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ASSAY FOR CATECHOL-O-METHYLTRANSFERASE IN ERYTHROCYTES USING A NEW FLUOROGENIC SUBSTRATE, 2-(3,4-DIHYDROXYPHENYL)NAPHTHO[1,2-*d*]THIAZOLE

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SUMMARY

A highly sensitive method for the assay of catechol-O-methyltransferase in erythrocytes is described, which employs high-performance liquid chromatography with fluorescence detection. A newly synthesized catechol compound, 2-(3,4-dihydroxyphenyl)naptho[1,2-*d*]-thiazole is used as a highly fluorogenic substrate for catechol-O-methyltransferase; the *m*- and *p*-methylated products formed enzymatically from the substrate under the optimum conditions, after extraction with *n*-hexane—chloroform, are separated by normal-phase chromatography on LiChrosorb Si 100. The limits of detection for *m*- and *p*-methylated products are 3 pmol per assay tube (60 fmol per injection volume of 20 µl) in each case. The ratio of *m*- and *p*-methylated products was 0.54. This method requires as little as 50 µl of human erythrocytes.

INTRODUCTION

Catechol-O-methyltransferase (COMT, EC 2.1.1.6) occurs in many tissues of mammals and catalyzes the transfer of the methyl group of S-adenosyl-L-methionine (SAM) to one of the phenolic groups of a catechol compound [1]. COMT plays an important role in the metabolism of catecholamines, and the

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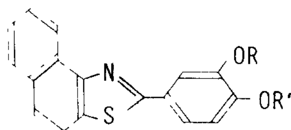
relationship between its activity in human erythrocytes and some diseases has therefore been investigated [2].

Many assay methods for COMT in biological materials have been reported: spectrophotometry [3–6], fluorimetry [1, 7–10], high-performance liquid chromatography (HPLC) with electrochemical [11] and ultraviolet [12] detection, and radioisotopic methods [13–18]. COMT activity in human erythrocytes is very low and can be assayed only by the radioisotopic methods. The methods, however, require radioisotopic substrate or SAM and do not permit the determination of the ratios of two O-methylated products. In the previous paper, we reported an assay method for COMT which allowed the determination of the product ratios [20]. This is based on the quantification of vanillin and isovanillin (*m*- and *p*-O-methylated products, respectively) formed enzymatically from the substrate 3,4-dihydroxybenzaldehyde by derivatizing these products with 2,2′-dithiobis(1-aminonaphthalene) (DTAN, a selective fluorogenic reagent for aromatic aldehydes [19]) to highly fluorescent 2-(3-methoxy-4-hydroxyphenyl)naphtho[1,2-*d*]thiazole (*m*-MNT; Fig. 1) and 2-(3-hydroxy-4-methoxyphenyl)naphtho[1,2-*d*]thiazole (*p*-MNT; Fig. 1), respectively, extracting the compounds into *n*-hexane–chloroform and separating the extract by normal-phase HPLC on LiChrosorb Si 100 [20]. The sensitivity of the method permits the assay of COMT in various tissues, but is not sufficient for assay of the enzyme in erythrocytes.



R=R'=H : DNT

R=CH_3, R'=H : m-MNT

R=H, R'=CH_3 : p-MNT

Fig. 1. Chemical structures of DNT, *m*-MNT and *p*-MNT.

We have synthesized 2-(3,4-dihydroxyphenyl)naphtho-[1,2-*d*]thiazole (DNT; Fig. 1) as a highly fluorogenic substrate for COMT by the reaction of 3,4-dihydroxybenzaldehyde with DTAN, and thus developed an HPLC method for the assay of the enzyme in erythrocytes. *m*- and *p*-MNT (*m*- and *p*-O-methylated products) formed enzymatically from DNT under the optimum conditions are determined as in the previous method. A COMT preparation from human erythrocytes was employed to establish the assay procedure.

EXPERIMENTAL

Material and reagents

All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. 3,4-Dihydroxybenzaldehyde was recrystallized from toluene. DTAN was purchased from Dojindo Laboratory (Kumamoto, Japan). *m*-MNT and *p*-MNT were prepared as previously described [20].

