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A New Fluorometric Assay for Human Serum Monoamine Oxidase[1)]

KIYOSHI ZAITSU, HIDEAKI NAGAI, KAZUYA KOHASHI,[2)] and YOSUKE OHKURA[2a)]

Faculty of Pharmaceutical Sciences, Kyushu University[2)]

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A new fluorometric method for the sensitive assay of human serum monoamine oxidase is presented. This is based on the determination of benzaldehyde produced from the substrate benzylamine under the optimal enzyme reaction conditions by means of the previously established fluorometric method for selective determination of aromatic aldehydes with 1,2-diaminonaphthalene sulfate. This method gives reliable results and is readily performed with a minimum amount of serum.

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

An increased human serum monoamine oxidase (S–MAO) activity was found in patients with chronic congestive heart failure,[3)] diabetes mellitus,[4)] hyperthyroidism[4)] and chronic liver disease.[5)] In particular, elevated S–MAO levels were found in the cases with advanced fibrosis and liver cirrhosis.[6)] Also, it was observed that S–MAO activity increased with the progression of hepatic fibrosis.[6)]

For the assay of S–MAO, the McEwen–Cohen method[7)] and its modified method,[8)] which measured spectrophotometrically benzaldehyde formed from the substrate benzylamine, have been widely utilized as conventional methods in the clinical evaluation. These methods require a large amount of serum, a long incubation time, deproteinization and extraction of benzaldehyde formed. Fluorometric, radio-chemical and colorimetric methods so far presented for S–MAO assay, which use kinuramine,[9)] ^{14}C-labelled amines (butylamine[10)] tyramine[11)] and benzylamine[11)]) and *p*-benzylaminoazo-β-naphthol[12)] as the substrates, respectively, also require a large amount of serum and deproteinization or extraction of the enzyme reaction product after deproteinization.

This paper describes a sensitive and simple method for the assay of S–MAO based on the enzymatic conversion of benzylamine under the optimal conditions to benzaldehyde, which is then determined by the previously established fluorometric method for the selective determination of aromatic aldehydes with 1,2-diaminonaphthalene sulfate (1,2-DNS).[13)]

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Experimental[14]

Reagents[15]——Phosphate Buffer (0.2M): Prepare by mixing 0.2M Na_2HPO_4 and 0.2M KH_2PO_4 in the usual way to be pH 7.2 at 37°.

Benzylamine·HCl Solution (0.01M): Dissolve 71.8 mg of benzylamine·HCl[16] in 50 ml of the phosphate buffer, store in a refrigerator and use within 2 days.

1,2-DNS Solution (45 μg/ml): Dissolve 9.0 mg of pure 1,2-DNS[13] in 3.0 ml of concentrated H_2SO_4 (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. The H_2SO_4 concentration of the solution is approximately 0.25M. This solution is usable for a week on kept frozen at −18° and for a day in a refrigerator (5°). A re-frozen solution should not be used.

NaOH Solution (100 g/l)

Benzaldehyde Standard Solutions: Prepare 2.5, 5.0, 7.5 and 10.0 nmol/0.4 ml benzaldehyde solutions by dissolving benzaldehyde[17] in the phosphate buffer. Use within a day.

Fluorescence Spectra and Intensities——Measured at a constant temperature of 25° with a Hitachi MPF-2A spectrofluorometer equipped with a 150 W xenon arc-lamp, a Hitachi QPD_{33} recorder and a quartz cell of 1×1 cm optical path lengths. In this instrument, the slit-widths in the exciter and the analyser in terms of wavelengths were fixed 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 0.05M 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.

Procedure——To 0.40 ml of the phosphate buffer placed in a glass-stoppered test-tube, add 0.10 ml of serum and preincubate at 37° for 5 min. Add 0.50 ml of benzylamine·HCl solution and incubate at 37° for 60 min. At the end of the incubation, add 2.0 ml of 1,2-DNS solution and heat in a boiling water-bath for 20 min. Cool in ice-water for about 5 min, and add 2.0 ml of NaOH solution. Prepare a blank in the same way, but add the substrate solution after adding 1,2-DNS solution. Keep standing the resulting mixtures at room temperature for about 10 min after adding NaOH solution, measure the fluorescence intensities of the test and the blank at 390 nm with the excitation at 356 nm within 5 hr after adding NaOH solution and calculate the net intensity. Read the nmol of benzaldehyde on the calibration curve described below.

