Determination of monoamines in urine by capillary electrophoresis with field-amplified sample stacking and amperometric detection

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Abstract

A simple, rapid and low-cost method using capillary electrophoresis coupled with field-amplified sample stacking and electrochemical detection was developed for the separation and determination of monoamines. In this present work, a systematic study of the parameters (pH value and concentration of electrophoretic buffer, composition of sample solvent, injection voltage and time) affecting separation and on-line concentration of monoamines has been performed enabling the detection sensitivity of these monoamines to be improved by 5000 times compared with the conventional electrokinetic injection. This developed method was applied to the direct analysis of these monoamines in human urine without off-line sample preconcentration. Due to the requirement for urine dilution to minimize the detrimental effects of high salt on analyte stacking, the real sensitivity improvement is about 50-fold when applying the optimized method to urine samples. In order to quantitate these monoamines accurately, internal standard calibration curves were constructed with standard monoamines in presence of salt with similar concentration as in human urine. In the method validation, the calibration curves were linear over a range of 1.0×10^{-9} to 2.5×10^{-8} mol/L for each monoamine and the limits of detection (signal to noise ratio of 3) for these monoamines were in the sub-nmol/L concentration range (6.0×10^{-10} mol/L).
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1. Introduction

The measurement of the level of monoamines such as catecholamines (dopamine, epinephrine, norepinephrine) and serotonin in biological fluids has an essential role in the diagnostics of diseases. Catecholamines in urine can help diagnosis of various diseases such as pheochromacytoma, ganglioneuroma, neuroblastoma and neurological disorders [1,2]. Measurement of urinary excretion of serotonin is useful for diagnosis of psychiatric disorders and carcinoid tumor [3]. Although many methods such as radioenzymic [4], enzyme-linked immunosorbent assay [5], voltammetry [6] and thermal lens spectrometry [7], have been reported for the determination of these monoamines, dominant technique is high-performance liquid chromatography (HPLC) [8] in combination with sensitive electrochemical [9,10] or fluorimetric detection [11,12]. However, due to the complex-

ity of the sample matrix and the very low level of catecholamines, purification treatment and preconcentration or derivatization are necessary before HPLC analysis. HPLC suffered from the disadvantages of cost, labor and analytes losing during the recovery steps.

Capillary electrophoresis (CE) has become a very important instrumental technique in the area of liquid-phase separation. The primary advantage of CE is its ability to provide extremely high separation efficiency in a short time with relatively simple instrumentation. However, due to a small sample volume, the reduced concentration sensitivity of CE compared to HPLC is a major limitation for the analysis of target analytes in very low concentration. Although some sensitive detectors, such as laser-induced fluorescence, electrochemical detectors had been demonstrated for direct determination of catecholamines and serotonin in single cells [13–15], CE is generally not sufficiently sensitive to quantify these monoamines in body fluids without extensive sample pretreatment. In order to improve the detection limit of catecholamines, Zhu and Kok [16] had developed a high sensitive luminescence method with post-column terbium complexation, however, a sample cleanup and preconcentration

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procedure was still required due to the complexity of electropherograms for raw urine samples in alkaline running buffer. Recently, Chen [17] reported an electrochemical detector of a parallel-opposed dual-electrode for CE, in which the redox cycling of the analytes between the two working electrodes can significantly improve the limit of detection. The method was successfully applied to determine catecholamines in diluted urine samples without purification. But, fabrication of the dual-electrode is a complicated work.

