[Chem. Pharm. Bull. 25(4) 748—753 (1977)]

UDC 577.152.02 : 543.426.06

A Novel Fluorometric Assay Method of Dopamine-β-hydroxylase Activity in Human Serum

Yosuke Ohkura, Kenji Ohtsubo, Kazuya Kohashi, and Kiyoshi Zaitsu

Faculty of Pharmaceutical Sciences, Kyushu University[1]

(Received July 10, 1976)

A fluorometric method is presented for the assay of dopamine-β-hydroxylase activity in human serum. This is based on the enzymatic conversion of β-phenylethylamine under the optimum conditions to phenylethanolamine, which is then oxidized to benzaldehyde and determined fluorometrically by means of the previously established method for selective determination of aromatic aldehydes with 1,2-diaminonaphthalene. The method is readily performed and is suitable to assay a large number of samples at the same time with 10 μl of sample.

Keywords——dopamine-β-hydroxylase; human serum; fluorometry; β-phenylethylamine; phenylethanolamine; 1,2-diaminonaphthalene; benzaldehyde determination; small sample size

Several presently available methods have been developed for the assay of dopamine-β-hydroxylase[2] (DBH) in serum and other biological materials. Those methods may be divided into two classes, enzymatic radiochemical methods and spectrophotometric methods, depending on their principles based on. In the enzymatic radiochemical methods,[3–5] phenylethanolamine or octopamine formed from the substrate β-phenylethylamine or tyramine in the DBH reaction is converted to the corresponding N-^{14}C-methyl derivative by reaction with partially purified bovine adrenal phenylethanolamine-N-methyltransferase (PNMT) in the presence of S-adenosylmethionine methyl-^{14}C (^{14}C-SAM). And the N-^{14}C-methyl derivative is separated by solvent extraction and its radioactivity is determined.[3–5] Although the methods are highly sensitive, they are rather complicated and require expensive PNMT and ^{14}C-SAM, and the enzyme reaction conditions are not optimal. In the spectrophotometric methods, octopamine formed from tyramine under the optimal conditions of the enzyme reaction is oxidized to *p*-hydroxybenzaldehyde with periodate after separation by ion-exchange chromatography and the absorbance due to the aldehyde is measured by conventional[6] or dual-wavelength spectrophotometry.[7] The conventional photometric method is sensitive enough to assay the DBH activity in human serum and the dual-wavelength method is devised to permit the assay of DBH preparations with low activities such as serum and brain from various animals, but they require micro-photometric technics because the final volume in their procedures is 1—1.2 ml.

We have developed a fluorometric assay method of DBH activity in human serum based on the measurement of phenylethanolamine formed from β-phenylethylamine under the optimal enzyme reaction conditions. In the method, the product phenylethanolamine is separated by ion-exchange column chromatographic technics and oxidized with periodate to benzaldehyde, which is then determined fluorometrically by the previously established

1) Location: *Maidashi, Higashi-ku, Fukuoka.*
2) 3,4-Dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (hydroxylating), EC 1.14.2.1.
3) R. Weinshilboum and J. Axelrod, *Circulation Research*, **28**, 307 (1971).
4) M. Goldstein, L.S. Freedman, and M. Bonnay, *Experientia*, **27**, 632 (1971).
5) P.B. Molinoff, R. Weinshilboum, and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **178**, 425 (1971).
6) T. Nagatsu and S. Udenfriend, *Clin. Chem.*, **18**, 980 (1972).
7) T. Kato, H. Kuzuya, and T. Nagatsu, *Biochem. Med.*, **10**, 320 (1974).

method for selective determination of aromatic aldehydes with 1,2-diaminonaphthalene.[8] Catalase and fumaric acid are used in the procedure as in the previously reported assay procedures[3–7] to protect DBH from hydrogen peroxide formed by non-enzymatic oxidation of ascorbic acid[9] and to accelerate the enzyme reaction,[10] respectively. And also, both N-ethylmaleimide and cupric ion are added to the enzyme reaction mixture to inactivate the endogenous inhibitors of DBH[11,12] as in the dual-wavelength method.[7] The method is readily performed and is precise and sensitive to assay the activity in as little as 10 μl of human serum.

