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Note

Assay of catechol O-methyltransferase activity by high-performance liquid chromatography with electrochemical detection

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The enzymatic methylation of catecholamines and their metabolites by catechol O-methyltransferase (COMT) is one of the important degradation routes of these compounds in the human body [1]. Recently, it has been stated that COMT activity might be closely related to affective disorders such as some cases of depression, and that the *meta/para* ratio of O-methylated products might be of clinical significance [2].

Of the methods for the assay of COMT activity, fluorimetric, radiochemical or colorimetric techniques only measure the total amount of products formed. On the other hand, high-performance liquid chromatographic (HPLC) [2—5] and gas chromatographic [6] techniques have been developed as a reliable procedure for the assay of *m*-methoxy and *p*-methoxy products. HPLC with electrochemical detection has proven to be sensitive and specific for the determination of phenolic compounds, including those arising from O-methylation by COMT.

The present paper describes a procedure for the assay of COMT activity, which involves the use of norepinephrine as a substrate, periodate oxidation, deproteinization by solvent extraction, and the separation of O-methylated isomers by HPLC with electrochemical detection.

EXPERIMENTAL

Chemicals

L-Norepinephrine bitartrate and DL-normetanephrine hydrogen chloride were obtained from Wako Junyaku (Osaka, Japan). Vanillin, isovanillin, *p*-hydroxyacetanilide and S-adenosyl-L-methionine hydrogen sulfate (SAM) were purchased from Tokyo Kasei (Tokyo, Japan).

Substrate (norepinephrine) solution (340 μg/ml) and SAM solution (1.6 mg/ml) were each prepared in 50 m*M* phosphate buffer (pH 7.5). Magnesium chloride ($MgCl_2$) solution was 9.4 mg/ml in water; and the internal standard solution was *p*-hydroxyacetanilide solution (5 μg/ml) in ethyl acetate. All other chemicals were of analytical grade.

Preparation of COMT

Adult Wistar rats (150—200 g) of either sex were decapitated and their livers were homogenated in 4 volumes of 50 m*M* phosphate buffer (pH 7.5). The homogenate was centrifuged at 100,000 *g* for 30 min at 4°C. The supernatant solution was used as a crude source of COMT. Protein determinations were carried out by the method of Lowry et al. [7] with bovine serum albumin as standard.

High-performance liquid chromatography

Apparatus for the HPLC in this work was constructed from a Kyowa Seimitsu mini micro pump Type KSU, a Kyowa Seimitsu damper Type KD-300, a Yanaco voltammetric detector Type VMD 101, and a column system. The HPLC column (15 cm × 4 mm I.D., stainless steel), was prepared with LiChrosorb 5 RP-18 (E. Merck, Darmstadt, G.F.R.) using a balanced density method. The mobile phase consisted of methanol—50 m*M* phosphate buffer (pH 7.2) (3:7, v/v) and the flow-rate was adjusted to 0.3 ml/min. All separations were performed at 40°C and the cell potential was maintained at +0.9 V vs. a Ag/AgCl reference electrode.

Standard curves

A standard curve for normetanephrine was prepared by the following procedure. To 0.8 ml of 50 m*M* phosphate buffer (pH 7.5) containing normetanephrine in the range 10—50 μg were added successively 0.2 ml of COMT preparation and 0.1 ml of 3.6 *N* perchloric acid. The mixture was treated according to the procedure described in assay of COMT activity. A standard curve for norparanephrine was prepared on the basis of that obtained above for normetanephrine, and calibration graphs were constructed for authentic vanillin and isovanillin using *p*-hydroxyacetanilide as internal standard.

Assay of COMT activity

A 0.5-ml volume of substrate solution, 0.25 ml of SAM solution and 0.05 ml of $MgCl_2$ solution were added successively to a 10-ml centrifuge tube. The tube was preincubated for 5 min at 37°C. The enzyme reaction was started by adding 0.2 ml of COMT preparation (10 mg of protein per ml). The standard incubation mixture (1 ml) consisted of 50 m*M* phosphate buffer (pH 7.5), 0.5