Sample stacking is one of the most common approaches to improving concentration sensitivity in CE, including field-amplified sample stacking (FASS) [18,19], dynamic pH junction [20,21], isotachophoretic stacking [22] and sweeping [23,24]. The simplest and most widely applied technique is FASS. FASS is based on the conductivity difference between the sample zone and the running buffer to effect preconcentration. The low conductive sample matrix will experience a high electric field strength, and analyte ions will move faster than the ions of the background electrolyte. Thus, a large amount of analyte ions can be injected into the capillary without compromising the separation efficiency. FASS has been shown to provide the greatest sensitivity enhancement and has been applied to determine a lot of compounds [25–30]. Hu [31] reported detection of catecholic compounds by CE with FASS and amperometric detection (AD). However, the developed method has not been applied to real samples. To our knowledge, FASS coupled with CE and amperometric detection (AD) has not been reported to determine monoamines in urine. In this work, FASS was applied for the first time to analyze nanomolar levels of monoamines in human urine using CE-AD. Noteworthy is that rapid analysis of monoamines content in human urine was realized without off-line sample preconcentration. The combination of the selectivity of amperometric detection with the sensitivity enhancement of FASS represents a simple, efficient and sensitive method to separate and detect monoamines from complex biological samples by CE.

2. Experimental

2.1. CE system

The CE separation system used in this work was similar to that described previously [32]. The system included a high-voltage power supply (Model 9323-*HVPS*, Beijing Institute of New Technology, Beijing, China) and a Faraday cage housing the electrochemical detector. The separations were performed using 66 cm × 25 μm I.D. fused-silica capillary (Yongnian Optical Conductive Fiber Plant, Yongnian, China). The amperometric detection at a constant potential was performed using the end-capillary approach with an electrochemical analyzer (Model CHI802A, CH Instruments, Austin, TX, USA). Electrochemical detection was carried out with a three-electrode system. It consisted of a carbon fiber microdisk array electrode (about 60 carbon fibers with 6 μm diameter) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a coiled Pt wire as the auxiliary electrode. The Pt wire also served as the ground for the high potential

drop across the capillary. All potentials were measured versus SCE.

2.2. Reagents and solutions

5-Hydroxytryptamine (5-HT), dopamine (DA), epinephrine (E), norepinephrine (NE), and isoproterenol (IP, used as the internal standard) were analytical grade and purchased from Sigma (St. Louis, MO, USA). The 5.00×10^{-2} mol/L stock solutions of each monoamine were prepared with 0.1 mol/L perchloric acid and stored at $-20\,^{\circ}$C in a refrigerator when not in use. On the day of analysis, they were diluted as needed. Other reagents were of analytical grade. The electrolyte solutions were buffered to the desired pH by drop-wise introduction of 4 mol/L NaOH. The reported pH of the solution was carefully measured with a pH meter (Model pHS-25, Jingke Instruments, Shanghai, China). All solutions were prepared with ultra-pure water from a Milli-Q water system (Millipore Corp., Bedford, MA, USA).

2.3. Construction of carbon fiber microdisc array electrode

The carbon fiber microdisc array electrodes were constructed with about 60 6-μm-diameter carbon fibers. First, the carbon fibers soaking up acetone were carefully inserted into a fused-silica capillary (200 μm I.D., 375 μm O.D., 1.5 cm length) until it protruded approximately 1 cm from the other end of the capillary (Fig. 1A), JE-101 epoxy glue (Ganyi Chemical Plant,
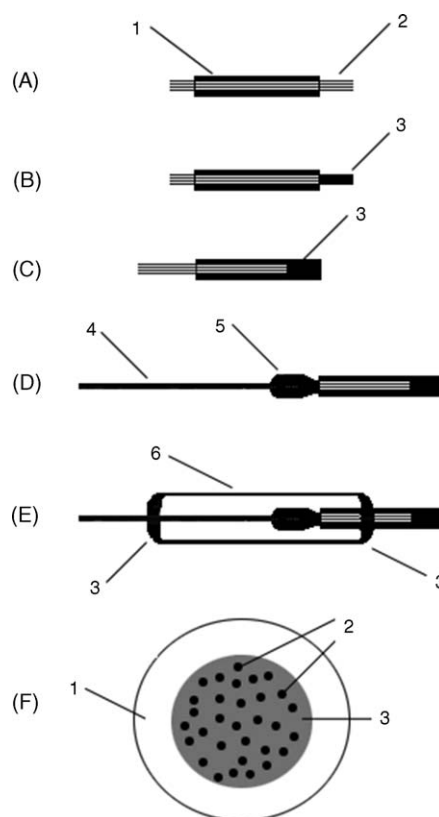

Fig. 1. Manufacturing process of the carbon fiber microdisc electrode: 1, fused-silica capillary; 2, carbon fiber; 3, JE-101 epoxy glue; 4, copper lead; 5, carbon powder conducting glue; 6, glass capillary.

