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Simultaneous determination of psychotropic drugs in human urine by capillary electrophoresis with electrochemiluminescence detection

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Abstract

Amitriptyline, doxepin and chlorpromazine are often used as psychotropic drugs in treatment of the various mental diseases, and are also partly excreted by kidney. This work developed a simple, selective and sensitive method for their simultaneous monitoring in human urine using capillary electrophoresis coupled with electrochemiluminescence (ECL) detection based on end-column ECL reaction of tris-(2,2′-bipyridyl)ruthenium(II) with aliphatic tertiary amino moieties. Acetone was used as an additive to the running buffer to obtain their absolute separation. Under optimized conditions the proposed method displayed a linear range from 5.0 to 800 ng mL^{-1} for the three drugs with the correlation coefficients more than 0.995 ($n = 8$). Their limits of detection were 0.8 ng mL^{-1} (3.6 fg), 1.0 ng mL^{-1} (4.5 fg) and 1.5 ng mL^{-1} (6.8 fg) at a signal to noise ratio of 3, respectively. The relative standard deviations for five determinations of 20 ng mL^{-1} amitriptyline, doxepin and chlorpromazine were 1.7%, 4.2% and 3.6%, respectively. For practical application an extract step with 90:10 heptane/ethyl acetate (v/v) was performed to eliminate the influence of ionic strength in sample. The recoveries of amitriptyline, doxepin and chlorpromazine at different levels in human urine were between 83% and 93%, which showed that the method was valuable in clinical and biochemical laboratories for monitoring amitriptyline, doxepin and chlorpromazine.

Keywords: Capillary electrophoresis; Electrochemiluminescence; Tris(2,2′-bipyridyl) ruthenium(II); Amitriptyline; Doxepin; Chlorpromazine; Simultaneous determination

1. Introduction

Amitriptyline, doxepin and chlorpromazine as shown in Fig. 1 are often used as psychotropic drugs in treatment of the various mental diseases. They are partly excreted by kidney. Amitriptyline and doxepin are tricyclic antidepressants showing sedative effect on anxious and psychomotor nervousness [1], and are used to treat several psychiatric disorders, including major depression, panic disorder, generalized anxiety disorder, obsessive-compulsive disorder, eating disorders, attention deficit hyperactivity disorder, and enuresis in children [2]. They act more effective when they are combined with tricyclic antipsychotic drugs (e.g. phenothiazine or thioxantene derivatives) in treatment of some psychosis [3,4]. Chlorpromazine is a phenothiazine derivative, its action mechanism is based on the blockade of nervous impulses from the central nervous system (CNS) as an antagonist of dopamine receptors [5]. Overdoses of these drugs may cause coma, miosis, delirium and respiratory depression, among other disorders [5,6]. Phenothiazines and tricyclic antidepressants usually exhibit low blood concentrations but often exhibit high- or medium-concentration levels in urine, about 2–10% of a dose is excreted in the urine unchanged [5,6]. On the other hand, therapeutic drug monitoring (TDM) is a practical tool that can help the physician to provide an effective and safe drug therapy in the patients who need medication. Thus a rapid, simple, sensitive and accurate method for the determination of these compounds in urine and monitoring their overdose and TDM has become significant.

Some methods such as spectrophotometry [3,7,8], spectrofluorimetry [9], chemiluminescence methods [10,11], flow injection analysis [12–15], gas chromatography (GC) [16], high performance liquid chromatography (HPLC) with ultraviolet [4,17], fluorescence [17] or mass spectrometric (MS) [18] detection have been developed for detection of one or two of these

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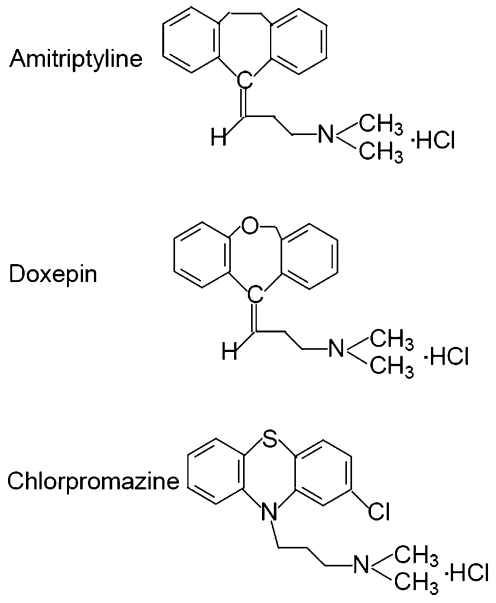

Fig. 1. Structures of amitriptyline, doxepin and chlorpromazine.

drugs. A GC–MS method with electron ionization or chemical ionization has been reported for simultaneous determination of these drugs in the hair of psychiatric patients [16].

