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ASSAY FOR MONOAMINE OXIDASES A AND B BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A sensitive method for the assay of monoamine oxidases A and B is described which employs high-performance liquid chromatography with fluorescence detection. Rat brain mitochondria were used as a preparation of the enzymes. *p*-Sulfamoylbenzaldehyde and benzaldehyde formed enzymatically from *p*-sulfamoylbenzylamine (the substrate of monoamine oxidase A) and benzylamine (the substrate of monoamine oxidase B), respectively, are converted simultaneously into fluorescent compounds with 2,2′-dithiobis(1-aminonaphthalene). These compounds are separated by reversed-phase chromatography on μBondapak CN. The limits of detection for *p*-sulfamoylbenzaldehyde and benzaldehyde formed enzymatically are 30 and 10 pmol per assay tube, respectively.

INTRODUCTION

Monoamine oxidase (MAO; monoamine:oxygen oxidoreductase, E.C. 1.4.3.4) catalyzes the oxidative deamination of monoamines in many tissues. The enzyme is classified into two types, MAO A and MAO B, by their substrate specificity and sensitivity to some irreversible inhibitors[1–4].

Many assay methods have been reported for the assay of MAO; spectrophotometric[5–7], fluorimetric[8–10], and radioisotopic[11,12] methods. High-performance liquid chromatography (HPLC)[13] and gas chromatography[14,15] have recently been introduced to the assay of MAO. These methods except for the radioisotopic methods do not permit the separate assay of the MAOs.

We have found recently that *p*-sulfamoylbenzylamine is a specific substrate of MAO A, and we have established a fluorimetric method for the separate assay of MAOs A and B using *p*-sulfamoylbenzylamine and benzylamine, the substrate of MAO B, respectively[16], based on the separate determination of *p*-sulfamoylbenzaldehyde and benzaldehyde formed enzymatically from the substrates by the fluorimetric method with 2,2′-dithiobis(1-aminonaphthalene) (DTAN)[17].

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We have developed an HPLC method with fluorescence detection for the microassay of MAOs A and B; the principle is basically the same as that of the fluorimetric method. The enzyme reactions of MAOs A and B are performed separately. *p*-Sulfamoylbenzaldehyde and benzaldehyde formed enzymatically are simultaneously converted into fluorescent compounds by the method with DTAN with minor modifications, and the compounds are separated by reversed-phase HPLC on μBondapak CN. Rat brain mitochondria were employed as a model enzyme preparation to establish the analytical procedure. The established method was examined to determine its usefulness in the assessment of the inhibitory potency of MAO A and MAO B inhibitors.

EXPERIMENTAL

Reagents and materials

All chemicals were of reagent grade, unless otherwise noted. Water was deionized and distilled. DTAN was purchased from Dojin Institute of Chemistry, Kumamoto, Japan. *p*-Sulfamoylbenzaldehyde was synthesized according to the method of Momose and Ueda[18]. The hydrochlorides of *p*-sulfamoylbenzylamine and benzylamine were repeatedly recrystallized from methanol–ethyl acetate and methanol–diethyl ether (peroxide free), respectively.

DTAN solution. DTAN (40 mg) was dissolved in 6.0 ml of methanol–6 *M* perchloric acid (9:1, v/v), then 0.1 ml of tri-*n*-butylphosphine was added. After the solution became colorless on standing for several minutes, 25.0 ml of 6 *M* perchloric acid were added, and the mixture was diluted with water to 100 ml. The resulting emulsified solution became clear and colorless on standing for 30–40 min. The solution was stable for at least one month when stored at 4°C.

Rat brain mitochondrial preparation. Donryu rats (male, 4 weeks old) were killed by decapitation, their brains were removed, and mitochondria were isolated by the method of Johnston[1] with minor modifications as follows. Brains were chilled on ice, weighed and homogenized with four volumes of ice-cold 0.25 *M* sucrose solution in a Potter–Elvehjem homogenizer. The homogenate was then centrifuged at 25,000 *g* for 15 min. The precipitate was suspended in water. The protein concentration was adjusted to *ca.* 0.5 mg/ml, and measured by the method of Lowry *et al.*[19], using bovine serum albumin as a standard protein.