Experimental[13]

Reagents[14]——Acetate Buffer (1.0M, pH 5.6): Prepare by mixing 1.0M NaOAc solution and 1.0M AcOH solution in the volume ratio of about 8: 1 so as to give pH of 5.6 at 25°.

β-Phenylethylamine Solution (0.5M): Dissolve 7.884 g of pure β-phenylethylamine·HCl[15] in H_2O and dilute to 100 ml. The solution is stable for several months when stored in a refrigerator.

Catalase Solution (3500 units[16]/ml): Prepare by dissolving appropriate amount of a catalase preparation[17] in cold H_2O, store in a refrigerator and use within a day.

Sodium Fumarate Solution (0.25M): Dissolve 3.45 g of monosodium fumarate in H_2O to make up 100 ml. The solution is usable for at least 2 months when stored in a refrigerator.

N-Ethylmaleimide Solution (0.15M): Dissolve 1.877 g of N-ethylmaleimide in about 80 ml of H_2O by warming and then dilute with H_2O to 100 ml. The solution is usable for at least 2 weeks when stored in a refrigerator.

Cupric Sulfate Solution (5 μM): Prepare first a 5 mM stock solution by dissolving 125 mg of $CuSO_4 \cdot 5H_2O$ in 100 ml of H_2O. Prepare the working solution by dissolving the stock solution 1000 times with H_2O before use.

Substrate·cofactor Solution: Mix the acetate buffer and the solutions of β-phenylethylamine, catalase, fumarate, N-ethylmaleimide and cupric sulfate in the volume ratio of 2: 1: 1: 1: 1: 1 prior to use.

Ascorbic Acid Solution (0.05M): Freshly prepare by dissolving 176 mg of ascorbic acid in 20 ml of H_2O degassed by boiling for about 5 min followed by cooling.

Trichloroacetic Acid Solution (3M): Dissolve 49 g of trichloroacetic acid in 100 ml of H_2O.

Ammonium Hydroxide Solution (4M): Dilute 270 ml of 28% NH_3 with H_2O to 1000 ml.

Sodium Periodate Solution (0.1%): Prepare an aqueous solution in the usual manner and use within a day.

Sodium Sulfite Solution (0.5%): Prepare an aqueous solution in the usual manner and use within a day.

Sulfuric Acid Solution (42.2 ml/1000 ml): Prepare using concentrated H_2SO_4 (above 95%).

1,2-Diaminonaphthalene Sulfate (1,2-DNS) Solution (90 μg/ml): Dissolve 18 mg of pure 1,2-DNS[8] in 3.0 ml of concentrated H_2SO_4 (above 95%) under mixing on a Vortex-Type mixer, cool in an ice-water bath and dilute with H_2O to 200 ml. Use within a day.

Sodium hydroxide solution (10%).

Phenylethanolamine Standard Solutions: Prepare first 1 μmol/0.1 ml solution by dissolving 137 mg purified phenylethanolamine[18] in H_2O to make up 100 ml. Diluting this solution with H_2O, prepare 1.0,

8) Y. Ohkura and K. Zaitsu, *Talanta*, **21**, 547 (1974).
9) E.Y. Levin and S. Kaufman, *J. Biol. Chem.*, **236**, 2043 (1961).
10) E.Y. Levin, R. Levenberg, and S. Kaufman, *J. Biol. Chem.*, **235**, 2080 (1960).
11) T. Nagatsu, H. Kuzuya, and H. Hidaka, *Biochem. Biophys. Acta*, **139**, 319 (1967).
12) D.S. Duch, O.H. Viveros, and N. Kirshner, *Biochem. Pharmacol.*, **17**, 255 (1967).
13) pH was measured by a Hitachi-Horiba M-7 pH meter. Centrifugation at 5° was made on a Marusan Refrigerated Centrifuge 45CFS. H_2O distilled after deionization was used throughout the work.
14) All reagents used were Reagent Grade unless otherwise stated. All reagent solutions should be prepared, stored and used avoiding contamination of atmospheric aldehydes, which especially come from tobacco-smoking in laboratory, otherwise a high blank value of the fluorescence intensity results.
15) β-Phenylethylamine was converted to its hydrochloride in the usual manner, which was recrystallized at least 5 times from C_6H_6–MeOH (about 95: 5) to colorless plates of mp 216° (uncorr).
16) The units are expressed as the μmol of H_2O_2 decomposed per min at 25° and pH 7.0 when assayed by the method: R.N. Feinstein, J.B. Howard, L.B. Ballonoff, and J.E. Seaholm, *Anal. Biochem.*, **8**, 277 (1964).
17) Crystalline catalase from bovine liver, 2400 units per mg (Sigma) was used.
18) Phenylethanolamine (Sigma) was recrystallized from C_6H_6 repeatedly to colorless needles of mp 56° (uncorr.).