Yichun, Jiangxi, China) was carefully applied on surfaces of the protruded each carbon fiber (Fig. 1B). Then, the coated carbon fibers with JE-101 epoxy glue were carefully drawn back into the capillary (Fig. 1C) and allowed to dry at room temperature for 24 h. The other end of carbon fibers was connected to a copper lead using carbon powder conductive glue (Fig. 1D). Finally, in order to protect the electrode, the fused-silica capillary with the copper lead was inserted into a glass capillary (ca. 1 mm I.D., 2 mm O.D. and 3.5 cm length), epoxy resin was applied to the two ends of the glass capillary to seal the fused-silica capillary and the copper leads to it (Fig. 1E). Before use, the electrode surface was polished with emery paper, and cleaned in ethanol and water for 5 min, respectively, by an ultrasonicator. The cross-sectional view of the electrode is shown in Fig. 1F.

2.4. Preparation of human urine sample

Twenty-four-hour urine sample from a healthy male(35-year-old, 52 kg) was collected in a bottle containing 15 mL of 6N hydrochloric acid as preservative. A 10-mL sample was stored at −20 °C refrigerator. Prior to analysis, the sample was thawed at room temperature and centrifuged for 10 min at 8000 rpm in order to remove precipitated proteins and other particulate matters.

2.5. Procedure

2.5.1. Capillary zone electrophoresis
Before every run, the capillary was flushed with 0.1 mol/L NaOH, water and the run buffer, respectively. The alignment between the working electrode and the capillary outlet was carried out with the aid of a microscope and a small mirror by adjusting a three-dimension adjustor. Then a voltage of 20 kV was applied across the capillary and the detection potential was applied at the work electrode. After the electroosmotic current reached a constant value (after about 10 min), the electrokinetic injection of the standard solution or samples was manipulated up. Then, the separation voltage was applied again and the electropherogram was recorded.

3. Results and discussion

3.1. Development of separation conditions for monoamines

Because the chemical structure and properties of these monoamines are very similar, separation and determination of these compounds is a challenging task. After our primary investigation, we found that peaks of 5-HT and DA, NE and E are two group of overlapped peaks. The pH value of the running buffer is considered as one of the most important parameters in CE because of its effect on the electroosmotic flow as well as the over charge of the analytes. So it is vitally important to select the optimum pH value of the running buffer. Fig. 2 depicts the effect of the running buffer pH on the resolutions of 5-HT and DA, E and NE with the running buffer containing 160 mmol/L sodium phosphate. As shown in Fig. 2, with the increase of pH value, the $R_{E, NE}$ increased and $R_{5\text{-HT, DA}}$ decreased, the optimum pH
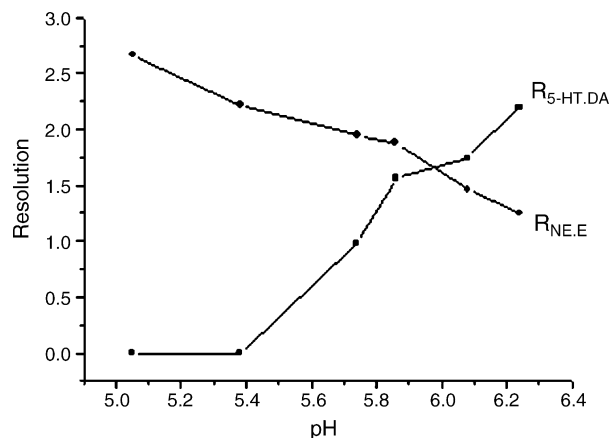

