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Improved assay for catechol-*O*-methyltransferase activity utilizing norepinephrine as an enzymatic substrate and reversed-phase high-performance liquid chromatography with fluorescence detection

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Abstract

We have previously established a rapid catechol-*O*-methyltransferase (COMT) assay using norepinephrine (NE) as a natural substrate and flow-injection analysis. In this study, the method is improved for screening of COMT inhibitors or activators using reversed-phase high-performance liquid chromatographic separation with fluorescence detection. The excess substrate, NE, was removed by the addition of borate in the eluent for HPLC to make an ionic complex with NE, which was eluted faster than the enzymatic product, normetanephrine. The method had good precision and accuracy, and was able to assay one sample in 5 min, showing the usability for screening of COMT inhibitors or activators.

Keywords: Catechol-*O*-methyltransferase; Norepinephrine as substrate; Borate complex; HPLC-fluorescence detection; Hypertension; Parkinson's disease

1. Introduction

Catechol-*O*-methyltransferase (COMT) catalyzes the methylation of *o*-hydroxyl group of compounds with catechol structure, using *S*-adenosyl-L-methionine (SAMe) as a methyl donor [1]. It plays an important role in the metabolism of catecholamines (CAs) and the deactivation of exogenous catechol compounds.

We have studied the blood pressure regulation in spontaneously hypertensive rats (SHR) in relation to CAs metabolism by COMT. As a result, it was found that compared to Wistar-Kyoto (WKY) rats, SHR had less ability for methylation of CAs, especially norepinephrine (NE) [2]. Also the activity and amount of membrane-bound COMT in liver, which should be an important isozyme in the metabolism of CAs, was lower in SHR [3]. When SAMe was given to SHR, blood pressure decreased because of the accelerated 3-*O*-methylation of CAs catalyzed by COMT to afford 3-*O*-methyl metabolites (especially normetanephrine (NMN)). Moreover, it was reported that COMT inhibition elicits a moderate, dose-dependent pressure response in the setting of severely impaired baroreflex buffering [4]. These results suggest that activation of COMT should cause blood pressure reduction. Thus, compounds that have the ability to activate COMT may be the candidates to control blood pressure under a new mechanism.

On the other hand, COMT inhibitors also attract attention because of their medical use for Parkinson's disease [5–7]. The brain of patients with Parkinson's disease is deficient of dopamine due to a defect in the dopamine nerves. L-dopa is, as its precursor, now administered to supply dopamine. To prevent the peripheral metabolism of L-dopa by COMT, the COMT inhibitor together with aromatic L-amino acid decarboxylase inhibitor is administered.

According to these observations, it was suggested that the search for the COMT activators and inhibitors is an important issue for the treatment of hypertension or Parkinson's

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disease, respectively. However, since only a few available compounds have been reported for the purpose, we have conducted a search for both the COMT activators and inhibitors by screening various compounds. Therefore, an assay was needed to examine the compounds which affect the reaction catalyzed by COMT.

COMT activities have been assayed by quantitating the methylated substrates with HPLC-ECD [8,9], radioactivity [10], fluorometric assay [11,12] and capillary electrophoresis [13]. In most cases, the method adopted the exogenous substrates, such as 3,4-dihydroxybenzoic acid (DBA) [8,9,13], which have a different affinity to COMT from the endogenous CAs. Thus, we have previously reported the assay for COMT activity using NE as a substrate instead of DBA, and found the results obtained with NE as the substrate reflected more physiological performance in vivo [14–18]. For our present purpose, the method should enable us to assay a large number of samples in a short time for screening. In the enzymatic reaction, the concentration of the substrate, NE, is about 1000 times higher than that of NMN, the enzymatic product by COMT. Therefore, in the previous report [19], NE was removed from the reaction medium by alumina followed by flow injection analysis, which enabled us to assay one sample in about 3 min. However, the procedure to remove NE was tedious and was not suitable for treating a large number of samples.

