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Short communication

Improved assay of reaction products to quantitate catechol-O-methyltransferase activity by high-performance liquid chromatography with electrochemical detection

Ilkka Reenilä, Päivi Tuomainen, Pekka T. Männistö*

Institute of Biomedicine, Department of Pharmacology and Toxicology, University of Helsinki, P.O. Box 8, Siltavuorenpenger 10, Fin-00014, Helsinki, Finland

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Abstract

We applied coulometric detection (three electrochemical electrodes in series) to quantitate vanillic acid and isovanillic acid using reversed-phase HPLC. The formation of these reaction products from dihydroxybenzoic acid was used as a precise and reproducible measure of catechol-O-methyltransferase (COMT) activity in striatal homogenates and recombinant membrane-bound COMT protein. This detection system has a higher sensitivity (0.5 pmol per injection) than a single-cell amperometric detection. As in a previous method, the deproteinized supernatants of the COMT assay could be injected directly onto the HPLC system allowing the handling of a large number of samples in one day.

1. Introduction

Catechol-O-methyltransferase (COMT, EC 2.1.1.6.), an enzyme present in various tissues, catalyzes the methylation of molecules containing a catechol moiety [1]. In the brain COMT participates in the inactivation of catecholamines [2,3]. In vitro the activity of the soluble form (S-COMT) predominates over the membrane-bound (MB-) COMT; however, at physiological substrate concentrations in vivo the importance of the MB-COMT is greatly accentuated [4]. In addition, MB-COMT may be the first enzyme to inactivate dopamine and noradrenaline after their release from neurons [5].

COMT activity in vitro can be measured by several methods [6–9]. Among those, reversed-phase HPLC with different detection systems is widely used to analyze the reaction products [10–13]. To improve the sensitivity of a previously published method [14], a dual-cell coulometric system (an oxidative conditioning cell and an analytical cell, consisting of oxidative and reductive electrodes in conjunction) was applied to detect the reaction products vanillic acid and isovanillic acid formed from the substrate, dihydroxybenzoic acid (DHBAc), by COMT.

COMT assays of recombinant MB-COMT [15] and 900-*g* supernatant from homogenized rat

* Corresponding author.

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striatal tissue are presented as practical examples.

2. Experimental

2.1. Materials

Ultrapure reagent-grade water was obtained with a Milli-Q system (Millipore/Waters, Milford, MA, USA). Vanillic acid (3-methoxy-4-hydroxybenzoic acid), isovanillic acid (4-methoxy-3-hydroxybenzoic acid), DHBAc and S-adenosyl-L-methionine iodide (AdoMet) were from Sigma (St. Louis, MO, USA). Perchloric acid, disodium ethylenediaminetetraacetic acid dihydrate (Na_2EDTA), disodium monohydrogen phosphate, magnesium chloride and dithiothreitol were purchased from E. Merck (Darmstadt, Germany). All reagents were of analytical grade and used without further purification. HPLC grade methanol was from Rathburn (Walkenburg, UK).

2.2. Recombinant membrane-bound COMT

The recombinant MB-COMT was made in *Spodoptera frugiperda* (Sf9) insect cells which were infected with a baculovirus containing MB-COMT-coding sequence [15]. The 100 000-*g* MB-COMT pellet was suspended in 10 m*M* sodium phosphate buffer containing 0.5 m*M* dithiothreitol and 5 m*M* $MgCl_2$. The COMT assay was performed with the diluted suspension (3 μg protein/reaction) using 200 μM of DHBAc as a substrate in the assay.

2.3. Preparation of rat striatal samples

Wistar/Kuo rats (Han/Kuo, Department of Pharmacology and Toxicology, University of Helsinki and Department of Physiology, University of Helsinki) were housed in cages under a 12-h light/dark cycle (light on at 7 a.m.). Food and water were available ad libitum.