Fig. 2. Effect of buffer pH value on the resolution of the two pairs of substance. Capillary column, 66 cm × 25 μm I.D.; separation voltage, 20 kV; buffer, 160 mmol/L salt phosphate, monoamines concentration, 1.00×10^{-8} mol/L, sample dissolved in water–ACN (30/70, v/v); electrokinetic injection 10 kV for 10 s.

value appeared to be in the region between 5.9 and 6.1, where both $R_{E, NE}$ and $R_{5\text{-HT, DA}}$ are larger than 1.5. So the pH 5.9 was chosen as the optimum pH value.

In addition to the pH value, the concentration of the running buffer is another important parameter affecting not only the resolution and migration time of the analytes, but also the peak current. The effect of the running buffer concentration on the separation was studied in the range from 50 to 180 mmol/L at the optimum buffer pH of 5.9. As the buffer concentration increased, the resolutions of analytes were gradually improved, when buffer concentration was 160 mmol/L, separation of all the analytes was achieved. Phosphate buffer of 160 mmol/L was chosen.

3.2. Optimization the nature and composition of the sample solvent

In FASS, the sample solution is of lower conductivity than the running buffer. Theoretically, the amount of stacking is proportional to the conductivity difference between the running buffer and the sample solution. Adding organic solvents to the sample solution can result in an increase in sensitivity due to the lower conductivity of the sample. Therefore, we studied the influence of the addition of two common organic solvents (methanol, acetonitrile (ACN)) to the sample solvent in FASS mode by injecting the analytes dissolved in water–methanol (30/70, v/v) or water–ACN (30/70, v/v). As shown in Fig. 3, the signal-to-noise ratio of each peak was drastically increased in the presence of 30/70 water–ACN mixture in comparison with the analytes dissolved in pure water. On the other hand, the water–methanol (30/70) induced a slightly lower signal-to-noise than with water. Thus, water–ACN mixture was selected as the sample solvent. The influence of the ACN content in the sample solvent was investigated with constant injection voltage and time of sample. Fig. 4 illustrates the variation in the peak currents when the ACN content in sample solvent increased from 30 to 100%. This effect is based on the changes in conductivity [33]. Although the
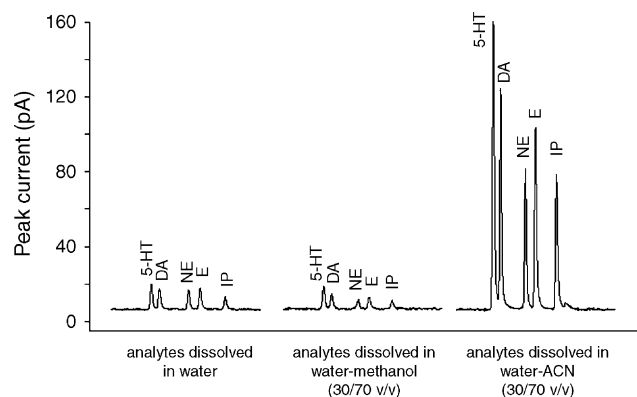
Fig. 3. Comparison of peak heights obtained from the analytes dissolved in water, in water–methanol (30/70, v/v) and in water–ACN (30/70, v/v) by FASS/electrokinetic injection. Other conditions are the same as Fig. 2.

highest sensitivity was obtained with the ACN content of 100%, the 90% of ACN was selected in this work considering the effects of injection parameters (discussed below).

