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Research Article

Simultaneous determination of six toxic alkaloids in human plasma and urine using capillary zone electrophoresis coupled to time-of-flight mass spectrometry

A novel capillary zone electrophoresis separation coupled to electro spray ionization time-of-flight mass spectrometry method was developed for the simultaneous analysis of six toxic alkaloids: brucine, strychnine, atropine sulfate, anisodamine hydrobromide, scopolamine hydrobromide and anisodine hydrobromide in human plasma and urine. To obtain optimal sensitivity, a solid-phase extraction method using Oasis MCX cartridges (1 mL, 30 mg; Waters, USA) for the pretreatment of samples was used. All compounds were separated by capillary zone electrophoresis at 25 kV within 12 min in an uncoated fused-silica capillary of 75 μm id × 100 cm and were detected by time-of-flight mass spectrometry. This method was validated with regard to precision, accuracy, sensitivity, linear range, limit of detection (LOD), and limit of quantification (LOQ). In the plasma and urine samples, the linear calibration curves were obtained over the range of 0.50–100 ng/mL. The LOD and LOQ were 0.2–0.5 ng/mL and 0.5–1.0 ng/mL, respectively. The intra- and interday precision was better than 12% and 13%, respectively. Electrophoretic peaks could be identified by mass analysis.

Keywords: Alkaloids / Capillary electrophoresis coupled to time-of-flight mass spectrometry / Plasma / Solid-phase extraction / Urine
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1 Introduction

Alkaloids, such as brucine, strychnine, anisodamine, atropine, scopolamine, and anisodine, are chemical compounds possessing pharmacological activities. Brucine and strychnine are both present in the seeds of traditional Chinese herb medicine Strychnos pierrian. They are frequently used as important ingredients in Chinese herbal medicines because of their anti-inflammatory properties to treat central nervous system (CNS) diseases, arthritic pain, and traumatic pain [1]. Tropane alkaloids: atropine, scopolamine, anisodamine, and anisodine are main effective ingredients in the Chinese herb Flos daturae, and other solanaceous plants. Tropane alkaloids for therapeutic purpose are isolated from plants and applied as salts such as atropine sulfate, scopolamine hydrobromide, and anisodamine hydrobromide. Atropine is normally used as a parasympatolytic, anticholinergic, spasmolytic, and antiemetic drug [2]. Scopolamine is an antispasmodic agent with antinausea properties and is extensively used in the treatment of motion sickness and in preoperative medication [3]. Anisodamine is a belladonna alkaloid that can reduce rigidity in Parkinsonism and is used as an antidote to poisoning with parasympathomimetic agents such as nerve gases and organophosphorus insecticides [4]. Anisodine, a tropane alkaloids extracted from the roots of Chinese traditional medicine *Anisodus tanguticus,* has important physiological activities such as spasmolytic, anesthetic, and ophthalmic effects [5, 6]. It is often used to treat transmissible shock, hepatitis, nephritis, and diabetes. However, these alkaloids are extremely toxic and unintentional ingestion of plants containing them may cause serious illness, injury, or even death. Analysis of alkaloids in plasma and urine is required for clinical toxicological purpose in cases of suicidal or accidental intoxications and acute poisoning. The chemical structures of the studied alkaloids are depicted in Supporting Information Fig. S1.

Several methods for the determination of these alkaloids have been described: thin-layer chromatography (TLC) [7], gas chromatography-mass spectrometry (GC-MS) [8, 9], high-performance liquid chromatography (HPLC) with ultraviolet detection [10], high-performance capillary electrophoresis (CE) [11–14], liquid chromatography-mass spectrometry (LC-MS) [15, 16], and LC-MS/MS [17, 18]. However, these

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Abbreviations: ER, extraction recovery; **ME**, matrix effect

methods have some drawbacks. TLC is inadequate due to either low resolution or poor reproducibility, while HPLC and CE with UV detection have relatively low sensitivity and cannot give accurate identification of samples. In addition, HPLC requires not only large amounts of toxic organic solvent, but also expensive chromatographic columns that are easily contaminated. Furthermore, unsatisfactory peak shapes are often obtained for alkaloids in the HPLC and LC-MS methods. Therefore, it is desirable to develop an alternative method to overcome these disadvantages.