Apparatus

A μBondapak CN column (Japan Waters, Tokyo, Japan) was used. A Hitachi 635A high-performance liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (20-μl loop) and a Hitachi 650-10S spectrofluorimeter fitted with a 20-μl flow cell setting the spectral bandwidths in the excitation and emission monochromators at 5 and 10 nm, respectively, and operating at an excitation wavelength of 345 nm and an emission wavelength of 410 nm. Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path-length, 10 × 10 mm); spectral bandwidths of 5 nm were used in both the excitation and emission monochromators. Mass spectra (electron-impact ionization) were measured with a JEOL JMS-01-SG mass spectrometer and infrared spectra with a Nihonbunko DS 710G infrared spec-

trometer in potassium bromide pellets. The pH was measured with a Hitachi-Horiba M-7 pH meter at 37°C. The melting point is uncorrected.

Procedure for the assay of MAOs A and B (Procedure A)

To 60 μl of 0.1 *M* Tris · HCl buffer (pH 8.5), 20 μl of the mitochondrial preparation were added. The mixture was preincubated at 37°C for 5 min, then incubated again after the addition of 20 μl of a substrate solution in the Tris · HCl buffer (76 m*M* *p*-sulfamoylbenzylamine hydrochloride for MAO A assay or 0.4 m*M* benzylamine hydrochloride for MAO B assay). The enzyme reaction was stopped by the addition of 20 μl of 3 *M* perchloric acid. The mixture was centrifuged at 1000 *g* for 10 min. Equal volumes of the supernatants from the reaction mixtures MAO A and MAO B were combined. To 100 μl of the resulting solution, 100 μl of DTAN solution were added, and the mixture was allowed to stand at 37°C for 40 min to derivatize the enzyme reaction products. To the mixture were added 100 μl of acetone to stabilize the resulting fluorescent products and 100 μl of 1.0 *M* sodium acetate to neutralize the reaction mixture. The fluorescence in the final reaction mixture was stable at room temperature for more than 6 h. The mobile phase was a mixture of methanol and 33 m*M* Na–Na phosphate buffer of pH 7.0 (6:4, v/v).

To prepare the blank, the same procedure was carried out except that the substrate solution was added after the addition of perchloric acid. For standard curves, 20 μl of a standard mixture of *p*-sulfamoylbenzaldehyde and benzaldehyde (1–50 nmol/ml, each) were used in place of 20 μl of the substrate solution. Net peak heights due to the individual aldehydes formed enzymatically were used for the quantitation.

Procedure for testing the effect of inhibitor concentration on MAO A and MAO B activities (Procedure B)

To 50 μl of 0.1 *M* Tris · HCl buffer (pH 8.5), 20 μl of the mitochondrial preparation were added. The mixture was preincubated at 37°C for 5 min, and incubated again at 37°C for 15 min after the addition of 10 μl of an MAO inhibitor solution (0–10^{-3} *M*) in the Tris · HCl buffer. At the end of the incubation, 20 μl of a substrate solution in the Tris · HCl buffer (see Procedure A) were added and then treated in the same way as for Procedure A.

Preparation of fluorescent compounds from p-sulfamoylbenzaldehyde and benzaldehyde

From p-sulfamoylbenzaldehyde. To 100 mg of *p*-sulfamoylbenzaldehyde dissolved in 100 ml of aqueous methanol (9:1, v/v) was added DTAN solution (100 mg of DTAN, 10 ml of methanol–6 *M* sulfuric acid (9:1, v/v), 0.2 ml of tri-*n*-butylphosphine, 25 ml of 6 *M* sulfuric acid and 65 ml of water). The mixture was allowed to stand at room temperature (20–25°C) for 1 h with stirring. The resulting yellow precipitate was filtered off, dried and recrystallized from ethyl acetate to pale yellow needles (compound I; m.p. 258–259°C; yield 124 mg). Since the fluorescent products in the reaction of DTAN with benzaldehyde, *p*-hydroxybenzaldehyde, vanillin and isovanillin have been characterized as 2-arylnaphtho[1,2-*d*]thiazole[20,21], compound I should be 2-(*p*-sulfamoylphenyl)naphtho[1,2-*d*]thiazole. This was confirmed by the following analytical data: calculated for $C_{17}H_{12}N_2O_2S_2$: 59.98% C, 3.55% H, 8.23% N; found, 59.91% C, 3.58% H, 8.23% N. Mass spectrum: *m/e* 340 (M^+, base peak),

