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Rapid assay for catechol-*O*-methyltransferase activity by high-performance liquid chromatography-fluorescence detection

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Abstract

A rapid assay for measuring the activities of catechol-*O*-methyltransferase (COMT) is described. The method is based on high-performance liquid chromatography (HPLC)-fluorescence detection, and includes on-line extraction of catecholamines with a precolumn, separation of norepinephrine (NE) and normetanephrine (NMN) on an ODS column, electrochemical oxidation, and post-column fluorogenic derivatization using ethylenediamine. The method took less than 25 min for one sample, which is half that of the previous method and the sensitivity was similar. The intra-day assay precisions were 0.52–1.6%, and the inter-day assay precisions were 3.6–5.8% for rat liver and cerebral cortex ($n = 5$). The method is suitable for the rapid measurement of COMT activities of many biological samples.

Keywords: Catecholamines; Catechol-*O*-methyltransferase; Norepinephrine; Normetanephrine

1. Introduction

In mammals, catecholamines, including dopamine, norepinephrine (NE), and epinephrine, play important roles as neurotransmitters and hormones. Especially, NE released from sympathetic nerve ending is a main factor of a rise in blood pressure and is inactivated by catechol-*O*-methyltransferase (COMT; EC2.1.1.6) and transformed into normetanephrine (NMN) [1–5]. COMT comprising two isoforms; soluble COMT (S-COMT) and membrane-bound COMT (MB-COMT) [1,6,7], exists in various tissues, and especially, has high activities in liver and kidney.

In the previous studies, it was found that in the liver of spontaneously hypertensive rats (SHR), both the activities and the amounts of MB-COMT were lower than those of Wistar-Kyoto (WKY) rats. It is worthy of note that MB-COMT in liver has a principal role in blood pressure regulation in rats [8,9]. Moreover, there are many studies indicating that low and high COMT activities affect various diseases, although in those studies, S- and MB-COMT activities were not determined separately. Low COMT activities seem to affect an obsessive-compulsive disorder (OCD), aggressive and highly antisocial impulsive schizophrenia, bipolar affective disorder, Parkinson's disease (PD), and breast cancer [1,10–16]. It was found that the reduction in COMT activities was associated with a recessive manner with susceptibility to OCD, particularly in males [10]. Schizophrenic patients who were homozygous for the low COMT activity allele seemed to have higher risks for aggressive and dangerous behavior than those who were homozygous for the high activity allele [11]. The low COMT activities also appeared to be risk factors for the development of rapid-cycling bipolar disorder [12,13]. It seemed that homozygosity for the low COMT activity allele constituted a genetic risk factor for PD among Japanese [14,15] and that postmenopausal women in low COMT activities had a greater than two-fold increased risk of developing breast cancer [16]. On the other hand, there are some studies that high COMT activities affect anorexia nervosa, polysubstance abuse, and suicide [17–19]. It was suggested that those who homozygous for the high activity allele had a two-fold increased risk of developing

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anorexia nervosa [17]. Individuals in high COMT activities seemed to have greater genetic vulnerability to drug abuse [18]. A high-activity COMT genotype was significantly less in male suicide completers than in male controls [19]. Thus, the estimation of COMT activities is necessary in clarifying the role of COMT in blood pressure regulation and the association of COMT activities with various diseases. In the future, the measurement of COMT activities may help both the diagnosis and the etiology identification of various diseases.

The methods for measuring COMT activities in rat tissues (erythrocyte, liver, kidney, adrenal and brain) and human erythrocyte have already been established with high-performance liquid chromatography (HPLC)-fluorescence or chemiluminescence detection using NE as a substrate [9,20–23]. They included on-line extraction of catecholamines with a precolumn, separation of NE and NMN on an ODS column, electrochemical oxidation, post-column fluorogenic derivatization using ethylenediamine, and fluorescence or chemiluminescence detection. Accordingly, the methods are very sensitive compared with other methods, such as a radiochemical assay [24–27] and HPLC-electrochemical detection [28–30]. However, the methods take about an hour for the analysis of one sample, and thus are not suitable for the measurement of many samples [9,20–23]. A rapid assay was developed employing flow injection analysis (FIA) to measure the activities of as many as 20 samples in an hour [31], but the method was not precise enough for the measurement of COMT activities of biological samples because of the lack of an internal standard. In this study, we therefore developed a method that enabled the measurement of COMT activities of biological samples in a shorter time.