3.3. Optimization of the injection parameters for FASS

Injection voltage and injection time are the factors increasing the stacking amount. To investigate the influence of different injection parameters on the injected sample amount in FASS method, a mixed standard sample in water–ACN (10/90 v/v) was used. As shown in Fig. 5A, the peak currents of the monoamines increased with the sample injection time from 4 to 13 s. However, peak-width broadened when injection time was greater than 10 s. Effect of the injection voltage on the peak currents was shown in Fig. 5B, the peak currents increased with the injection voltage from 10 to 15 kV, however, when the injection voltage of 17.5 kV was applied, the peak currents decreased rapidly. The reason may ascribe to generation of gas bubble at the inlet of capillary when higher injection voltage was applied. This means that when higher ACN content in sample solvent was used, only lower injection voltage can be used. So the selected injection time and voltage were 10 s and 15 kV, respectively. After the
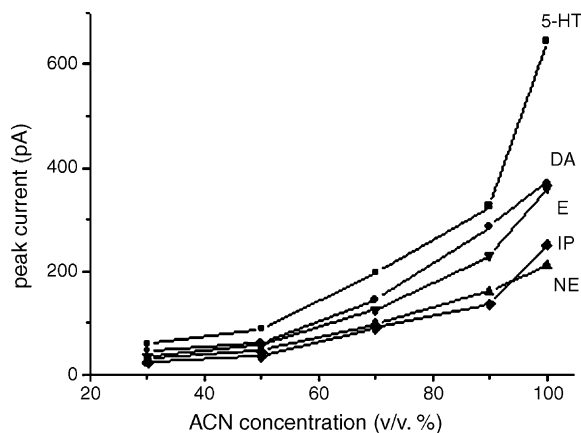

Fig. 4. Effect of the volume fraction of ACN in the sample solvent on the peak current of the five monoamines. Capillary column, 66 cm × 25 μm I.D.; separation voltage, 20 kV; buffer, 160 mmol/L salt phosphate (pH 5.9); monoamine concentration, 1.00×10^{-8} mol/L, electrokinetic injection 10 kV for 10 s.
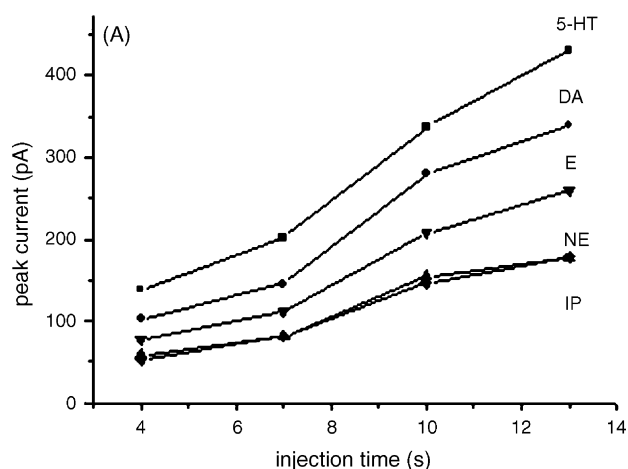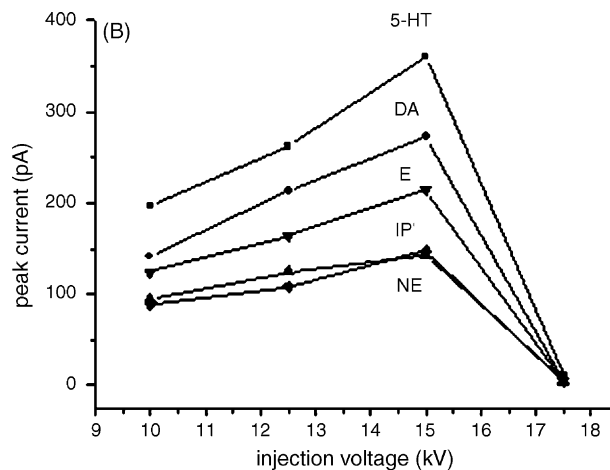

Fig. 5. Effect of sample injection time (A) and voltage (B) on the peak current of the five monoamines. (A) Electrokinetic injection voltage, 15 kV. (B) Electrokinetic injection time, 10 s. Other conditions are the same as Fig. 4.

effect of each single parameter on the separation or the injected amount had been studied, the measurement were carried out by combining the optimum values of each parameter, namely, 160 mmol/L sodium phosphate, pH 5.9, electrokinetic injection with 15 kV for 10 s, and sample dissolved in water–ACN (10/90, v/v) mixture.

