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Simultaneous determination of catecholamines and polyamines in PC-12 cell extracts by micellar electrokinetic capillary chromatography with ultraviolet absorbance detection

Guanshu Liu, Junnan Chen, Yinfa Ma*

Department of Chemistry, University of Missouri-Rolla, 1870 Miner Circle, Rolla, MO 65409, USA

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Abstract

A method for simultaneous determination of polyamines and catecholamines in cell extracts by micellar electrokinetic capillary chromatography with UV detection at 254 nm was established at the first time. The polyamines (putrescine, spermidine and spermine) and catecholamines (dopamine, serotonin, norepinephrine and epinephrine) were extracted from PC-12 cells and were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. Different derivatization conditions such as temperature, ratio of derivatization reagents and incubation time were investigated to find the best reaction condition which gave the highest detection sensitivity for polyamines and catecholamines. The influence of running buffer and additives on the separation such as pH, sodium dodecyl sulfate (SDS) concentrations and various additives was also investigated. Separation was achieved within 20 min with good repeatability in a 100 mM boric acid buffer containing 10 mM SDS and 10 mM 18-crown-6 at a pH of 9.5. The detection limit ranged from 1.0×10^{-7} to 9.0×10^{-7} M, which is sufficient for determination of polyamines and catecholamines in many cell extracts. This technique can be easily applied to polyamine-related anticancer drug studies or clinical follow-ups after each dosage of these anticancer drugs, since these drugs not only have great inhibition on polyamine levels in blood, but also have a large influence on catecholamine levels in blood.

Keywords: PC-12 cell extracts; Catecholamines; Polyamines

1. Introduction

Polyamines spermine (Spm), spermidine (Spd) and putrescine (Put), as shown in Fig. 1, are ubiquitous components in eukaryotic and prokaryotic cells. They play an important role in cell proliferation, cell growth and synthesis of protein and nucleic acids [1–3]. Rapid tumor growth has been associated with remarkable elevation of polyamine biosynthesis and accumulation [4], which leads to higher concentrations of polyamines in urine or serum of almost all cancer patients [4–10]. Despite of the limitations of polyamines as markers for malignant tumors [11,12], polyamines now are still considered as one group of the tumor markers in humans (although not as a sole marker) and as tracers for evaluating the effectiveness of anticancer drugs [13,14]. Catecholamines, such as epinephrine, norepinephrine, serotonin and dopamine (their structures are shown in Fig. 1), occur naturally in the body and serve as hormones or as neurotransmitters in the sympathetic nervous system [15]. Changes in catecholamine levels have been correlated with stress, heart disease, changes in blood pressure and thyroid hormone levels, catecholamine-secreting tumors, neuromuscular disorders and various mental diseases [16–20]. Recent studies found that certain polyamine inhibitor-related anticancer drugs such as α-difluoromethylornithine (DFMO) and methylglyoxal bis(guanylhydrazone) (MGBG) have strong impact on the levels of catecholamine while they inhibit the production of polyamines by cells [21,22]. Therefore, development of a method that can simultaneously assess the levels of polyamines and catecholamines in cells will greatly benefit polyamine-related anticancer researches.

A number of papers have been published for quantitative analysis of polyamines, including gas chromato-

* Corresponding author. Tel.: +1-573-341-6220; fax: +1-573-341-6033.
E-mail address: yinfa@umr.edu (Y. Ma).

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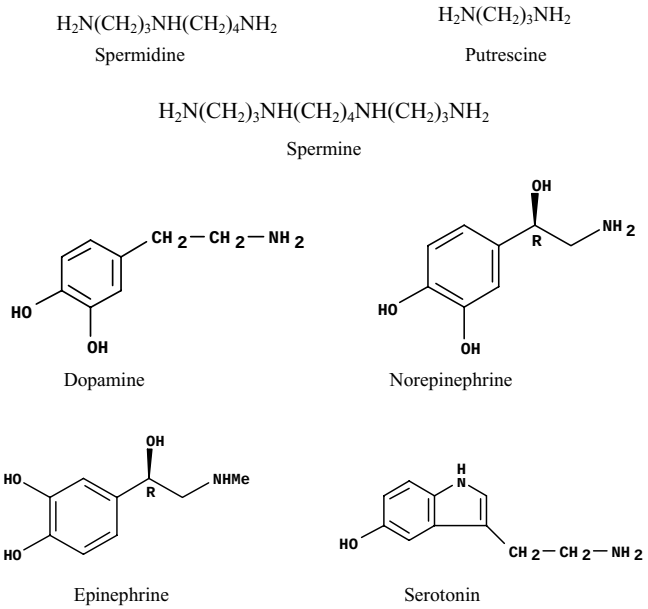