2.0, 5.0, 10.0, 15.0 and 20.0 nmol/0.1 ml solutions. These solutions remain usable for several weeks when stored in a refrigerator.

Quinine Sulfate Solution (1.0 μg/ml): Prepare in the usual manner by dissolving quinine sulfate in 0.1N H_2SO_4.

Human Serum Samples——Obtain blood by venipuncture, place on ice for 30—60 min, then centrifuge at about $10000 \times g$ at 5° for 10 min and remove serum. The DBH activity in serum is fairly stable and remains unchanged for at least 2 weeks when stored frozen at −20°.

Ion-Exchange Column——Pack 0.2 ml of Dowex 50 W×4 (H^+, 200—400 mesh) into a glass column (0.5×13 cm) in the usual manner and wash the column successively with 2 ml of 4N NH_4OH, two more times with each 2 ml of H_2O and with 2 ml of 6N HCl. The used column can be regenerated by washing in the same way and so remains usable for more than 10 times.

Fluorescence Spectra and Intensities——Measured at a constant temperature of 25° by a Hitachi MPF-2A Spectrofluorometer equipped with a Hitachi QPD_{33} Recorder in a quartz cell of 1×1 cm light path. In this fluorometer, the slit-widths in the exciter and the analyser in terms of wavelength were fixed at 10 nm, respectively. The sensitivity of the fluorometer was checked each time fluorescence intensities were determined by measuring the fluorescence intensity of quinine sulfate solution 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 0.19 ml of H_2O placed in a 10 ml centrifuge-tube, add 10 μl of serum and 0.70 ml of the substrate·cofactor solution and pre-incubate in a water bath at 37° for 5 min. At zero time, add 0.10 ml of ascorbic acid solution, incubate at 37° for exactly 30 min in air with continual shaking and add 0.2 ml of trichloroacetic acid solution to stop the enzyme reaction. Centrifuge at 2500 rpm for 10 min, transfer 1.0 ml of the supernatant solution to the ion-exchange column, wash the column two more times with each 2 ml of H_2O and then elute the adsorbed amines with 1.0 ml of NH_4OH solution into a 20 ml glass-stoppered test-tube. Add 0.1 ml of $NaIO_4$ solution, allow to stand at room temperature for about 5 min[19] and add 0.1 ml of Na_2SO_3 solution. Add 2.0 ml of 1,2-DNS solution to the resulting mixture after neutralizing with 2.8 ml of H_2SO_4 solution and heat in a boiling water bath for 20 min. Cool in an ice-water bath and add 4.0 ml of NaOH solution (the final volume, 10 ml). Prepare a blank in the same way, but add ascorbic acid solution after the incubation. Measure the fluorescence intensities of the test and the blank at 390 nm with the excitation at 356 nm and calculate the net intensity. Read the nmol of phenylethanolamine on the calibration curve described below.

Calibration Curve and Enzyme Units——Treat 0.10 ml of each phenylethanolamine standard solution (and of H_2O for blank), 0.09 ml of H_2O, 10 μl of a pooled serum,[20] 0.70 ml of the substrate·cofactor solution and 0.10 ml of ascorbic acid solution in the same way as described in the procedure without the incubation, and prepare a calibration curve in the usual manner. There observed a linear relationship between the measured fluorescence intensity and the concentration of phenylethanolamine. The fluorescence intensity obtained for each concentration of phenylethanolamine agreed within ±3%.

The units of DBH activity are defined as the μmol of phenylethanolamine formed by the enzyme in 1000 ml of serum per min at 37°, similarly to the manner of representation of the international units. Therefore, when the enzyme reaction is carried out with 10 μl of serum for the incubation time of 30 min as prescribed in the procedure, the units are calculated by the nmol of phenylethanolamine formed × 3.33.