In recent years, capillary electrophoresis (CE) has been widely applied for the analysis of pharmaceutical compounds because of its minimal sample volume requirement, efficiency, flexibility, accuracy and high resolution. The analysis of these drugs by CE with ultraviolet detection has been reported [19–24]. Electrochemiluminescence (ECL) is another sensitive detection method, which has advantages of high sensitivity, good selectivity, a wide dynamic linear range, simplicity and inexpensive instrumentation [25] and has been used for sensitive detection of chlorpromazine [26,27]. Two HPLC-ECL methods for determination of some tricyclic antidepressants have also been developed [28,29]. CE coupled with ECL using tris(2,2′-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) has been studied for separation and determination of different compounds containing tertiary amine [30]. A series of methods based on CE coupled with ECL detection of $Ru(bpy)_3^{2+}$, wherein a chemiluminescence reaction is initiated from reagents in the vicinity of the working electrode surface when potential is applied, has been developed for tertiary amines and their derivatives [31–38]. However, to our best knowledge, the CE-ECL determination of these three compounds has not been reported yet.

This work proposed a rapid CE-ECL method for simultaneous determination of amitriptyline, doxepin and chlorpromazine. The metabolites of these drugs gave much lower ECL signal than unchanged drugs and did not interfere with their determination. Furthermore, the metabolites of these drugs could be separated from its original drugs. By using acetone as an additive to the running buffer the absolute separation among these compounds were achieved under optimal conditions. The optimal conditions were obtained by orthogonal experiment design (OAD). The application in monitoring amitriptyline, doxepin, and chlorpromazine in the urine samples showed that the method was valuable in clinical and biochemical laboratories for monitoring amitriptyline, doxepin and chlorpromazine.

2. Materials and methods

2.1. Materials

Pure powders of amitriptyline hydrochloride, doxepin and chlorpromazine hydrochloride were obtained from Nanjing Institute for Drug Control (Nanjing). Tris(2,2′-bipyridyl) ruthenium (II) chloride was purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. All other chemicals were of analytical reagent grade. All solutions were prepared with water purified in a Milli-Q System (Millipore, Bedford, MA). A 2.0 mg mL^{-1} stock solutions were stored in the refrigerator (4 °C). Standard solutions were prepared by serial dilution with purified water. The buffer solutions were prepared with sodium dihydrogen phosphate and disodium hydrogen phosphate. All standard solutions and phosphate-buffered saline (PBS) were weekly prepared and filtered through 0.22 μm cellulose acetate filters (Shanghai Xinya Purification Material Factory) before use.

2.2. Apparatus and procedures

All separation and detection were done on a Model MPI-A CE-ECL Analyzer Systems (Xi'an Remax Electronic High-Tech Ltd., Xi'an, China). A programmable high-voltage power supply (0–20 kV) was applied to perform the electrokinetic sample injection and electrophoretic separation. An uncoated fused-silica capillary with 50 cm length, 25 μm i.d. and 360 μm o.d. was used for separation (Yongnian Optical Fiber Factory, Hebei, China). The new capillary was filled with 0.1 M NaOH and placed overnight before use. A three-electrode system comprising a platinum wire as auxiliary electrode, an Ag/AgCl (3.0 M NaCl) electrode as reference electrode and a 500 μm platinum disk as working electrode was used in the electrochemical measurements in CE-ECL experiments. The ECL emission was detected with a Model BPCL Ultraweak Chemiluminescence Analyzer (Institute of Biophysics, Beijing) in a pulse mode, which was sensitive to photons with a wavelength range of 200–800 nm. The working electrode was adjusted and fixed by three screws from three different directions to align with the capillary under the microscope. The gap between working electrode and capillary was controlled at 70 ± 5 μm [39,40]. The reference and counter electrodes were inserted into the solution above both the capillary and the working electrode. The lower layer of cell was made of a piece of optic glass through which the photons were captured by photomultiplier tube, which was biased at 800 V. A 450 μl, 50 mM, pH 7.0 phosphate buffer containing 5.0 mM $Ru(bpy)_3^{2+}$ was added to the cell for CE-ECL detection.