2. Experimental

2.1. Reagents

NE, NMN, *S*-adenosyl-L-methionine (SAMe) chloride salt and 4-methoxytyramine (4-MT), 4-hydroxy-3-methoxybenzylamine (HMBA) were from Sigma Chemical Co. (St. Louis, MO, USA). Imidazole and 1,4-dithiothreitol were obtained from Merck (Darmstadt, Germany). Ethylenediamine was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Acetonitrile and ethanol were from Kanto Chemical Co. (Tokyo, Japan). All reagents in this study were of analytical grade.

2.2. Sample preparation

Male Sprague–Dawley rats (6–7 weeks old, 200–220 g) were obtained from Charles River Japan Inc. (Kanagawa, Japan) and housed under controlled environment (22–24 °C and a 12 h light–dark cycle) with human care according to the National Institute of Health guideline. The rats were anesthetized with diethyl ether. After blood was quickly removed from abdominal aorta, liver and cerebral cortex were immediately removed and chilled on ice. Tissues were homogenized with 50 mM sodium phosphate buffer (pH 7.5) containing 0.5 mM 1,4-dithiothreitol. The homogenates were centrifuged at 100,000 × *g* at 4 °C for 30 min and the supernatant was stored as S-COMT sample. The pellet was washed with 2 ml of homogenizing buffer (pH 7.5) and re-centrifuged at 100,000 × *g* at 4 °C for 30 min. Then the pellet was suspended in 2 ml of homogenizing buffer for MB-COMT sample. These fractions were frozen at −80 °C until the COMT assay.

2.3. Procedure for COMT activity measurement

In S-COMT assay, 10 μl of the liver sample or 40 μl of the cerebral cortex sample were incubated with 290 μl or 260 μl (for liver or cerebral cortex, respectively) of 50 mM sodium phosphate buffer (pH 7.8) containing 1.5 mM NE, 2 mM $MgCl_2$ and 200 μM SAMe [9,22]. While, in MB-COMT assay, 10 μl of the liver sample or 40 μl of the cerebral cortex sample were incubated with 290 μl or 260 μl (for liver or cerebral cortex, respectively) of 50 mM sodium phosphate buffer (pH 7.4 or pH 7.0 for liver or cerebral cortex, respectively) containing 200 μM NE, 2 mM $MgCl_2$ and 200 μM SAMe [9,22]. After incubation at 37 °C for 10–30 min, the reaction was stopped by adding 30 μl of 4.0 M perchloric acid and keeping reaction mixtures on an ice-bath for 2 min. The mixtures were centrifuged at 4000 × *g* at 4 °C for 10 min. In the case of liver sample, 10 μl aliquot of the supernatant was added to 190 μl of the sample dilution buffer [10 mM glutathione, 10 mM citric acid, 100 mg/l EDTA-2Na and 6.25 μM HMBA (internal standard) (pH 4.5)], and for cerebral cortex sample, 30 μl aliquot of the supernatant was added to 170 μl of the sample dilution buffer and 150 μl of 200 μl aliquots were injected into the HPLC system. Protein concentrations of the samples were determined according to Bradford [32] with bovine serum albumin as the standards and calibration control samples.

2.4. HPLC conditions

COMT activities were valued by measuring the amount of NMN, which is a metabolite of NE by COMT. To determine NMN, HPLC-fluorescence detection system (Fig. 1) was used [9,22,33]. The system included on-line extraction of amines including NE and NMN on a cation-exchange precolumn with pretreatment buffer, separation of NE and NMN on an ODS column, electrochemical oxidation, post-column fluorogenic derivatization, and fluorescence detection. Three HPLC pumps (PU-2080 and PU-980, JASCO, Tokyo, Japan), an autosampler (950-AS, JASCO), a rotatory six-way switching valve (HV-992-01, JASCO), an electrochemical coulometric reactor (Coulochem 5100A, ESA) and a fluorescence detector (2025-FP, JASCO) were used. A pre-column, CAPCELL PAK MF-SCX cartridge, 10 mm × 4.0 mm i.d. (SHISEIDO, Tokyo, Japan) and a separation column, Unison UK-

