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Simultaneous determination of five toxic alkaloids in body fluids by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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ABSTRACT

A novel analytical method was developed and validated for the rapid and simultaneous analysis of five toxic alkaloids: Brucine, Strychnine, Ephedrine, Aconitine and Colchicine, in blood and urine using high-performance liquid chromatography–electrospray ionization tandem mass spectrometry in the multiple reaction monitoring (HPLC–ESI-MRM) mode. The linear range was 0.05–50.0 ng mL^{-1} for Brucine, 0.1–50.0 ng mL^{-1} for Strychnine and Ephedrine, 0.01–10.0 ng mL^{-1} for Aconitine and Colchicine. The limits of quantification for Brucine, Strychnine, Ephedrine, Aconitine and Colchicine were found to be 0.03, 0.05, 0.20, 0.05, 0.01 ng mL^{-1}, respectively. The average extraction recoveries in urine ranged from 96.0 to 114.0% and in whole blood were 94.0 to 113.0%. The intra-day and inter-day RSDs were less than 8.3 and 10.6%, respectively. The five alkaloids could be well separated within 7 min in a single run. The established method should be suitable for the determination of trace alkaloids in body fluids.

1. Introduction

Alkaloids such as Brucine, Strychnine, Ephedrine, Aconitine and Colchicine have interesting pharmacological profiles. Strychnine and Brucine are frequently used as important ingredients in Chinese herbal medicines to treat Central Nerve System (CNS) diseases, arthritic and traumatic pains [1]. However, Strychnine and Brucine are extremely toxic alkaloids that exist in the seed of *Strychnos nux-vomica* L. and other species in genus *Strychnos*. The lethal dose (LD) of Brucine in mice is 3.03 mg kg^{-1}. The literature [2–4] indicated that blood concentrations of Strychnine below 2 mg/L are toxic, blood concentrations between 2 and 10 mg L^{-1} may be lethal, and blood concentrations above 10 mg L^{-1} are fatal. The LD of Strychnine is reported to be between 50 and 100 mg kg^{-1} for human [2]. Aconitine has been demonstrated excellent efficacy against rheumatosis, rheumatoid arthritis and some other inflammative diseases. However, it is a major toxic Aconitum alkaloid, Aconitine induces several arrhythmias which sometime leads to death. LD_{50} of Aconitine for mice is 1.8 mg kg^{-1} (single dose, orally) [1] and in humans, the reported minimum LD of orally ingested Aconitine ranges from 2 to 6 mg. Ephedrine is contained in various herbal preparations (e.g. Chinese traditional medicine preparations) and has been utilized for respiratory, antitussive, CNS stimulant, antipyretic and anti-inflammatory purposes [5]. However, Ephedrine has also been associated with adverse health effects, ranging from mild hypertension and palpitations to stroke and even death [6,7]. Colchicine is naturally occurring alkaloid in the *Colchicum autumnale* plant that has been used for the treatment of gout and arthritis for centuries. Although it is quite effective in the management of gout, Colchicine can also be quite toxic to humans [8]. Several cases of Colchicine poisoning (accidental and purposeful) have been reported [9,10]. Medical reports indicate that for adults, more than 0.8 mg kg^{-1} Colchicine is most likely fatal, suggesting a lethal dose of 40–60 mg for a 50–75 kg adult [11]. Thus, the determination of the five alkaloids in body fluids is very important in toxicological and forensic analysis.

Several methods have been proposed for determination of Brucine, Strychnine, Ephedrine, Aconitine and Colchicine in body fluids as well as in herbal medicines, including the use of HPLC [12,13], liquid chromatography–mass spectrometry (LC–MS) [14,15], gas chromatography (GC) [16], GC–MS [17,18], capillary electrophoresis (CE) [19], etc. However, some shortcomings exist in these methods, such as long analytical time, complex procedures. Moreover, the low sensitivity of methods mentioned above

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limits their usage in analyzing these alkaloids in body fluids accurately [20–23]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) performing on-line composition and structural analyses provides a wealthy information that is unsurpassed by other techniques. MS detection in multiple reaction monitoring (MRM) can offer a more sensitive and selective detection technique in comparison to UV and fluorescence detection, so it is more suitable for toxicological analysis purpose. To our knowledge, no LC–MS/MS method was reported for the detection and separation of all of those five alkaloids.

The objective of this work was to develop a simple and sensitive LC–MS/MS analytical method for the detection and determination of five toxic alkaloids, Brucine, Strychnine, Ephedrine, Aconitine and Colchicine, in blood and urine. The whole blood was extracted by 1% trifluoroacetic acid–acetonitrile, and the urine samples were acidified with hydrochloric acid, and followed by clean-up by SPE using Oasis MCX cartridges. After pretreatment, the samples were analyzed by a high-performance liquid chromatography with electrospray ionization tandem mass spectrometry in the multiple reaction monitoring (HPLC–ESI-MRM) mode. The proposed method was validated by evaluating selectivity, recovery, linearity and precision, and has been applied for analyses of five alkaloids in blood and urine.

2. Experimental

2.1. Chemicals and solvents

The acetonitrile (MeCN), methanol (MeOH) and trifluoroacetic acid (CF_3COOH), Merk (Darmstadt, Germany), were HPLC grade. Purified Water was supplied by a Milli-Q water purification system from Millipore (Molsheim, France). The control articles of Brucine, Strychnine, Ephedrine, Aconitine and Colchicine (molecular structure are shown in Fig. 1) were obtained from the National Institute for the Control of Pharmaceutical Products (Beijing, China). Ammonium bicarbonate, aqueous ammonia and hydrochloric acid were purchased from Shanghai Reagent Co. (Shanghai, China). All solvents were filtered through a 0.45-μm membrane before use.