Electrophoresis in the capillary was driven by a high-voltage power supply (12 kV, 4 μA), which was applied at the injection end with the detection cell held at ground potential through the separation capillary guide. During the experiment, the separation voltage was applied at the injection end, with the reservoir in the

ECL detection cell held at ground potential, and the detection potential was applied at the working electrode. In all experiments, sample introduction was accomplished by electrokinetic injection for 10 s at 10 kV (about 4.5 nL) [39]. Before use, the capillary was flushed with purified water and the running buffer for 15 min by means of a syringe. The running buffer (pH 7.2) contained 20 mM phosphate and 60% acetone. After each run the electrode was treated with a cyclic voltammetric scan in a potential range of −0.5 to 0 V at 100 mV s^{-1} for 2 min [34,39], ascertaining to get better resolution and reproducibility. After a stable baseline ECL signal was reached, electromigration injection was used for sample introduction, and the electropherogram was recorded. The sample concentrations were quantified by ECL peak intensities.

2.3. Sample preparation

The blank urine samples of healthy people collected from student volunteers in the laboratory were used as matrix to spike amitriptyline, doxepin and chlorpromazine. The extraction procedure of these drugs used here was a modification of the technique reported by Wu et al. [20,21]. Urine sample (200 μL) or the spiked sample was pipetted into clean 2.0 mL centrifugation, and 1.0 mL of extraction solvent (heptane:ethyl acetate = 90:10, v/v) was added to the sample and the tube was capped. The sample was vortexed using a medium motion on a shaker for 1 min, and then centrifugated at 2000 rpm for 10 min. The top organic layer was separated and transferred into a clean centrifugation tube. This procedure was repeated and the obtained organic layers were mixed, which was evaporated to dryness under a gentle stream of nitrogen at 35 °C in a water bath. The residue was reconstituted with 200 μL water and vortexed for 60 s. Finally the sample solution was injected into the electrophoresis system by electrokinetic injection for 10 s at 10 kV (about 4.5 nL). The extraction efficiency was estimated by measuring the peak intensity of nonextracted standard solution compared with that of corresponding spiked sample after extracting at each analyte concentration.

3. Results and discussion

3.1. Optimization of ECL detection

Several factors influencing the intensity of ECL, including the applied potential, the distance between the end of the capillary and the working electrode [41], the pH value of detection solution and the concentration of $Ru(bpy)_3^{2+}$. According to the previous results [40–42] the gap between working electrode and capillary was controlled at 70 ± 5 μm for 25 μm i.d. capillary, and the concentration of $Ru(bpy)_3^{2+}$ was choose as 5.0 mM. The preliminary experimental results showed the detection solution at pH 7.0 could give a good ECL response and stable baseline, which was used for ECL detection.

The applied potential was carefully evaluated to achieve a maximum ECL signal. As shown in Fig. 2, the applied potential for ECL detection was investigated towards amitriptyline, doxepin and chlorpromazine by changing from +1.10 to +1.40 V.

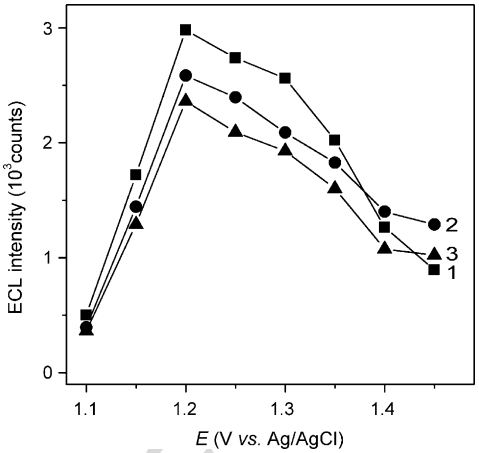

Fig. 2. Effect of applied potential on ECL intensity of 0.2 μg mL^{-1} amitriptyline, doxepin and chlorpromazine. Injection, 10 kV for 10 s; running buffer, 20 mM PBS + 60% acetone at pH 7.2; separation voltage, 12 kV; ECL cell, 5.0 mM $Ru(bpy)_3^{2+}$ + 50 mM PBS at pH 7.0; PMT voltage, −800 V.

The ECL responses were the mean of five measurements. When the applied potential was less than +1.10 V, light emission was not observed since $Ru(bpy)_3^{2+}$ was not oxidized on the electrode. With the increasing applied potential from +1.1 V the ECL intensities for amitriptyline, doxepin and chlorpromazine increased and reached the maximum values at +1.20 V. At low concentration of these compounds the optimum ratio of signal to noise was also obtained at this potential. Therefore, +1.20 V was selected as the applied potential for ECL detection.

