4-*bis*-2(4-methyl-5-phenyl-oxazolyl)benzene (dimethyl–POPOP).

For the assay of radioactivity in protein solutions, 1 ml of methanol containing 1 M Hyamine hydroxide (Packard Instrument Co.) was added to 0·2 ml of sample and heated for 4 hr at 60° to dissolve the protein and assayed with the addition of 10 ml of toluene–PPO solvent with an efficiency of 32 %.

Counting efficiencies of the various solvents were determined by the internal standard method using a standard solution of ^{14}C-toluene containing 10,000 dpm/0·1 ml. All the counting was done in low-potassium 5-dram glass vials (Wheaton Glass Co.).

Diamine oxidase activity was assayed according to the procedure of Okuyama and Kobayashi.[6]

General incubation and extraction procedure

All enzyme assays were done in a screw-cap culture tube (10 × 1·5 cm) in a final volume of 2·0 ml in air at 37° in a Dubnoff metabolic shaker. At the end of the incubation period 0·4 ml of 2 M citric acid was added to the culture tube followed by 10 ml of the toluene- or anisole–PPO solution. The mixture was shaken vigorously for about a minute, centrifuged at 1,000 rev/min in an International centrifuge, model PR-1, and allowed to stand at −20° until the lower aqueous phase was frozen. The upper layer was then poured into a counting vial and assayed in a liquid scintillation counter.

Standard test solution

A standard test solution for extraction studies was prepared as follows: 1 ml of kidney monoamine oxidase preparation was incubated with 40 μg of ^{14}C-tyramine and 10^{-3} M ethylenediamine tetraacetate (EDTA) in 20 ml of 0·1 M sodium phosphate buffer, pH 7·5, at 38°. After 60 min, the reaction mixture was heated for 5 min in a boiling-water bath and centrifuged. Aliquots of the supernatant portion were used in the extraction studies.

TABLE 1. EFFECT OF ETHYLENEDIAMINE TETRAACETIC ACID ON MONOAMINE OXIDASE REACTION

	Radioactivity extracted (cpm)
Enzyme alone	4,812
Enzyme + EDTA	5,533
Boiled enzyme	37
Boiled enzyme + EDTA	37

Four μg ^{14}C-tyramine was incubated in 2 ml of 0·1 M phosphate buffer, pH 7·4, with 0·1 ml hog kidney monoamine oxidase preparation or a heat-inactivated preparation (the enzyme was kept in boiling-water bath for 5 min) with or without 10^{-3} M EDTA. Time was 60 min in air at 37°.

The radioactive products were extracted with anisole–PPO as described under Methods.

EDTA was found to accelerate the enzyme reaction as estimated by the extraction method (Table 1). EDTA was also used to prevent further oxidation of the reaction product.

Paper chromatography

Samples were applied on strips of Hengar tube filter paper ($\frac{3}{4}$ in. width) and developed in a mixture of *n*-butanol–acetic acid–water (4:1:1, by volume). The paper was scanned on an Actigraph II, model C-100B (Nuclear Chicago Corp.) under the following conditions: collimeter of $\frac{1}{4}$ in., scan speed of 12 in./hr, time constant of 10 sec, and count range of 1,000 counts.