Electropherograms under the above FASS and conventional injection of samples are shown in Fig. 6 for comparison. For the conventional injection mode, the samples were dissolved in running buffer and then electrokinetically injected for 5 s at 10 kV. It should be noted that the concentrations of the five monoamines in FASS are 5.00×10^{-10} mol/L, while in conventional injection mode their concentrations are 1.00×10^{-6} mol/L. The stacking efficiency (peak height enhancement factors) could be calculated by multiplying the peak height ratios with the concentration dilution factors. Compared with the conventional injection, the FASS gave a stacking efficiency of 7942-, 7350-, 5239-, 5657- and 5779-fold for 5-HT, DA, NE, E and IP, respectively; it also could be observed in Fig. 6 that the peak widths in FASS were narrower than those in the conventional injection mode. Peak efficiencies of the monoamines range from 284,000 to 359,000 theoretical plates in FASS mode and from 146,000 to 166,000

Table 1
Analytical parameters of the assay of the four monoamines

Monoamines	Regression equation $Y = a + bX$[a]	Correlation coefficient (r)	Concentration range (mol/L)	Limit of detection (mol/L)[b]
5-HT	$Y = 0.167 + 1.66X$	0.9993	1.00×10^{-9} to 2.50×10^{-8}	2.98×10^{-10}
DA	$Y = 0.378 + 1.21X$	0.9997	1.00×10^{-9} to 2.50×10^{-8}	4.42×10^{-10}
NE	$Y = 0.0179 + 0.812X$	0.9992	1.00×10^{-9} to 2.50×10^{-8}	6.00×10^{-10}
E	$Y = 0.0268 + 1.06X$	0.9995	1.00×10^{-9} to 2.50×10^{-8}	5.22×10^{-10}

Experimental conditions were the same as in Fig. 7.

[a] Where Y is the peak height ratios based on internal standard and X is the concentration (10^{-8} mol/L) of the analyte.

[b] Detection limits corresponding to the concentrations giving signal to noise ratio of 3.

theoretical plates in conventional injection mode. The detection limit in the range of 4.17×10^{-11} to 1.63×10^{-11} mol/L for 5-HT, DA, NE, E and IP in standard solution were obtained with FASS mode. Because the FASS mode provided the greatest sensitivities and the highest peak efficiencies for the detection of these monoamines, its conditions were taken consequently as the method for the determination of these monoamines in human urine. However, due to the high salt content in real urine, the method optimization was performed on standard solutions and the sensitivity enhancement and LOD achieved are not amenable with real urine samples.