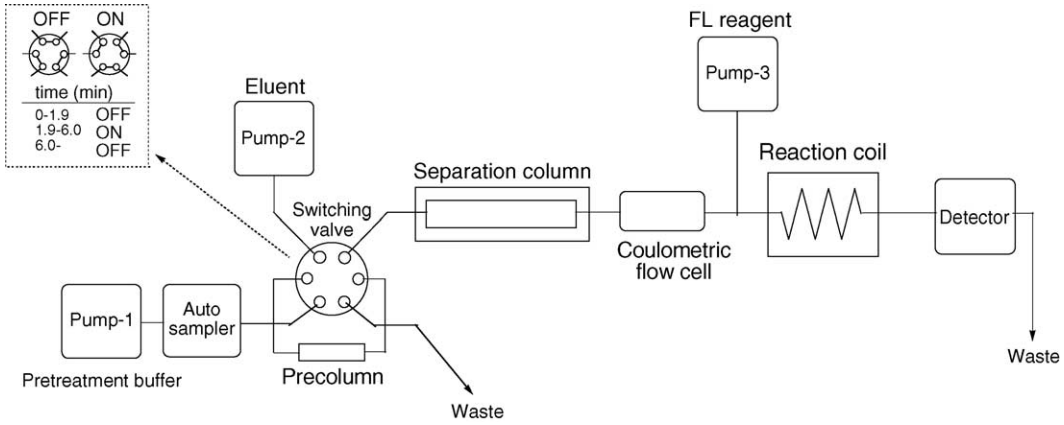

Fig. 1. Block diagram for the column-switching HPLC-fluorescence detection system.

C18, 30 mm × 4.6 mm i.d. (Imtakt, Kyoto, Japan) kept at 40 °C in a column oven were used. The fluorescence detection was carried out at an emission wavelength of 505 nm with excitation at 430 nm. The pretreatment buffer consisted of 10 mM potassium phosphate buffer (pH 3.2) and was pumped at a flow rate of 1.5 ml/min. The eluent was 75 mM potassium acetate buffer (pH 3.2)/50 mM potassium phosphate buffer (pH 3.2)/acetonitrile (93.1/4.9/2, v/v/v) containing 10 mM sodium 1-hexanesulfonate and the flow rate was 0.5 ml/min. The fluorogenic (FL) reagent, 105 mM ethylenediamine and 175 mM imidazole in acetonitrile/ethanol/water (80/10/10, v/v/v), was pumped at a flow rate of 0.32 ml/min.

2.5. Optimization for fluorescence derivatization

The effects of the length of a reaction coil were investigated from 2.5 to 10.0 m. The temperature of the reaction coil was varied from 80 to 105 °C when the length of the coil was fixed at 2.5–10.0 m. During investigation of the length of the coil and the reaction temperature, the flow rates of the eluent and the FL reagent were 0.5 and 0.32 ml/min, respectively. We determined the peak area of the reaction product of NMN after electrochemical oxidation, and post-column fluorogenic derivatization using ethylenediamine.

2.6. Measurement of K_m values

The K_m values for NE in cerebral cortex were determined by varying the concentrations of the NE (0.050–2.0 mM and 0.5–200 μM, for S and MB-COMT, respectively) using the Lineweaver Burk plot [34]. Values of all the data in this paper are described as the mean ± standard deviation.

3. Results and discussion

3.1. Optimization for the HPLC conditions

We previously established the methods to measure COMT activities of rat tissues (erythrocyte, liver, kidney, adrenal and brain) and human erythrocyte using HPLC-fluorescence or chemiluminescence detection [9,20–23]. The system included an on-line extraction of catecholamines with a precolumn, separation of NE and NMN on an ODS column, electrochemical oxidation, post-column fluorogenic derivatization using ethylenediamine, and fluorescence or chemiluminescence detection. However, it took about 60 min to measure the COMT activity in one sample. To shorten the analysis time for the measurement of COMT activity, in this study, the internal standard, the length of the separation column and the length of a reaction coil were changed and the HPLC-conditions were re-optimized.

First, the internal standard was changed from 4-MT to HMBA because the retention time of 4-MT was much longer than those of the analytes and thus the most effective way for shortening the analysis time was by changing the internal standard. The retention times of 4-MT and HMBA were 54.3 and 25.1 min, respectively. At that stage, the peak of HMBA overlapped with that of the decomposed product of NE. Second, by shortening the length of a separation column, the retention times of all the analytes were shorter. And then, we examined the HPLC conditions for the sufficient separation between NE and NMN. To shorten the reaction time without the reduction of the sensitivity, the peak area of NMN after electrochemical oxidation and post-column fluorogenic derivatization with ethylenediamine was investigated by varying the temperature of the coil from 80 to 105 °C with the coil length fixed at 2.5, 5.0, 7.5, 10.0 m, respectively.