Preparation of DNT

To 100 mg of 3,4-dihydroxybenzaldehyde dissolved in 100 ml of water was added DTAN solution (200 mg of DTAN, 30 ml of methanol, 0.4 ml of tri-*n*-butylphosphine and 50 ml of 6 *M* sulphuric acid). The mixture was stirred at room temperature for 3 h. Yellow resinous substance was extracted with ethyl acetate, and the solvent was removed in vacuo. The residue was crystallized from *n*-hexane—ethyl acetate (about 9:1, v/v) to give yellowish needles (m.p. 116—117°C) which seemed to have crystal solvent. The crystals were further purified by sublimation in vacuo at 100—110°C, and yielded colourless needles (m.p. 212°C; yield 20 mg). Analytical data were as follows. Calc. for $C_{17}H_{11}O_2NS$: 69.61% C, 3.78% H, 4.78% N. Found: 69.50% C, 3.73% H, 4.79% N. Mass spectrum: *m/z* 293 (M^+, base peak), 247 (M^+ —H_2O—CO), 246 (M^+ —H_2O—CHO). ^{1}H-NMR spectrum δ (ppm): 6.48—7.92 (9 H, complex multiplet, aromatic protons), 8.93 (2H, broad doublet, hydroxyl protons, disappeared on adding a small amount of heavy water). IR spectrum: ν_{max} 3440 (OH), 1595 (aromatic C=C and/or C=N).

COMT preparation from human erythrocytes

Venous blood (5 ml) obtained from a normal volunteer was collected into a 10-ml glass tube containing 100 μl of 0.1 *M* EDTA · 2Na. The blood was centrifuged for 10 min at 1500 *g* and the plasma removed. The erythrocytes were washed twice with isotonic phosphate buffer (pH 7.4) and stored at —75°C until used. A 1-ml volume of the erythrocytes was lysed with 9.0 ml of ice-cold water and centrifuged at 1500 *g* for 10 min. The supernatant was used as the COMT preparation.

Apparatus

The column for HPLC was prepared by packing LiChrosorb Si 100 (particle size 5 μm; Japan Merck, Tokyo, Japan) into a stainless-steel tube (150 × 4 mm I.D.) by the slurry technique using tetrabromoethane—tetrachloroethylene (3:2, v/v) as solvent.

A Hitachi 635A liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injection valve (20-μl loop) and a Hitachi 650-10S spectrofluorimeter fitted with a 20-μl flow-cell operating at an emission wavelength of 390 nm and an excitation wavelength of 348 nm; spectral bandwidths of 10 nm and 5 nm, respectively, were used. Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical pathlength, 10 × 10 mm); spectral bandwidths of 5 nm were used in both the excitation and emission monochromators. The mass spectrum was measured with a JEOL JMS-01-SG mass spectrometer. The ^{1}H-NMR spectrum was obtained with JEOL JNM-PS-110 spectrometer at 100 MHz using an approximately 15% (w/v) solution in dimethylsulfoxide-d_6 containing tetramethylsilane as internal standard. The infrared (IR) spectrum was taken with a Nihonbunko DS 701G spectrometer using a potassium bromide pellet. The melting points are uncorrected.

Procedure for COMT assay (procedure A)

To 0.5 ml of the enzyme preparation (corresponding to 50 μl of

erythrocytes) were added 200 μl each of 0.1 *M* phosphate buffer (pH 6.5) and 0.5 m*M* SAM and 50 μl of 40 m*M* magnesium chloride. The mixure was preincubated at 37°C for 5—15 min, then incubated again at 37°C for 30 min after the addition of 50 μl of 1 m*M* DNT in isopropanol—water (1:1, v/v). The reaction was stopped by adding 1.0 ml of 0.2 *M* hydrochloric acid. To the mixture, 1.0 ml of *n*-hexane—chloroform (4:1, v/v) was added and the resulting *m*- and *p*-MNT were extracted with mechanical shaking (approximately 300 rpm) for 10 min. A 20-μl volume of the upper organic layer was injected into the chromatograph. The organic layer could be used for more than a week when stored at room temperature (approximately 20°C).

The mobile phase comprised 25 m*M* acetic acid in *n*-hexane—chloroform (4:1, v/v), and the flow-rate was 2.0 ml/min. Column temperature was ambient (20—25°C).

For the blank, the same procedure was carried out except that DNT solution was added after the addition of 0.2 *M* hydrochloric acid. To prepare the calibration curves, 50 μl of DNT solution were replaced with 50 μl of DNT solution containing *m*- and *p*-MNT (each 0.05—1.0 nmol per 50 μl) and the same procedure as for the blank was carried out.

The peak heights in the chromatogram were used for the quantification of *m*- and *p*-MNT.