The rats were decapitated, the striata were dissected, cooled in liquid nitrogen and stored at −80°C. The striata were homogenized in 10 m*M* Na_2HPO_4 buffer (pH 7.4) containing 0.5 m*M* dithiothreitol. The homogenates were centrifuged 900 *g* for 10 min at 4°C. The 900-*g* supernatants were assayed for COMT activity using 240 μM of DHBAc as a substrate.

2.4. COMT assay

The COMT assay was performed according to the procedure described by Nissinen and Männistö [14] by incubating the enzyme preparation at 37°C in 100 m*M* phosphate buffer (pH 7.4) containing 5 m*M* $MgCl_2$, 0.2 m*M* AdoMet and 200 or 240 μM DHBAc as final concentrations in a volume of 250 μl.

2.5. Preparation of calibration samples

Vanillic acid and isovanillic acid were dissolved in 0.1 ml of dimethyl sulfoxide and made up to 10 ml for a 2 m*M* stock solution with 10 m*M* Na_2HPO_4 buffer (pH 7.4) and stored at −20°C. The stock solution could be stored at −20°C for several months. For each run the stock solution was diluted usually to 0.01–2.0 μM with water.

2.6. High-performance liquid chromatography

Aliquots (10 μl) of the samples were injected by a Waters 712 Wisp autosampler with cooler (Waters) onto the HPLC system which consisted of an isocratic Waters Model 6000 A pump and a LiChrospher 100 RP-18 column (5 μm, 125 × 4 mm I.D., Merck) guarded with precolumn. Vanillic acid and isovanillic acid were detected with a coulometric ESA Coulochem Model 5100 A, cell Model 5011 detector, potential set to +0.10 V (detector 1), −0.30 V (detector 2) and a conditioning cell set to +0.40 V (ESA, Bedford, MA, USA). The current response of detector 2 (gain: 30 × 100) was recorded with a Hewlett-Packard HP 3396 Series II integrator. The mobile phase was 0.1 *M* Na_2HPO_4 (pH 3.2), 0.15 m*M* EDTA and 15% methanol with a flow-rate of 1.0 ml/min.

2.7. Protein measurement

Proteins were measured with a Ultrospec III Spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) utilizing the dye binding method [16] and BioRad protein assay reagent (BioRad, Richmond, CA, USA). Bovine serum albumin (Pierce, Rockford, IL, USA) served as a protein standard.

2.8. Quantitation and calculations

For each run the method was calibrated, usually with 0.01–2.0 μM calibration samples. A calibration curve was prepared by linear regression of the peak heights of the calibration samples with Quattro Pro software (Borland International, Scotts Valley, CA, USA). The concentrations of the reaction products produced by the samples were calculated from the peak-height values utilizing the calibration curve. The concentrations of the samples are expressed as picomoles of analyte per 10-μl injection. Specific activity of COMT is expressed as picomoles vanillic acid formed in one minute per mg protein in the sample.

3. Results

3.1. Specificity

Typical chromatograms obtained with calibration and COMT samples are shown in Fig. 1. The vanillic acid and isovanillic acid eluted with retention times of 13.2 and 15.2 min, respectively (Fig. 1A). No interfering peaks were detected when the substrate was omitted from the recombinant MB-COMT assay (Fig. 1B). Incubation with 200 μM DHBAc resulted in the formation of reaction products with authentic retention times (Fig. 1C). Production of vanillic acid and isovanillic acid was also obtained with striatal 900-g supernatants (Fig. 1D). Sometimes one small peak eluting between the vanillic acid and isovanillic acid peaks was found in the striatal samples (data not shown). This peak has not yet been identified, but it is obviously a catecholamine metabolite. The unknown peak at 4.8 min was present in all samples and no attempts were made to identify it.

3.2. Limit of detection, linearity and range

The detection limit of both analytes was 0.1 pmol per injection at a signal-to-noise ratio of 3.