CE-ESI-TOF-MS is a rapidly developing analytical technology for several routine applications [19, 20]. This method has several distinct advantages including high separation efficiency, small sample size, minimal solvent consumption, high detected sensitivity, and strong qualitative identification for analytes. TOF-MS is useful for screening unknown samples because it can provide accurate mass information of the compounds. TOF-MS was formerly unsuitable for quantitative determination owing to its narrow dynamic range. However, TOF-MS has become more advanced in recent years and has achieved a dynamic range thus it is pretty useful when quantifying any amount of compounds simultaneously [19, 21]. Qualitative identification is also expected to improve by integration of accurate mass and isotopic pattern.

2 Experimental

2.1 Instruments

2.1.1 Capillary electrophoresis

CE separation was conducted on a P/ACE System MDQ (Beckman Instruments, Fullerton, CA, USA), equipped with a diode array detector (DAD) and coupled to the MS detector by an orthogonal electrospray interface (ESI). The CE instrument was operated by a PC running 32 Karat System Software from Beckman.

2.1.2 Mass spectrometry

Mass spectrometry experiments were performed on a micro-TOF (Bruker Daltonics, Bremen, Germany) that was equipped with an orthogonal interface (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and data processing were carried out using the MicroTOF Control and Data Analysis 4.0 software (Bruker Daltonik GmbH)

2.2 Reagents and materials

Reference materials of (i) brucine, (ii) strychnine, (iii) atropine sulfate, (iv) anisodamine hydrobromide, (v) anisodine hydrobromide, (vi) scopalamine hydrobromide, and (vii) berberine hydrochloride were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP, Beijing, China). The purity of each compound was determined to be >98% by HPLC analysis. Acetonitrile, methanol, formic acid, and isopropanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Acetic acid (HPLC grade), ammonium formate, and ammonium acetate (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were analytical grade.

2.3 Stock and working solutions

All standard stock solutions were prepared in methanol at a concentration of 1.0 mg/mL. A series of working solutions were prepared by diluting the stock solution with a mixed solution of methanol and deionized water (1:4, v/v) to the appropriate concentration. Berberine hydrochloride as internal standard substance was added to each mixed working solution to a final concentration of 1 μg/mL. The stock solutions and working solutions were all stored at 4°C until use.

2.4 Calibration standards and quality control samples

Mixed standard solutions were prepared by spiking blank plasma and urine with appropriate amounts of above working solutions to yield final concentrations of 0.50–50 ng/mL for atropine, anisodamine hydrobromide, strychnine, and scopolamine hydrobromide, and 1.0–100 ng/mL for brucine and anisodine hydrobromide. Quality control (QC) samples at lower limit of quantification (LLOQ), low, middle, and high concentrations (0.5, 1.0, 5.0, 20 ng/mL) for atropine, anisodamine hydrobromide, strychnine, and scopolamine hydrobromide, and (1.0, 2.0, 10, 50 ng/mL) for brucine and anisodine hydrobromide were prepared with human plasma and urine for the determination of interday, intraday accuracy and precision, matrix effect, room temperature, and freeze–thaw stability.

2.5 SPE procedure

Both plasma and urine samples were pretreated before analysis. Plasma samples (600 μL) were acidified with 300 μL of 0.4% phosphoric acid, spiked with 50 μL of drugs in concentrations of 0.50–100 ng/mL, mixed with 50 μL (1 μg/mL) of the internal standard in 2-mL tubes then vortexed for 2 min, and centrifuged at 12 000 rpm/min for 5 min. The pretreatment for urine samples (600 μL) included in mixing with 300 μL of 0.05 mmol/L aqueous hydrochloric acid, spiked with 50 μL of drugs in concentrations of 0.50–100 ng/mL mixed with 50 μL (1 μg/mL) of the internal standard in a vortex-mixer. Subsequently, the samples were enriched by SPE on Oasis MCX cartridges. Samples were loaded onto individual SPE cartridges and washed with 1 mL of 2% aqueous formic acid followed by methanol and then 1 mL of

5% ammonium in methanol/water (55:45, v/v). The samples were eluted with 1 mL of 5% ammonia in methanol/water (95:5, v/v). The eluates were evaporated to dryness at 50°C under an air gas stream. The residues were reconstituted in 100 μL of methanol/water (1:4, v/v) for CE-TOF-MS analysis.

