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Determination of urine catecholamines by capillary electrophoresis with dual-electrode amperometric detection

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Abstract

Demonstrated in this study is that without pretreatment and preconcentration nanomolar-level catecholamines in human urine samples can be quantitatively determined with ease by utilizing capillary electrophoresis coupled with amperometric detection. The detector employs a parallel-opposed dual-electrode scheme assembled with an on-capillary electrode and a disk electrode and takes advantage of the redox cycling of analytes between the two working electrodes to improve the limit of detection. The matrix effect of urine samples significantly decreases the detection sensitivity from that obtained in standard solutions. Therefore, calibration curves derived from standard solutions cannot be used in quantitative determination of catecholamines. Methods of standard addition and internal standard have been studied. The results suggest that isoproterenol is a good internal standard to facilitate the measurements of dopamine, epinephrine, and norepinephrine in human urine samples.

Keywords: Detection, electrophoresis; Catecholamines; Dopamine; Epinephrine; Norepinephrine

1. Introduction

Quantitative analysis of dopamine, epinephrine (adrenaline), and norepinephrine (noradrenaline) is important in screening for diagnosing and monitoring treatment of pheochromocytoma, neuroblastoma, and ganglioneuroma which are tumors occurring in the adrenal gland and in many areas of the body that cause excess release of these hormones. Measurements of the individual catecholamines, rather than the total catecholamine amount, are necessary, because, for example, certain pheochromocytomas only secret epinephrine [1–3] whose quantity is very small relative to dopamine and norepinephrine. When only total catecholamine levels are determined, these tumors can be easily missed. Although catecholamine levels can be determined in the blood samples, the blood test is not as sensitive as the 24-h urine test because the blood samples are drawn from specific areas and thus sometimes lead to false results and because urine test reflects the production rates of the catecholamines over the collection period. For 24-h urine samples collected from healthy adults, the reference values of total catecholamines, epinephrine, and norepinephrine are less than 100 μg, 10 μg (55 nmole), and 100 μg (591 nmole), respectively

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[4]. Even at abnormal levels, the elevated quantities of these catecholamines are still very small. Therefore, extremely high sensitivity is required for methods suitable for routine analysis of these catecholamines.

Due to the sensitivity and specificity, HPLC-ED (high-performance liquid chromatography coupled with electrochemical detection) and ELISA (enzyme-linked immunosorbent assay) are the most prevailing among the methods used for the quantitative determination of catecholamines [5–7]. However, prior to HPLC analysis, purification treatment [5,8] and preconcentration are necessary due to the complexity of the sample matrix and the very low level of catecholamines [9–11]. The pretreatment usually involves selectively extractive removal of the catecholamines from the urine matrix, e.g. by employing a cation-exchanger [12,13], acid-washed alumina (Al_2O_3) at alkaline pH [10,11,14], or a boric acid affinity gel [15–17]. For immunoassay-based measurements of urine samples, the competitive technique is generally adapted. Good specificity relies on a *cis*-diol-specific affinity gel to selectively extract catecholamines from a complex matrix. The subsequent steps involve derivatization of the extracted catecholamines for antibody recognition, competition between the derivatized analytes and antigen linked with an enzyme for a fixed number of antibody binding sites on the ELISA microtiter plate, and quantification of the enzyme-linked antigen by monitoring the enzyme–substrate reaction. The amount of enzyme-linked antigen is inversely proportional to the catecholamines of the urine sample. The ELISA test involves multiple incubations and washes. Although these procedures for HPLC and ELISA are routinely practiced in clinical laboratories, a method requiring no sample workup such as clean-up steps and preconcentration would have advantages in diagnostic time, cost, labor, and having no analytes lost during the recovery steps.