260 ($M^+ - SO_2NH_2$). IR spectrum: ν_{max} 3420 and 3320 (NH_2), 1600 (aromatic C=C and/or C=N), 1329 and 1160 (SO_2).

From benzaldehyde. The compound (compound II) was prepared as previously reported[20].

RESULTS AND DISCUSSION

The conditions for the respective reactions of MAOs A and B are optimal[16]. If the enzyme reactions are simultaneously carried out using a mixture of *p*-sulfamoylbenzylamine and benzylamine as substrate solution, the amounts of *p*-sulfamoylbenzaldehyde and benzaldehyde formed enzymatically are reduced to one second, respectively, when compared with those attained by the separate enzyme reactions, for unknown reasons. Therefore, the enzyme reactions should be carried out separately.

Fig. 1 shows typical chromatograms obtained with Procedure A. DTAN derivatives of *p*-sulfamoylbenzaldehyde and benzaldehyde and the reagent blank (DTAN) can be completely separated within 5 min. The retention times for DTAN, *p*-sulfamoylbenzaldehyde and benzaldehyde are 2.2, 3.0 and 3.8 min, respectively. The eluates from peaks 2 and 3 in Fig. 1a have fluorescence excitation (maxima, 358 and 345 nm, respectively) and emission (maxima, 435 and 410 nm, respectively) spectra which are identical with those of compounds I and II dissolved in the mobile phase, respectively. The excitation and emission maxima for benzaldehyde were tentatively used for monitoring the fluorescence of the eluate.

Small peaks in the chromatogram of the blank (peaks 2 and 3 in Fig. 1b) have the same retention times as those for *p*-sulfamoylbenzaldehyde and benzaldehyde,

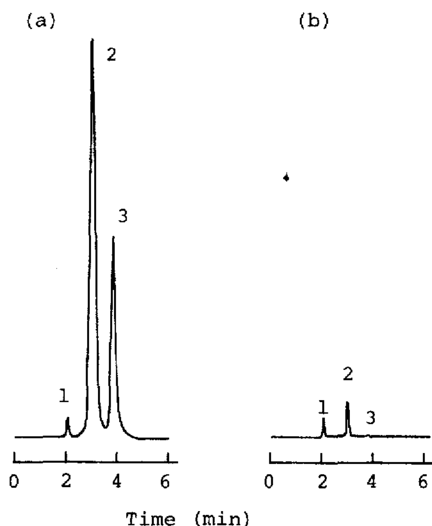

Fig. 1. Chromatograms obtained with (a) rat brain mitochondrial preparation (10 μg protein per 20 μl) and (b) the blank, according to Procedure A. Peaks: 1 = the reagent blank; 2 = *p*-sulfamoylbenzaldehyde; 3 = benzaldehyde. MAO A and MAO B activities were 0.68 nmol *p*-sulfamoylbenzaldehyde and 1.09 nmol benzaldehyde per min mg protein, respectively.

respectively, and the fluorescence excitation and emission spectra of the eluates from these peaks are identical with those from peaks 2 and 3 in Fig. 1a, respectively, in shapes and maxima. The peaks increase in height when the blank added with compounds I and II was subjected to HPLC. Their heights are also proportional to the concentrations of the substrates. When the enzyme preparation was treated as in the procedure without the substrates, no peaks were observed at the retention times for the aldehydes. These facts indicate that the peaks are due to *p*-sulfamoylbenzaldehyde and benzaldehyde occluded as contaminents in the substrates used. The aldehydes cannot be completely removed from the substrates by recrystallization.

