Focusing Review

Development of an Analytical Method for Catecholamines with HPLC–Chemiluminescence Detection and its Application

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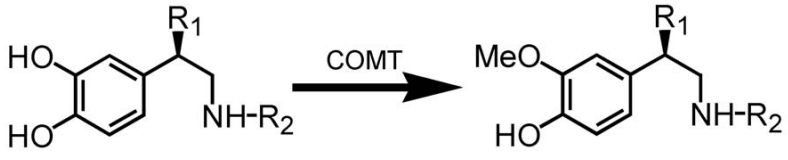
Abstract

Catecholamines play important roles as neurotransmitters and hormones, and are metabolized by catechol–*O*–methyltransferase (COMT) and inactivated. We have developed the highly selective and sensitive determination methods for catecholamines (and their 3–*O*–methyl metabolites) with high–performance liquid chromatography–peroxyoxalate chemiluminescence reaction detection. The developed methods were applicable to the clarification of the roles of catecholamines and their 3–*O*–methyl metabolites in blood pressure regulation.

Keywords: peroxyoxalate chemiluminescence; high–performance liquid chromatography (HPLC); catecholamines; catechol–O–methyltransferase (COMT); blood pressure

1. Introduction

Catecholamines (norepinephrine (NE), epinephrine (E) and dopamine (DA)) play important roles as neurotransmitters or hormones, and are known to be metabolized by catechol–*O*–methyltransferase (COMT) and monoamine oxidase. As shown in Figure 1, catecholamines are metabolized into their respective 3–*O*–methyl metabolites (normetanephrine (NMN), metanephrine (MN) and 3–methoxytyramine (3–MT)) by COMT. Catecholamines and/or their metabolites concentrations in plasma and/or urine are used as an index for several diseases such as hypertension and pheochromocytoma. Therefore, many methods have been developed for the determination of catecholamines and/or their metabolites. Most of them adopted high–performance liquid chromatography (HPLC)–electrochemical detection or fluorescence detection. However, there are many endogenous substances in bio–samples such as plasma or urine. Therefore, these methods have been used in combination with complicated sample pre–treatment such as solid phase extraction or liquid–liquid extraction to determine catechola-



R_1	R_2		
OH	H	Norepinephrine (NE)	Normetanephrine (NMN)
OH	CH_3	Epinephrine (E)	Metanephrine (MN)
H	H	Dopamine (DA)	3-Methoxytyramine (3-MT)

Figure 1. Metabolic pathway of catecholamines by COMT.

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mines selectively. Furthermore, since the plasma catecholamine concentrations are very low (pmol order per 1 ml of plasma), 1 ml of plasma is needed for the sensitivity of electrochemical or fluorescence detection.

In this article, the development of analytical methods for both catecholamines and catecholamines and their 3–*O*–methyl metabolites with HPLC–chemiluminescence detection is described. Application of the developed methods to the clarification of the roles of the sympathetic nervous system and COMT in blood pressure regulation is also described.

2. Development of an analytical method for catecholamines

Considering the background as mentioned above, Higashidate et al. applied HPLC–peroxyoxalate chemiluminescence (POCL) reaction detection to the determination of catecholamines [1], since higher sensitivity should be obtained with POCL detection than with fluorescence detection [2]. This method was as follows: (1) pre–treatment of samples with alumina extraction; (2) separation of catecholamines on an ODS column; (3) post–column derivatization of catecholamines with ethylenediamine, which produced fluorescence derivatives; (4) on–line POCL reaction detection. As expected, the limits of detection for catecholamines were 1 fmol on column, which were better than those previously reported. Prados et al. modified the method and reported the column–switching system for the determination of catecholamines [3].

We have adopted a semi–micro column (1.5 mm I.D.) to improve the sensitivity [4]. The block–diagram of the system is shown in Figure 2. Figure 3 shows the chromatograms of a mixture of standard catecholamines and of a rat plasma sample. As a result, 3–10 times higher sensitivity was obtained compared with the method with conventional column.

3. Investigation of baroreflex–mediated sympathetic nervous system with the developed methods

Blood pressure is regulated by the integrated action of cardiovascular, renal, neural and endocrine system, and the sympathetic nervous system is important for momentary regulation. Sympathetic nerve outflow is controlled by baroreflex sensor systems. In response to a sudden fall in arterial pressure, afferent signals are sent to central nervous system to activate sympathetic nervous system resulting in increased NE outflow.

We investigated the role of baroreflex–mediated sympathetic nervous system in blood pressure regulation in conscious rats [5]. When blood pressure was reduced by the administration of a calcium antagonist, blood pressure reduction correlated with the increase of plasma NE and E concentrations. Further studies of administration of a calcium antagonist to spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats revealed the reduced response of baroreflex–mediated sympathetic nerve system in SHR compared with WKY rats.