3 Results and discussion

3.1 Optimization of CE condition

3.1.1 Influence of buffer pH and concentration

Both ammonium acetate and ammonium formate are commonly used as BGEs in most CE-MS, and consequently the two volatile electrolytes were also used for the preparation of CE-MS running buffer solutions in this study. In this paper, ammonium acetate was selected as the final buffer electrolyte.

The separation buffer pH influences not only the net charge of the compounds but also the electro-osmotic flow inside the capillary, which affects the separation of compounds. When a buffer pH from 4 to 7 was used, the peak of compounds overlapped completely, and IS and anisodine hydrobromide peaks shape were weak. When the pH was increased to 7.60, the peaks of compounds separated partially, but the maximum separation was achieved at pH 8.40 (as shown in Fig. 1.) With the aim of option for resolution and peaks shape, pH 8.40 was selected for this method.

Different concentrations (20–80 mmol/L) of ammonium acetate buffer (pH 8.40) containing 10% acetonitrile were investigated to study the effect of buffer concentration on the resolution. The results are presented in Fig. 2, where it can be seen that with increased concentration of buffer, the separation of the samples was improved. However, high buffer concentration had a significant negative effect on the peak shape and resulted in an increase in migration time. Considering the separation and migration time as a whole, 40 mmol/L ammonium acetate was chosen for subsequent investigation.

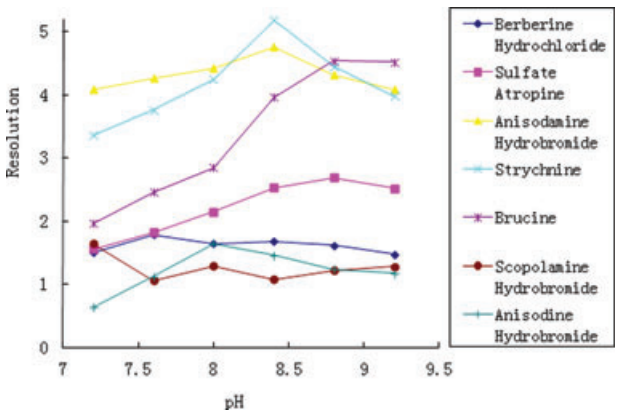

Figure 1. Influence of buffer pH on the resolution of the tested alkaloids.

3.1.2 Influence of organic additives

Under the optimal pH (pH 8.40) and buffer concentration (40 mmol/L ammonium acetate), it was still not possible to separate tested samples completely. Organic reagents were added to the buffer to improve the efficiency of separation. In order to determine the influence of organic solvents, we studied the addition of methanol, acetonitrile, and isopropanol to the buffer. Methanol and isopropanol were found to have little effect on separation of the samples, while the addition of acetonitrile resulted in improving resolution. Figure 3 shows the effect of acetonitrile concentration in BGEs on the separation of the tested alkaloids. However, as the concentration of acetonitrile increased, the migration time became longer. Finally, 10% acetonitrile in the buffer was selected.

3.1.3 Influence of separation voltage

Increasing the voltage will reduce migration time. However, higher voltage may result in higher Joule heat that will directly affect the separation efficiency and peak shape of the tested alkaloids. The effect of voltage was investigated in the range of 10–30 kV. It was found that the separation voltage of 25 kV was the most satisfactory.

3.1.4 Influence of sample solvent

Initially, the sample was dissolved in the water or methanol and then introduced into the capillary. It was found that current was unstable and peak shape was poor. In order to improve peak shape, an online electrophoretic preconcentration method, namely field-amplified sample stacking (FASS), was performed. Briefly, this method relies on the injection of a relatively large volume of a sample to boost the sensitivity. The ionic analyte is then electrokinetically concentrated at the boundary between two zones of high and low conductivity created by the prior injection of a plug of solvent without the electrolyte. When employing this method, the sample was dissolved in a mixture of methanol and water. It was observed that peak shape became increasingly sharp with a mixture of methanol and water at a ratio of 1:4 (v/v).

3.2 Optimized SPE procedure

Although mass spectrometry provides both high sensitivity and selectivity, due to the very low concentration of the analytes in plasma and urine, preconcentration of the samples before CE-MS analysis was usually required. In this work, SPE procedure was used using Oasis MCX cartridges. Oasis MCX cartridges provide two retentive modes including ion exchange and reverse phase retention resulting in higher selectivity and sensitivity for alkaline compounds.