3.4. Quantification of the monoamines in urine

3.4.1. Reproducibility and linear range

For analysis real samples by FASS, the detrimental effects of high salt content of urine on sensitivity enhancement should be considered. In order to analysis of urine directly without off-line sample pretreatment, dilution of urine sample is needed. It was found that when 100-fold dilution of urine with 10/90 (v/v) water–ACN was applied, a higher sensitivity could be achieved. Therefore, 100-fold dilution was selected in our experiments. Due to averagely 200 mmol/L salt existed in real urine, after 100-fold dilution, the sample still have about 2 mmol/L salt, so
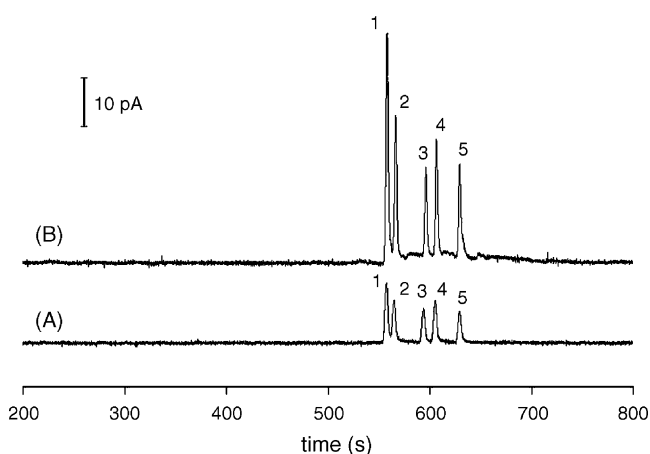
standard monoamines in presence of 2 mmol/L NaCl was used for constructing the internal standard calibration graphs. Under the selected conditions, the calibration graphs were obtained by plotting the relative peak height (peak height of an analyte divided by that of the internal standard) versus the concentration of the analyte. The concentration of the internal standard was 1.00×10^{-8} mol/L. The data presented in Table 1 revealed that a good linear relationship was obtained in the study. The method was validated for the reproducibility of the relative migration times and the relative peak heights of the substance. The result obtained was listed in Table 2. R.S.D. values of the relative migration times and relative peak heights for six replicate injections ranged from 0.13 to 0.28% and from 1.9 to 4.2%, respectively. These experimental results attest the reliability of FASS method to analyze monoamines by CE.

3.4.2. Application

Ten microliters of health human urine was diluted to 1 mL with 10/90 (v/v) water–ACN solution containing the internal standard with the final concentration 1.00×10^{-8} mol/L. The sample mixture was injected and separated under the optimum conditions described above. The electropherograms resulting from the analysis of the real urine sample are shown in Fig. 7. Peaks were identified by comparison with the migration time of standard solutions and by spiking the sample with the standard stock solutions. From the Fig. 7, it was found that 5-HT, DA and IP were well resolved from sample matrix. However, NE and E could be partially separated from background interferents. Because these monoamines carry positive charge in sample solvent, using FASS sample injection, a large fraction of negatively charged interferents can be precluded from these monoamines. Therefore, there are just a few peaks other than those of the monoamines within the migration time necessary for one run. The concentrations of catecholamines and serotonin in urine can be calculated according to the internal standard calibration curve based on peak height other than peak area due to overlapped peaks. The results obtained from urine sample are listed in

Fig. 6. Comparison of electropherograms of the monoamines obtained by routine injection and FASS. Capillary column, 66 cm × 25 μm I.D.; separation voltage, 20 kV; buffer, 160 mmol/L salt phosphate (pH 5.9); (A) monoamine concentration, 1.00×10^{-6} mol/L, sample dissolved in running buffer; electrokinetic injection 10 kV for 5 s; (B) monoamine concentration, 5.00×10^{-10} mol/L, sample dissolved in water–ACN (10/90, v/v); electrokinetic injection 15 kV for 10 s. Peak identification: 1, 5-HT; 2, DA; 3, NE; 4, E; 5, IP.

Table 2
R.S.D. (%) of 1.00×10^{-8} mol/L 5-HT, DA, E and NE ($n = 6$)

	5-HT	DA	NE	E
$t_m/t_{m,IP}$	0.27	0.28	0.19	0.13
$i_p/i_{p,IP}$	3.9	3.0	4.2	1.9

$t_m/t_{m,IP}$ and $i_p/i_{p,IP}$ are the relative migration times and relative peak heights, respectively. Experimental conditions were the same as in Fig. 7.