As shown in Fig. 2, the highest fluorescence intensity was achieved with the coil conditions, 10.0 m and 85 °C. However, our aim was shortening the analysis time, and hence we selected the coil conditions, 5.0 m and 95 °C. In fact, the sensitivity with this coil condition was sufficiently efficient to measure COMT activity.

3.2. Chromatographic separation

The representative chromatograms are shown in Fig. 3, using the samples of rat liver and cerebral cortex. Suffi-

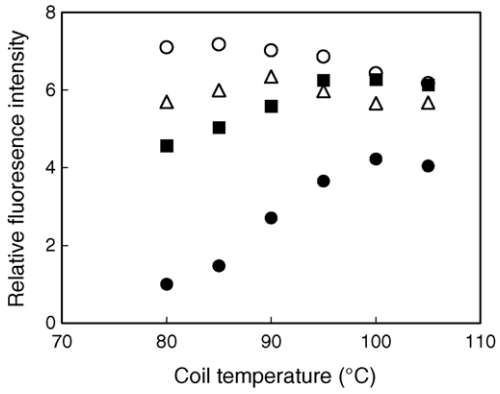

Fig. 2. Effect of the length of the reaction coil and temperature on the fluorescence intensity under the block diagram shown in Fig. 1. Coil lengths were varied from 2.5 to 10.0 m (2.5 m (●), 5.0 m (■), 7.5 m (△), 10.0 m (○)).

cient separation between NE (the excessive substrate) and NMN was achieved, and no other significant peaks except for 4-methoxy compound of NE were observed in the chromatograms. We were able to measure one sample in less than 25 min.

3.3. Validation of the proposed method

Table 1 shows the intra- and inter-day assay precisions of the proposed method for S- and MB-COMT of rat liver and cerebral cortex. All the values were within 2 and 6% for intra- and inter-day analyses, respectively ($n = 5$). Thus, this method achieved little fluctuation in both intra- and inter-day assay, and was more precise than the previous method, which included a troublesome double alumina extraction of the excess amount of NE before the FIA and determined without an internal standard [31]. Therefore, the proposed method is suitable for the routine assay of COMT activities in rat liver and cerebral cortex.

3.4. K_m values to norepinephrine

The K_m values for both S-COMT and MB-COMT to NE in rat cerebral cortex were 371 ± 60 and 11.8 ± 3.8 μM, respectively ($n = 3$). The results show that in cerebral cortex, MB-COMT has a higher affinity to NE than S-COMT. The trend was similar to that in the previous study [22].

Table 1
Precisions of the proposed method for the determination of rat liver and cerebral cortex COMT activities

	Intra-day variation (%) ($n = 5$)	Inter-day variation (%) ($n = 5$)
Liver		
S-COMT	1.3	3.6
MB-COMT	0.52	4.6
Cerebral cortex		
S-COMT	1.6	4.5
MB-COMT	1.2	5.8