In order to establish the optimum conditions for the SPE procedure, the composition of washing solution and eluting solution were investigated. Different proportions of the wash solution (formic acid, methanol, and 5%

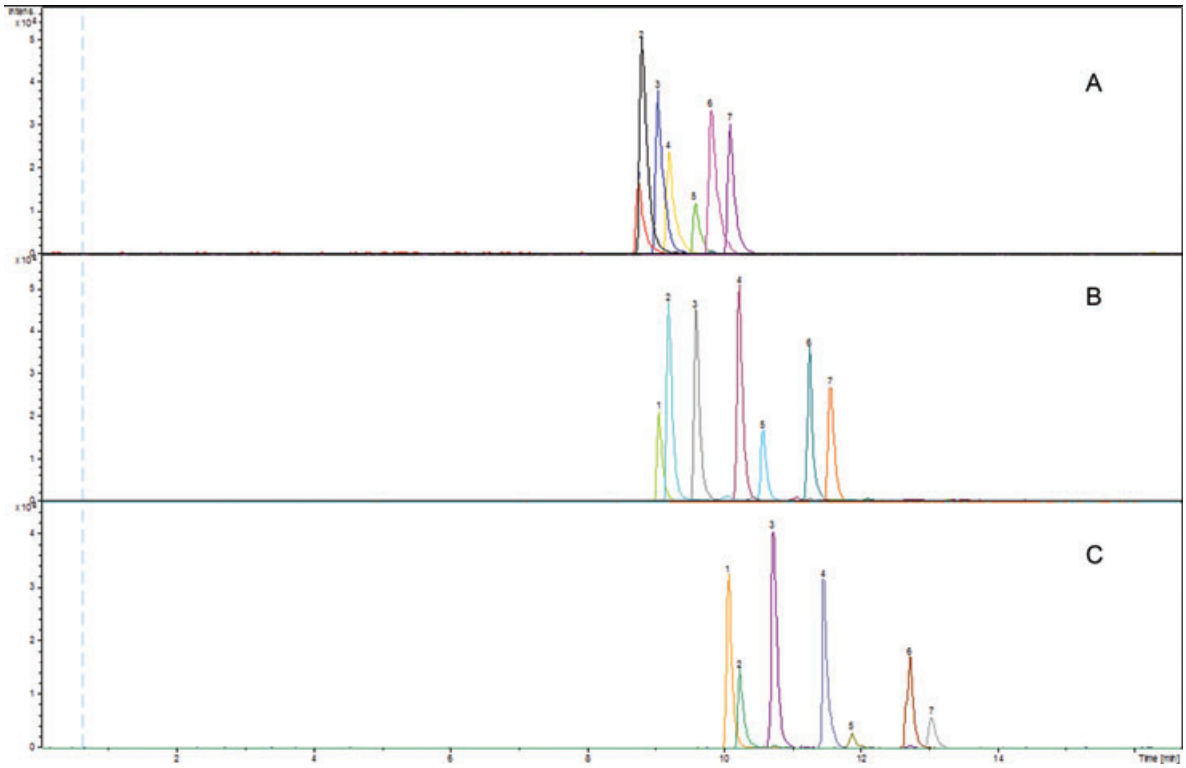

Figure 2. The influence of buffer concentration (A) 20 mM, (B) 40 mM, and (C) 60 mM on the separation of the tested alkaloids.