Fig. 1. Structures of polyamines and catecholamines.

graphy [23–28], high-performance liquid chromatography (HPLC) with derivatization [29–37], enzymatic assay [38], and capillary electrophoresis (CE) with indirect absorption detection or fluorescence detection with precolumn derivatization [39–44]. Assessment of catecholamines was generally performed by using liquid chromatography with electrochemical detection, UV absorption and fluorescence detection after derivatization [37,45–47]. Due to the unique advantages of CE in bioanalysis, determination of catecholamines in biological samples with CE has been demonstrated by using various detection techniques [48–54]. Amperometric detection of polyamines [55–57] and catecholamines [58–63] have been accomplished after HPLC or CE separations. However, the simultaneous detection of both polyamines and catecholamines were not performed due to their different detection conditions, such as buffer types, pH values and applied voltages. Actually, simultaneous determination of polyamines and catecholamines in biological samples by using CE or HPLC has never been demonstrated up to date due to the different sample extraction processes for polyamines and catecholamines and/or different detection schemes.

In this paper, we demonstrated a successful separation and detection of catecholamines and polyamines in PC-12 cell extracts by using micellar electrokinetic capillary chromatography (MECC) with UV absorption detection. The method is simple, rapid and quite sensitive. The method had a detection limit of 10^{-7} M for catecholamines and polyamines. Effects of some factors on separation of catecholamines and polyamines including pH, sodium dodecyl sulfate (SDS), derivatization conditions and additives have been tested and discussed in detail in the paper.

2. Experimental

2.1. Chemicals

Three authentic polyamines and four authentic catecholamines, including putrescine, spermidine, spermine, dopamine, norepinephrine, epinephrine, and serotonin were purchase from Sigma (St. Louis, MO, USA). Solutions (10 μM) of each were prepared by dissolving into 0.1 M HCl containing 0.4 mM sodium sulfite to prevent oxidation and stored at 4 °C for short term or −20 °C for long-term storage. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) and AccQ reagents were obtained from Waters (Milford, MA, USA). Sodium dodecyl sulfate and 18-crown-6 were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals used for preparation of the running buffers such as boric acid were also purchased from Sigma. Deionized water was obtained through a Milli-Q system (Millipore Corp, Bedford, MA, USA) and had a resistance larger than 15 MΩ.

2.2. Preparation of running buffer solution

The optimized running buffer solution was prepared by dissolving 100 mM boric acid, 10 mM 18-crown-6 and 10 mM SDS into deionized water and the solution pH was adjusted to 9.5 by using 1 M NaOH before diluting to final volume. In the study of effects of running buffer components, the concentrations of studied component were varied and the details are introduced in the later relevant sections. All buffer solutions were filtered through a 0.45 μm membrane filter before use.

2.3. Cell culture of PC-12 cells

The rat pheochromocytoma (PC-12) tumor cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and was cultivated with RPMI-1640 medium supplemented with 5% fetal calf serum and 10% heat inactivated horse serum, 1% glutamine and 1% penicillin/streptomycin (Invitrogen Co., Carlsbad, CA, USA). Cells were maintained at 75 cm^2 flasks until use.

2.4. Extraction of polyamines and catecholamines from PC-12 cells

When the PC-12 cells were ready to be harvested, the cell number was counted. Then, the growth media were removed by aspiration and the cells were washed three times with 15 ml of 0.3 M sucrose (pH 7.4). A 500–600 μl aliquot of 15% iced trichloroacetic acid (TCA) was added to the cell pellet. The samples were stored frozen at −20 °C until further analysis. The frozen samples were thawed at room temperature and immediately centrifuged at 10,000 × *g* for 10 min at 5 °C. The supernatant was extracted three times with 1.5 ml diethyl ether to remove the extra TCA. After the

pH was increased to pH 1–2 through ether extraction, the supernatant was immediately used for derivatization.