p-Sulfamoylbenzaldehyde and benzaldehyde have been derivatized with DTAN in the presence of sulfuric acid[10,17]. The sulfuric acid, if used in the derivatization reaction under the present assay conditions, causes the formation of poorly soluble sodium sulfate in the mobile phase on neutralizing the reaction mixture with sodium acetate. Perchloric acid gives the same rate of derivatization as sulfuric acid, and sodium perchlorate is fairly soluble in the mobile phase. Thus perchloric acid was employed, in which DTAN dissolved.

Various columns for reversed-phase HPLC were examined for the separation of the neutralized final mixture. μBondapak C_{18}, LiChrosorb RP-18 (Merck Japan, Tokyo, Japan) and LiChrosorb RP-8 (Merck) did not give a sufficient separation of the peak for *p*-sulfamoylbenzaldehyde from that for the reagent blank. With a μBondapak Phenyl column (Waters), the peak for benzaldehyde broadened. A μBondapak CN column gave the most satisfactory separation of the peaks.

The separation of the final reaction mixture can be made by using a mixture of Na–Na phosphate buffer and methanol as mobile phase. The pH values (5.0–8.0) and the concentrations (10–50 m*M*) of the phosphate buffer have no effect on the retention times and heights of all the peaks; 33 m*M* Na–Na phosphate buffer of pH 7.0 was employed in the procedure. The phosphate buffer can be replaced by water though the peak height of the reagent blank results in five times that obtained with the phosphate buffer. Na–K phosphate buffer (33 m*M*, pH 7.0), if used in place of Na–Na phosphate buffer, causes the formation of poorly soluble potassium perchlorate on mixing with the final reaction mixture. The concentration of methanol in the mobile phase affects the retention times of the peaks (Fig. 2). With a higher concentration of methanol, the peaks of DTAN derivatives shift to shorter retention times; 60% was selected for adequate separation of DTAN derivatives.

The standard curves for *p*-sulfamoylbenzaldehyde and benzaldehyde were linear up to the concentrations examined (50 nmol/ml each). The recoveries of 0.5 nmol of *p*-sulfamoylbenzaldehyde and benzaldehyde added to the enzyme reaction mixture of the blank in Procedure A were 100 ± 1.4 and $100 \pm 2.0\%$ (mean $\pm$ standard deviation, $n = 10$ in each case), respectively.

The limits of detection for *p*-sulfamoylbenzaldehyde and benzaldehyde formed enzymatically were 30 and 10 pmol/assay tube (MAO A and MAO B activities of 100 and 30 pmol/min mg protein), respectively. The limit was defined as the concentration giving a peak height twice that of the blank.

The precision was established with respect to repeatability. The coefficients of variation of Procedure A were 2.3 and 2.8% for mean activities of 0.68 nmol *p*-sulfamoylbenzaldehyde and 1.09 nmol benzaldehyde per min mg protein, respectively ($n = 10$ in each case).