4. Development of an analytical method for catecholamines and their 3–*O*–methyl metabolites in plasma

Simultaneous determination of catecholamines and their 3–*O*–methyl metabolites was thought to be useful for the examination of sympathoadrenal activity as well as metabolic activity of catecholamines by COMT. Hence, we attempted to develop the simultaneous determination method for catecholamines and their 3–*O*–methyl metabolites in rat plasma [6]. However, since the fluorescence reaction of catecholamines with ethylenediamine is selective for catechol compounds, catecholamines metabolites were not able to react with ethylenediamine. With the coulometric oxidation of catecholamines metabolites into *o*–quinone compounds followed by fluorescence reaction with ethylenediamine, the simultaneous determination of catecholamines and their 3–*O*–methyl metabolites

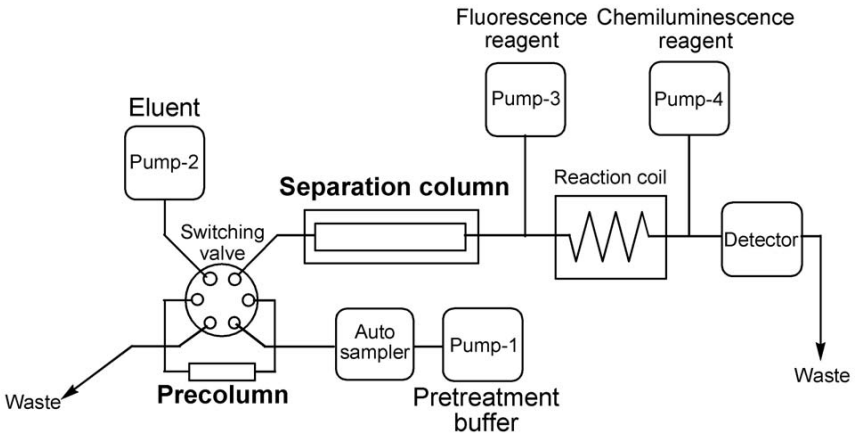

Figure 2. Block diagram of the column–switching HPLC system for catecholamine analyzer.

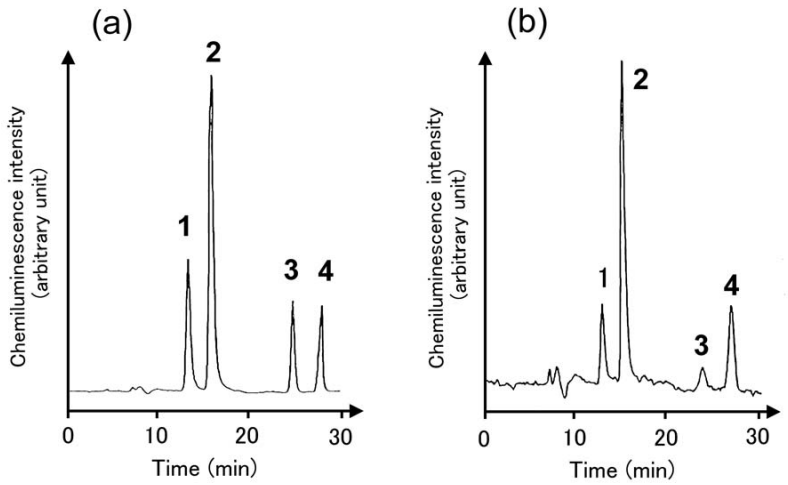

Figure 3. (a) Chromatogram of a standard mixture of catecholamines (250 fmol each). (b) Chromatogram obtained from a Sprague–Dawley rat plasma sample. Peaks: 1, norepinephrine; 2, epinephrine; 3, dopamine; 4, *N*–methyldopamine (internal standard).