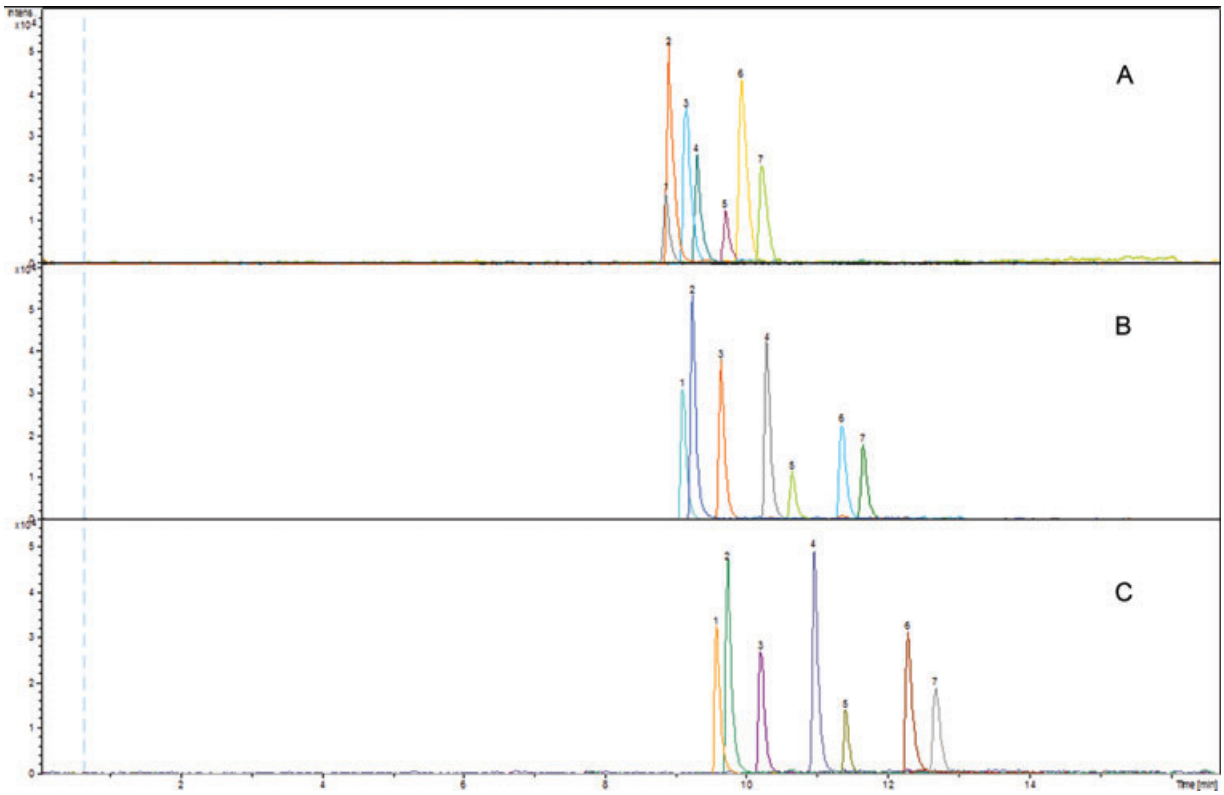

Figure 3. The influence of acetonitrile concentration (A) 5%, (B) 10%, and (C) 20% on the separation of the tested alkaloids.

ammonium methanol) were evaluated. Finally, 1 mL of 2% aqueous formic acid, 1 mL of 100% methanol, and 1 mL of 5% ammonium in methanol/water (55:45, v/v) were selected as the wash solution. Different proportions of eluting solution also were studied. A mixture of 5% ammonium in methanol/water (95:5, v/v) provided the best results.

3.3 Optimized the CE-ESI-TOF-MS coupling

Some MS parameters played a key role in obtaining accurate and reliable experimental results. Therefore, these parameters were optimized when searching for the best CE-ESI-MS analytical conditions.

3.3.1 Optimization of the coaxial sheath liquid

According to previous studies [22], the nature, composition, and flow rate of the sheath liquid have a critical effect on the performance of CE-MS analysis. In this work, three different kinds of mixed solution (methanol/water, acetonitrile/water, and isopropanol/water) were investigated. It was found that an isopropanol/water mixture (50:50, v/v), in the presence of 0.5% formic acid gave the most stable and highest MS signal. The make-up flow rate of sheath liquid was studied between 2 and 6 μL/min. While higher flow rates resulted in a lower signal-to-noise (S/N) ratio due to the dilution of the separated compounds, lower flow rates affected the stability of the spray negatively. Thus, a compromise was obtained at a flow rate set at 3 μL/min (shown in Fig. 4).

3.3.2 Optimization of nebulizer gas pressure

Nebulizer gas can sustain droplet formation and obtain stable spray at the interface of the CE outlet tip and ESI. Different nebulizer gas pressures in the range of 0.2–1.0 bar were tested. Increasing the nebulizer gas pressure could improve the stability of spray, result in lower sensitivity and resolution, likely due to an aspirating effect [23]. Taking into account all of these conditions, a nebulizer gas pressure of 0.4 bar was observed to be optimal without a noticeable effect on CE resolution and electrospray stability (shown in Fig. 5).