Procedure for COMT assay in the presence of pyrogallol (procedure B)

To 0.5 ml of the enzyme preparation were added 200 μl of 0.1 *M* phosphate buffer (pH 6.5), 100 μl of $5 \cdot 10^{-5}$ *M* pyrogallol and 50 μl each of DNT solution and 40 m*M* magnesium chloride. The mixture was preincubated at 37°C for 5 min, then incubated again at 37°C for 30 min after the addition of 100 μl of 1 m*M* SAM, and then treated in the same way as in procedure A.

RESULTS AND DISCUSSION

The optimum pH values for the enzymatic *m*- and *p*-methylation reactions were 6.5 and 6.5—8.0, respectively (Fig. 2A). The pH affects the product ratio of *m*- and *p*-MNT and the ratio increases with increasing pH, particularly in the pH range 6.0—8.5 (Fig. 2B); pH 6.5 was used in the standard procedure.

Maximum and constant velocities of the *m*- and *p*-methylation reactions were attained in the presence of 20—70 μ*M* DNT in the incubation mixture; DNT at a concentration greater than 70 μ*M* caused inhibition of the enzyme (Fig. 3). The Michaelis constant (K_M) values for DNT were 2.1 ± 0.5 μ*M* and 3.2 ± 0.6 μ*M* (mean ± S.D.) when measured on the basis of the formation of *m*- and *p*-MNT, respectively (n = 7 in each case). This indicates that the affinity of COMT is fairly high for DNT. Therefore, 50 μ*M* DNT was used as saturating concentration in the procedure. DNT is hardly soluble in water, so it had to be dissolved in isopropanol. The alcohol did not affect COMT activity at a concentration range of 0.5—5% (v/v) in the incubation mixture; aqueous isopropanol (1:1, v/v) was used for the preparation of DNT solution (the concentration in the incubation mixture was 2.5%).

Almost maximum and constant velocities of the *m*- and *p*-methylation reactions were achieved over SAM concentrations of 50—200 μ*M*, with the K_M

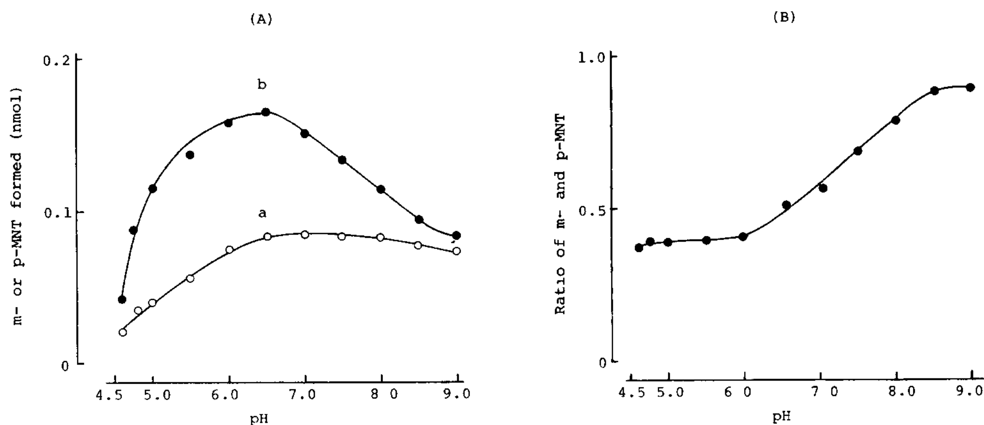


Fig. 2. Effect of pH on (A) the amounts of *m*- and *p*-MNT formed and (B) the ratio of *m*- and *p*-MNT. Curves: a = *m*-MNT; b = *p*-MNT. Buffers: pH 4.6—5.0, 0.1 *M* acetate buffer; pH 5.0—8.0, 0.1 *M* phosphate buffer; pH 8.0—9.0, 0.1 *M* Tris—hydrochloric acid buffer.