Compared to HPLC, CE (capillary electrophoresis) is a relatively new separation technique which is less expensive and instrumentally simple, requiring only a high-voltage power supply and a separation capillary with inner diameters ranging from 100 to 2 μm. Additionally, CE requires exceptionally small sample volumes, consumes less solvent, and shows an order-of-magnitude better performance in numbers of theoretical plates than HPLC. However, the applications of CE for trace analyses are limited, primarily due to the lack of sensitive detection systems for nanoliter sample volumes. Although many sensitive methods, such as fluorescence and electrochemical detection, have been demonstrated for CE to determine catecholamines, the detection sensitivity in concentration is sufficient for single cell studies but not satisfactory for the quantitative measurements in body fluids. In 1997, the detection limit of epinephrine was improved to the nanomolar scale by Zhu and Kok [18] with a terbium post-column reaction scheme for a luminescence method in boric acid buffer at pH 10. However, they found that the electropherograms for raw urine samples in an alkaline solution were so complicated that a sample cleanup and preconcentration procedure was still required.

We recently reported an electrochemical detector of a parallel-opposed dual-electrode for CE [19] in which the oxidation–reduction recycling mechanism can significantly enhance amperometric signals only for electrochemically reversible substances. This detection scheme provides great advantages both in signal enhancement and in signal isolation of electrochemically reversible catecholamines from the sample matrix. Further isolation of catecholamines from the urine sample can be accomplished by electrokinetic sample injection (using an electric field to drive analytes into the capillary) in an acidic medium. Electrophoresis, by definition, means that analytes migrate under the influence of a high DC voltage across the separation capillary. Cations and anions are attracted, respectively, to the cathode (the negative terminal) and the anode. When the sample solution is buffered to an acidic pH, the catecholamines will carry positive charge. Using electrokinetic sample injection, a large fraction of negatively charged interferants will be precluded from migrating into the separation capillary. With a highly sensitive detector and proper selection of the separation conditions, we will demonstrate in this article that CE can be utilized to quantitatively determine the amounts of dopamine (DA), epinephrine (Epi), and norepinephrine (NE) directly from raw 24-h human urine samples.

2. Experimental

2.1. Apparatus

A high-voltage DC power supply (model CZE 1000R, Spellman High-Voltage Electronics, Plainview, NY, USA) was used to apply potentials for injection and separation. Two digital lab-bench power supplies (model E3615a, Hewlett-Packard) were used to regulate the output voltage of the high-voltage power supply so as to improve the reproducibility of the experiments. The dual EC detection was carried out on a potentiostat (model LC-4C, Bioanalytical Systems, West Lafayette, IN, USA) with a four-electrode configuration including an on-capillary working electrode [20,21], a disk working electrode, an Ag-wire coated with chloride as a quasi-reference electrode, and a Pt counter electrode. The detailed procedures for the preparation of the electrodes and the EC cell were described in the literature [19]. To minimize the interference of external electric noise, the electrochemical cell was housed in a home-made Faraday cage. All CE data were collected by a PC with data acquisition software ChemLab (Scientific Information Service, Taipei, Taiwan, ROC).

2.2. Reagents and solutions

All catecholamines and reagents for the preparation of buffer solutions were analytical reagent grade and were purchased from Sigma or Aldrich. Solutions were prepared with purified water (18 MΩ cm, Millipore-Q, Millipore). The electrolyte solutions were buffered to the desired pH by drop-wise introduction of 5 *M* NaOH (Aldrich, semiconductor grade) or 1.67 *M* sodium acetate. The reported pH of the solution was carefully measured with a pH meter (model SP-2200, Suntex, Taiwan, ROC). Standard solutions of catecholamines with the desired concentrations were freshly made daily by serial dilution in the separation electrolyte. Twenty-four-hour urine samples were collected in a bottle containing 15 ml of 6 N hydrochloric acid as preservative. Prior to injection, all solutions were filtered through a 0.45-μm PVDF filter (Lida Manufacturing).