Table 3
Analytical results of 5-HT, DA, E and NE in a urine sample ($n = 3$)

Analyte	Average concentration (mol/L)	Concentration added (mol/L)	Concentration detected (mol/L)	Recovery (%)
5-HT	$(4.20 \pm 0.171) \times 10^{-7}$	1.00×10^{-6}	$(1.35 \pm 0.051) \times 10^{-6}$	93
DA	$(5.66 \pm 0.198) \times 10^{-7}$	1.00×10^{-6}	$(1.63 \pm 0.062) \times 10^{-6}$	106
NE	$(1.21 \pm 0.069) \times 10^{-7}$	1.00×10^{-7}	$(2.23 \pm 0.087) \times 10^{-7}$	102
E	$(3.70 \pm 0.214) \times 10^{-8}$	1.00×10^{-7}	$(1.47 \pm 0.064) \times 10^{-7}$	110

Experimental conditions were the same as in Fig. 7. The values are mean ± standard deviation of the mean.

Table 3. The concentrations of 5-HT, DA, NE and E in the human urine obtained by the internal standard calibration method were 4.20×10^{-7} mol/L for 5-HT, 5.66×10^{-7} mol/L for DA, 1.21×10^{-7} for NE and 3.70×10^{-8} mol/L for E, which were close to the values (9.7×10^{-7} mol/L for DA, 2.1×10^{-7} mol/L for NE and 8.0×10^{-8} mol/L for E) reported in literature [34]. It should be noted that the values of NE and E detected in the diluted urine sample were near or below the reported LOD of the method. This is because the LOD is obtained with standard monoamines in the presence of 2 mmol/L NaCl. According to the theory of FASS, the amount of stacking is proportional to the conductivity difference between the running buffer and the sample solution. If the concentration of salts in the dilution urine was smaller than 2 mmol/L, then the stacking efficiency for these monoamines in urine was higher than those in standard solution. The recoveries of the method by the analysis of samples spiked with known quantities of standard stock solutions for 5-HT, DA, NE and E were between 93, 106, 102 and 110%, respectively. This method could detect lower levels of analytes and provide some information for clinical use. Compared with conventional HPLC-amperometric detection, the current FASS-CE-AD has advantage of higher separation efficiency, shorter analysis time, relatively simple instrumentation and without off-line sample pretreatment. However, sensitivity is relatively lower due to a small sample volume. FASS-CE-AD combining with off-line sample cleanup and/or further preconcentration could reduce interference and improve sensitivity of the current method.

4. Conclusions

A FASS technique combined with CE-AD for the on-line improvement of detection sensitivity of the monoamines in human urine has been developed in the work. With the method, a stacking efficiency of about 5000-fold with respect to the conventional sample injection for standard solutions was obtained. For real urine samples, about 50-fold improvement in sensitivity was achieved due to the need for dilution prior to sample injection. Internal standard calibration curves used for quantitation were constructed with standard monoamines in presence of an amount of salt, where the salt concentration is similar to that in human urine. Nanomolar-level monoamines in human urine have been detected by FASS-CE-AD without off-line sample preconcentration. Compared with HPLC-amperometric detection, the method is simple, rapid, and low-cost, which can be used in clinical analysis.
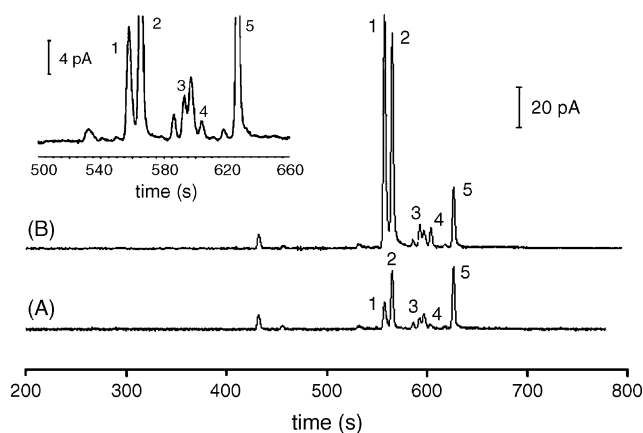
Fig. 7. Electropherograms of urine sample (A) from a health man and spiked sample (B) under optimum conditions. The inset shows an expanded portion of the curve (A) electropherogram. Experimental conditions and compounds identification is the same as in Fig. 6.

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