2.5. Derivatization of standards and cell extracts

The derivatization process of catecholamines and polyamines was similar to the reported methods with minor modification [40,64]. Briefly, 20 μl of extracts or standards was added to 40 μl borate buffer (provided with AccQ reagent kit) and briefly vortexed. Twenty microliters of AccQ-Fluor reagent (10 mM in acetonitrile) was added to the sample. The mixtures were vortexed and heated at 65 °C in a heating block for 20 min.

2.6. Instrumentation and electrophoretic procedures

All the experiments were carried out on a Beckman P/ACE MDQ UV capillary electrophoresis system (Fullerton, CA, USA). Fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA) with 50 μm i.d. (50 cm in length with 40 cm from inlet to detection window) was used for the separation.

Prior to each analysis, the capillary was sequentially rinsed with 0.1 M NaOH, deionized water and buffer (1 min each at 50 psi or 3.4 atm). The sample was then introduced into the capillary electrokinetically for 5 s at 5 kV. Separation was carried out under constant voltage of 20 kV at 25 °C. An ultraviolet detector was used with the filter setting at 254 nm due to the maximum absorption of derivatizations of amino compounds and the data were collected and processed by the Beckman P/ACE 32 karat software version 4.0.

3. Results and discussion

Fig. 2 demonstrates the separation of standard mixtures of catecholamines and polyamines at optimal conditions: 100 mM boric acid + 10 mM SDS + 10 mM 18-crown-6 with a pH of 9.5. The influence factors for separation of these analytes are investigated in the following sections.

3.1. Effect of buffer pH

It is well known that the buffer pH is crucial for MECC separations since it can not only affect micelle–solute interactions by changing the acid or base functionalities of solutes, but also affect surface characteristics of the capillary wall. In this study, a pH range of the running buffer from 7.5 to 10.5 was examined for standard mixtures of polyamines and catecholamines and the results are shown in Fig. 3. The separation of polyamines and catecholamines was also conducted at the pH values <7.5. We found that polyamines and catecholamines could not be separated at all and the signals were hardly detected. We suspected that the polyamines and catecholamines did not form micelles

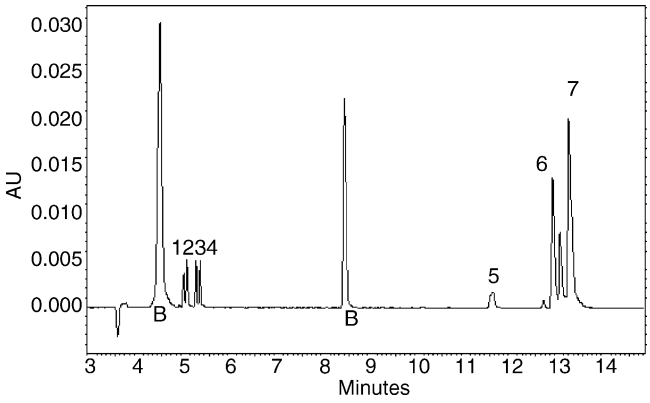

Fig. 2. Electropherogram of standard mixtures of three polyamines and four catecholamines by MECC with UV detection at 254 nm. Conditions: 50 cm × 50 μm i.d. fused-silica capillary (40 cm to the detector); 5 s injection electrokinetically at 5 kV; 20 kV operating voltage at 25 °C; running buffer is 100 mM boric acid buffer + 10 mM SDS + 10 mM 18-crown-6 at pH 9.5. The concentration is 1.0 μM for each standard. Peak identification: (1) norepinephrine; (2) epinephrine; (3) dopamine; (4) serotonin; (5) putrescine; (6) spermidine; (7) spermine; (B) system peak from AccQ.

with SDS at those pH values and the AccQ derivatives of polyamines and catecholamines might not be stable at lower pH values. The detailed reason will be further investigated. It was clear that the separation efficiency increased as increases of running buffer pH. However, the migration times of analytes increased dramatically when the buffer pH was over 9.5. The major reason was that the electroosmotic flow became greater and greater as the increase of buffer pH. Since micelles migrated against the electroosmotic flow under our experimental conditions and migration rate also increased as pH increased, the solutes transferring into the micelles would stay longer in column and had a longer retention time. This phenomenon was unusual compared to routine MECC separations because the analytes eluted slower as buffer pH increased. In routine MECC separations, analytes elutes faster as pH increases due to a greater electroosmotic flow. In our study, it seemed that the micelles migrated faster toward the