m*M* norepinephrine, 1 m*M* SAM, 5 m*M* $MgCl_2$ and 2 mg of COMT. The mixture was incubated for 1 h except for the study of the time course. Incubation was stopped by adding 0.1 ml of 3.6 *N* perchloric acid. After centrifugation, all the supernatant fluid was decanted into another 10-ml tube containing 3 ml of 1 *M* phosphate buffer (pH 9.5). A 0.3-ml volume of 0.01 *N* sodium metaperiodate ($NaIO_4$) was added in order to oxidize O-methylated products and the mixture was allowed to stand for 10 min at room temperature. Excess $NaIO_4$ was destroyed by adding 1 ml of 0.1 *N* sodium metabisulfate ($Na_2S_2O_5$). The mixture was saturated with sodium chloride and the resulting O-methylated products (vanillin and isovanillin) were extracted with 3 ml of ethyl acetate containing *p*-hydroxyacetanilide as internal standard. The extract was evaporated to dryness under reduced pressure and the residue was redissolved in 1 ml of 1% acetic acid. A 10-μl aliquot of the solution was determined with the HPLC system as described above. Each sample was injected in duplicate. Peak height ratios of products to internal standard were measured and the amount of methylated products (normetanephrine and norparanephrine) in an unknown sample was calculated from the standard curves.

RESULTS AND DISCUSSION

Norepinephrine, one of the important catecholamines in biological fluids, was chosen as a substrate for an assay of COMT activity, because the structure of the substrate used causes marked differences in COMT activity and in its evaluation. In vitro normetanephrine and norparanephrine have been known to be enzymatically formed from norepinephrine by the COMT reaction [3]. Both O-methylated isomers can be separated by HPLC, but in this case we encountered two problems. One was the problem of deproteinization. Incomplete deproteinization often caused rapid degeneration in the resolution of the column, so a sample clean-up procedure was considered using DEAE-Sephadex A-25 chromatography [4] or Bio-Rex 70 ion-exchange resin [2]. The other problem is that the commercial availability of norparanephrine, one of the metabolites, is limited. On the other hand, it is known that normetanephrine is easily converted to vanillin by periodate oxidation [8]. This reaction attracted our attention because vanillin and isovanillin are commercially available, so normetanephrine and norparanephrine can be measured by conversion to vanillin and isovanillin, respectively. Complete deproteinization can also be expected by extracting vanillin and isovanillin with a suitable organic solvent from the reaction mixture. The technique has been successfully applied to an assay of COMT activity. The optimum conditions for the periodate oxidation of normetanephrine as described under Experimental were fixed from preliminary experiments consulting the method of Pisano [8]. Under these conditions it was expected that the reaction of norparanephrine with periodate proceeds in a similar manner to normetanephrine. Vanillin, isovanillin and *p*-hydroxyacetanilide (internal standard) were quantitatively extracted with 3 ml of ethyl acetate below pH 7.5 when saturated with sodium chloride. Vanillin and isovanillin were not very stable above pH 7.0, so they were treated in 1% acetic acid solution.

The separation of vanillin, isovanillin and *p*-hydroxyacetanilide on a LiChro-

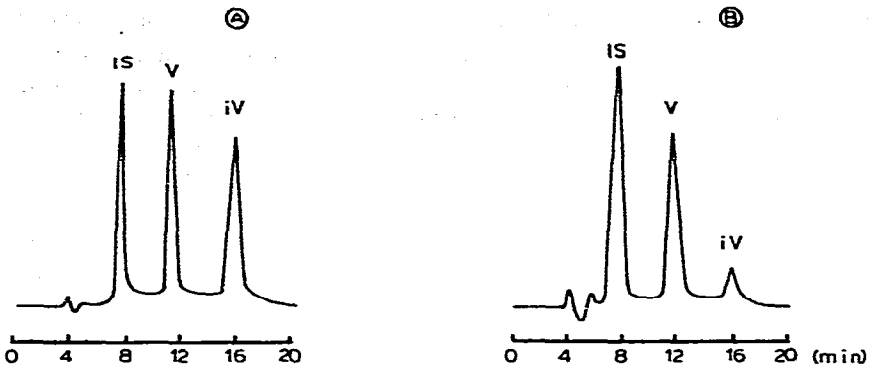

Fig. 1. Liquid chromatographic separation of vanillin (V) and isovanillin (iV) with *p*-hydroxyacetanilide (IS) as internal standard. Chromatographic conditions: column, 15 cm × 4 mm I.D. filled with LiChrosorb 5 RP-18; column temperature, 40°C; mobile phase, 30% methanol in 0.05 *M* phosphate buffer (pH 7.2); pressure, 1 MPa; flow-rate, 0.3 ml/min; detector, voltammetric +0.9 V; injection volume, 10 µl. (A) Separation of standard mixture; (B) separation of O-methylated isomers formed by COMT reaction after periodate oxidation.