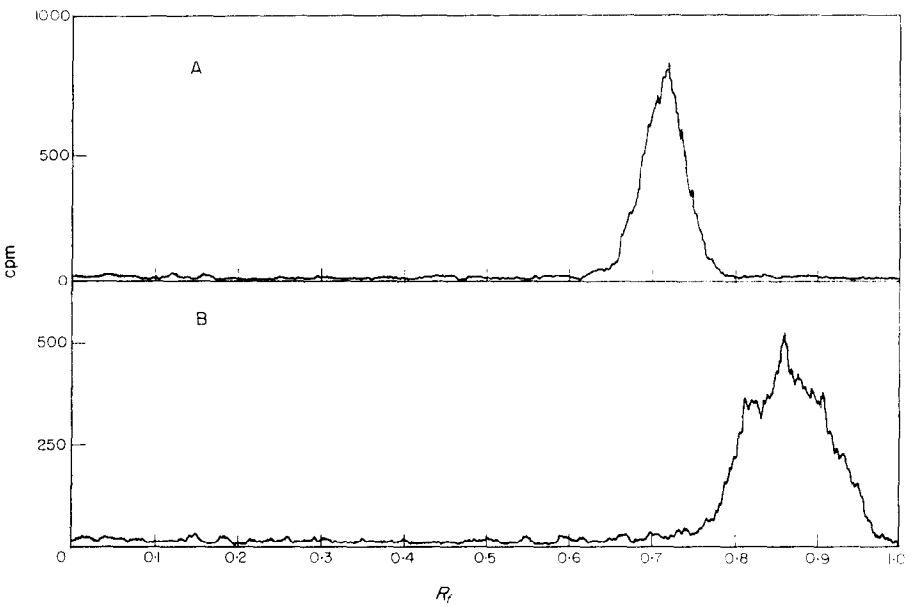

FIG. 1. Paper chromatography of ^{14}C-tyramine and its metabolite. (A) ^{14}C-Tyramine, alone. (B) ^{14}C-Tyramine metabolite after extraction with solvent. Conditions as described in text.

EXPERIMENTAL

Paper chromatography of products

The standard aqueous solution of reaction product, described in the previous section, was chromatographed directly on paper and found to yield two major peaks of radioactivity: one at the origin and the other at R_f 0·90. Neither peak corresponded to that of the substrate, ^{14}C-tyramine (Fig. 1A). The peak at the origin was probably due to the adsorption of some radioactivity by the protein. This was indicated by the fact that the peak at the origin disappeared when the product was extracted with solvent and chromatographed (Fig. 1B).

Extraction of reaction product

Extraction efficiencies of both tyramine and reaction product with toluene–PPO and anisole–PPO at various pH values of the aqueous phase were tested as follows: 0·1 ml of the standard reaction mixture or ^{14}C-tyramine (4 μg) was mixed with 1·8 ml of phosphate–HCl or phosphate–NaOH solutions at various pH values. The solutions were extracted twice and 10-ml portions of solvent and the total radioactivity extracted were plotted against pH.

With anisole–PPO, the recovery of the product was almost constant within the entire range of pH tested (1–11·5). However, at pH 6 or above, tyramine was also

extracted. The addition of 0·4 ml of 0·2 M citric acid to 2·0 ml of the aqueous phase brought the pH value to about 3 for selective extraction of the reaction product (Fig. 2). Under these acid conditions the recoveries of the reaction product were approximately 60% and 82%, respectively, with toluene- and anisole–PPO.

With toluene–PPO, the pH range of 2 to 7 appeared suitable for the extraction of the product. Tyramine was also extracted at alkaline pH greater than 6. The extraction pattern was similar to that for anisole–PPO (Fig. 2).