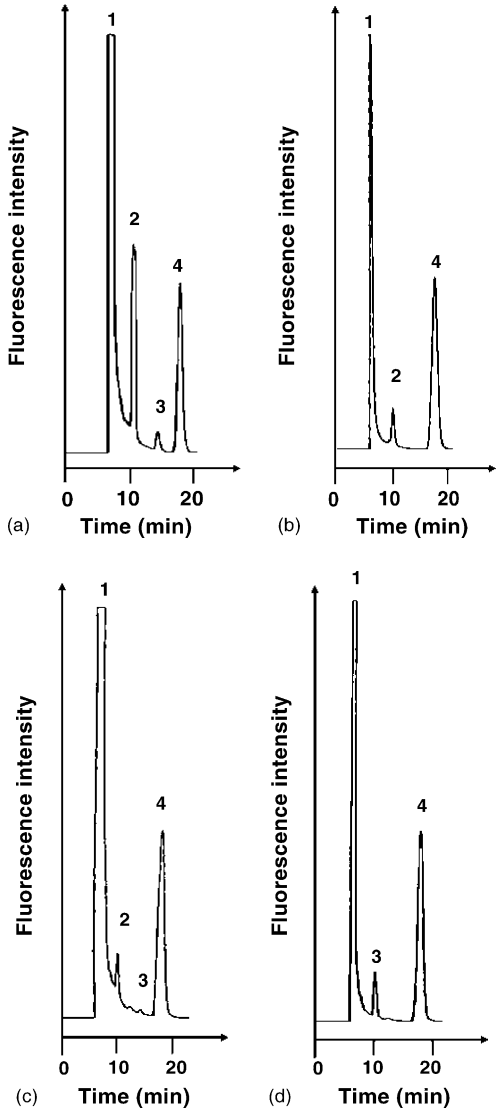

Fig. 3. Chromatograms of the reaction mixtures of (a) S-COMT or (b) MB-COMT sample in liver and (c) S-COMT or (d) MB-COMT sample in cerebral cortex of male Sprague–Dawley rats. Peaks: 1, NE; 2, NMN; 3, 4-methoxy compound of NE; 4, HMBA (internal standard). HPLC conditions are described in Section 2.

3.5. Rat liver and cerebral cortex COMT activity

It was reported that the activities of S-COMT was higher than those of MB-COMT in rat tissues (erythrocyte, liver, kidney, adrenal and brain) and human erythrocyte with the previous method [9,20–23]. Table 2 shows the S- and MB-COMT activities, evaluated by the amount of NMN (nmol)/reaction

Table 2
S- and MB-COMT activities in rat liver and cerebral cortex

	S-COMT, V_{max} (nmol/min/mg protein)	MB-COMT, V_{max} (nmol/min/mg protein)
Liver	6.21 ± 0.14	0.21 ± 0.04
Cerebral cortex	0.028 ± 0.004	0.012 ± 0.004

Values shown are the mean ± standard deviation ($n = 3$).

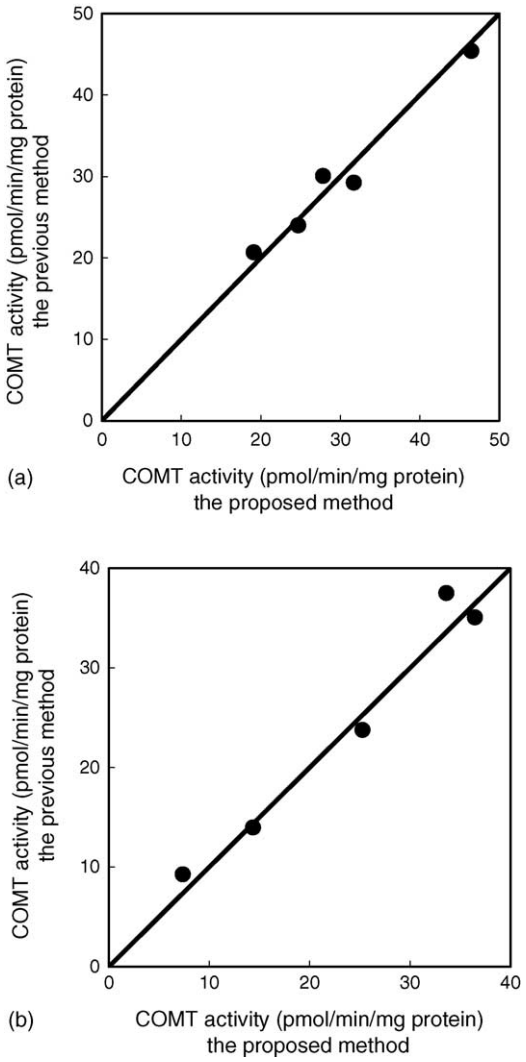

Fig. 4. Comparison of (a) S-COMT or (b) MB-COMT activities in cerebral cortex of male Sprague–Dawley rats ($n = 5$) between the proposed method and the previous one.

time (min)/mg protein, in rat liver and cerebral cortex with the proposed method. The results were similar with those obtained by the previous method [9,22].

3.6. Comparison between the proposed method and the previous method

In this study, to examine if the proposed method is an alternative to the previous one [22], we measured S- and MB-COMT activities of rat cerebral cortex with both methods. In Fig. 4 (a, S-COMT; b, MB-COMT), the straight lines indicate $y = x$ and each point almost locates on these lines. It shows that the COMT activities were similar with both methods. The present method is therefore a good alternative to the previous one.

In conclusion, we have developed a rapid and precise method for the measurement of COMT activities in rat liver and cerebral cortex. This rapid method enables us to examine the COMT activities in various tissues (erythrocyte, kidney, and individual brain areas; cerebellum, hippocampus, brain stem, hypophysis, hypothalamus) and those are currently under investigation. We propose that this method provides further understanding of the role of COMT in the regulation of blood pressure and the association of COMT activities with various diseases in both brain and periphery.

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