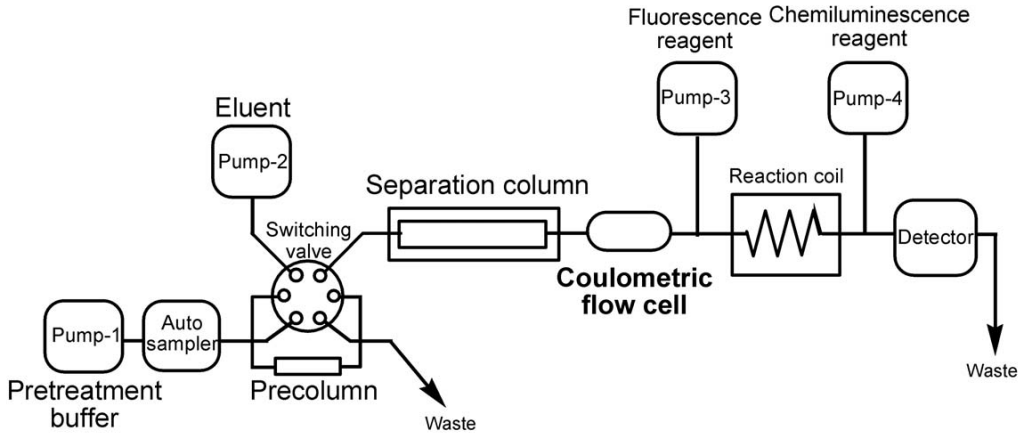

Figure 4. Block diagram of the column–switching HPLC system for the automated analyzer of catecholamines and their 3–*O*–methyl metabolites.

was achieved. Figure 4 shows the block diagram for the system, which included on–line extraction of amines using a precolumn packed with cation exchange resin, separation of catecholamines and their 3–*O*–methyl metabolites on an ODS column, coulometric oxidation of metabolites, fluorogenic derivatization with ethylenediamine, and postcolumn POCL detection.

A typical chromatogram of 250 fmol of NE, E, DA, NMN, MN, 3–MT, and 4–MT (internal standard) is shown in Figure 5 (a). Figure 5 (b) shows a typical chromatogram obtained from a Sprague–Dawley rat plasma sample (50 µl). With the exception of 3–MT, the peaks for NE, E, DA, NMN, and MN in the rat plasma were clearly shown with no interference from endogenous compounds. This implies the high selectivity of the proposed method, since it includes on–line selective extraction of amines by the pre–column and a selective ethylenediamine condensation for catechol compounds. The concentrations in rat plasma were 1.05 ± 0.03, 0.64 ± 0.02, 0.19 ± 0.01, 0.51 ± 0.02, 0.26 ± 0.01 pmol/ml (n=3, mean ± SEM) for NE, E, DA, NMN and MN, respectively.

Improved sensitivity could be obtained with the use of a semi–micro column [7]. To avoid extra–column peak diffusion, a micro coulometric flow cell was developed. It enabled to lower the detection limits for catecholamines and their 3–*O*–methyl metabolites to 0.3–2.0 fmol.

The developed method was applicable to the determination of catecholamines in 15 µl of mouse plasma [8]. Figure 6 shows the chromatograms of a standard mixture of catecholamines and their 3–*O*–methyl metabolites and obtained from a mouse plasma sample. Previously, due to the lack of the sensitivity, we were not able to determine plasma catecholamines concentrations continuously from a mouse. However, we could obtain some plasma samples

from a mouse with the developed method, and we have found that after the administration of minoxidil, which is an antihypertensive agent, plasma catecholamines were significantly increased by the baroreflex–mediated response in mouse.

5. Development of an analytical method for catechol–*O*–methyltransferase activity

COMT catalyses the transfer of the methyl group of *S*–adenosyl–L–methionine (SAMe) to catechol compounds including catecholamines. COMT is distributed in most tissues, and there are two forms, one is soluble form (S–COMT) and the other is membrane–bound form (MB–COMT).

There are many measurement methods for COMT activity. However, many adopted exogenous substrates, and we have developed an analytical method for COMT activity with catecholamines as the endogenous substrate. COMT activities were evaluated by the quantitation of catecholamines metabolites produced by the enzymatic reaction. The analytical method for catecholamines metabolites was the same as that for plasma sample. COMT activities in rat erythrocytes [9], rat kidney and liver [10, 11], rat adrenal gland [12], rat brain [13] and human erythrocytes [14] were measured. Typical chromatograms obtained from the reaction solution of rat erythrocyte S–COMT and MB–COMT, with NE as the substrate, are shown in Figure 7.

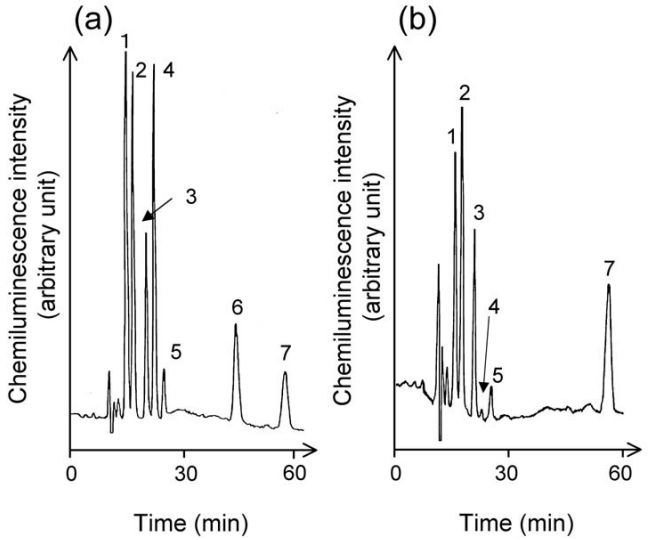

Figure 5. Chromatograms (a) of a standard sample of catecholamines and their 3–*O*–methyl metabolites (each 250 fmol) and (b) obtained from rat plasma sample (50 μl). Peaks: 1, norepinephrine; 2, epinephrine; 3, normetanephrine; 4, dopamine; 5, metanephrine; 6, 3–methoxytyramine; 7, 4–methoxytyramine (internal standard).