Good linearity between the vanillic acid and isovanillic acid calibration sample concentrations and peak heights was observed from 0.1 pmol to 20 pmol per injection. The slopes for vanillic acid and isovanillic acid were 0.009451 ± 0.00322 and 0.007156 ± 0.00249 (mean $\pm$ S.D., $n = 13$), respectively, with mean ($\pm$ S.D.) y-intercepts of 0.002059 ± 0.00701 and 0.002463 ± 0.0063, respectively ($n = 13$).

3.3. Precision and accuracy

Under the present chromatographic conditions, the accuracy of the calibration samples was better than the precision. Since at the lowest concentrations (0.1 and 0.2 pmol per injection) the coefficient of variation (C.V.) was $>20\%$, the limit of reliable quantitation was set to 0.5 pmol per injection. Within the range of 0.5–20 pmol per injection the precision of the calibration for the vanillic acid was between 0.28 and 6.64% and the accuracy was between -0.47 and 2.89% (expressed as %error, $n = 9$–14). The C.V. for isovanillic acid was between 0.58 and 4.90% with the error between -0.92 and 1.72% ($n = 10$–13).

The within-day precision and accuracy were followed by measuring the same calibration sample several times within the same run. The representative C.V.s within the range 0.5–20 pmol per injection were between 0.65 and 6.7% for vanillic acid and between 2.8 and 5.68% ($n = 5$–8) for isovanillic acid, respectively. At lower concentrations the C.V. was less than 15% but the error exceeded 40%.

The between-day precision of the measurement for one recombinant MB-COMT sample was 10.4% for vanillic acid and 14.9% for isovanillic acid ($n = 14$). One sample of striatal tissue 900-g supernatant was also measured with-

