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A Sensitive Fluorimetric Assay for Human Serum Monoamine Oxidase Activity

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A sensitive fluorimetric method for the assay of human serum monoamine oxidase is described, in which benzaldehyde, formed enzymatically from the substrate benzylamine, is quantitated by means of the fluorimetric method for selective determination of aromatic aldehydes with 2,2′-dithiobis(1-aminonaphthalene). The limit of detection for the benzaldehyde formed enzymatically is 100 pmol. This method is readily performed with good precision and a minimum amount of serum. Serum monoamine oxidase activity in patients with various thyroid diseases was assayed by this method.

Keywords——fluorimetry; human serum monoamine oxidase; benzylamine hydrochloride as substrate; benzaldehyde determination; 2,2′-dithiobis(1-aminonaphthalene); thyroid diseases

Human serum monoamine oxidase [S-MAO; monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4] activity is increased in patients with hyperthyroidism,[1–3] chronic congestive heart failure,[2] diabetes mellitus[1] and chronic liver disease.[4]

S-MAO has usually been assayed by the McEwen–Cohen method[5] or a modification of it,[6] in which benzaldehyde formed from the substrate benzylamine is measured spectrophotometrically. These methods have been widely utilized as conventional methods in clinical evaluation. However, these methods require a large amount of serum (0.6 ml), a long incubation time (3 h) and extraction of benzaldehyde formed. Therefore, several highly sensitive and rapid methods for the assay of S-MAO which use benzylamine as the substrate have been developed. The radiochemical method,[7] which uses ^{14}C-labelled benzylamine as the substrate, also requires a large amount of serum (0.2 ml) and extraction of the enzyme reaction product after deproteinization. The fluorimetric method,[8] which is based on the conversion of benzaldehyde, formed enzymatically from the substrate benzylamine, to a fluorescent product, is more sensitive.

We have developed a sensitive and simple fluorimetric method for the assay of S-MAO in human serum. The method is based on the enzymatic conversion of benzylamine under the optimum conditions to benzaldehyde, which is then determined by the previously reported method[9,10] for selective and sensitive determination of aromatic aldehydes with 2,2′-dithiobis-(1-aminonaphthalene) (DTAN). S-MAO activity in patients with various thyroid diseases was assayed by the established method.

Experimental

Reagents and Solutions——All chemicals were of reagent grade, unless otherwise noted. Double-distilled water was used. DTAN was prepared as described previously.[9]

Benzylamine·HCl Solution (0.01 M): Benzylamine·HCl (71.8 mg) was dissolved in 50 ml of 0.2 M phosphate buffer (pH 7.2). The solution was stored in a refrigerator and used within 2 d.

DTAN Solution: DTAN (40 mg) was dissolved in 6.0 ml of methanol, then 1.0 ml of methanolic 80 mg/ml tri-*n*-butylphosphine and 25.0 ml of 30% (v/v) H_2SO_4 were added, and the mixture was diluted with water to 100 ml. The solution was stable for at least 1 week when stored at 4°C.

2-Mercaptoethanol Solution: 2-Mercaptoethanol (5.0 ml) was added to 33.5 ml of 30% (v/v) H_2SO_4 and diluted with water to 50 ml. The solution was usable for 3 d.

Apparatus——Fluorescence spectra and intensities were measured with a Hitachi MPF-3 spectrofluorimeter using quartz cells of 1×1 cm optical pathlength. The slit widths in terms of wavelength were set at 10 nm in both the exciter and analyzer. The fluorescence spectra are uncorrected. pH was measured with a Hitachi-Horiba M-7 meter at 25°C. Centrifugation at low temperature was carried out in a Hitachi 05 PR-22 refrigerated centrifuge.