In this study, we used a purified porcine liver COMT as a model COMT and try to assay its activity more easily and in a shorter time. The excess substrate, NE, was removed by the addition of borate into the HPLC mobile phase to make an ionic complex with NE, which was eluted faster than the enzymatic product, NMN.

2. Experimental

2.1. Reagents

Norepinephrine (+)-bitartrate salt monohydrate, catechol-*O*-methyltransferase purified from porcine liver, DL-normetanephrine hydrochloride, and *S*-adenosyl-L-methionine chloride salt were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, HPLC grade, was purchased from Kanto Kagaku (Tokyo, Japan). Boric acid was from Wako (Osaka, Japan). 3,5-Dinitrocatechol was from Tocris (Ballwin, MO, USA). In this study, water was used after the purification with Milli-Q reagent system (Nihon Millipore, Tokyo, Japan).

2.2. Apparatus

The chromatographic system was composed of system controller (LCSS-900, JASCO, Tokyo, Japan), pump (PU-1580, JASCO), auto sampler (AS-950, JASCO), column oven (CO-1560, JASCO), fluorescence detector (F-1080, Hitachi, Tokyo, Japan) and integrator (D-7500, Hitachi). Separation was carried out using an Unison UK-C_{18} column (30 mm × 4.6 mm I.D., 3 μm) (Imtakt, Kyoto, Japan). Fluorescence spectra were measured by a Hitachi F-4500 fluorescent spectrometer.

2.3. Enzymatic reaction

The purified COMT (1.3 mg) was dissolved in 21 ml of 25 mM sodium phosphate buffer (pH 7.8) containing 0.5 mM dithiothreitol. A 50 μl aliquot of the enzyme solution was incubated in 50 mM sodium phosphate buffer (pH 7.8) containing 2 mM $MgCl_2$, 200 μM SAMe and 1500 μM NE in a volume of 125 μl. The mixture was incubated for 1 h at 37 °C. The reaction was terminated by adding 12.5 μl of 1 M perchloric acid. The samples were centrifuged at 3000 × *g* for 5 min at 4 °C, and the supernatant was used as a sample solution.

2.4. Separation of NE and NMN on an ODS column

To examine the effect of borate on the separation of NE and NMN, mobile phase consisted of 75 mM potassium acetate buffer (pH 3.2)/50 mM potassium phosphate buffer (pH 3.2)/acetonitrile (90.25/4.75/5, v/v/v) containing 4 mM sodium 1-hexanesulfonate was used. The flow rate was 0.5 ml/min. The wavelengths for the fluorescence detection were 283 and 315 nm for excitation and emission, respectively.

The optimization of the content of the mobile phase was investigated; pH (6.6–7.6), sodium phosphate buffer concentration (10–50 mM) and the concentration of borate (0–80 mM).

Optimized mobile phase conditions were as follows, 10 mM sodium phosphate buffer (pH 7.0)/acetonitrile (95/5, v/v), containing 80 mM boric acid and 4 mM sodium 1-hexanesulfonate, 0.8 ml/min.

2.5. Optimization of excitation and emission wavelength

One μM of NE or NMN was dissolved in 10 mM sodium phosphate buffer (pH 7.0)/acetonitrile (95/5, v/v) containing 80 mM boric acid and 4 mM sodium 1-hexanesulfonate, and excitation and emission spectra were measured.

2.6. Validation

2.6.1. Calibration curves, precision and accuracy

To 250 μl of enzyme reaction solution, 0–400 pmol of NMN were added. The calibration curves for the peak height vs. the concentration of NMN were obtained ($n = 3$). The intra- and inter-day precision of the system was evaluated by analyzing the same enzymatic reaction solution ten consecutive times or for five successive days, respectively. The accuracy was estimated with the comparison of the slopes of the calibration curves for standard samples and those obtained from the spiked experiments.

2.6.2. Kinetics measurement

Kinetic parameters for the purified COMT were determined by varying the concentration of NE (0–2 mM) or SAMe (0–200 μM). The data was analyzed by fitting the Michaelis–Menten equation to the initial velocities. K_i values for COMT inhibitor 3,5-dinitrocatechol were determined by varying the concentrations of NE and COMT inhibitors. Enzyme preparations containing 0.25, 0.5, or 1.5 mM of NE were incubated with inhibitor concentrations from 0 to 25 nM 3,5-dinitrocatechol. The data were analyzed by the method of Dixon [20].