3. Results and discussion

3.1. Selection of carrier electrolytes

Acetate, borate, CAPS [3-(cyclohexylamino)propanesulfonic acid], citrate, MES [2-(*N*-morpholinoe)thanesulfonic acid], and phosphate are the carrier electrolytes examined in this study for preliminary evaluation of the search for optimal separation and detection conditions. These electrolytes are commonly used buffers and have been applied in the analysis of catecholamines in previous HPLC [10,22,23] or CE [18,19,24–30] literature. Because dilution will be the only pretreatment step for the raw urine samples, the solution pH should be close to its maximum buffer capacity so that the experimental environments do not deviate significantly from the optimal conditions. Suitable carrier electrolytes should provide high sensitivity, short experimental times (i.e. migration times of the analytes), a good theoretical number of plates, and a reasonable resolution (*Rs*) between the analytes. These properties are affected by interrelated factors, such as solution pH and conductivity, electrolyte concentration, voltages for sample injection and separation, duration of sample loading time, and detection potentials at the dual working electrodes.

Optimization in the sensitivity and the detection limit would be meaningless if the analytes are not well-resolved. Therefore, the search for a suitable electrolyte for the resolution of analyte peaks larger than 1.5 is the most important task which is difficult due to the similarity of their structures and thus the migration times. Our results show that the *Rs* of NE and Epi peaks larger than 1.5 can be found only for those electrolytes with low pK_a values, such as phosphoric acid (pK_{a1} 2.1), citric acid (pK_a 3.1), and acetic acid (pK_a 4.7), or electrolytes with high pK_a values, such as CAPS (pK_a 10.4). The performance of borate and CAPS is not superior to the others in sensitivity and reproducibility due to the deterioration taking place easily for catecholamines in alkaline solutions. Addition of anti-oxidants into the sample vials is required for high pH electrolytes. Because the goal of this study is to simplify the necessary steps in the sample workup, alkaline electrolytes are thus not under further consideration.

For low pH buffers, these systems generally suffered from the disadvantages of the very long migration times and insufficient sensitivities, such that only in an acetic acid buffer could the electrochemical signals of urinary catecholamines be detected. Fig. 1(A) is a representative electropherogram of a urine sample diluted 5-fold and separated in a 0.15 *M* acetic acid buffer (pH 5.59). The inset of Fig. 1(A) shows that the analyte signals are apparent and well-resolved but the background noise is relatively large, probably due to the property of high ionic strength of this buffer system.

After an exhaustive survey of the aforementioned electrolytes in terms of adjustment of variables such as separation voltages, detection potentials at both working electrodes, electrolyte concentrations and pHs, we have found that satisfactory sensitivity can be obtained in MES, in good agreement with literature reports [28]. Fig. 1(B) shows the electropherogram of the same 24-h urine sample as that of Fig. 1(A). The sample is diluted 5-fold by 0.15 *M* MES buffered to pH 5.57 with dropwise addition of NaOH. The migration times of the analytes are within 7 min. The inset of Fig. 1(B) demonstrates that the sensitivity of the dual-electrode detection is adequate for diluted urine samples without pretreatment and preconcentration. The peak resolution, however, is not satisfactory. An electropherogram of a urine sample diluted 5-fold from the same raw sample as that of Fig. 1(A) and (B) is shown in Fig. 1(C). The resolution is greatly improved by utilizing sodium acetate, rather than NaOH, to adjust the pH of 0.15 *M* MES solution to pH 5.57 as the separation electrolyte. The migration times are longer than those in MES [Fig. 1(B)] by about 4 min. We would like to emphasize that the sample is diluted with the NaOH buffered solution, the same as that used in Fig. 1(B). Also noted is that the constituents and conductivity of the electrolytes for Fig. 1(A) and (B) are different although the solutions have similar pH values. Sodium acetate is a weaker base than sodium hydroxide and thus requires a greater amount to buffer MES to the desired pH. The sensitivity of Fig. 1(C) is significantly enhanced, probably resulting from an increase in the number of redox cycles of analytes between the electrodes due to the relatively slow flow velocity [19] in this electrolyte.