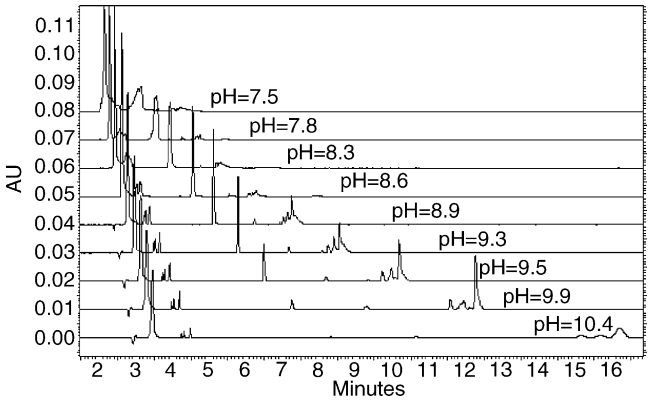

Fig. 3. The pH effect on the separation of catecholamines and polyamines by MECC. The experimental conditions were the same as those of Fig. 2, except the variation of pH values and without 18-crown-6.

injection end as pH increased and did not maintained a constant electrophoretic mobility at different pH values. Since the average velocity of analytes in MECC system is related to several factors [65], the detailed mechanism needs to be further investigated. This phenomenon on one hand will enhance the separation of analytes within certain pH values, but on the other hand will increase the retention times of analytes and cause band broadening at too high pH values. In addition, we found that the reproducibility was poor at higher pH values since the buffer pH had a strong tendency to decrease when exposed to air. Therefore, the optimized pH for this study was maintained at 9.5 for a good separation of all the catecholamines and polyamines with a good reproducibility under reasonable separation time.

3.2. Effect of SDS concentrations

The separation of polyamines and catecholamines was not complete at all without SDS because the polyamine derivatives themselves bore no charge [40]. Therefore, MECC was applied for improving the separation. The effect of SDS concentrations on separation and migration time of each analyte was investigated. A concentration range of 2, 5, 10, 20, and 50 mM of SDS was examined while the boric buffer concentration was maintained at 100 mM with a pH of 9.5. The results are shown in Fig. 4. It was interesting that different effects of SDS concentrations on the separation of polyamines and catecholamines were observed. The resolution of polyamines was improved greatly as SDS concentration was increased. Of course, when SDS concentration was smaller than 5 mM, it would not improve the separation since the SDS concentration was below the critical micelle concentration (CMC), which is 8.1 mM. At the same time, the retention times of polyamines were also increased significantly. The optimal SDS concentrations for polyamines alone were at 50 mM or higher. This effect can be explained by the increases in electroosmotic flow due to the increases in buffer conductivity, by the changes in surface charge due to the presence of counter ions and by the increased ratio of volume of the micelle phase to that of aqueous phase [66]. However, the separation of catecholamines was not significantly affected by the increases of SDS concentrations. The major reason was probably that the –OH group on the aromatic ring was partially ionized at pH 9.5 (the pK_a values for most phenols range from 9.3 to 9.9) and the negative changes carried by the catecholamine derivatives had strong repulsions with the SDS micelles, which inhibited transferring catecholamines into SDS micelles. The detailed mechanism on degree of dissociation, distribution coefficient of catecholamines in SDS micelles, and others are under investigation in a separate study. At higher concentrations of SDS (>20 mM), the separation of catecholamines was totally ruined due to the significant changes in electroosmosis, relative volumes of micelles and surface character of inside capillary wall. In order to maintain a good separation of both polyamines and catecholamines, 10 mM SDS concentration, which was close to the CMC of SDS, was chosen in further studies and analysis of cell extracts.

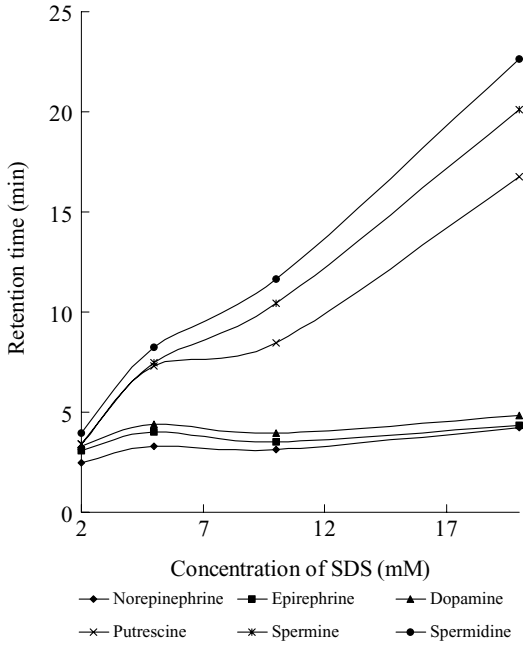

Fig. 4. Effect of SDS concentrations on the separation of catecholamines and polyamines by MECC. The experimental conditions were the same as those of Fig. 2, except the variation of SDS concentrations and without 18-crown-6.