3. Results and discussion

3.1. Separation of NE from NMN using dual columns

For an easy removal of the excess NE, we first used the dual columns. One was packed with an alumina to remove the excess NE, and the other was with an ODS to separate and quantitate NMN. However, good result was not obtained because of the instability of alumina under the elution condition (50 mM sodium phosphate buffer (pH 8.5)), and thus it was difficult to obtain a reproducible removal of NE. In the next step, a borate-gel column was adopted under the elution condition with 50 mM sodium phosphate buffer (pH 8.5). As a result, there was a broadening of the NMN peak, which affected its quantitation with sufficient sensitivity.

3.2. Separation of NE from NMN on an ODS column

In the next step, the short time separation of NE from NMN was attempted with the use of an ODS column only.

We have previously reported a method for COMT activity measurement, which included a column-switching system using ion exchange pre-column for trapping NE and NMN, and ODS column for separation and quantitation of NMN. It took about an hour to analyze one sample [14–18]. In this study, column switching was omitted for a more rapid assay. Further, the length of the ODS column was changed to 30 mm instead of 150 mm, which was used in our previous study.

Fig. 1a shows the chromatogram of NE and NMN with the mobile phase (75 mM potassium acetate buffer (pH 3.2)/50 mM potassium phosphate buffer (pH 3.2)/acetonitrile (90.25/4.75/5, v/v/v) containing 4 mM sodium 1-hexanesulfonate). Then, based on the idea that the retention time of NE-borate complex should be short because of the ionic charge of the complex [21,22], borate was added to the mobile phase. The retention time of NE was shortened from 1.6 to 1.1 min, while that of NMN was unchanged (Fig. 1b).

pH is an important factor to make complex NE with borate, and therefore, the effect of pH of the mobile phase was investigated from 6.6 to 7.6. As a result, good separation was obtained at pH 7.0 (data not shown).

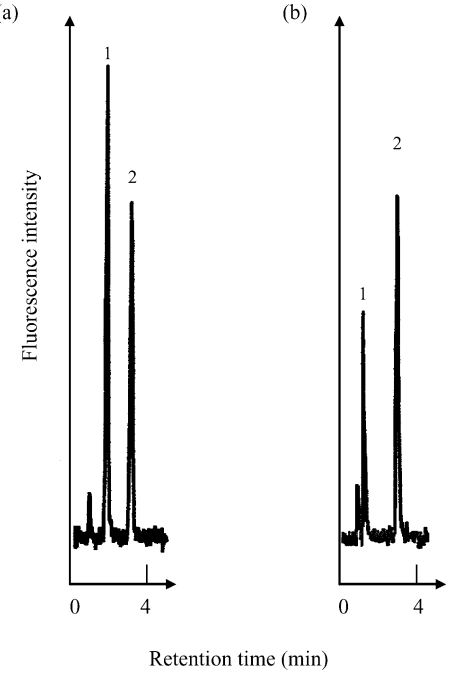

Fig. 1. Representative chromatograms of standard NE and NMN (10 pmol each), obtained by using the same mobile phase as the previous HPLC method [14] (a), and the same mobile phase added with 50 mM boric acid and adjusted to pH 7.0 (b). 1, NE; 2, NMN. The chromatographic conditions are described in the text.

Next, the component of the mobile phase was optimized. Using sodium phosphate buffer, optimized concentration was 10 mM. The concentration of borate was investigated, and with the higher concentration the retention time of NE was shorter (from 2.5 to 1.5 min with the borate concentration from 0 to 80 mM), while the retention time of NMN was not changed. Therefore, the concentration of borate in the mobile phase was selected to be 80 mM.

3.3. Optimization of excitation and emission wavelength

In the optimized mobile phase (10 mM sodium phosphate buffer (pH 7.0)/acetonitrile (95/5, v/v) containing 80 mM boric acid and 4 mM sodium 1-hexanesulfonate), excitation and emission maximum wavelengths were 278 and 311 nm, respectively.