Besides of all these above-mentioned conditions, the effect of drying gas was investigated on the experimental results. The drying gas temperature was found to be optimal at 200°C with the best drying gas flow rate at 4 L/min.

Based on the above optimizations, the optimal experimental results were obtained by using the following CE–MS conditions: the running buffer was composed of 40 mmol/L ammonium acetate/ammonia, pH 8.40, 10% acetonitrile, voltage 25 kV, 10 s injection time. A sheath liquid isopropanol/water (50:50) with 0.5% formic acid delivered at a flow rate of 3 μL/ min, a drying gas flow rate at 4 L/min at 200°C, and a nebulizer gas pressure of 0.4 bar was supplied for ESI formation. Under these conditions, six toxic alkaloids and IS could be separated within 12 min (shown in Supporting Information Fig. S2) and accurate molecular weight was

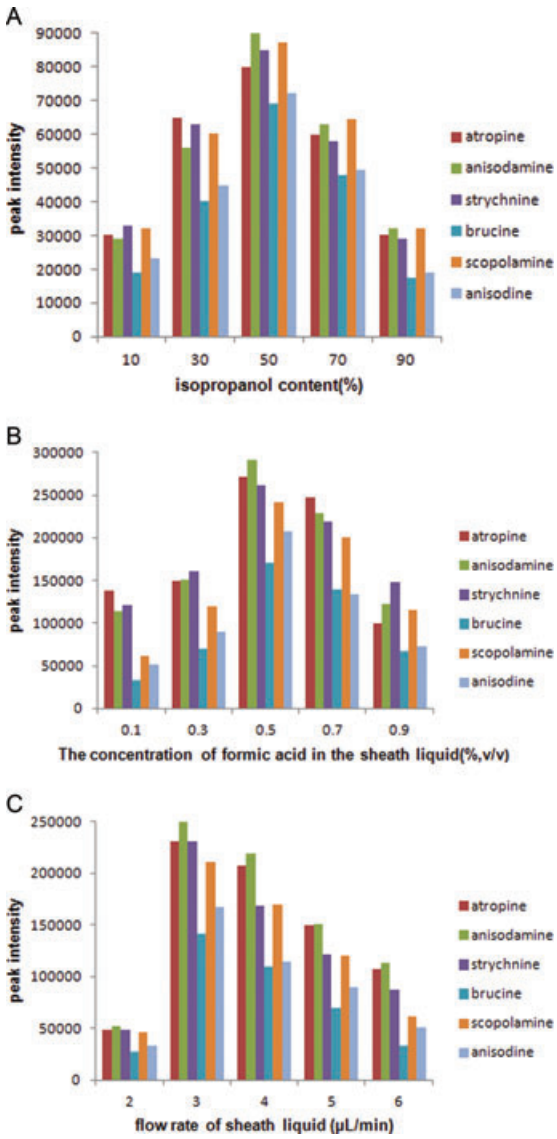

Figure 4. (A) Influence of isopropanol–water composition of the sheath liquid on the peak intensity of six alkaloids. Studies were performed in the presence of 0.5% formic acid (v/v). (B) Influence of formic acid concentration in the sheath liquid on peak intensity of samples. (C) Influence of sheath liquid flow rate on peak intensity of samples.

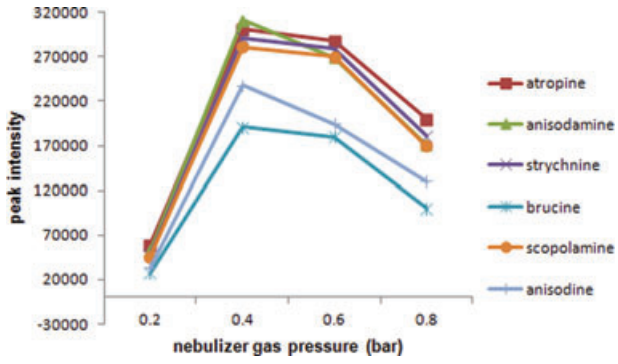

Figure 5. Influence of nebulizer gas pressure on peak intensity of samples.

detected by TOF/MS (shown in Supporting Information Fig. S3). Good mass accuracy was evaluated by the error values (shown in Supporting Information Table S1).