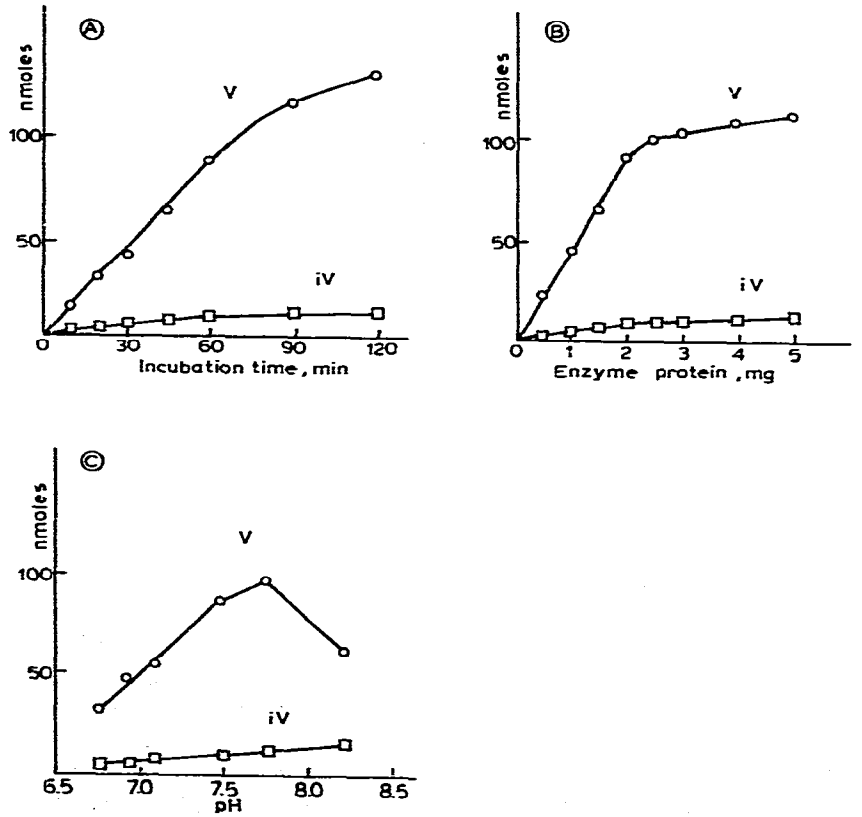

Fig. 2. The kinetic parameters of COMT from rat liver using norepinephrine as substrate. ○, Vanillin (V); □, isovanillin (iV).

sorb 5 RP-18 column is shown in Fig. 1A. To demonstrate the ability of this method, we measured the COMT activity present in the soluble fractions of rat liver (Fig. 1B). There were neither interfering compounds nor the peak derived from the substrate. In the blank tests performed by omitting SAM or norepinephrine, no O-methylated products were formed.

Some kinetic parameters of rat liver COMT were also measured by this method. The results are shown in Fig. 2. Fig. 2A shows the time course for formation of O-methylated products in enzyme incubation mixtures and Fig. 2B shows the influence of the enzyme concentration on the COMT activity. The enzyme activity was linear up to 60 min incubation time and up to about 2.0 mg of enzyme protein.

The *meta*- and *para*-methylated products were simultaneously measured in the same incubation mixture and the effect of pH on the enzyme activity and the *meta/para* ratios was also studied. As is shown in Fig. 2C, the COMT activity changed depending on the pH of the incubation mixture. As the pH was increased from 7.50 to 8.23, the *meta/para* ratio rapidly decreased from 10.31 to 5.02. These results are similar to those reported by Creveling et al. [9].

The COMT activities were measured from a series of five separately prepared samples using the same rat liver preparation. The relative standard deviations for the *meta*- and *para*-O-methylated isomers were 3.97% and 4.29%, respectively. The correlation coefficient for the standard curve obtained using normetanephrine according to the procedure described under standard curves was 0.997. Quantitative assay was possible on the chromatogram even for the injection of 1 ng (6.6 pmoles) of vanillin or isovanillin. The specific activity of the rat liver COMT was 43.3 nmoles of vanillin and 4.2 nmoles of isovanillin per mg of protein per h at pH 7.5.

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