3.3. Effect of concentrations of 18-crown-6 and other additives

Previous research indicated that the resolution of primary amino compounds was improved greatly by incorporating crown ethers in the running buffer [67]. Therefore, the effect of 18-crown-6 concentrations to the separation of polyamines and catecholamines was investigated in our study. The results are shown in Fig. 5. The resolution of polyamines was increased greatly when 18-crown-6 concentrations were increased from 2 to 10 mM. However, the effect on separation of catecholamines was quite weak. The major reason was that the primary amino group can form a selective host–guest complex with the non-chiral 18-crown-6 in the running buffer solution [67]. All polyamines we were interested in this paper contained two primary amino groups and one primary amino group was still free to form complex with crown ether molecules after derivatization, which caused improvement in resolution of polyamines. However, catecholamines contained only one primary amino group to undergo the derivatization. No primary amino group left to form complex with crown ether after derivatization and thus the effects of 18-crown-6 on catecholamine resolutions was not significant. Since we could not use optimum SDS concentration due to its negative effects on catecholamine separations and the addition of crown ether improved the separation of polyamines,

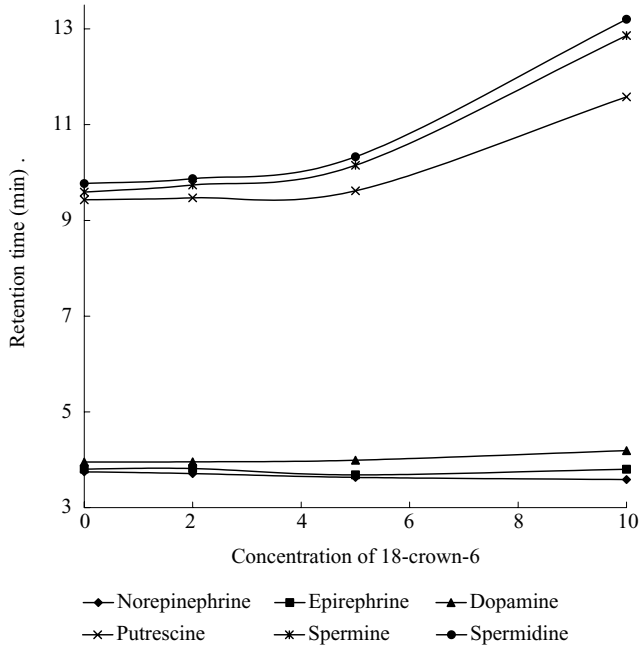

Fig. 5. Effect of 18-crown-6 concentrations on the separation of catecholamines and polyamines by MECC. The experimental conditions were the same as those of Fig. 2, except the variation of 18-crown-6 concentrations.

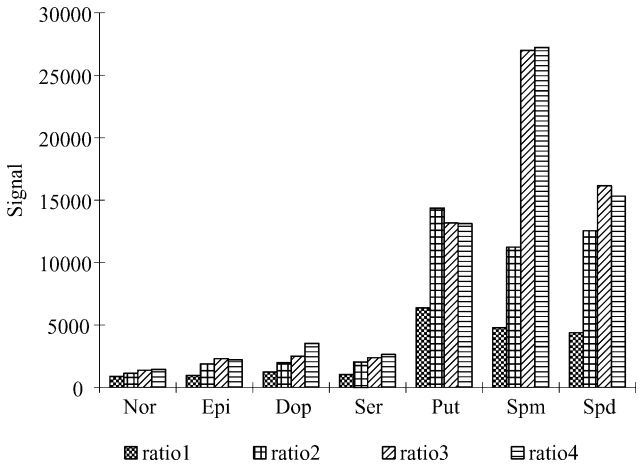

Fig. 6. Effect of derivatization conditions on analysis of catecholamines and polyamines by MECC. The experimental conditions were the same as those of Fig. 2, except the variation of derivatizing volume ratios in microliters (buffer:sample:AccQ reagent): ratio 1, 10:70:20; ratio 2, 10:30:10; ratio 3, 10:20:10; ratio 4, 40:20:20. *Abbreviations*: Put, putrescine; Spd, spermidine; Spm, spermine; Dop, dopamine; Nor, norepinephrine; Epi, epinephrine; Ser, serotonin.

