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ASSAY FOR CATECHOL-O-METHYLTRANSFERASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A sensitive method for the assay of catechol-O-methyltransferase in preparations from rat liver by determination of the *m*- and *p*-O-methylated products (vanillin and isovanillin, respectively) of the substrate 3,4-dihydroxybenzaldehyde is described. High-performance liquid chromatography with fluorescence detection is employed. Vanillin and isovanillin are converted into fluorescent compounds with 2,2′-dithiobis(1-aminonaphthalene). These compounds, after extraction with *n*-hexane–chloroform, are separated by normal-phase chromatography on LiChrosorb Si 100. The limits of detection for vanillin and isovanillin formed enzymatically are 5 and 7 pmol, respectively. The product ratio of vanillin to isovanillin obtained with the enzyme in rat liver was 2.1.

INTRODUCTION

Catechol-O-methyltransferase (COMT, EC 2.1.1.6) plays an important rôle in the metabolism of catechols, and catalyzes the transfer of the methyl group of S-adenosyl-L-methionine (SAM) to the phenolic group of catechol or substituted catechol[1]. The O-methylation *in vivo* occurs almost exclusively on the hydroxyl group at the *m*-position of substituted catechols[1,2], but *in vitro* it takes place at one of the *m*- or *p*-hydroxyl groups[2,3].

Many methods have been reported for the assay of COMT which employ radiochemical[4–9], fluorimetric[1,10–13] or spectrophotometric[14–17] techniques, which in most cases measure the sum of the *m*- and *p*-O-methylated products or only the *m*-O-methylated product. Thin-layer chromatography[18] and high-performance liquid chromatography (HPLC) with UV absorption[19] and electrochemical[20] detection have been used for the determination of the ratios of the *m*-O- to the *p*-O-methylated products.

We have developed an HPLC method with fluorescence detection for the assay of COMT, which measures the formation of *m*- and *p*-O-methylated products. 3,4-Dihydroxybenzaldehyde is used as the substrate for the enzyme reaction, and vanillin

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and isovanillin (*m*- and *p*-O-methylated products, respectively) are converted into fluorescent compounds by the reaction with 2,2′-dithiobis(1-aminonaphthalene) (DTAN; fluorogenic reagent for aromatic aldehydes[21]). The compounds are extracted into an organic solvent and separated by normal-phase HPLC. A COMT preparation from rat liver was employed to establish the assay procedure.

EXPERIMENTAL

Materials and reagents

All chemicals were of analytical reagent grade, unless otherwise noted. Deionized and distilled water was used. DTAN was prepared as described previously[21]. 3,4-Dihydroxybenzaldehyde was recrystallized from *n*-hexane, and vanillin and isovanillin from water.

DTAN solution. DTAN (40 mg) was dissolved in 6.0 ml methanol, then 0.1 ml tri-*n*-butylphosphine was added. After standing for several min the solution became clear, whereupon 25.0 ml of 30% (v/v) sulphuric acid 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) sulphuric acid and diluted with water to 50 ml. The solution could be kept for 3 days.

COMT preparation from rat liver. The supernatant from rat liver homogenate was used as a COMT preparation, and was prepared by a modification of the method of Parvez and Gripois[22]. Adult (9–10 week-old) Donryu male rats were stunned and exsanguinated, and the livers were immediately removed and chilled on ice. All further procedures were carried out at 0–5°C. The livers were trimmed free of fat and 36 g were homogenized with 144 ml of 1.15% (w/v) potassium chloride solution in a Potter Elvehjim homogenizer. The homogenate was centrifuged at 5500 *g* for 30 min. The protein concentration of the supernatant was measured by the method of Lowry *et al.*[23] using bovine serum albumin as a standard protein, and adjusted to $\leqslant 40$ μg per 10 μl with 1.15% (w/v) potassium chloride solution.

Column for HPLC

A stainless-steel tube (150 × 4.0 mm I.D.) was packed with LiChrosorb Si 100 (particle size, 5 μm; Japan Merck, Tokyo, Japan) by the slurry technique described[24]. The column can be used for more than 500 injections with only a small decrease in the theoretical plate number.

Apparatus

A Hitachi 635A liquid chromatograph was equipped with a Rheodine 7120 syringe-loading sample injector valve (20-μl loop) and a Hitachi fluorescence monitor (primary filter, EX-360; secondary filter, EM-440). Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (10 × 10 mm); spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. Mass spectra were measured with a JEOL-01-SG spectrometer and IR spectra with a Nihonbunko DS 701G spectrometer in potassium bromide pellets. The melting points are uncorrected.