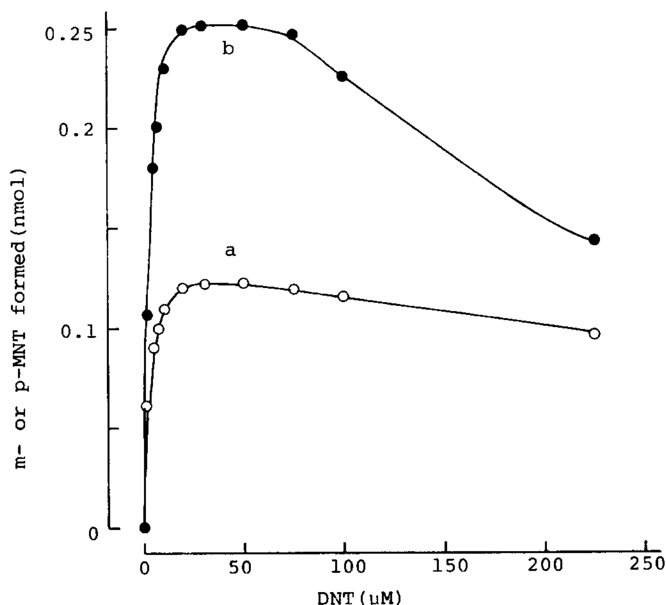


Fig. 3. Effect of DNT concentration on the amounts of (a) *m*-MNT and (b) *p*-MNT formed.

value at 3.0 ± 0.2 μM (mean ± S.D.) in both the methylation reactions; 100 μM SAM was used for convenience. Magnesium chloride gave maximum activity at 2 mM in the incubation mixture. Calcium ion has been described to inactivate human erythrocyte [17] and rat liver [21] COMT. In our study, however, the removal of the ion from the enzyme preparation by adding Chelex-100 (particulate chelating agent of divalent cations; Bio-Rad, Richmond, CA, U.S.A.) according to the method of Raymond and Weinshilboum [17] had no effect on the enzyme activity.

The amounts of *m*- and *p*-MNT formed enzymatically were proportional to the volume of erythrocytes in the enzyme preparation up to at least 200 μl

when the enzyme reaction mixture was incubated at 37°C for 30 min (Fig. 4), and to the incubation time up to at least 120 min; 50 μl of erythrocytes and 30-min incubation were used in the procedure.

Hydrochloric acid (0.2 *M*) was used for the termination of the enzyme reaction, otherwise *m*- and *p*-MNT could not be reproducibly extracted with *n*-hexane—chloroform mixture. When the incubation mixture was deproteinized with perchloric acid or trichloroacetic acid, the recovery of *m*- and *p*-MNT in the organic layer was almost nil, probably due to the coprecipitation of the products with the denatured protein.

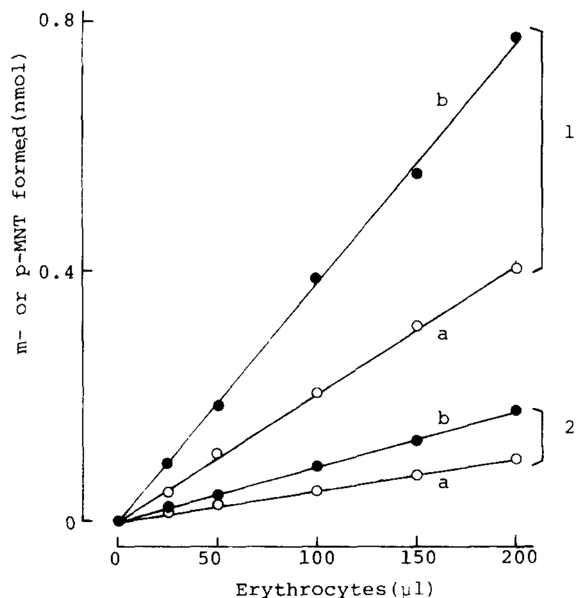


Fig. 4. Effect of the volume of erythrocytes obtained from two healthy men (1 and 2) on the amounts of (a) *m*-MNT and (b) *p*-MNT formed

Pyrogallol was reported to be a potent inhibitor of COMT [22—24], and thus its inhibitory action was examined according to procedure B. Pyrogallol inhibited COMT in a competitive mode against DNT (Fig. 5) and the observed inhibitory constant value calculated according to the method of Dixon [25] was 7 m*M* in both the *m*-and *p*-O-methylation reactions. This value is almost identical to that obtained with rat liver COMT using a natural substrate, norepinephrine [24].

The HPLC conditions in this method were the same as those in the previous method [20]. Fig. 6 shows a typical chromatogram obtained using procedure A. No peak was observed in the chromatogram of the blank. The eluates from peaks 1 and 2 in Fig. 6 had fluorescence excitation (maximum, 349 and 347 nm, respectively) and emission (maximum, 393 and 389 nm, respectively) spectra that were identical with those of authentic *m*- and *p*-MNT, respectively, dissolved in the mobile phase.