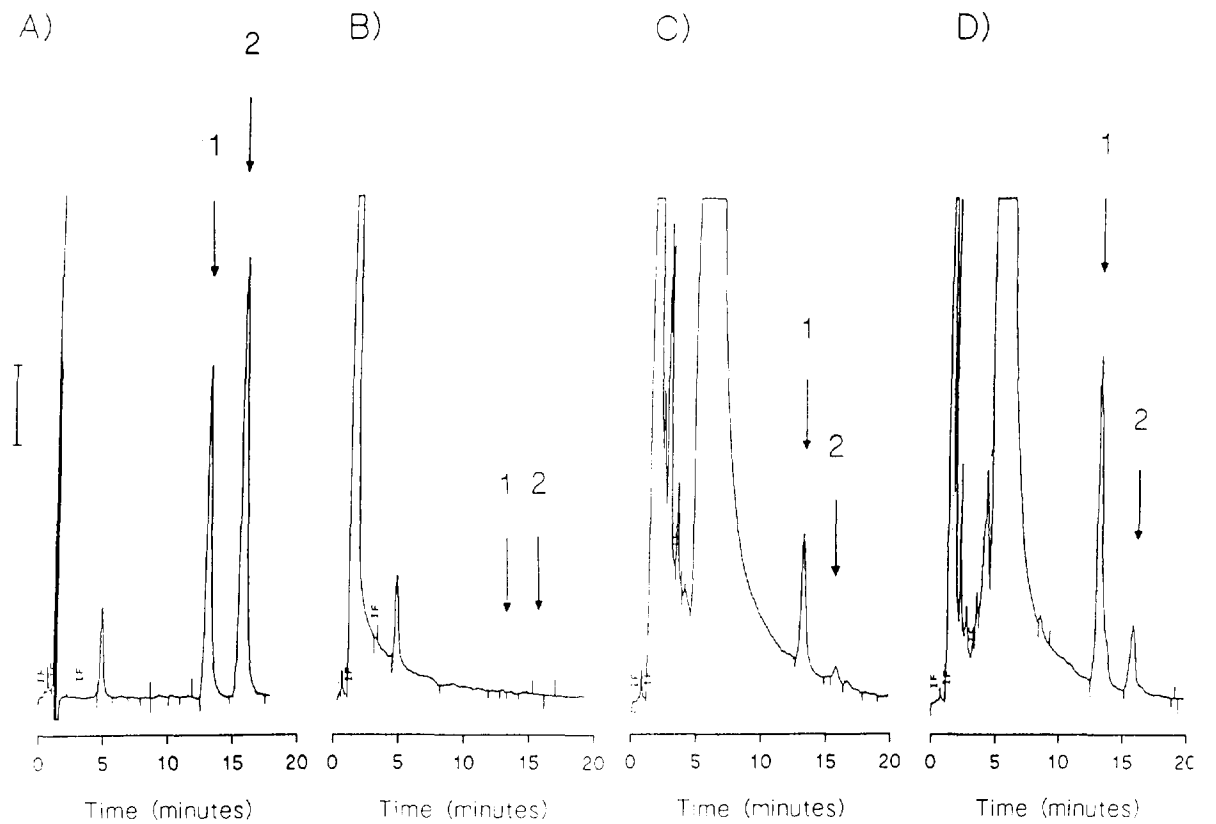

Fig. 1. Chromatograms obtained from the samples. (A) Calibration sample containing 10 pmol of both vanillic acid and isovanillic acid, (B) recombinant MB-COMT assayed without the substrate, (C) assayed reaction products from recombinant MB-COMT (substrate: 200 μM DHBAc) and (D) the reaction products from the sample from the rat striatal 900-g supernatant (substrate: 240 μM DHBAc). The bar at y-axis denotes 10 nA in (A)–(C) and 5 nA in (D). Peaks: 1 = vanillic acid and 2 = isovanillic acid. In each case the injection volume was 10 μl.

in the following runs. The mean vanillic acid concentration found (± S.D.) was 19.65 ± 0.318 pmol (C.V. = 1.62%) and for the isovanillic acid 3.109 ± 0.091 pmol (C.V. = 2.93%, $n = 8$).

The ratios of 3- to 4-methylated products (*meta*/*para* ratio) for recombinant MB-COMT and the striatal 900-g supernatant were 15.5 ± 1.69 ($n = 10$) and 6.32 ± 0.152 ($n = 8$), respectively, expressed as mean ± S.D.

3.4. Stability

The stock substrate solution (1 mM DHBAc) could be stored at least for two weeks at 4°C. Calibration samples kept overnight in the cooler of the autosampler cooler (4°C) did not show loss of vanillic acid or isovanillic acid. However, the reaction products of the recombinant MB-COMT reaction supernatants decreased in concentration during overnight runs, thereby increasing the variation of replicate samples.

The supernatants from the COMT assays stored at −80°C did not show any change in concentration over a two-months (recombinant MB-COMT) or three-months (striatal aliquots) period, respectively. The 900-g tissue supernatant also did not show any clear change over a two-months storage period at −80°C.

3.5. COMT assay

The recombinant MB-COMT assay was linear at least up to 45 min of incubation (Fig. 2A). Similar results were reproduced twice.

Diluted recombinant MB-COMT enzyme-pool aliquots gave variable activities in repeated assays performed on different days, probably due to the low stability of the dilute enzyme suspension. When all the analyses were performed with fresh enzyme dilutions, less day-to-day variation in the COMT activity was observed. The within-day variation was usually less than 20% (data not shown) including the error caused by the storage at 4°C in the cooler (1–10%).