3.2. Orthogonal design for the running buffer

In general, mono-variate investigation method, namely when investigating a certain factor, the other factors are fixed as constant, is utilized to optimize the separation conditions. This method can achieve certain satisfying performance. However, the best combination of every experimental parameter cannot be obtained when the interaction between some factors occurs. Combination investigation of all possible factors needs large number of experiments [43]. In order to reduce the experiment number and gain the desired experimental results, orthogonal experimental design (OAD), also known as Taguchi design, can be very efficient to quickly generate useful information on key variable by arranging different factors for effective optimization of experimental conditions. The CE separation buffer involved three factors: pH, concentration of PBS and percentage of organic additive. The preliminary experiments showed the addition of acetone to the running buffer could better improve the separation of these three drugs than other several solvents and additives, such as acetonitrile and cyclodextrin. Furthermore its presence did not affect the ECL detection of $Ru(bpy)_3^{2+}$ system. Thus it was used as an efficient additive for this work. Three factors were studied in the following ranges, respectively. The pH value was from 5.2 to 9.2, PBS was in the concentration range of 10–30 mM and acetone was between 40% and 60% (v/v). The OAD experiments were arranged according to a L_9 (3^4) orthogonal design table (Table 1), and the resolution among

Table 1
Factors and levels for L_9 (3^4) OAD experiments

No.	Buffer pH	Buffer concentration (mM)	Acetone concentration (%)
1	5.2	10	40
2	5.2	20	50
3	5.2	30	60
4	7.2	10	50
5	7.2	20	60
6	7.2	30	40
7	9.2	10	60
8	9.2	20	40
9	9.2	30	50

amitriptyline, doxepin and chlorpromazine peaks was taken as optimization target function. The resolution (R_s) was calculated according to $R_s = 2(t_{r2} - t_{r1})/(w_1 + w_2)$, where t_{r1} and t_{r2} are the migration times of two adjacent peaks, w_1 and w_2 are the baseline width of the two adjacent peaks, respectively [37].

The electrophoregrams of amitriptyline, doxepin and chlorpromazine performed under the buffer conditions according to the L_9 (3^4) table were shown in Fig. 3. Clearly, the optimum conditions were 60% acetone in pH 7.2 20 mM PBS, under which the completely baseline separation with a retention time less than 18 min at a separation voltage of 15 kV could be obtained.

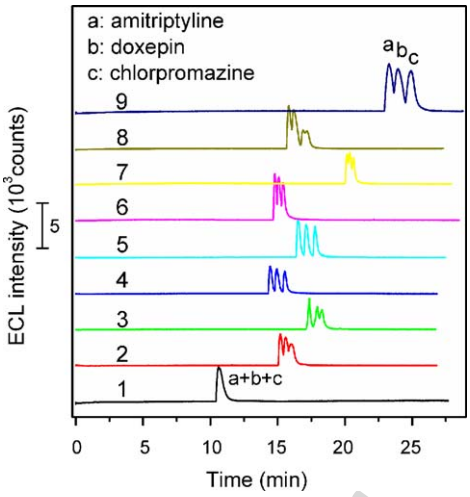

Fig. 3. CE-ECL electropherograms for orthogonal design for the buffer. Injection, 10 kV for 10 s; separation voltage, 15 kV; ECL cell, 5.0 mM $Ru(bpy)_3^{2+}$ + 50 mM PBS at pH 7.0; PMT voltage, −800 V.

3.3. Effect of separation voltage on CE-ECL

Both electroosmotic and electrophoretic velocities were proportional to the field strength. So the increase of separation voltage would shorten the analysis time and increase the efficiency. However, higher voltage would cause higher current and lead to more Joule's heat, which affected the separation of the tested analytes. Meanwhile, with the increasing separation voltage, the EOF increased, more analyte arrived in the diffusion layer of working electrode within a given time to produce higher ECL signal and better peak shape. Thus the applied voltage would affect the ECL signal. A series of operating voltages of 8, 10, 12, 15, 18 and 20 kV were used for optimization of separation voltage. The separation voltages of 12 and 15 kV showed similar resolution. Although the separation voltage of 15 kV could obtain complete separation in a relatively short time, high separation voltage would produce high Joule's heat, which would affect the long-term performance of this method. In addition high voltage was easy to produce discharge in a high humidity air. Thus the separation voltage of 12 kV was selected for following experiments and CE-ECL detection at which a good separation with the analysis time less than 21 min could be achieved.