DNT dissolved in the mobile phase had only a weak fluorescence with an excitation maximum at 350 nm and an emission maximum at 404 nm; the

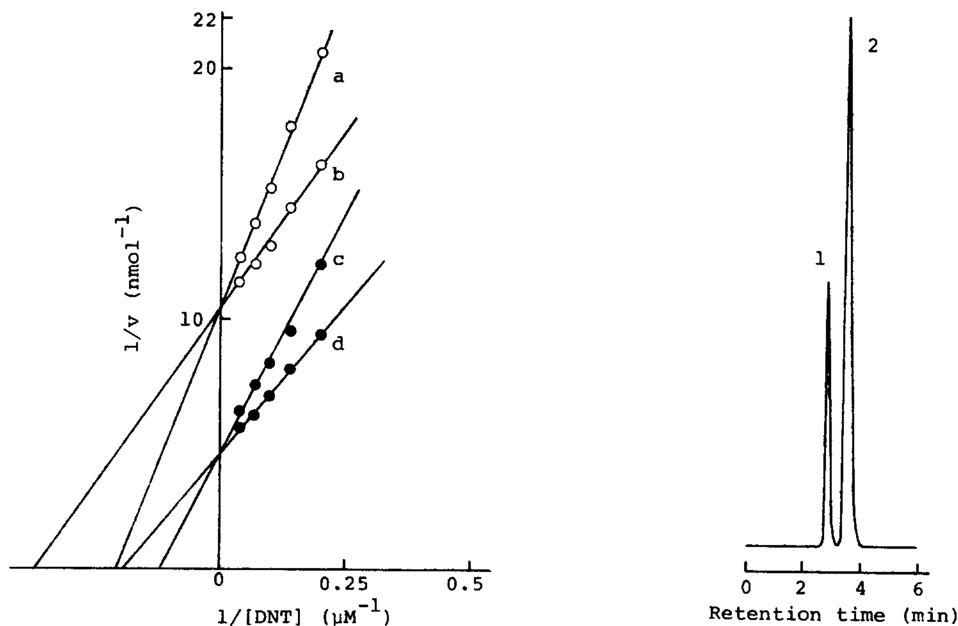


Fig. 5. Inhibition of human erythrocyte COMT by pyrogallol, examined according to procedure B. The data were plotted by linear regression analysis. Lines: a and b, *m*-MNT; c and d, *p*-MNT. Pyrogallol in the incubation mixture: a and c, 5 m*M*; b and d, absent.

Fig. 6. Chromatogram obtained with COMT preparation (50 μl of human erythrocytes per 0.5 ml) according to procedure A. Peaks: 1 = *m*-MNT; 2 = *p*-MNT. COMT activity: 6.2 nmol *m*-MNT and 11.6 nmol *p*-MNT per ml erythrocytes per h.

intensity was less than 1% of that of *m*- or *p*-MNT. DNT was strongly retained on the silica gel column for HPLC and did not elute with the mobile phase. However, the column was deactivated when the sample solution was repeatedly applied on to it for a long time. This was probably caused by small amounts of water contained in the sample solutions. In this case, DNT eluted and interfered with the quantification of *m*- and *p*-MNT. Therefore, the column should be washed every 300—500 injections of the sample by passing approximately 100 ml of chloroform containing a small amount of acetic acid (0.15%, v/v) through it. The retention times for *m*- and *p*-MNT were reproducible even in the deactivated column.

The recoveries of *m*- and *p*-MNT added to the incubated mixture of the blank in amounts of 0.25 nmol were 67.3 ± 2.1% and 71.2 ± 1.6% (mean ± S.D., n = 12 in each case), respectively. The lower limits of detection for *m*- and *p*-MNT formed enzymatically were both 3 pmol per assay tube (60 fmol per injection volume of 20 μl). The precision was established with respect to repeatability. The coefficients of variation were 2.0% and 1.9% for mean activity of 2.7 nmol *m*-MNT and 5.2 nmol *p*-MNT per ml erythrocytes per h, respectively (n = 14).

COMT activities in normal human erythrocytes assayed by the present method were 5.6 ± 1.6 nmol *m*-MNT and 10.5 ± 3.2 nmol *p*-MNT per ml erythrocytes per h (mean ± S.D., n = 12); the product ratio of *m*- and *p*-MNT was 0.54 ± 0.02.

This study provides the first method for the assay of COMT in erythrocytes that does not use radioisotopic substrate or cofactor. This method may also permit the assay of COMT in preparations obtained from other tissues such as liver, kidney, brain and heart muscle; the results will be published elsewhere. This method is simple and precise, and should be useful for biological and biomedical investigations.

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