Results and Discussion

The fluorescence spectra of the final reaction mixture in the procedure had the excitation and emission maxima at 356 and 390 nm, respectively, and were identical to those observed in the determination of benzaldehyde.[8]

The elution of phenylethanolamine from the ion-exchange column with 1 ml of 4N ammonium hydroxide was complete, but an incomplete elution was obtained with 1 ml of ammonium hydroxide lower than 2.5N.

The concentrations of sodium periodate in the oxidation of phenylethanolamine and of sodium sulfite for the reduction of the excessive periodate had a correlated effect on the fluorescence development, which was examined by adding 2—20 nmol of the amine to the eluate obtained in the column chromatography of the blank incubation mixture. A maximum and constant fluorescence intensity was observed at concentrations of the periodate ranging from 0.02 to 0.13% when the sulfite was used at concentrations (%) of 4—6 times each con-

19) The standing time is not critical.

20) A mixture of usually available normal or pathological sera can be used.

centration of the periodate used, and concentrations of the sulfite higher or lower than those ratios caused decreased fluorescence intensities. The fluorescence intensity of the blank was minimum and constant when the periodate was used at concentrations of 0.08—0.2%. Thus, 0.1% sodium periodate and 0.5% sodium sulfite were employed as the optima, respectively. Sodium metabisulfite interfered with the fluorescence development though this reagent was used to remove the periodate in the previously reported procedures.[6,7]

Blank has been prepared with heated enzyme preparation in the methods so far proposed.[3-7] The fluorescence intensity of a blank prepared by incubating with intact serum in the absence of ascorbic acid as described in the procedure was identical to that of a blank obtained by treating serum heated at 95° for 5 min as in the test in the procedure. Therefore, intact serum was used for easy preparation of the blank.

The enzyme reaction was investigated so as to be performed under the opimum conditions to avoid the factors unfavorably affected the amount of phenylethanolamine formed by DBH.

As shown in Fig. 1, a 200 mM sodium acetate buffer in the incubation mixture gave the maximum enzyme activity at pH 5.6, though the enzyme in human serum was reported to be most active at pH 5.0 in the same buffer when tyramine was used as the substrate.[6] A tris-(hydroxymethyl)-aminomethane buffer was used in the enzymatic radiochemical method at pH 6,[5] at which pH the enzyme activity decreased by about 30% than at pH 5.6. A 200 mM phosphate buffer gave the same enzyme activity as that given by the 200 mM acetate buffer at pH 5.6 (Fig. 1). Thus, the acetate buffer of pH 5.6 was employed for its easy preparation.

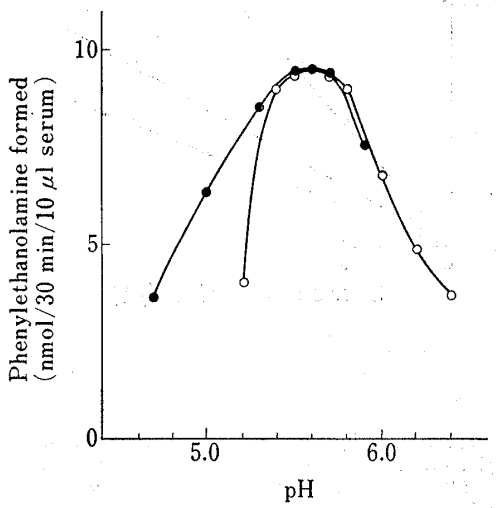

Fig. 1. Effect of pH on the Enzyme Activity

●—●, 0.2 M sodium acetate buffer; ○—○, 0.2 M potassium phosphate buffer in the incubation mixture.

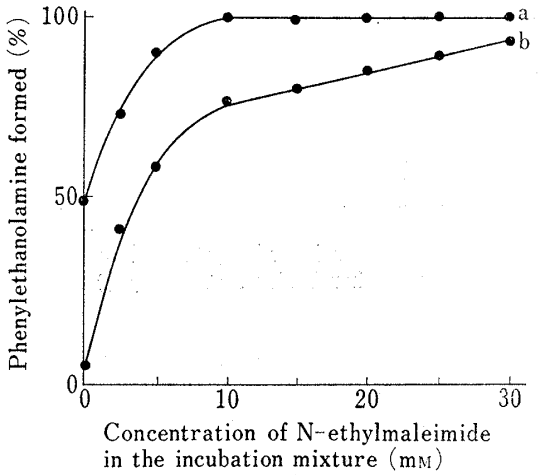

Fig. 2. Effect of N-Ethylmaleimide Concentration on the Enzyme Activity

a, with 0.5 μM cupric sulfate,; b, without cupric sulfate. Each plot was the mean value obtained by duplicate determinations on each of 10 sera with DBH activities of 21.5—64.8 units, where the amount of phenylethanolamine formed in the presence of 15 mM N-ethylmaleimide and 0.5 μM cupric sulfate was taken as standard 100. The coefficient of variation for the each value was below 3.1%.