3.4. Detection limit, linearity and reproducibility

Under the optimized conditions: 20 mM phosphate buffer (pH 7.2) containing 60% (v/v) acetone, 50 mM phosphate detection solution (pH 7.0) containing 5 mM $Ru(bpy)_3^{2+}$, applied detection potential of 1.2 V (versus Ag/AgCl) and separation voltage of 12 kV, the calibration curve for simultaneous detection of amitriptyline, doxepin and chlorpromazine was obtained. The typical electropherograms of the three analytes at different concentrations were shown in Fig. 4A. In the concentration range of 5.0–800.0 ng mL^{-1}, the ECL intensity was proportional to their concentrations with the correlation coefficients of 0.9971, 0.9976 and 0.9984 for amitriptyline, doxepin and chlorpromazine. The linear

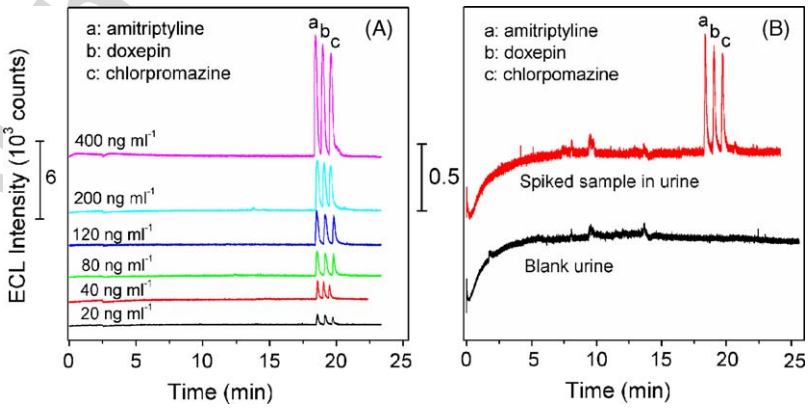

Fig. 4. CE-ECL electropherograms for separation of amitriptyline, doxepin and chlorpromazine at different concentrations (A) and blank urine sample and the urine sample spiked with 0.05 μg mL^{-1} amitriptyline, doxepin and chlorpromazine (B) under optimal conditions.

regression equations were $I = 18.9(\pm 2.6) + 243.3(\pm 10.6)c$ ($\mu g\,mL^{-1}$), $I = 17.0(\pm 3.2) + 227.0(\pm 12.4)c$ ($\mu g\,mL^{-1}$)and $I = 11.3(\pm 3.6) + 221.5(\pm 14.1)c$ ($\mu g\,mL^{-1}$), respectively. Their limits of detection were 0.8 ng mL^{-1} (3.6 fg), 1.0 ng mL^{-1} (4.5 fg) and 1.5 ng mL^{-1} (6.8 fg) at a signal to noise ratio of 3, respectively. The relative standard deviations of the ECL intensity and the migration time for five continuous injections of samples were 1.7% and 0.52% for 20 ng mL^{-1} amitriptyline, 4.2% and 0.54% for 20 ng mL^{-1} doxepin and 3.6% and 0.75% for 20 ng mL^{-1} chlorpromazine, respectively.

3.5. Application

Because an electrokinetic injection mode was employed in this work, the ionic strength of sample matrix would influence the injection of samples. On the other hand, some organic compounds in urine samples might influence the ECL reaction. Therefore, an extraction procedure described in Section 2.3 was performed to remove the ions and some organic compounds in urine to obtain a clear electrophoretic sample profile, high detection sensitivity and good reproducibility. The developed method was applied to the detection of amitriptyline, doxepin, and chlorpromazine extracted from the urine samples. The electrophoregrams were shown in Fig. 4B. The recoveries were 85–92%, 88–93% and 83–89%, respectively. The relative standard deviations for five extraction and CE-ECL procedures at three concentrations were less than 7.3%, showing good reproducibility. This proposed method could therefore be applied to analysis of amitriptyline, doxepin, and chlorpromazine in urine samples.

4. Conclusions

This work proposes a simple, rapid and sensitive method for simultaneous determination of amitriptyline, doxepin and chlorpromazine using CE-ECL detection by end-column mode. To obtain a good separation of these drugs with analogous structures, acetone is used as a running buffer additive. The optimized conditions are 20 mM PBS (pH 7.2) containing 60% (v/v) acetone, 50 mM phosphate detection solution (pH 7.0) containing 5 mM $Ru(bpy)_3^{2+}$, applied detection potential of 1.2 V (versus Ag/AgCl) and separation voltage of 12 kV. This method is practical and valuable in clinical and biochemical laboratories for the determination for amitriptyline, doxepin and chlorpromazine.

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