Calibration Curve and Enzyme Units——To 0.10 ml of pooled serum[18] placed in a glass-stoppered test-tube, add successively 0.40 ml of each 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 heat in a boiling water-bath for 20 min. Cool in ice-water for about 5 min and add 2.0 ml of NaOH solution. Prepare a calibration curve in the usual way. A linear relationship was observed between the measured fluorescence intensity and the concentration of benzaldehyde.

Units of S-MAO activity were defined as the nmol of benzaldehyde formed at 37° for 1 hr per 1 ml of serum as in the modified McEwen-Cohen method.[8] One tenth nmol of benzaldehyde formed in the present procedure corresponded to 1 unit.

Results and Discussion

The clinical evaluation of S–MAO activity assayed by means of the McEwen–Cohen method[7] using benzylamine as the substrate has been compiled, and therefore benzylamine was employed as its stable hydrochloride in the present procedure as in the modified McEwen–Cohen method.[8]

The fluorescence spectra of the final reaction mixture in the procedure had the excitation maximum at 356 nm and the emission maximum at 390 nm, and were identical to those observed in the determination of benzaldehyde.[13]

The enzyme reaction conditions were investigated so as to be optimal. The pH optimum for the enzyme reaction was investigated using phosphate buffer solutions. The optimum varied in the range of pH 7.0—7.2, depending on serum used. The reaction rate

14) pH was measured by a Hitachi-Horiba M-7 pH meter equipped with a Horiba 6028-10T electrode. H_2O distilled after deionization was used throughout the work.
15) All reagents used were Reagent Grade unless otherwise noted.
16) A freshly distilled benzylamine was converted to its hydrochloride in the usual way, which was recrystallized at least twice from EtOH.
17) Benzaldehyde freshly distilled *in vacuo* in a stream of N_2 was used.
18) A mixture of usually available normal or pathological sera was used.

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increased with increasing ionic strength in 0.01—0.40 M phosphate buffers, as shown in Fig. 1. A 1.0 M phosphate caused turbidity in the final reaction mixture, which interfered with the measurement of fluorescence. A 0.2 M phosphate buffer of pH 7.2 was adopted in the present method as in the McEwen–Cohen and its modified methods.

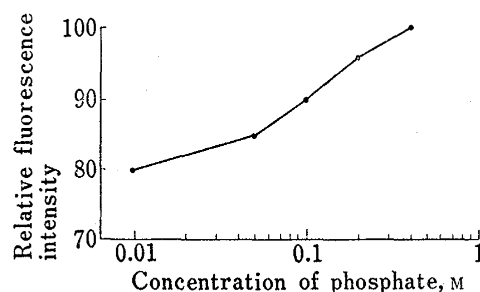

Fig. 1. Effect of the Phosphate Concentration in the Buffer of pH 7.2 on the Enzyme Activity

Each plot was the mean value of triplicate determinations on serum with S-MAO activity of 20.3 units which was a mixture of 50 sera with the activities of 7.3—48.2 units. A 2 hr incubation was employed.

The effect of benzylamine hydrochloride concentration on the reaction rate was studied over the range of 0.10—25 mM in the incubation mixture. The maximum rate was obtained in a range of 2.0—5.0 mM and the inhibition evidently occurred at concentrations more than 10 mM. Michaelis constant (*Km*) for the substrate was obtained as 0.24 mM. Thus, the prescribed concentration, 5.0 mM was employed as a saturated concentration. The substrate was calculated to be consumed only by about 0.2% even when a serum with a very high S-MAO activity of 100 units was treated under the conditions of the procedure, indicating that the consumption might not affect the measured S-MAO activity. Under the prescribed conditions described above, the enzyme activity was found to be linear with the incubation period for at least 4 hours at 37° and with the amount of benzaldehyde formed up to about 10 nmol, as shown in Fig. 2. The linearity was deviated, when the aldehyde was formed in amounts more than that concentration, by the decreased fluorescence intensities due to inner filter effects[19] (Fig. 2, e and d). These observations suggested that the

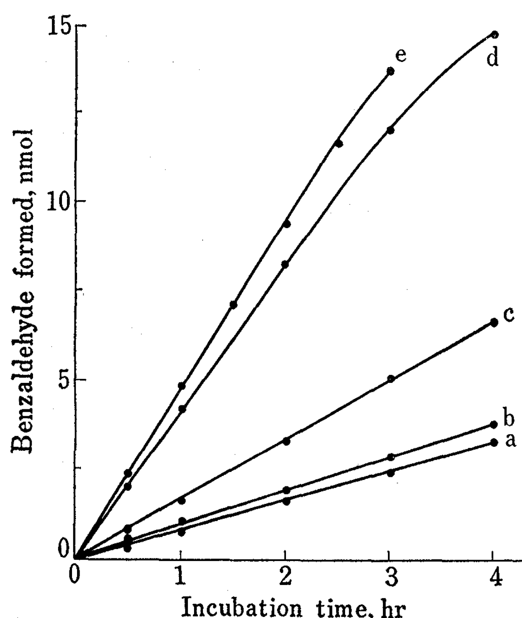

Fig. 2. Relationship between the Incubation Time and the Amount of Benzaldehyde Formed

a, 8.2; b, 9.5; c, 15.5; d, 44.2; e, 49.8 units serum. Each plot was the mean value of duplicate determinations.