3.4 Method validation parameters

3.4.1 Linearity, precision, and limits of detection

Calibration curves for each alkaloid were obtained by preparing blood and urine samples as described above and performing an electrophoretic separation under the above optimized condition. All compounds were linear over a concentration range of 0.50–50 ng/mL for atropine, strychnine, scopolamine, and anisodamine, and 1.00–100 ng/mL for brucine and anisodine. The linearity of each alkaloids was confirmed by plotting the mean peak area ratio of the drug and IS versus the concentration of the drug. The peak areas were taken from extracted ion electrophegrams of respective alkaloids. Every measurement was calibrated with tune mix in each run to minimize the errors of m/z. The results are listed in Tables 1 and 2.

The LOD was defined as the lowest concentration of the calibration standards fortified in human plasma and urine yielding an S/N of at least 3. The LLOQ was defined as the lowest concentration of the calibration standards fortified in human plasma and urine with both accuracy and precision within ±20%. The results are listed in Tables 1 and 2.

The intraday accuracy and precision (RSD) was evaluated by analysis of the QC samples (LLOQ, A, B, and C) with five determinations per concentrations in the same day. The interday accuracy and precision (RSD) was measured by analyzing the QC samples with five determinations per concentrations on five consecutive days. The method shows that the accuracy was 94.68–107.54% with an RSD of 2.50–11.85% in urine and 91.35–112.46% with an RSD of 3.62–12.37% in plasma.

3.4.2 Recovery and matrix effect

QC samples (LLOQ, A, B, and C) were prepared for evaluating the recovery and ionization suppression or enhancement. Set A was prepared to evaluate the MS response in pure solutions. Set B consisted of three plasma and urine samples, respectively, spiked with working standard solutions after SPE. Set C was composed of plasma and urine samples spiked with working standard solutions before SPE. Three replicates of each QC samples were used for the determination of extraction recovery and absolute matrix effect. The matrix effect and extraction recovery were evaluated by calculating the mean area of set B solution versus the mean area of set A and the mean area of set C solution versus the mean area of set A. Results from study of ME and RE are summarized in Tables 3 and 4. Despite a significant matrix effect, the sensitivity of the assay was still sufficient to detect the six alkaloids in the human plasma and urine. Recovery results indicated that matrix effect does not affect the precision of determination for six alkaloids.

3.4.3 Sample stability testing

The stability of QC plasma and urine samples kept at room temperature for 12 h was evaluated. For the freeze-thaw stability, QC plasma and urine samples were stored at −70°C and thawed at room temperature at three cycles. Three replications for each concentration were performed. It was observed that the six alkaloids in the human plasma and urine at three typical concentrations are stable at the testing conditions for

Table 1. Calibration curves, LOD, and LLOQ of toxic alkaloids in urine

Compound	Linear range (ng/mL)	Regression equation	r	LOD (ng/mL)	LLOQ (ng/mL)
Atropine	0.50–50	$Y = 2.878X + 0.016$	0.9952	0.2	0.5
Anisodamine hydrobromide	0.50–50	$Y = 2.743X + 0.034$	0.9981	0.2	0.5
Strychnine	0.50–50	$Y = 1.636X + 0.065$	0.9984	0.2	0.5
Brucine	1.0–100	$Y = 0.680X - 0.0094$	0.9992	0.4	1.0
Scopolamine hydrobromide	0.50–50	$Y = 1.268X - 0.011$	0.9965	0.2	0.5
Anisodine hydrobromide	1.0–100	$Y = 2.560X - 0.019$	0.9936	0.4	1.0

Table 2. Calibration curves, LOD, and LLOQ of toxic alkaloids in plasma

Compound	Linear range (ng/mL)	Regression equation	r	LOD (ng/mL)	LLOQ (ng/mL)
Atropine	0.50–50	$Y = 3.025X - 0.0092$	0.9953	0.2	0.5
Anisodamine hydrobromide	0.50–50	$Y = 3.163X + 0.0036$	0.9936	0.2	0.5
Strychnine	0.50–50	$Y = 2.731X + 0.0220$	0.9935	0.2	0.5
Brucine	1.0–100	$Y = 1.708X - 0.0186$	0.9982	0.5	1.0
Scopolamine hydrobromide	0.50–50	$Y = 1.209X - 0.0029$	0.9965	0.2	0.5
Anisodine hydrobromide	1.0–100	$Y = 0.287X - 0.0116$	0.9923	0.5	1.0