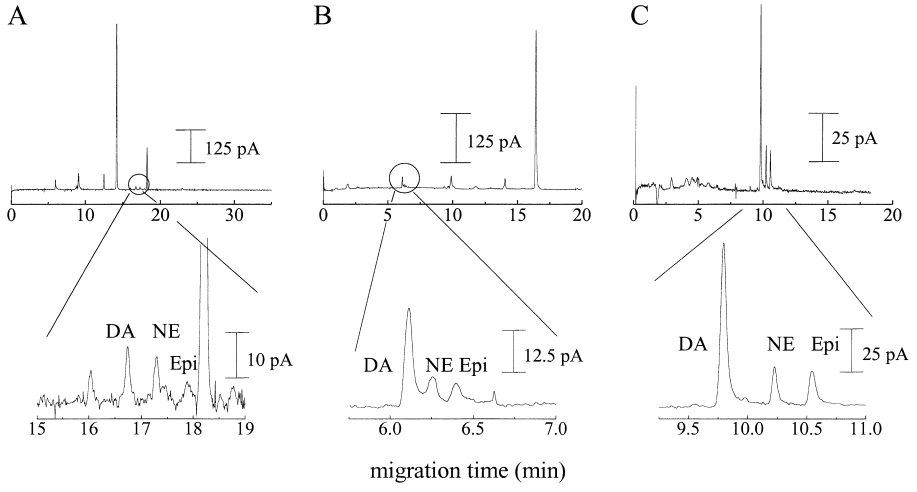

Fig. 1. Electrophoretic separation of a 5-fold-diluted 24-h urine sample without preconcentration and further pretreatment. The separation electrolytes are: (A) 0.15 *M* acetic acid buffered to pH 5.59 with NaOH; (B) 0.15 *M* MES buffered to pH 5.57 with NaOH; and (C) 0.15 *M* MES buffered to pH 5.57 with sodium acetate. The raw sample is diluted with the corresponding separation electrolytes for (A) and (B). For (C), the raw sample is diluted with electrolyte (B). Other conditions: fused-silica capillary 60 cm long (10 μm I.D.×375 μm O.D.); sample injection, 16 kV for 3 s; separation voltage, 16 kV; detection potential, −0.5 V (vs. $E_{Ag/AgCl}$) at the on-capillary electrode, +0.6 V at the disk electrode.

3.2. Selection of detection potentials for electrochemical measurements

Selection of detection potentials at the on-capillary electrode and the disk electrode is decided from the hydrodynamic voltammograms such as those shown in Fig. 2. Although cyclic voltammetry can easily provide information of redox potentials of the analytes for the settings of the working potentials, the conditions are, after all, not the same as when a high separation voltage is applied, especially for the end-capillary detection scheme where no decoupler is employed [31,32]. In Fig. 2, under the influence of separation voltage, the current at the disk electrode is measured and normalized as a function of the potential at the on-capillary electrode (Fig. 2) and at the disk electrode [Fig. 2(B)]. While the potential at one of the working electrodes is a variable, the potential at the other electrode is held constant. For all three analytes at the separation voltage of 16 kV, the maximum currents of reduction and oxidation are reached at −0.45 V and +0.55 V, respectively.

