Improved Extraction Method for Catecholamines Using Monolithic Silica Disk-Packed Spin Column

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Abstract
We have previously reported analytical methods for the quantification of catecholamines (norepinephrine, epinephrine, and dopamine) via simple pretreatment using a monolithic silica disk-packed spin column with an attached phenylboronate moiety. However, under certain conditions, splitting in the dopamine peak was observed. In this study, we investigated the reason for this peak splitting and found that anions in the basic buffer solution used in the extraction influenced the peak shape. The extraction could be improved via additionally washing the column with a low-concentration buffer. The extraction recoveries of the catecholamines via the improved method were in the range of 95.9–100.8%. Thus, the improved method is expected to be more reliable for the quantification of catecholamines in biological samples.

Keywords: Dopamine; HPLC; Phenylboronate; Peak splitting; Pretreatment

1. Introduction
Catecholamines such as norepinephrine (NE), epinephrine (E), and dopamine (DA) (Fig. 1) are important neurotransmitters and hormones that mediate various neurophysiological processes (stress, anxiety, and depression). The quantification of catecholamines in the physiological systems facilitates a better understanding of disease mechanisms, thereby aiding the diagnosis of many diseases. Thus, the analysis of biological compounds including catecholamines in biological samples is of great importance [1-4].

Numerous techniques have been utilized for the determination of catecholamines. Although high-performance liquid chromatography (HPLC)-peroxoxyxalate chemiluminescence detection methods are highly sensitive and selective for the quantification of catecholamines [5-8], HPLC-electrochemical detection methods are commonly adopted for the analysis of these compounds in biological fluids [9-12]. Previously, we developed an analytical method involving the selective extraction of catecholamines in monolithic silica disk-packed spin columns containing a phenylboronate moiety, followed by HPLC-electrochemical detection, which was applied to urine sample [13]. These methods could indeed clarify some significant roles of catecholamines in diseases [14-16]. However, a technical problem—the peak splitting of DA—was encountered occasionally, wherein a single peak was observed when standard DA solution was injected directly, while peak splitting occurred when DA solution extracted via solid-phase extraction was injected.

In this study, we aimed to investigate the reason for the peak splitting and thereby find a procedure to obtain a single peak of DA using solid-phase extraction. Anions in the sample buffer (phosphate or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer) were found to influence the peak splitting; thus, an improved extraction procedure was developed on the basis of this observation.

2. Experimental
2.1. Materials
Norepinephrine and epinephrine were obtained from Tokyo Chemical Industry (Tokyo, Japan). Dopamine, sodium acetate, citric acid monohydrate, sodium 1-octanesulfonate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were purchased from FUJIFILM Wako Pure Chemical (Osaka,
Japan). Sodium hydroxide was from Kanto Chemical (Tokyo, Japan). MeCN (HPLC grade) was from Merck KGaA (Darmstadt, Germany). A Milli-Q system (Merck) was used for water purification.

![Chemical structures of catecholamines](image)

**Fig. 1.** Chemical structures of catecholamines.

2.2. Standard solution

Standard samples of 500 µM NE, E, and DA were prepared in 1% aqueous acetic acid by diluting the respective standard solutions with the solvent.

2.3. Extraction method

Solid-phase extraction of catecholamines was performed using monolithic silica disk-packed spin columns containing a phenylboronate moiety (GL Sciences, Tokyo, Japan).

2.3.1. Original extraction method

Previously, 200 µL of 1% aqueous acetic acid was poured into the spin column and centrifuged at 3,000 × g for 5 min at 4°C; the same centrifugal conditions were employed in this study. Subsequently, 200 µL of 100 mM phosphate buffer (pH 8.0) was poured into the spin column, following which centrifugation was repeated. This operation was conducted to prevent compounds from not being retained. A premixed solution of 100 µL standard sample and 100 µL 1 M phosphate buffer (pH 8.0) was then poured into the preactivated spin column and centrifuged. The column was washed via centrifugation with 200 µL 100 mM phosphate buffer (pH 8.0). Finally, catecholamines were eluted via centrifugation using 200 µL of 1% aqueous acetic acid. Ten microliters of the eluent was injected into the HPLC system.

2.3.2. Improved extraction method

As part of the improved extraction method, the column was additionally rinsed with 200 µL of 10 mM phosphate buffer (pH 8.0) after being washed with 100 mM phosphate buffer (pH 8.0). Subsequently, the catecholamines were eluted as per the original method. In another set of experiments, HEPES buffer of the same concentration was used instead of the phosphate buffer.