The measured DBH activity did not change over a concentration range of β-phenylethylamine of 35—60 mM in the incubation mixture, and the prescribed concentration, 50 mM was selected as a saturated concentration for DBH. The Michaelis constant (K_m) for β-phenylethylamine was obtained as 7.6 mM, and therefore the prescribed concentration of the amine was about 6.6 times the K_m value. Ascorbic acid gave almost constant value of the enzyme activity in a concentration range of 3—8 mM in the incubation mixture, and slightly lower value in a range of 10—30 mM. Thus, 5 mM was selected as the optimum.

Catalase intensified the DBH activity by approximately 1.5 times when added to the incubation mixture over a wide range of 180—600 units, and 350 units was used in the procedure. Sodium fumarate stimulated the enzyme activity by approximately 1.25 times in a concentration range of 18—40 mM in the incubation mixture. Therefore, 25 mM was selected as a sufficient concentration.

The concentrations of N-ethylmaleimide and cupric sulfate had a correlated effect of the inactivation of the endogenous inhibitors. In the presence of 0.5 μM cupric sulfate, the maximum enzyme activity was obtained at N-ethylmaleimide concentrations ranging from 10 to at least 30 mM, as shown in Fig. 2, a. While, in the absence of cupric sulfate, the enzyme activity did not reach the maximum even at the imide concentration of 30 mM, as shown in Fig. 2, b, indicating that the inhibitors in 10 μl of human serum could not be completely inactivated by N-ethylmaleimide alone. Cupric sulfate gave the maximum enzyme activity at concentrations of 0.3—0.7 μM in the presence of 15 mM N-ethylmaleimide as shown in Fig. 3, and higher concentrations of cupric sulfate were inhibitory. In the absence of N-ethylmaleimide, however, the optimum concentration of cupric sulfate to give the maximum activity varied with each serum sample as previously reported.[6] Therefore, both N-ethylmaleimide and cupric sulfate were employed in the procedure at the concentrations of 15 mM and 0.5 μM, respectively.

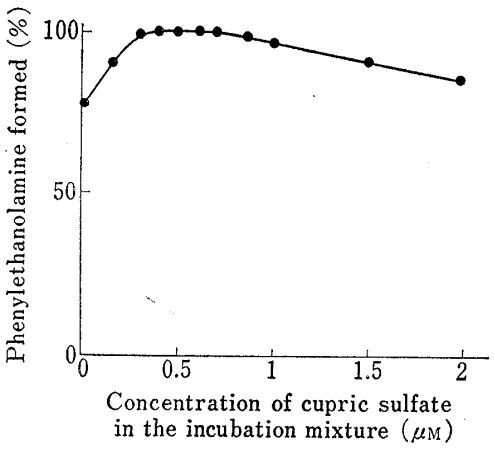

Fig. 3. Effect of Cupric Sulfate Concetration on the Enzyme Activity in the Presence of 15 mM N-Ethylmaleimide

Each plot was the mean value obtained by triplicate determinations on each of 11 sera with DBH activities of 18.5—64.8 units, where the amount of phenylethanolamine formed in the presence of 0.5 μM cupric sulfate was taken as standard 100. The coefficient of variation for the each value was below 3.5%.