In the solution without borate, the fluorescence intensity of NMN was similar to that of NE. While, in the mobile phase containing borate, the fluorescence intensity of NMN was five times higher than that of NE because NE only made complex with borate and it should decrease the fluorescence derived from the catechol moiety [23].

3.4. Chromatogram

The representative chromatogram obtained from the reaction mixture under the optimized conditions is shown in Fig. 2. It took only 5 min to assay 1 sample. The peak

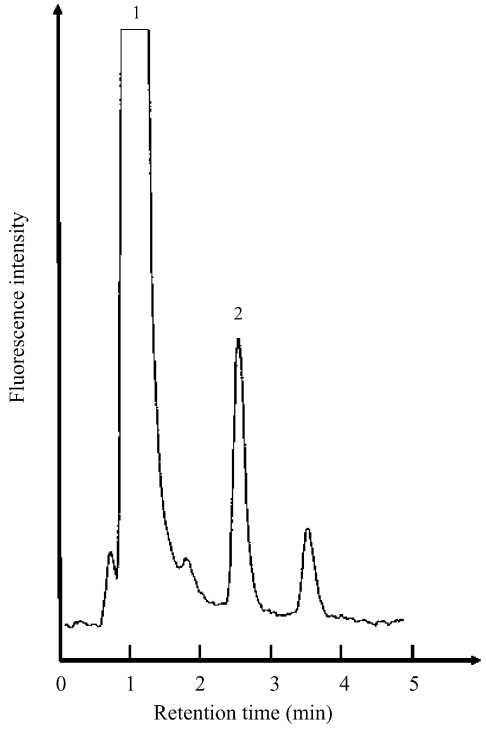

Fig. 2. Representative chromatogram obtained from an incubation medium of purified porcine liver COMT with NE as the substrate; 1, NE, 2, NMN. The chromatographic conditions are described in the Section 2.

retained at 3.6 min should be the 4-methoxy metabolite because it is known to be produced in in vitro enzymatic reaction, whereas not in vivo [24]. We measured the molecular weight by mass-spectrometry after HPLC fractionation. When we took the fraction of the peak retained at 3.6 min and subjected to mass-spectrometry, and found that the molecular weight (183.2) was the same as NMN. Furthermore, the peak retained at 3.6 min was not influenced by the addition of borate, which indicated that the compound has no catechol moiety.

3.5. Validation of the developed method

A linear relationship ranged from 0 to 400 pmol for NMN with correlation coefficients of 0.999. Intra-day precision and inter-day precision were 2.12% ($n = 10$) and 4.99% ($n = 5$), respectively. The accuracy for the method was 93.7%. These results suggest that the developed assay should be useful for the screening of COMT activators and inhibitors.

3.6. Assay for the purified COMT activity

V_{max} values measured with the developed assay were 4.36 ± 0.08 nmol/min/mg protein, which was similar with those measured with previous HPLC method (3.99 ± 0.24 nmol/min/mg protein) [14] and smaller than those measured with FIA method (5.80 ± 0.20 nmol/min/mg protein) [19]. This is probably because the values with FIA method include not only the amount of NMN but also the 4-methoxy metabolite, while NMN and 4-methoxy metabolite were separated and quantitated by HPLC methods.

K_m value for NE was 488 ± 51 μM. K_i value for 3,5-DNC was 30.7 ± 2.5 μM. These were consistent with the values measured with FIA methods (432 ± 10 and 19.0 ± 9.5 μM, respectively) [19].

In conclusion, the assay was improved for the rapid screening of COMT inhibitors or activators with HPLC-fluorescence detection. The enzymatic reaction solution was directly injected into the HPLC system without the time-consuming pre-treatment, and because of the addition of borate to the mobile phase to make a complex with NE, it enabled us to assay 1 sample in 5 min. The method was superior to the previous FIA method [19] and HPLC method [14–18] in terms of analysis time and ease of operation.

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