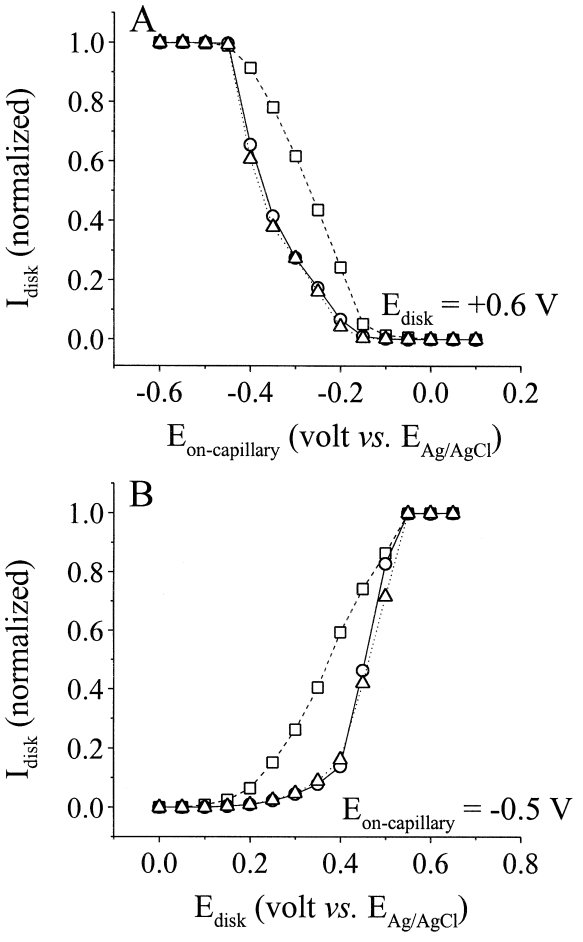

Fig. 2. Hydrodynamic voltammograms of normalized redox current at the disk electrode vs. potential variation (A) at the on-capillary electrode where the potential at the disk electrode is held constant at +0.6 V, and (B) at the disk electrode where the potential at the on-capillary electrode is −0.5 V for DA (□), NE (○), and Epi (△). Analytes: 5 μ*M* for DA, NE, and Epi; separation voltage: 16 kV; other conditions as for Fig. 1(C).

The bipotentiostat (LC-4C) used in this study is not a true dual-potentiostat system. Only one of the two detection channels has a high detection sensitivity. The numbers of theoretical plates at the disk electrode are always better than those obtained at the on-capillary electrode due to the relatively small size of the disk electrode. Therefore, the disk electrode is connected to the channel with better detection sensitivity and is used for quantitative analysis. The potential settings for the on-capillary and disk electrodes are −0.50 V and +0.60 V, respectively. At −0.50 V reduction of solution dioxygen takes place and contributes to the background current. Amperometric signals arising from reduction of a trace amount of analytes will be diminished. In order to obtain a good detection limit, the potential of the disk electrode is designated at +0.60 V, where the oxidation current of catecholamines is measured.

3.3. Validation of quantitative determination of catecholamines in urine samples

Fig. 3(A) shows the electropherogram of a standard solution with a concentration of 0.64 n*M* for all the analytes, approaching the detection limit under optimized conditions. A sample loading time of 3 s appears to be the best in terms of the theoretical number of plates and the resolution of the peaks. A sampling time longer than 4 s does not increase the amperometric signal, but significantly broadens the peaks, suggesting that the sample zones are longer than the radius of the disk electrode when the analytes migrate out of the separation capillary.

Demonstrated in Fig. 3(B)–(F) is an example of quantitative determination of catecholamines in a 24-h urine sample. Similar to Fig. 1(C), within the