2.4. HPLC system

The HPLC setup (Hitachi, Tokyo, Japan) was equipped with a 5110 pump, 5310 column oven, and 5430 diode array detector. The analytes were separated on an Inertsil ODS-4 column (150 × 3.0 mm, 5.0 µm, GL Sciences) and detected at 260 nm. Twenty millimolar sodium acetate-citrate buffer/MeCN (85/15, v/v) containing 1 g/L of sodium 1-octanesulfonate as an ion-pairing reagent was used as the mobile phase. A flow rate of 0.3 mL/min was maintained, while the column temperature was maintained at 40°C.

2.5. Recovery

Extraction recoveries of the catecholamines were calculated by dividing the peak areas of the samples after solid-phase extraction by those of the standard samples.

3. Results and discussion

3.1. Investigation of peak splitting

First, we confirmed the peak splitting of DA. Based on our previous report [6], the three catecholamines were separated on an ODS column using acetate-citrate buffer/MeCN with 1-octanesulfonate being used as the mobile phase. When a standard sample of catecholamines dissolved in 1% acetic acid was injected, the three catecholamines were well separated with properly resolved peaks. (Fig. 2a). However, when the standard sample was injected after the solid-phase extraction (extraction methods are described in the experimental section), the peak for DA was split, as shown in Fig. 2b.

Since no peak splitting of DA was observed with the standard sample in 1% aqueous acetic acid, whereas the peak of the same compound was split after solid-phase extraction, it was speculated that the sample obtained after solid-phase extraction contained some compounds that influenced the peak shape of DA. We hypothesized that a small amount of buffer remained in the spin column after washing was conducted with 100 mM phosphate buffer, and this got mixed in the extracted solution. Hence, the effect of phosphate buffer on the peak shape was examined.

Standard samples in different concentrations of phosphate buffer (10, 25, and 50 mM) were chromatographically analyzed. It is evident from Fig. 3 that the extent of peak splitting was higher for higher buffer concentrations.

![Chromatograms of (a) standard sample and (b) extracted standard sample. Peaks: 1, NE; 2, E; 3, DA split into two peaks.](image)

**Fig. 2.** Chromatograms of (a) standard sample and (b) extracted standard sample. Peaks: 1, NE; 2, E; 3, DA split into two peaks.
The result indicated that the peak splitting of DA was probably caused by the phosphate buffer (phosphate ions). To confirm this, the HEPES buffer was examined (instead of phosphate buffer) as it was used as the wash buffer during the solid-phase extraction. Similar results were obtained in this case as compared to those for phosphate buffer. It appears that the anions in the phosphate or HEPES buffer interact with the ion pair reagent (1-octanesulfonate) in the mobile phase and induce peak splitting, and the interaction depends on the kind of anion.

3.2. Improvement of the extraction procedure

As mentioned above, the anion in the injected sample causes the peak splitting of DA. In order to prevent the peak splitting, the extraction using monolithic silica disk-packed spin column was re-investigated with the aim of decreasing the anion content in the extracted sample. As part of the new protocol, the spin column was additionally washed with a lower concentration of phosphate buffer (10 mM) before extraction to reduce the phosphate ion content in the spin column. Chromatograms of the extracted sample, with and without the additional washing step being conducted, are shown in Fig. 5a and 5b. A single peak of DA was successfully obtained upon the additional washing step being conducted. The chromatograms obtained using HEPES buffer were better than those obtained using phosphate buffer. These observations confirmed that the peak splitting was caused by anions.

As the additional washing process was performed, the recovery of the extraction with the spin column was calculated. The recoveries for NE, E, and DA were 100.8%, 99.1%, and 100.0%, respectively, with phosphate buffer and 97.5%, 95.9%, and 97.0%, respectively, with HEPES buffer. The values were similar to those obtained using the original protocol, suggesting that the additional washing step did not influence the recoveries of the catecholamines.

4. Conclusion

In this study, anions in the phosphate or HEPES buffer in the extracted sample solution were found to cause the peak splitting of DA. The extraction procedure was reexamined, and the inclusion of an additional washing step using a lower concentration buffer was observed to prevent the peak splitting for the cases of both the buffers. This improved method is therefore expected to be more reliable for the quantification of catecholamines in biological samples.

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