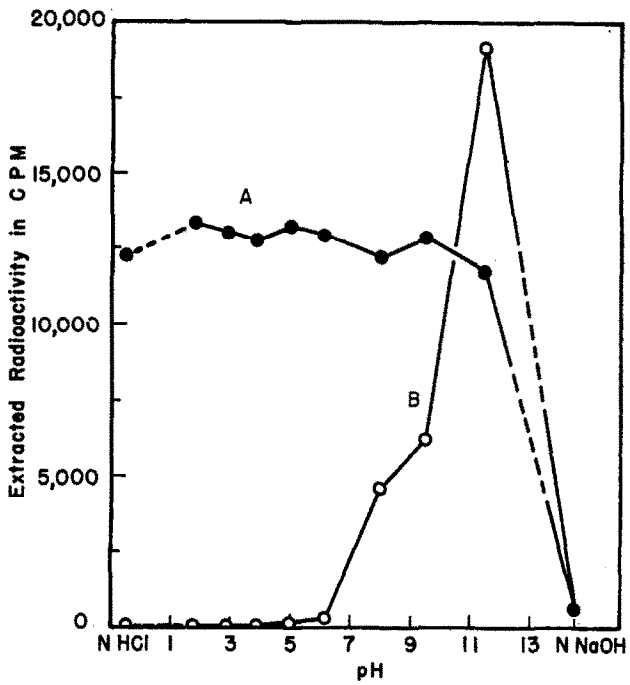

FIG. 2. Extraction efficiency of anisole–PPO solvent as a function of the reaction mixture pH. Curve A is the amount of radioactive end product extracted by the solvent. Curve B is the amount of radioactive tyramine extracted by the solvent. Conditions as described in text.

The relation between the number of extractions and the recovery of radioactivity extracted under various conditions is shown in Fig. 3. It can be seen that anisole and citric acid gave the highest extraction efficiency.

The relation between the amount of reaction product and radioactivity extracted by one and two extractions with anisole–PPO are shown in Fig. 4.

These data (Fig. 2 and 3) showed that anisole–PPO was superior to toluene–PPO for the extraction of the reaction product(s) from the action of monoamine oxidase on tyramine. Therefore, the toluene–PPO system was abandoned for further study and the anisole–PPO system was adopted as the extraction solvent.

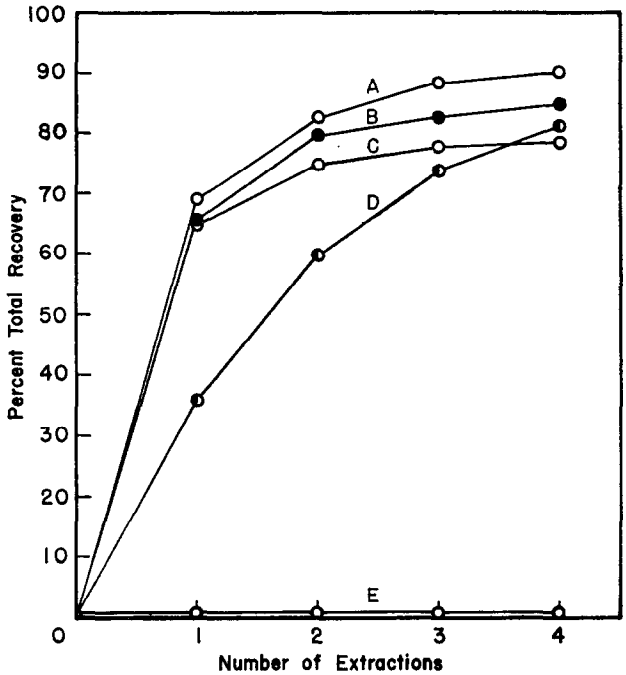

FIG. 3. Extraction efficiency as a function of the pH and the number of extractions. A, Reaction product: adjusted to a final pH of 2·5 by adding 0·4 ml 2 M citric acid before extraction with anisole–PPO. B, Reaction product: adjusted to a final pH of 2·8 with 0·1 M sodium phosphate–N HCl mixture before extraction with anisole–PPO. C, Reaction product: adjusted to a final concentration of N HCl (pH 1·0) before extraction with anisole–PPO. D, Reaction product: adjusted to a final pH of 2·5 by adding 0·4 ml 2 M citric acid before extraction with toluene–PPO. E, ^{14}C-Tyramine substituted for reaction product in A.