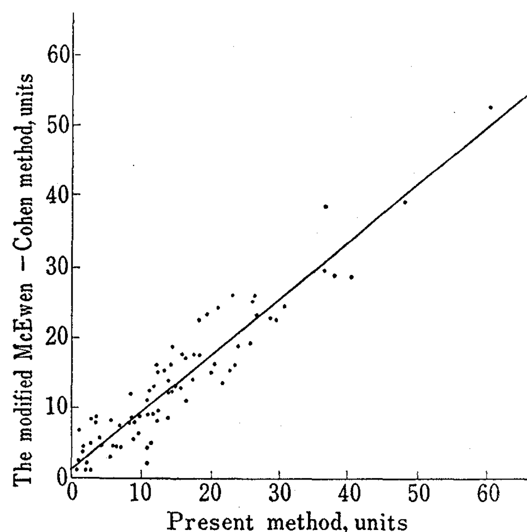

Fig. 3. Correlation between the Values obtained by the Present Method and the Modified McEwen–Cohen Method

19) This was confirmed by the fact that a linear correlation was observed between the measured fluorescence intensities and the incubation times when the final reaction mixtures in Fig. 2, e and d were diluted 2 times with the sodium hydroxide solution used in the procedure.

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present procedure permitted the assay of highly elevated S–MAO activity up to 100 units for the prescribed incubation time of 1 hour. And also, the amount of benzaldehyde formed was proportional to serum sample size over the range of 0.02—0.12 ml until the amount reached at least 10 nmol.

Interfering substances were examined in regard to their effects on the enzyme activity on sera with the activities of 25—26 units. Bilirubin decreased the activity only by 1.1 units, even when added to serum at the concentration of 25 mg/dl. Acetaldehyde and L-cysteine reduced the activity by 0.9 units at the concentrations of 1.0 and 12.5 mg/dl in serum, respectively. L-Methionine, D-glucuronolactone and salicylic acid caused decrease in the activity by 0.8 units, respectively, if present at the concentration of 25 mg/dl in serum, respectively. These substances seemed to be non-interfering substances even when occurred in serum at unusually high concentrations. Other substances tested did not interfere with the assay if present in serum at the concentrations described. They were urea (100 mg/dl), acetoacetic acid and acetone (10 mg/dl, respectively), lactic acid, pyruvic acid, α-ketoglutaric acid, ascorbic acid, citric acid, acetylsalicylic acid, creatinine, creatine, glycine, 15 different L-α-amino acids (25 mg/dl, respectively) and L-cystine (12.5 mg/dl).

Effect of serum protein on S–MAO activity was examined by adding S–MAO-free human serum albumin to a serum with 35.0 S–MAO units (total protein, 7.1 g/dl) at a concentration of 3.0 g/dl. No effect was found on the measured S–MAO activity. When the enzyme reaction mixture added with 5.0 or 10.0 nmol of benzaldehyde was incubated for 1 hour in the absence of benzylamine, the fluorescence intensity was not changed by the incubation, suggesting that enzymes, which decreased benzaldehyde formed by S–MAO, did not affect the measured S–MAO activity if present in serum.

The parallel tests with the modified McEwen–Cohen method[8] and the present method were performed with 77 different sera. The results are shown in Fig. 3. The correlation coefficient for both methods was obtained as 0.945. The units obtained by the present method (x) could be converted to the units in the modified McEwen–Cohen method (y) by a linear regression equation, $y=0.80x+1.6$.

The precision of the present method was studied by performing 12 determinations at the same on 3 sera with the mean activities of 2.4, 27.4 and 40.2 units. The standard deviations were 0.18, 0.49 and 1.12, respectively (the coefficient of variation, 7.6, 1.8 and 2.8%, respectively).

The present method required only 0.2 ml of serum for the test and the blank and 1 hour incubation, while the McEwen–Cohen method and its modified method required 6-fold amount of serum and 3-fold incubation time of the present method. The entire procedure required only about 2 hours. The method might be suited for clinical use.

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