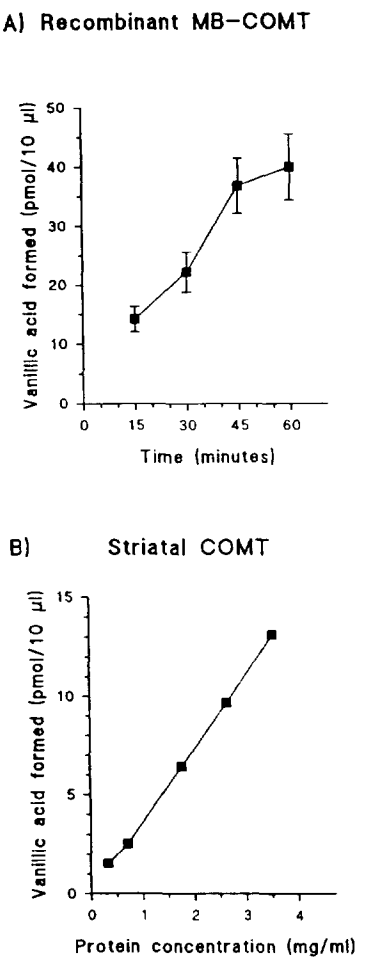

Fig. 2. Some characteristics of the COMT assay. (A) The formation of vanillic acid by recombinant MB-COMT at different incubation periods. The recombinant MB-COMT was assayed with 200 μM of DHBAc as a substrate and the corresponding mean (± S.D.) concentration of vanillic acid was calculated ($n = 7$–8). (B) The COMT activity of rat striatal 900-g supernatant with increasing protein concentration in the sample. Different amounts of the 900-g supernatant were assayed with 240 μM of DHBAc as a substrate. The vanillic acid concentration is expressed against each protein concentration.

The C.V. of the aliquots of the same striatal 900-g supernatant analyzed at different days, was 10.7 and 10.4% for vanillic acid and isovanillic acid, respectively ($n = 4$). The *meta*/*para* ratio was 7.23 ± 0.07 (mean ± S.D.).

The mean (± S.D.) specific activity of seven different striatal tissue supernatants was 45.8 ± 17.3 pmol min^{-1} mg^{-1} protein when these samples were assayed within two months. The intra-assay variation of specific activity for individual tissues was usually less than 20% (data not shown).

The effect of an increasing amount of protein in the 900-g supernatant on the COMT activity was tested with three striatal samples. The COMT assay was linear at least up to 3.5 mg/ml protein concentration in the sample (Fig. 2B).

4. Discussion

Analysis of COMT activity is conveniently made by determination of the reaction products. Our previous COMT assay utilized reversed-phase HPLC with single-cell amperometric detection [14]. Our improved three-electrode coulometric system lowers the detection limit about four times (0.5 pmol/10 μl compared to 1.0 pmol/20 μl). The sensitivity may still be improved if the gain is increased. So far, the present range is suitable for the analysis of the reaction products generated by both the striatal 900-g supernatants and even by the subcellular fractions of striatal samples (data not shown).

With a higher sensitivity lower COMT activities may be detected allowing the use of lower substrate concentrations. Thus, the suggested

overestimation of the tissue S-COMT activity, relative to the MB-COMT activity, is reduced [4]. At 240 μM of DHBAc, the concentration used to characterize the COMT assay, the rat brain MB-COMT is supposed to be saturated, while S-COMT is half-saturated [4,10]. Based on this, the COMT assay performed with the 900-*g* supernatant quantitates mainly the soluble COMT activity of the striatal tissue. This is supported by the lower *meta*/*para* ratio of S-COMT [10], whereas the ratio is higher for the recombinant MB-COMT which has the same characteristics as the native membrane-bound form [15].

In general, the present COMT assay is precise, accurate and gives a linear response for the activities found in our samples. The previous COMT assay method introduced the direct non-radioactive analysis of the deproteinized reaction supernatant without any further handling [14] enabling simple and rapid analysis of the COMT activity.

When 15% methanol was used as organic component in the mobile phase, good separation is achieved without lengthening of the retention time. Stabilization of the detector between subsequent mobile-phase batches took approximately one hour and no extra washing of the system was needed. Under the present conditions high substrate concentrations (about 400 μM of DHBAc) increase the width of the front peak leading to difficulties in the analysis of the reaction products. DHBAc contains no detectable impurities (Fig. 1) as did dihydroxybenzylamine in the previous method [14]. Since the chromatographic conditions are the same for the striatal tissue samples and the recombinant MB-COMT (Fig. 1C and D), the COMT assay using coulometric determination of the reaction products is evidently applicable to various sources of COMT.

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