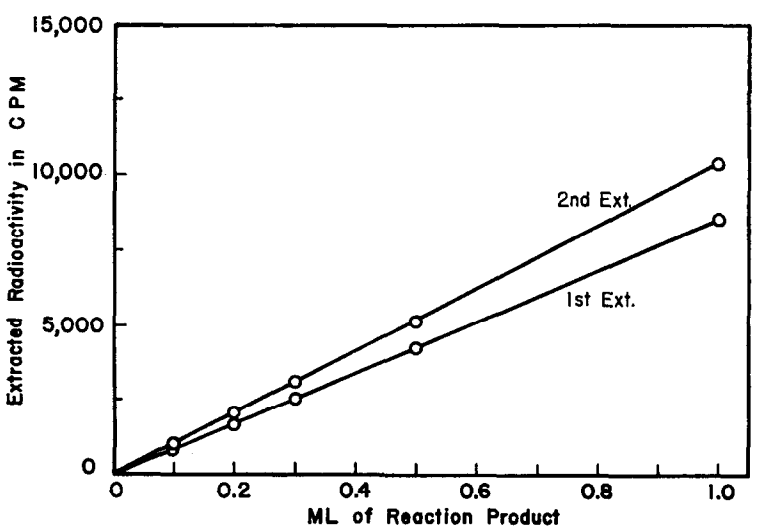

FIG. 4. Relationship between the amount of reaction product and the amount of radioactivity extracted with anisole–PPO. Conditions: Aliquots of the standard test solution were diluted to a final volume of 2·0 ml, acidified by adding 0·4 ml 2 M citric acid, and extracted with anisole–PPO. Theoretical recovery of end product from 1 ml standard test solution was 13,750 cpm.

Conditions for the enzyme reaction

The formation of the end product was found to be roughly proportional to incubation time during the first hour and deviated from linearity with longer incubation periods (Fig. 5). Only a small increase in extractable radioactivity was found after a 240-min incubation, indicating the completion of the reaction.

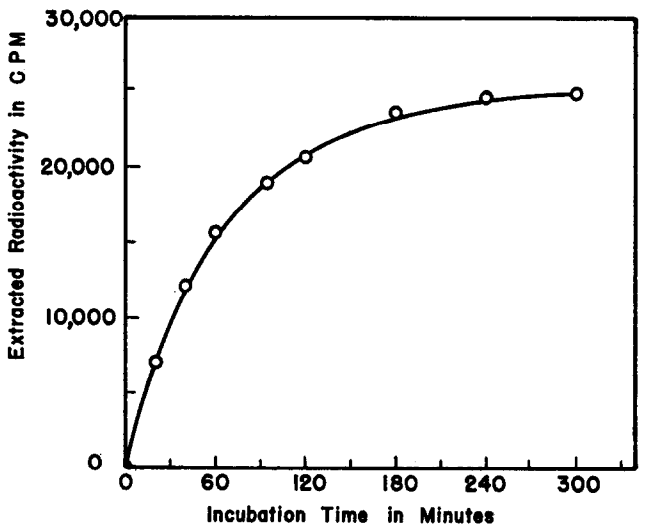

FIG. 5. Production of reaction product from tyramine as a function of time; 0·5 ml hog kidney monoamine oxidase preparation and 40 μg ^{14}C-tyramine were incubated in 0·1 M phosphate buffer, pH 7·4 (total volume, 20 ml), with 10^{-3} EDTA. Two-ml aliquots were pipetted out from the reaction mixture at the times indicated. The radioactive products were extracted with anisole–PPO as described under Methods.

Figure 6 shows the effect of enzyme concentration on the reaction rate. In this experiment, 4 μg ^{14}C-tyramine was incubated with varying amounts of diluted enzyme in phosphate buffer to make the final volume of 2 ml. It can be seen that the oxidation of tyramine proceeds at a rate approximately proportional to the enzyme concentration within the range tested. Deviation from linearity of the extracted radioactivity was observed with higher enzyme concentration. This may have been due to the interference of enzyme protein with the extraction process or to the rapid depletion of substrate.

Stoichiometry of monoamine oxidase reaction

The stoichiometry of monoamine oxidase reaction was determined by the estimation of the remaining tyramine, the product formed, and oxygen consumed during the incubation.