6. Investigation of catechol–*O*–methyltransferase in blood pressure regulation

As mentioned above, in blood pressure regulation, catecholamines play important roles, and therefore, COMT is also important factor for the regulation of catecholamines. However, so far, relationship between COMT and blood pressure has not been examined. We determined plasma catecholamines and their 3–*O*–methyl metabolites concentrations after the administration of a calcium antagonist with the developed method, and found that methylation of catecholamines by COMT in SHR was reduced as compared to WKY rats [15]. Furthermore, we found the reduced amounts and activity of liver MB–COMT in SHR [16]. These results indicate that liver MB–COMT plays an important role in blood pressure

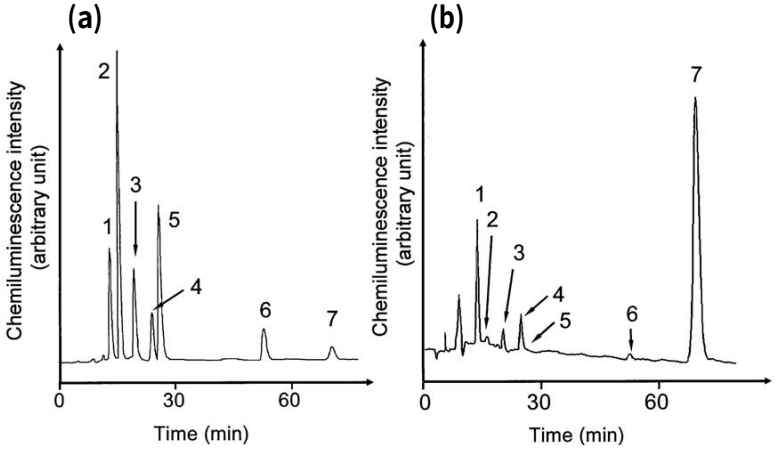

Figure 6. Chromatograms (a) of a standard mixture of catecholamines and their 3–*O*–methyl metabolites (each 250 fmol), and (b) obtained from a mouse plasma when 30 μL of blood were collected from veins in both orbitae. Peaks: 1, norepinephrine; 2, epinephrine; 3, normetanephrine; 4, dopamine; 5, metanephrine; 6, 3–methoxytyramine; 7, 4–methoxytyramine (internal standard).

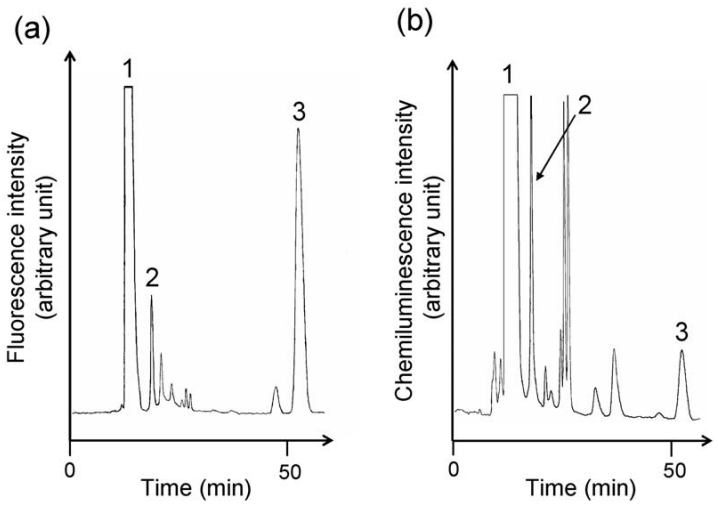

Figure 7. Chromatograms obtained from reaction solution of rat erythrocytes (a) S−COMT and (b) MB−COMT. Peaks: 1, norepinephrine; 2, normetanephrine; 3, 4−methoxytyramine (internal standard).

regulation.

7. Conclusions

We have developed the simultaneous and sensitive determination methods for catecholamines and their 3−*O*−methyl metabolites with HPLC−POCL detection [17]. These methods enabled us the clarification of the role of COMT in blood pressure regulation. In the near future, further clarification of the physiological role of catecholamines and their metabolism should be expected.

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