Procedure——To 0.40 ml of 0.2 M phosphate buffer (pH 7.2), 50 μl of serum was added. The mixture was preincubated at 37°C for 5 min, then incubated again at 37°C for 30 min after addition of 0.50 ml of benzylamine·HCl solution. The reaction was stopped by the addition of 0.20 ml of 3 M trichloroacetic acid. The mixture was centrifuged at *ca.* $1000 \times g$ for 10 min. To the supernatant (1.0 ml), 2.0 ml of DTAN solution and 0.5 ml each of 0.05% (w/v) Na_2SO_3 solution and 15% (w/v) $Na_2HPO_3 \cdot 5H_2O$ solution (both freshly prepared; accelerators of the fluorescence reaction) were successively added. The mixture was allowed to stand at 37°C for 30 min to develop the fluorescence. 2-Mercaptoethanol solution (1.0 ml) was added to stop the fluorescence reaction. The fluorescence intensity of the final mixture was measured at 475 nm with excitation at 360 nm. For the blank, the same procedure was carried out except that the benzylamine·HCl solution was added after the addition of trichloroacetic acid.

To prepare a standard curve, 0.40 ml of the phosphate buffer in the procedure was replaced with 0.40 ml of a solution of benzaldehyde (0.05—10.0 nmol) dissolved in the phosphate buffer. One unit of S-MAO activity was defined as that producing one nmol of benzaldehyde at 37°C in 1 h per 1 ml of serum as in the modified McEwen–Cohen method. Benzaldehyde (25 pmol) formed in the present procedure corresponded to 1 unit.

Results and Discussion

S-MAO in human serum is generally most active at pH 7.0—7.2 in 0.2 M phosphate buffer. Phosphate buffer gave a maximum and constant activity at concentrations of 0.2—0.4 M. Therefore, 0.2 M phosphate buffer of pH 7.2 was used in this procedure, as in the McEwen–Cohen method and its modifications.

A maximum and constant activity was obtained in the presence of 2.0—6.0 mM benzylamine hydrochloride with an observed K_m value of 0.27 mM. Thus, 5.3 mM benzylamine hydrochloride was used as a saturating concentration for the enzyme reaction.

The reaction rate was linear with time up to 4 h on incubation at 37°C and with the amount of benzaldehyde formed up to about 10.0 nmol, as shown in Fig. 1. The plot deviated from linearity when the aldehyde was formed in greater amounts as a result of decreased fluorescence intensities due to inner filter effects (Fig. 1, e); this was confirmed by the fact that a linear correlation between the measured fluorescence intensity and the incubation time was observed when the final mixture in Fig. 1, e was diluted 2-fold with the final solution for the blank. These observations suggested that the present procedure permitted the assay of highly elevated S-MAO activity (up to 400 units) in the prescribed incubation time of 30 min. The amount of benzaldehyde formed was proportional to the human serum sample size up to 200 μl, when sera with S-MAO activities of 15—60 units were used.

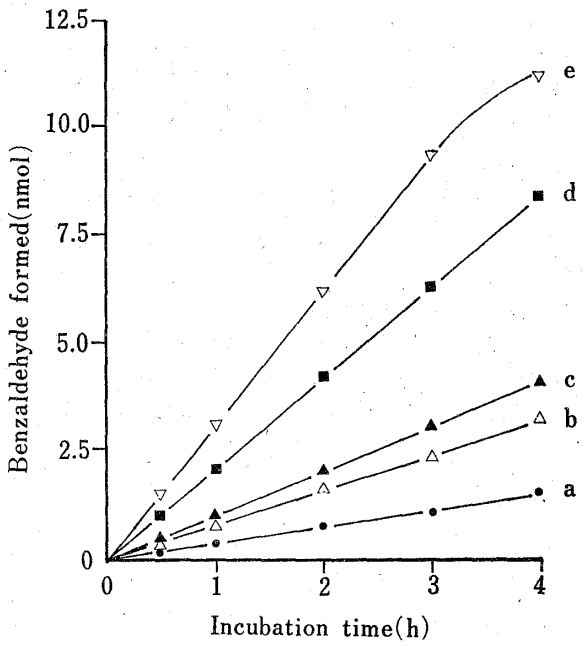

Fig. 1. Effect of Incubation Time on the Enzyme Activity at 37°C

a, 7.8; b, 16.2; c, 20.8; d, 42.4; e, 62.5 units serum. Each point is the mean value of duplicate determinations.