Four μg ^{14}C-tyramine, 10 μmoles nonisotopic tyramine, 0·25 or 0·50 ml of enzyme and 10^{-3} M EDTA were incubated in 0·1 M sodium phosphate buffer, pH 7·5, in a total volume of 2·0 ml. After 1-hr incubation at 38°, the product was extracted eight times with 10-ml portions of anisole–PPO. The amount of product was calculated from the total radioactivity of the combined extracts. The aqueous phase was concentrated to about one-tenth volume by evaporation and extracted twice with ethanol–toluene

mixture containing PPO. The aqueous phase was now evaporated to dryness and heated for 4 hr at 60° with 1 ml of 1 M Hyamine hydroxide in methanol to dissolve the protein. The Hyamine solution was taken up with 20 ml of the ethanol–toluene mixture for liquid scintillation counting. The unchanged tyramine was assumed to be equivalent to the total radioactivity contained in the ethanol–toluene extracts and the Hyamine solution.

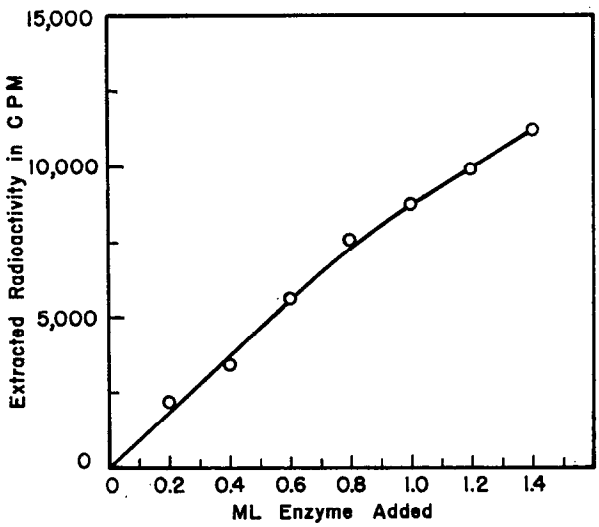

FIG. 6. Enzyme concentration vs. enzyme activity. Original enzyme preparation was diluted 20 times. Aliquots of the diluted enzyme preparation were added to 4 μg of ^{14}C-tyramine and 0·2 ml of 10^{-2} M EDTA in a total volume of 2 ml with 0·1 M phosphate buffer, pH 7·4. Reaction mixture was incubated in air at 38° for 1 hr.

The oxygen consumption of a reaction mixture identical with that above, with the exception of the ^{14}C-tyramine, was measured manometrically with 0·1 ml of 20% potassium hydroxide in the center well of the Warburg vessels. The results of both the manometric and isotopic determinations are given in Table 2.

Isolation and identification of tyramine metabolite

Ten ml of hog kidney monoamine oxidase, 34·8 mg tyramine, 288 μg ^{14}C-tyramine (0·94 μc), and 1 ml of 2×10^{-2} M EDTA were incubated in 0·1 M sodium phosphate buffer, pH 7·5, in a final volume of 15·2 ml for 2 hr at 38° in air. At the end of the incubation period the reaction mixture was acidified with 3·0 ml of 2 M citric acid and extracted four times with 30-ml aliquots of toluene. The combined toluene extracts were found to contain a total of 593,380 dpm indicating the metabolism of 28·8% of the tyramine. The toluene extract was evaporated to dryness in the presence of 2,4-dinitrophenylhydrazine. The resulting wet crystals were recrystallized, first from benzene and then from alcohol. The crystals were collected by centrifugation and dried *in vacuo*. The orange crystals melted with decomposition between 182° and 187°; lit. value 183° to 186°,[11] and was therefore presumed to be the 2,4-dinitrophenylhydrazone of *p*-hydroxyphenylacetaldehyde. The total yield of the hydrazone was

dissolved in toluene and assayed for its radioactive content in a liquid scintillation counter. The counting efficiency of the highly quenched solution was determined by the addition of an internal standard, ^{14}C-toluene, and the hydrazone was found to contain 232,338 dpm. This indicated a 39% yield of purified 2,4-dinitrophenylhydrazone from the original combined toluene extract. Although the yield is low, the losses can be attributed to the difficulty in recovering small amounts of material in good yield. The fact that a radioactive hydrazone was formed from the toluene extract containing the tyramine metabolite is presumptive evidence that the product is *p*-hydroxyphenylacetaldehyde.