Procedure

The substrate–cofactor solution comprised 10 μl of 0.5 *M* phosphate buffer (pH 6.6), 5 μl of 0.15 *M* magnesium chloride solution and 25 μl of 0.75 m*M* 3,4-dihydroxybenzaldehyde solution. To this were added 10 μl of the COMT preparation. The mixture was preincubated at 37°C for 5 min, then incubated again at 37°C for 30 min after addition of 25 μl of 0.9 m*M* SAM chloride solution. The reaction was stopped by heating the mixture at 100°C for 5 min. Then 1.0 ml water was added and the solution was mixed on a Vortex-type mixer. The mixture was centrifuged at 800 *g* for 10 min. To 0.5 ml of the supernatant (which contained vanillin and isovanillin as products), were added 1.0 ml of DTAN solution and 250 μl each of 0.05% (w/v) sodium sulphite solution and 15% (w/v) sodium phosphite pentahydrate solution (both freshly prepared; accelerators of the fluorescent derivatization reaction). The mixture was allowed to stand at 37°C for 60 min. 2-Mercaptoethanol solution (0.5 ml) was then added to stop the reaction and to stabilize the resulting fluorescence. To the reaction mixture, 0.5 ml *n*-hexane–chloroform (4:1, v/v) were added and the fluorescent products were extracted with shaking mechanically (260–360 rpm) for 10 min. A 20-μl volume of the upper organic layer was injected on to the chromatograph.

The organic layer could be used for more than 1 day when stored in the dark. 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 (72 kg/cm^2). The column temperature was ambient (20–25°C). For the blank, the same procedure was carried out except that SAM chloride solution was added just before heating the incubation mixture. For calibration curves, the 25 μl of SAM chloride solution were replaced with 25 μl of a standard solution of vanillin and isovanillin (each 100–750 pmol per 25 μl). The peak heights in the chromatogram were used for the quantitation of vanillin and isovanillin.

Preparation of fluorescent compounds from vanillin and isovanillin

From vanillin. To 152 mg vanillin dissolved in 100 ml water were added DTAN reagent solution [200 mg DTAN, 30 ml methanol, 0.4 ml tri-*n*-butylphosphine, 50 ml of 30% (v/v) sulphuric acid and 120 ml water] and 50 ml of an aqueous solution containing 25 mg sodium sulphite and 7.5 g sodium phosphite pentahydrate. The mixture was warmed at 37°C for 1 day with stirring. The resulting yellow precipitate was filtered off, dried and dissolved in a small amount of ethyl acetate. The solution was poured on to a glass column (50 × 1.2 cm I.D.) packed with 5 g of silica gel 60 (Japan Merck) and eluted with benzene–ethyl acetate (4:1, v/v). The largest fraction was concentrated almost to dryness *in vacuo* and the residue was recrystallized from ethyl acetate–benzene (*ca.* 1:4, v/v) as pale yellow prisms (I; m.p. 183–184°C; yield 92 mg). Since the fluorescent products in the reactions of DTAN with benzaldehyde and *p*-hydroxybenzaldehyde have been characterized as 2-arylnaphtho[1,2-*d*]thiazole[25], compound I should be 2-(3-methoxy-4-hydroxyphenyl)naphtho[1,2-*d*]thiazole. This was confirmed by the following analytical data. Calc. for $C_{18}H_{13}O_2NS$: 70.36% C, 4.23% H, 4.56% N. Found: 70.32% C, 4.20% H, 4.45% N. Mass spectrum: *m/e* 307 (M^+, base peak), 292 ($M^+ - CH_3$), 278 ($M^+ - CHO$), 264 ($M^+ - CH_3 - CO$). IR spectrum: $\nu_{max.}$ 3500 (OH), 1608 and 1590 (aromatic C=C and/or C=N), 1213 and 1030 cm^{-1} (C–O–C, phenyl methyl ether).

From isovanillin. Isovanillin (66 mg) was treated in the same way as for vanillin. Pale yellow needles (II; m.p. 156–157°C; yield 51 mg) were obtained. Compound II should be 2-(3-hydroxy-4-methoxyphenyl)naphtho[1,2-*d*]thiazole. This was established by the following data. Calc. for $C_{18}H_{13}O_2NS$: 70.36% C, 4.23% H, 4.56%N. Found: 69.46% C, 4.36% H, 4.38% N. Mass spectrum: *m/e* 307 (M^+, base peak), 292 ($M^+ - CH_3$), 278 ($M^+ - CHO$), 264 ($M^+ - CH_3 - CO$). IR spectrum: $\nu_{max.}$ 3440 (OH), 1608 and 1590 (aromatic C=C and/or C=N), 1213 cm^{-1} (C–O–C, phenyl methyl ether).

RESULTS AND DISCUSSION

The conditions for the enzyme reaction are optimal[13].

The fluorescent compounds can be extracted from the reaction mixture with a mixture of *n*-hexane and chloroform, identical in composition with the mobile phase except for acetic acid. The recoveries of the compounds from vanillin and isovanillin were ⩾99%. When the extract was allowed to stand in daylight for several hours, a peak caused by an unknown substance was observed in the chromatogram at the retention time of 1.8 min, and the peaks due to vanillin and isovanillin increased in height for unknown reasons. Therefore the extract should be kept in the dark when left to stand for long periods.