10 mM 18-crown-6 was added into the running buffer for enhancing separations of polyamines in the cell extracts.

We found during our experiment that current leakage occurred very frequently when 18-crown-6 concentrations were higher than 10 mM. When its concentration reached to 15 mM, the experiments could hardly be performed because of current leakage. The reason for this phenomenon will be further investigated. One possible reason for this phenomenon was due to the precipitation of the non-polar crown ether in the capillary at high concentrations. In addition, various concentrations of different organic modifiers in running buffer, such as 5–15% of acetonitrile and 5–15% of methanol, were also examined for their effects on separations of polyamines and catecholamines. None of these showed a significant improvement in separation of catecholamines and polyamines (data not shown).

After completion of these studies, the optimal condition for separation of catecholamines and polyamines was 100 mM boric acid + 10 mM SDS + 10 mM 18-crown-6 with a pH of 9.5. All seven standards of polyamines and catecholamines were well separated even though there was a retention gap between catecholamine group and polyamine group. The main reason for this gap is due to the different interactions between catecholamine–SDS micelles and polyamine–SDS micelles as explained in Section 3.3.

3.4. Derivatizing conditions

The derivatization conditions were critical for improving the detection sensitivities of analytes and for quantitative studies. The 20 min incubation was used in all experiments to assure complete reaction of all catecholamines and polyamines in the standard samples and cell extracts with the derivatizing reagent, even though studies showed that a derivatizing time of 10 min was sufficient [64]. Higher incubation temperature has shown an improvement in derivatization efficiency in our study, therefore the temperature of 65 °C rather than 55 °C was used at all studies. Under these derivatization conditions, the volume ratios of borate buffer, sample and AccQ-Fluor reagent were also investigated. In the case of the concentrations of each compound in sample at lower level (less than 10 μM), increase of the ratio of sample to AccQ reagent improved the detection sensitivity greatly and the results are shown in Fig. 6. The optimal ratio was 40:20:20 (volume in microliters) for buffer:sample:AccQ reagent, respectively. The conditions were used for calibration and for the studies of PC-12 cell extracts.

3.5. Reproducibility, linearity and detection limit

A complete study on reproducibility, linearity and detection limit for determination of catecholamines and polyamines has been conducted and all the data are shown in Table 1. The reproducibilities, which were expressed as a percentage relative standard deviation (R.S.D.) for relative migration time (RMT) and relative peak areas (RPA), were calculated by using catecholamine and polyamine standards for a total of six consecutive analyses. The R.S.D. values of RMT were lower than 2.41% and the R.S.D. values of RPA were lower than 4.13%, indicating good reproducibility.

The linearity of the method was determined for each compound by using authentic standards in the range of 1–100 μM. The relative standard deviations for peak areas

Table 1
The reproducibility, linearity and detection limit of MECC method for determinations of polyamines and catecholamines

Compound	R.S.D. (%)		Linearity		Detection limit (μM; S/N = 3)
	RMT	RPA	Slope	R^2	
Norepinephrine	1.61	2.715	143960	0.9995	0.8
Epinephrine	0.92	1.77	121780	0.9983	0.7
Dopamine	1.78	3.56	95711	0.9997	0.8
Serotonin	0.93	2.38	496230	0.9996	0.95
Putrescine	2.41	4.13	178070	0.9925	0.45
Spermine	1.02	2.73	1057570	0.9924	0.2
Spermidine	1.77	3.41	2544610	0.9999	0.15

The experimental conditions were the same as those of Fig. 2. *Abbreviations*: R.S.D., relative standard deviation; RPA, relative peak area; S/N, signal-to-noise ratio; RMT, relative migration time; R^2, correlation coefficient. The data were calculated by using catecholamine and polyamine standards for a total of six consecutive analyses.