TABLE 2. STOICHIOMETRY OF MONOAMINE OXIDASE REACTION

Enzyme added (ml)	Tyramine remaining (μmoles)	Product (A) (μmoles)	Oxygen consumed (B) (μmoles)	Ratio A/B
0·5	5·74	2·50	1·36	1·83
0·25	8·52	1·59	0·82	1·94
0	9·90	0	0	0

Conditions: 4 μg ^{14}C-tyramine contained in 10 μmoles nonisotopic tyramine, 0·5 and 0·25 ml of enzyme, and 10^{-3} M EDTA, final concentration, made up in 0·1 M phosphate buffer, pH 7·4, to a volume of 2·2 ml.

Incubation time was 60 min in air at 37° .

Application of monoamine oxidase assay

Two ml of oxalated plasma was assayed for monoamine and diamine oxidase activity. Diamine oxidase and monoamine oxidase inhibitors, listed under Materials, were used in a final concentration of 10^{-4} M to characterize the amine oxidase activity. The results are shown in Table 3. Each value is an average of duplicate determinations. Plasma 1 and 2 were from male subjects and plasma 3 and 4 were pooled pregnancy plasmas.

Parnate and aminoguanidine appeared to be the most specific inhibitors for mono- and diamine oxidase respectively. These two inhibitors were serially diluted to find a concentration that would be completely inhibitory to one enzyme without influencing the other. It was found that 10^{-5} M aminoguanidine inhibited diamine oxidase activity about 97% and monoamine oxidase activity less than 10%. Parnate, at a concentration of 10^{-5} M, inhibited monoamine oxidase activity of plasma completely without any measurable effect on diamine oxidase activity.

All monoamine oxidase determinations were done with only a single extraction with anisole–PPO.

DISCUSSION

The classification of monoamine oxidase activity found in plasma and tissues is difficult because there is no established convention concerning nomenclature to follow in this field.[1,9] Plasma monoamine oxidase activity, as described by Tabor *et al.*[12] and by Yamada and Yasunobu[13] have little to no activity against the usual biogenic monoamines and are inhibited by carbonyl reagents. Tabor *et al.* have classified the

TABLE 3. AMINE OXIDASE ACTIVITY IN HUMAN PLASMA

	Monoamine oxidase activity as measured by tyramine metabolized								Diamine oxidase activity as measured by putrescine metabolized			
	PLASMA 1		PLASMA 2		PLASMA 3		PLASMA 4		PLASMA 3		PLASMA 4	
	(cpm)	Inhib. (%)	(cpm)	Inhib. (%)	(cpm)	Inhib. (%)	(cpm)	Inhib. (%)	(cpm)	Inhib. (%)	(cpm)	Inhib. (%)
Plasma alone	68	0	329	0	225	0	242	0	1,791	0	1,137	0
Plasma with aminoguanidine*	53	22	252	23	166	26	190	21	27	98	48	96
Plasma with semicarbazide	63	7	281	15	194	14	179	26	715	60	592	48
Plasma with Marsilid	3	96	41	88	32	86	22	91	1,586	11	873	23
Plasma with Nardil					0	100	2	99	362	80	363	68
Plasma with Parnate					0	100	0	100	1,688	6	892	22
Plasma with Catron					3	99	4	98	1,478	17	832	27
Plasma with INH							221	9			950	16