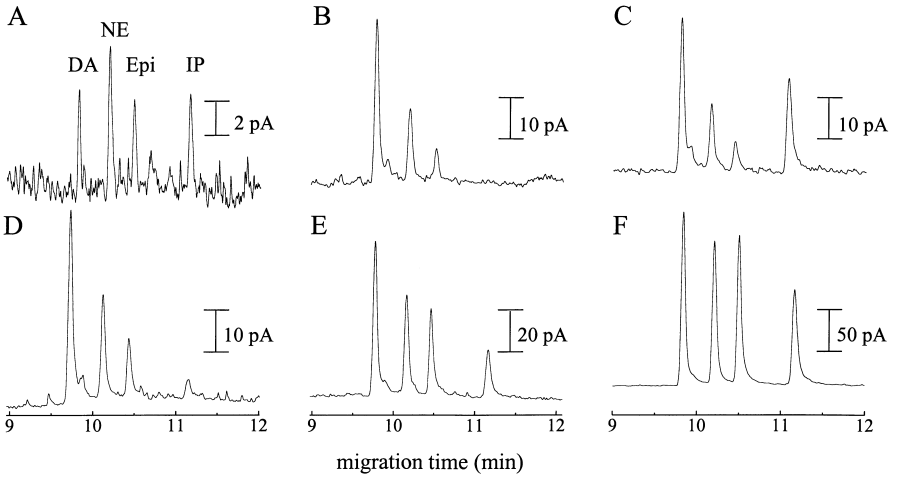

Fig. 3. Electropherogram obtained under optimized conditions. (A) Standard solutions containing 0.64 n*M* for all four analytes. (B) 5-fold-diluted urine sample. (C) Addition of 16 n*M* IP into 5-fold-diluted urine sample as the internal standard. Standard addition of (D) 3.2 n*M*, (E) 16 n*M*, and (F) 80 n*M* analytes into the 5-fold-diluted urine sample. Conditions as for Fig. 1(C).

migration time necessary for one run, there is no peak other than those of the catecholamines. As stated in Section 1, a large fraction of negatively charged interferants can be precluded from the catecholamines in acidic solutions during the sampling injection. Fig. 3(B) is obtained from a sample with 5-fold dilution. For routine HPLC-ED analysis of catecholamines in clinical laboratories, the most prevailing method of quantitative analysis is addition of DHBA (3,4-dihydroxybenzylamine) into the sample as the internal standard. DHBA structurally resembles dopamine (3,4-dihydroxyphenethylamine) with only one methylene unit short in the amine side chain. Unfortunately, the resolution of DHBA and DA peaks is smaller than 1.5 under our optimized conditions. IP (isoproterenol, 1-[3′,4′-dihydroxyphenyl]-2-isopropylaminoethanol) has been used as an internal standard for quantitative measurements of catecholamines in human blood plasma and urine [33]. This compound appears to be a good alternative because this synthetic compound is not produced in the human body, structurally related to Epi, and is less expensive than DHBA. Fig. 3(C) is an electropherogram of the diluted real sample containing 16.0 n*M* of IP which is well-resolved from Epi. The numbers of theoretical plates for DA, NE, Epi, and IP are 153 000, 162 000, 159 000, and 128 000, respectively. For 10 consecutive measurements, the results of the internal standard method indicate that the concentrations of DA, NE, Epi are 17.45 ± 0.19, 7.89 ± 0.07, and 3.12 ± 0.14 n*M*, respectively, corresponding to 20.29, 10.12, and 4.28 μg/24 h. The results are in good agreement with the reference values [4]. The accuracy of the measurements is examined using the standard addition method. Representative electropherograms are shown in Fig. 3(D)–(F). The relative error, the difference between the two methods divided by the values derived from the method of standard addition, for DA, NE, Epi are, respectively, 4.2%, 4.7%, and 5.1%, suggesting that IP is eligible to serve as the internal standard for quantitative determination of the catecholamines. The analytical recoveries, calculated from the addition of 3.2 n*M* catecholamines into real samples [Fig. 3(D)], are 90.1%, 102.6%, and 106.7%, respectively, for DA, NE, and Epi. A calibration curve derived from standard solutions shows that the limits of detection ($S/N = 3$) for DA, NE, Epi are as low as 0.41, 0.16, and 0.14 n*M*, respectively. However, the results obtained from the calibration curve method are more than 50% larger than those obtained using the internal standard method. The large deviation is due to the fact that the samples measured in this study are five-fold diluted only, unlike the pretreated samples for HPLC-ED or ELISA. The analytical signal is strongly affected by the urine matrix so that

to extract concentrations of analytes from a calibration curve becomes unrealistic.

4. Conclusions

We have demonstrated that trace concentration of catecholamines in human urine can be quantitatively determined by CE-ED. Because dual-electrode detection can offer great amperometric sensitivity, real samples can be analyzed by simple 5-fold dilution, without pretreatment or preconcentration. Compared to techniques such as HPLC-ED and ELISA, the ease in sample preparation can tremendously reduce the labor efforts and time. The commonly employed internal standard, DHBA, is not suitable in our optimized electrophoretic system because its migration time is close to that of DA. Alternatively, IP is an appropriate internal standard because quantitative analysis of urine catecholamines is validated by standard addition and recovery rate methods.

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