Various substances were examined for interfering effect on the enzyme activity in pooled serum with a mean activity of 27.8 units. Bilirubin, L-cystine, L-methionine, D-glucurono-

lactone and salicylic acid decreased the activity by only 0.4—0.5 units, even when added to serum at the concentration of 15 mg/dl. These substances seem to be essentially non-interfering even when present in serum at unusually high concentration. Other substances tested did not interfere with the assay if present in serum at the concentrations described; these were urea (100 mg/dl), acetoacetic acid, acetone, lactic acid, pyruvic acid, α-ketoglutaric acid, citric acid, ascorbic acid, acetylsalicylic acid, creatine, glycine and 15 different L-α-amino acids (each 15 mg/dl).

The fluorescence spectra for the final reaction mixture had the excitation maximum at 360 nm and the emission maximum at 475 nm, and were identical to those observed in the determination of benzaldehyde.[9]

The standard curve was linear up to 10.0 nmol of benzaldehyde and passed through the origin. The limit of detection for benzaldehyde formed enzymatically was 100 pmol, which gave a fluorescence intensity of twice the blank. This is a better result than that of the previously reported fluorimetric method.[8]

Comparison with the modified McEwen–Cohen method[6] using normal and pathological sera (*ca.* 10—*ca.* 60 units) gave a correlation coefficient of 0.963 ($n=32$) and the regression equation for the present method (x) against the modified McEwen–Cohen method[6] (y) was $y=0.87x+1.32$. The within-day precision was examined using sera with mean S-MAO activities of 22.6 and 48.0 units. The coefficients of variation were 1.3 and 2.2% ($n=20$ each), respectively.

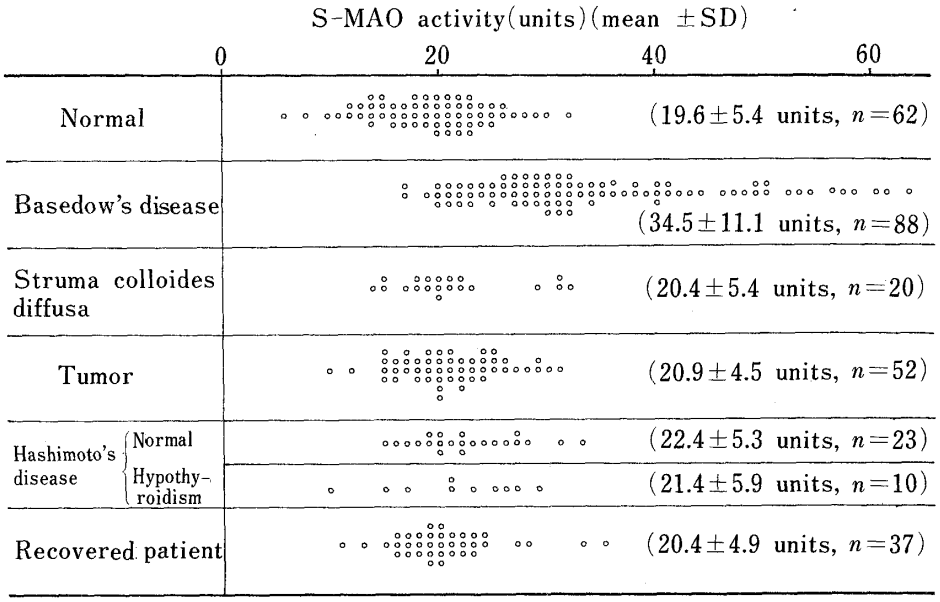

Fig. 2. S-MAO Activities in Normal Persons and in Patients with Thyroid Diseases

S-MAO activities in normal sera and in sera of patients with various thyroid diseases are shown in Fig. 2. The data are in agreement with previously published results[1-3] obtained by the colorimetric method using benzylamine as a substrate.

The present method required only 0.1 ml of serum for the test and blank, and 30 min incubation, while the McEwen–Cohen method[5] and its modification[6] require 12 times as much serum and a 6-fold longer incubation time than the present method. The method is very rapid and precise, and is the most sensitive of the S-MAO activity assay methods based on benzylamine as a substrate. The method should be useful for clinical and biological investigations.

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References and Notes

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