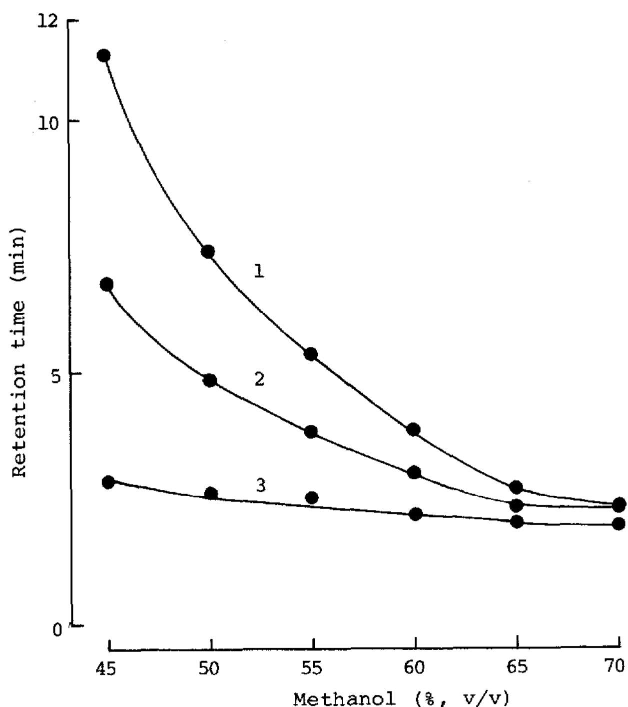

Fig. 2. Effect of methanol concentration in the mobile phase on the separation of the peaks in the chromatogram. Curves: 1 = benzaldehyde; 2 = p-sulfamoylbenzaldehyde; 3 = the reagent blank.

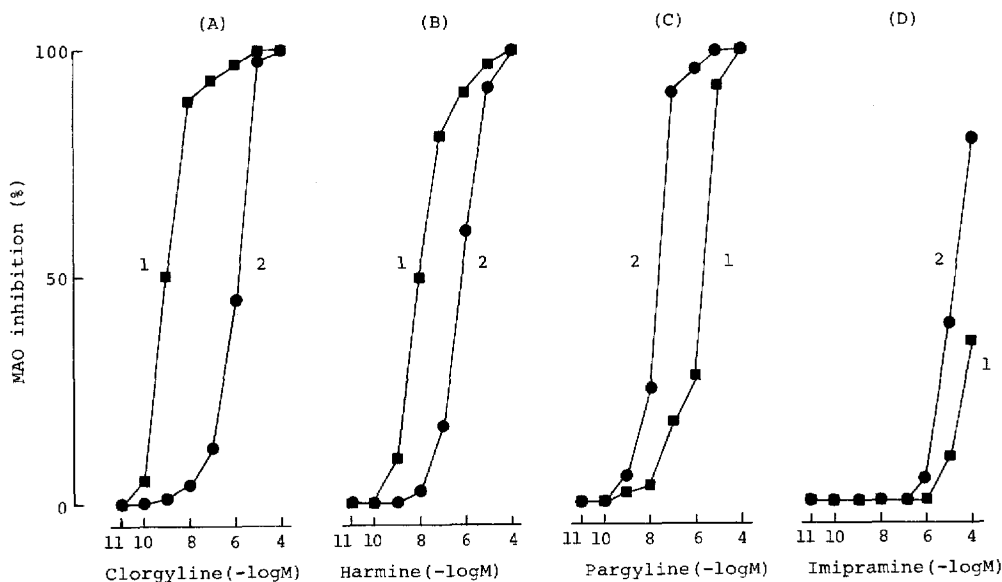

Fig. 3. Effect of the concentrations of (a) clorgyline, (b) harmine, (c) pargyline and (d) imipramine on the inhibition of MAO A and MAO B. Portions (20 μl) of rat brain mitochondrial preparation (10 μg protein per 20 μl) were treated according to Procedure B. Curves: 1 = MAO A; 2 = MAO B.

MAO A and MAO B activities in the rat (Donryu, male, 4 weeks old) brain mitochondrial preparations were 0.67 ± 0.15 nmol *p*-sulfamoylbenzaldehyde and 1.10 ± 0.29 nmol benzaldehyde per min per mg protein (mean ± standard deviation, $n = 5$ in each case), respectively.

Clorgyline and harmine are known to be inhibitors of MAO A and pargyline and imipramine of MAO B[2–4]. The effect of these inhibitors on MAO A and MAO B activities was tested according to Procedure B (Fig. 3). Clorgyline and harmine inhibit MAO A activity and pargyline and imipramine inhibit MAO B activity at relatively low concentrations. The inhibition patterns with clorgyline and pargyline are identical with those obtained by the fluorimetric method[16].

This study provides the first HPLC method for the assay of both MAOs A and B. The method is simple and sensitive, and should be useful for the assay of the MAOs in other biological samples and for screening of MAO-inhibitory potency of newly developed drugs.

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