Fig. 1 shows typical chromatograms obtained according to the procedure. The

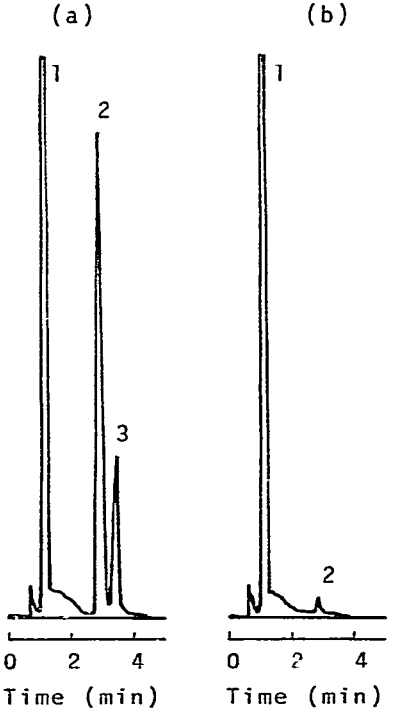

Fig. 1. Chromatograms obtained with COMT preparation (25 μg protein per 10 μl) (a) and the blank (b), according to the procedure described. Peaks: 1 = DTAN; 2 = vanillin; 3 = isovanillin. The activity of COMT was 28.3 nmol vanillin and 13.5 nmol isovanillin per 30 min per mg protein.

fluorescent compounds and the components of the reagent blank can be completely separated within 5 min. The retention times for DTAN, vanillin and isovanillin are 1.2, 2.8 and 3.5 min, respectively. The eluates from peaks 2 and 3 in Fig. 1a have fluorescence excitation (maximum, 349 and 347 nm, respectively) and emission (maximum, 393 and 389 nm, respectively) spectra which are identical with those of compounds I and II dissolved in the mobile phase, respectively.

A small peak in the chromatogram of the blank (peak 2 in Fig. 1b) has exactly the same retention time as that of vanillin and increased in height when the blank added with compound I was subjected to HPLC. This indicates that the peak is caused by occlusion of aldehyde as contaminant.

A small peak at the retention time of 0.7 min and a small broad (and complex) peak around 1.0–2.4 min (Fig. 1) are caused by the COMT preparation and the components other than DTAN in DTAN solution. 3,4-Dihydroxybenzaldehyde gives a small peak at the retention time of 1.6 min, but this is not discernible under our conditions.

Acetic acid in the mobile phase in the concentration range 18–85 m*M* provides a good separation of vanillin and isovanillin; 25 m*M* was used in the procedure recommended. In absence of acetic acid, the peaks could not be separated. The magnitude of the *n*-hexane–chloroform ratio in the mobile phase is important. With increasing ratio, the peaks shift to longer retention times (Fig. 2). The peak heights (fluorescence intensities) of vanillin and isovanillin are also affected by this ratio, maximum (and constant) peak heights being obtained in the range 3.8–5.4. A ratio of 4.0 was selected for the standard procedure as it resulted in rapid and satisfactory separation of the peaks.

The peak height of vanillin was 1.36 times greater than that of isovanillin at equimolar concentration. Linear relationships were obtained between the peak heights of vanillin and isovanillin and the amounts of these aldehydes added in the range 100–750 pmol to the enzyme reaction mixture in place of SAM chloride so-

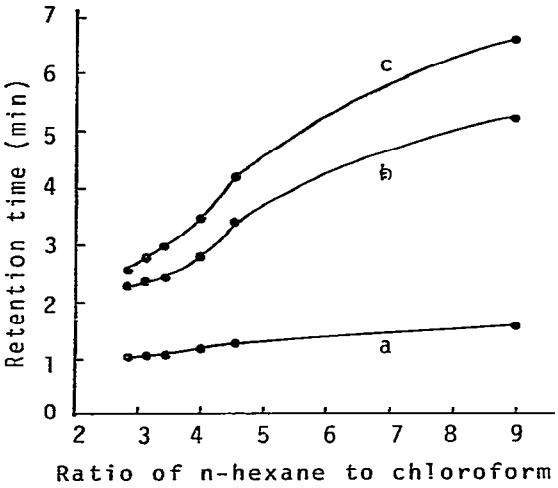

Fig. 2. Effect of the *n*-hexane–chloroform ratio in the mobile phase on the separation of the peaks in the chromatogram. Curves: a = DTAN; b = vanillin; c = isovanillin.

lution. The recoveries of vanillin and isovanillin added to the incubated mixture of the blank in amounts of 100, 300 and 750 pmol were 99 ± 2% (mean ± standard deviation, $n = 5$ in each case).

The product ratio of vanillin to isovanillin obtained with the COMT preparation by this method was 2.1. This value is in good agreement with that reported by Creveling *et al.*[18], whereas Katz and Jacobson[26] predicted a value of 1.6 from theoretical considerations. The precision was established with respect to repeatability. The coefficients of variation were 1.7% (for both vanillin and isovanillin) for a mean activity of 28.3 nmol vanillin and 13.5 nmol isovanillin per 30 min per mg protein ($n = 15$).

The limits of detection for vanillin and isovanillin formed enzymatically were 5 and 7 pmol, respectively. This sensitivity may also permit the assay of COMT in preparations obtained from other tissues, *e.g.*, kidney, spleen, small intestine, lung, brain and heart muscle, but is not sufficient for assay of the enzyme in preparations from erythrocytes which have usually very low activity.

This method is sensitive and precise, and should be useful for biological investigations.

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