Table 3. The ME, ER, and precision of six alkaloids in plasma ($n = 5$)

Compound	Added (ng/mL)	ME (%)	ER (%)	RSD%	
				Intraday	Interday
Atropine	0.5	88.25	106.52	6.06	8.93
	1	93.26	75.01	6.35	7.68
	5	81.48	79.06	9.03	12.06
	20	87.06	82.5	7.82	9.46
Anisodamine hydrobromide	0.5	88.32	108.93	12.16	9.74
	1	86.19	105.22	7.58	11.39
	5	92.47	92.06	10.43	8.02
	20	113.05	113.06	13.17	10.17
Strychnine	0.5	91.05	105.22	8.09	10.47
	1	85.48	85.45	9.63	8.52
	5	83.72	81.38	7.58	8.36
	20	89.07	73.18	12.16	9.57
Brucine	1.0	93.08	112.06	6.47	9.16
	2.0	87.05	105.48	7.69	6.33
	10	86.33	82.09	8.43	8.05
	50	109.73	86.39	10.03	13.05
Scopolamine hydrobromide	0.5	86.15	109.75	9.48	4.79
	1	84.39	106.26	4.37	6.93
	5	88.49	109.42	5.62	7.06
	20	93.16	110.08	9.73	11.79
Anisodine hydrobromide	1.0	87.16	105.38	12.48	12.05
	2.0	92.08	82.09	8.65	3.98
	10	83.57	86.42	5.47	7.43
	50	106.38	71.58	10.05	12.68

Table 4. The ME, E, and precision of six alkaloids in urine ($n = 5$)

Compound	Added (ng/ mL)	ME (%)	ER (%)	RSD%	
				Intraday	Interday
Atropine	0.5	91.06	109.07	12.36	8.37
	1	89.38	104.39	9.48	7.03
	5	90.42	87.36	10.95	8.35
	20	84.36	112.09	7.09	9.63
Anisodamine hydrobromide	0.5	83.49	106.42	8.47	10.32
	1	80.17	110.06	6.05	7.85
	5	85.23	87.35	13.96	5.43
	20	81.95	81.56	9.78	11.85
Strychnine	0.5	87.06	109.42	11.15	9.52
	1	83.19	86.37	9.47	7.83
	5	85.38	79.42	10.08	5.39
	20	91.08	73.17	12.25	10.85
Brucine	1.0	89.02	85.06	7.83	10.06
	2.0	94.25	70.37	5.36	10.18
	10	101.48	78.16	13.97	7.54
	50	110.53	73.59	7.56	9.76
Scopolamine hydrobromide	0.5	94.06	108.25	12.25	8.92
	1	86.69	110.47	11.07	8.39
	5	113.27	102.06	6.29	8.17
	20	85.18	87.04	9.65	11.08
Anisodine hydrobromide	1.0	92.17	112.48	5.59	9.38
	2.0	105.36	106.14	13.52	6.17
	10	87.05	87.05	7.59	11.05
	50	81.02	81.79	10.67	10.16

up to 24 h, the repeated freeze and thawing (three cycles) did not affect the stability of all analytes.

3.5 Application to a real plasma sample

This method was successfully applied to determine strychnine and brucine in human plasma. A girl died of the poisoning of these two alkaloids. A plasma sample provided by a forensic engineer named Zhongping Wu of Shanghai Forensic Institute was tested using this method (shown in Supporting Information Fig. S3). In this study, we just did a qualitative test due to the lack of blood, urine, and other related tissues.

4 Concluding remarks

In this paper, a novel CE-ESI-TOF-MS method was established for the simultaneous determination of six alkaloids in human plasma and urine. Under the desired conditions, six alkaloids can be determined and identified. The linearity, accuracy, precision, sensitivity, recovery, and matrix effect were determined in a series of validation experiments and indicated that the developed method is very highly selective. This method represents a valuable tool for the identification of the alkaloids in biological matrix. It is suitable for application in forensic investigation as well as for determining the course of first-aid treatment in accidental poisonings. Hence, the developed method will certainly complement the already existing LC-MS and GC-MS techniques.

The authors have declared no conflict of interest.

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