* All inhibitors were used in a final concentration of 1×10^{-4} M.

preparation as spermine oxidase; Yamada and Yasunobu call their preparation monoamine oxidase. An enzyme(s) of major interest in the psychopharmacology field is that associated with the destruction of biogenic monoamines which is also classified as monoamine oxidase. This latter monoamine oxidase, which is associated with tissues rather than plasma, is not inhibited by carbonyl reagents and is usually inactive against spermine.[1, 14, 15] The assay described in this paper measures the plasma enzyme that attacks the biogenic monoamines and is not the one that attacks spermine. This contention is supported by the observation that the carbonyl reagent, aminoguanidine, in a concentration that completely inhibits putrescine metabolism, does not influence the destruction of tyramine. In view of the confusion which has arisen because of the general use of the term monoamine oxidase, it may be desirable to identify monoamine oxidase assays according to the substrate used until a suitable convention in nomenclature is adopted.

The stoichiometry of the monoamine oxidase reaction was found, experimentally, to average 1·88 moles of product formed per mole of oxygen consumed (Table 2). This approximates the expected 2:1 ratio for monoamine oxidase in the absence of aldehyde oxidase and the presence of catalase in the enzyme preparation.[16] The absence of aldehyde oxidase was shown by its inability to oxidize acetaldehyde. It therefore appeared reasonable to assume that monoamine oxidase with catalase activity was being measured by our assay procedure. This conclusion is also supported by the paper chromatographic data and the isolation of the 2,4-dinitrophenylhydrazone of *p*-hydroxyphenylacetaldehyde.

The sensitivity of this method is a function of the specific activity of the tyramine available. In our diluted preparation, the metabolism of 17 ng tyramine could be measured with ease. Without dilution, the metabolism of 1·4 ng tyramine (100 cpm at 50% efficiency) can be detected. Therefore, this method appears to compete favorably with other available assays for monoamine oxidase.

The critical consideration in judging solvents for a particular extraction is not always the efficiency of extraction of the desired material but the selectivity of the extraction process. The quantitative aspects of the extraction process are automatically determined by the partition coefficients of the material in the solvent system employed. Although the data presented show anisole–PPO to be more efficient than toluene–PPO in extracting the end product, toluene–PPO appears to result in a sharper separation of the end product from tyramine. In some instances, therefore, toluene–PPO may be preferred over anisole–PPO as the extracting solvent.

An application of this method was demonstrated by its use in the determination of monoamine oxidase activity in human plasma (Table 3). The monoamine oxidase activity was found to be very weak. However, this was expected because this enzyme has been reported to be absent from human plasma.[1,9] The plasma from the first two male subjects tested indicated that monoamine oxidase was present because Marsilid was the most potent of the inhibitors tested. Third and fourth analyses were done on pooled pregnancy plasma both with tyramine and putrescine as substrates and a larger selection of inhibitors. Only the monoamine oxidase inhibitors Marsilid, Nardil, Parnate, and Catron effectively inhibited tyramine metabolism. Against putrescine, the two diamine oxidase inhibitors, aminoguanidine and semicarbazide, and the monoamine oxidase inhibitor, Nardil, were found to be effective inhibitors. Our finding that either mono- or diamine can be inhibited completely without influencing

the other enzyme is in harmony with the report of Burkard *et al.*[17] We suggest that monoamine oxidase and diamine oxidase of human plasma are two different enzymes.

These data show that the monoamine oxidase assay described here offers a simple means of evaluating *in vivo* the effectiveness of monoamine oxidase inhibitors in the circulation as well as a method for determining the biological half-life of these drugs. This assay may then be a useful adjunct to the existing methods for monoamine oxidase determinations. These comments also apply to the diamine oxidase determination described previously.[6] This monoamine oxidase assay has been used routinely in these laboratories to determine the monoamine oxidase contamination in purified diamine oxidase preparations; the results will be presented elsewhere.

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