and retention times, the linearity which were indicated by correlation coefficient of six consecutive injections were calculated and are listed in Table 1. The R^2 values were ranged between 0.9924 and 0.9999, which demonstrated a good linearity in this concentration range. The detection limits for each compound were ranged from 0.15 to 0.95 μM in final solutions, which were corresponding to 15–95 fmol absolute detection limits with a 100 nl sample injection. Due to much lower concentrations of catecholamines than polyamines in many cell extracts, the detection limits for catecholamines are very crucial. The detection limits of catecholamines in this study were ranged 0.7–0.95 μM, close to the reported sensitivities of catecholamines of 0.4–0.7 μM [50] and 0.5–4 μM [49] after solid-phase extraction (SPE) purification by UV–CE without derivatization. It is confident that the sensitivity of this method is high enough that it can be used for many biological applications.

Table 2
The MECC results of amino compounds level at PC-12 cell samples

Amino compound	Concentration (pmol/10^6 cells)	Recovery rate (%)
Putrescine	2242 ± 107	97.6 ± 8.22
Spermidine	635 ± 82	89.3 ± 8.22
Spermine	880 ± 94	93.5 ± 6.98
Dopamine	7.33 ± 2.34	86.2 ± 9.43
Norepinephrine	1.75 ± 0.54	88.1 ± 9.7

The experimental conditions were the same as those of Fig. 2. *Note*: Amounts reported were expressed as the mean ± standard deviation. Number of data collection was 3.

3.6. Analysis of PC-12 cells

Under optimal separation conditions, the determination of catecholamines and polyamines in PC-12 cell extracts was demonstrated. A representative electropherogram of the sample separation is shown in Fig. 7. The peaks were identified by standard addition under the exactly same conditions. The results obtained through our newly developed HPCE methods are shown in Table 2. The results were comparable to those reported in HPLC and other CE studies [44,68].

4. Conclusion

We have first time demonstrated a simple, sensitive and less time consuming MECC method to simultaneously determine both polyamines and catecholamines in cell extracts. The methods can allow researchers to quantify each of the

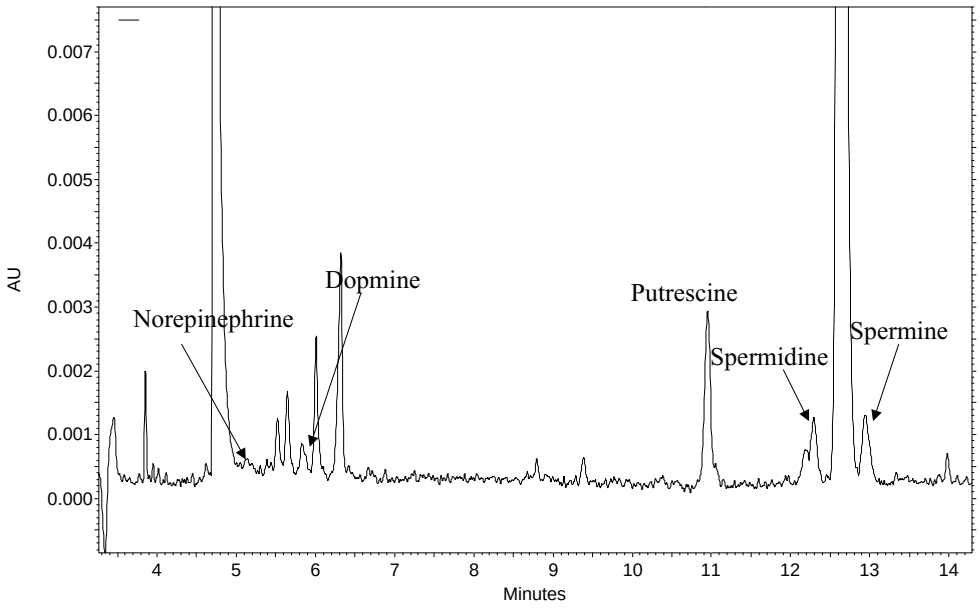

Fig. 7. A representative electropherogram of catecholamines and polyamines in PC-12 cell extracts. The experimental conditions were the same as those of Fig. 2. The peaks were labeled with names on the top of the peaks.

polyamines and catecholamines for polyamine-related anticancer drug studies and for mechanism studies for all biological systems involving polyamines and catecholamines. The method is suitable for high-throughout discovering and screening of polyamine-related anticancer drugs due to its automatic character of CE instrument.

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