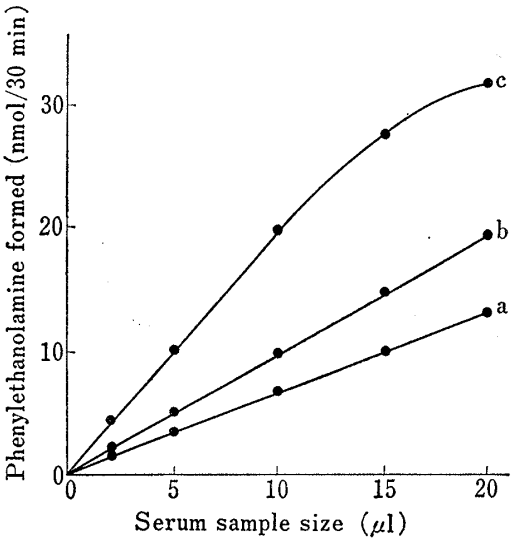

Fig. 4. Relationship between Serum Sample Size and the Amount of Phenylethanolamine Formed

a, 21.9 units, b, 31.9 units; c, 64.8 units serum. Each plot was the mean value of triplicate determinations and the coefficient of variation for the each value was below 3.5%.

Pargyline, a monoamine oxidase (MAO) inhibitor, has been used at a concentration of approximately 1 mM in the DBH assay procedures so far proposed[3-7] to suppress extraneous oxidation of the substrate amine to the corresponding phenylacetaldehyde with generation of hydrogen peroxide by the action of MAO present in DBH preparations. Pargyline produced neither stimulation nor suppression of measured DBH activity at concentrations of 1—4 mM in the incubation mixture even when explored on sera with high MAO activity,[21] suggesting that MAO activity in 10 μl of human serum was negligible under the conditions of the present procedure. Therefore, pargyline was not employed in the procedure.

21) Sera with 30—46 units of MAO were used. The MAO activity was assayed by the method: C.M. McEven and J.D. Cohen, *J. Lab. Clin. Med.*, **62**, 766 (1963).

Under the prescribed conditions described above, the enzyme activity was linear with the incubation time for at least 60 min at 37° and with the amount of phenylethanolamine formed up to 20 nmol. When the amine was produced in amounts more than 20 nmol, the measured fluorescence intensities deviated from the linearity due to inner filter effects.[22] This fact indicated that the present procedure might permit the assay of DBH activity up to 67 units for the incubation time of 30 min, which was prescribed in the procedure to assay usually encountered DBH activities in human sera.[23] The range might be extended to higher activities by employing shorter incubation time.

As shown in Fig. 4, the amount of phenylethanolamine formed was proportional to the serum sample size up to 20 μl until the amount reached 20 nmol, suggesting that the sample size might be varied within a range of 2—20 μl if necessary.

Recovery of phenylethanolamine was tested by adding 2.0, 8.0, or 16.0 nmol of the amine to the incubated enzyme reaction mixture in the procedure. Complete recoveries, 98±2%, were obtained.

The precision of the method was studied with respect to repeatability and reproducibility. The repeatability was examined by performing 30 assays at the same time on 2 sera with the mean activities of 33.3 and 62.8 units. The standard deviations were 0.5 and 2.2 units, respectively (the coefficient of variation, 1.5 and 3.5%, respectively). The reproducibility was obtained by repeating the assay 14 times on different days on a serum stored frozen at −20° with the mean activity of 41.5 units. The standard deviation was 1.8 (the coefficient of variation, 4.3%).

The proposed method is the first fluorometric DBH assay method which measures the amount of phenylethanolamine formed from β-phenylethylamine under the optimal enzyme reaction conditions. β-Phenylethylamine hydrochloride used as the substrate is very stable in air and in aqueous solution while tyramine or its hydrochloride is unstable in aqueous solution even when stored in a refrigerator, though the K_m value for β-phenylethylamine is about 2.5 times larger than that for tyramine (3 mM).[6] The limit of sensitivity of the method is 0.8 nmol of phenylethanolamine when defined as a concentration which gives a fluorescence intensity more than 1.5 times that of the blank. The entire procedure requires 3—4 hours and more than 20 samples can be assayed at the same time. Studies on the assay of DBH activity in sera or tissues of experimental animals are in progress.

Acknowledgement The authors express their gratitude to the staff of Central Clinical Laboratory of Kyushu University Hospital for the supply of serum sample.

22) This was confirmed by the fact that a linear relationship was observed between the incubation time and the measured fluorescence intensity until the amount of phenylethanolamine formed up to at least 30 nmol when the final reaction mixture was diluted 3 times with the sodium hydroxide solution used in the procedure.

23) It was reported that DBH activity of normal human serum was 42.6±27.0 units (mean±standard deviation) when tyramine was used as the substrate.[6]