Urine and blood were taken from healthy human volunteers.

2.2. Instruments

An Agilent 1100 series LC/MSD Trap SL (Palo Alto, CA, USA) was used for the method development and validation, consisting of a quaternary pump (G1311A), a column thermostat (G1316A), a degasser unit (G1379A), an autosampler (G1313A), and a diode array detection (DAD) system, and data were analyzed on a computer equipped with LC/MSD Trap Software 4.2 from Bruker Daltonic Inc. (Bruker, Germany).

2.3. Analyte preparation

A standard stock solution of 1 mg mL^{-1} of each alkaloid (Brucine, Strychnine, Ephedrine, Aconitine and Colchicine) was prepared in methanol and stored in the dark at −20 °C stable for more than 3 months. Working standard solutions were prepared in 25-mL calibrated volumetric flasks by mixing the five alkaloid solutions in volumes to obtain analyte concentrations ranging from 1 up to 5000 ng mL^{-1}.

2.4. Solid-phase extraction procedure

Both blood and urine were pretreated in a specific manner. Blood samples (200 μL) were mixed with 300 μL of 1% trifluoroacetic acid–acetonitrile solution on a WH-1 vortex-mixer from Huxi Analytical Instrument Factory (Shanghai, China) for 2 min followed by ultrasonication for 10 min and were centrifuged at 10,000 rpm min^{-1} for 10 min. The pretreatment for urine sample (500 μL) involved in mixing with 500 μL of 0.05 mol L^{-1} HCl in vortex-mixer. Subsequently, the analytes were cleaned up by solid-phase extraction on Oasis® MCX column (Waters Co., USA). The column was first conditioned with 2 mL methanol followed by 2 mL of water. The analytes were loaded onto individual SPE columns,

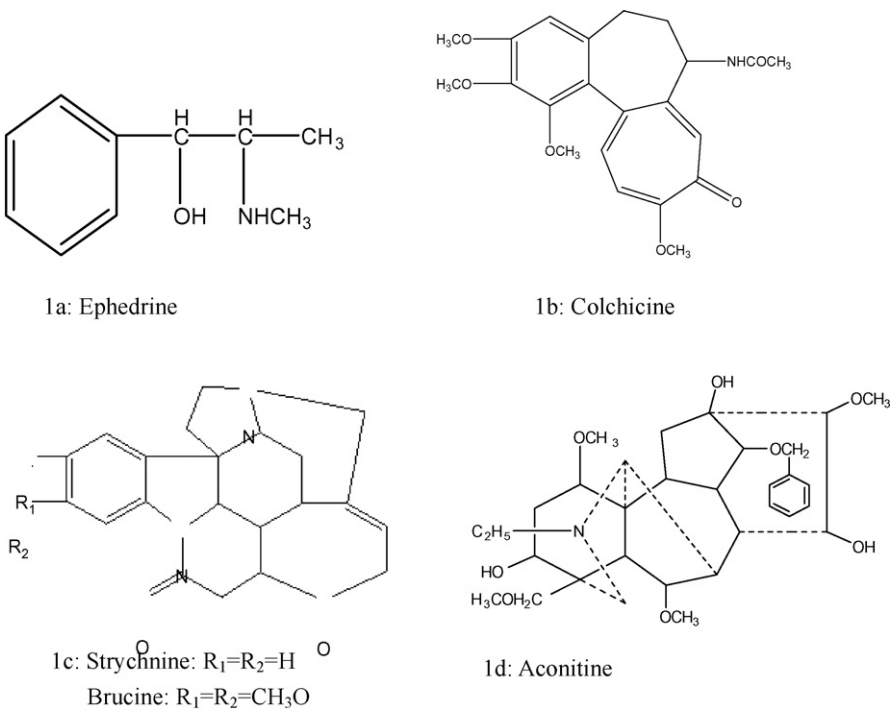

Fig. 1. Molecular structure of Brucine; Strychnine; Ephedrine; Aconitine and Colchicine.

Table 1
MRM parameters for the five alkaloids.

Parameters	Brucine	Strychnine	Ephedrine	Aconitine	Colchicine
Parent ions (*m*/*z*)	395	335	166	646	400
Fragmental ions (*m*/*z*)	324	264	148	586	382
Confirmatory ions (*m*/*z*)	367,282	184,234	133,117	526,368	358,326
Peak base wide (*m*/*z*)	2.0	2.0	2.0	2.0	2.0
Deadline mass number	108	91	43	179	111
CID (V)	1.35	1.35	0.85	0.90	1.10
RT (min)	3.47	4.41	3.81	5.86	3.20

and were eluted with 2 mL of 5% aqueous ammonia/methanol (20/80, V/V). The elutes were evaporated to dryness under nitrogen gas. The residues were reconstituted in 1 mL of methanol, then the methanol solution was transferred into HPLC vials containing limited volume inserts and 10 μL of each sample was injected onto the HPLC column.

2.5. Chromatographic and MS conditions

Separation was performed on a XBridge™ Shield RP18 column (250 mm × 3.0 mm i.d., 5 μm, Waters Co., USA) using the following mobile phase: (A) acetonitrile and (B) 10 mmol L^{-1} of ammonium bicarbonate (aqueous ammonia was used to adjust pH 10.5). An

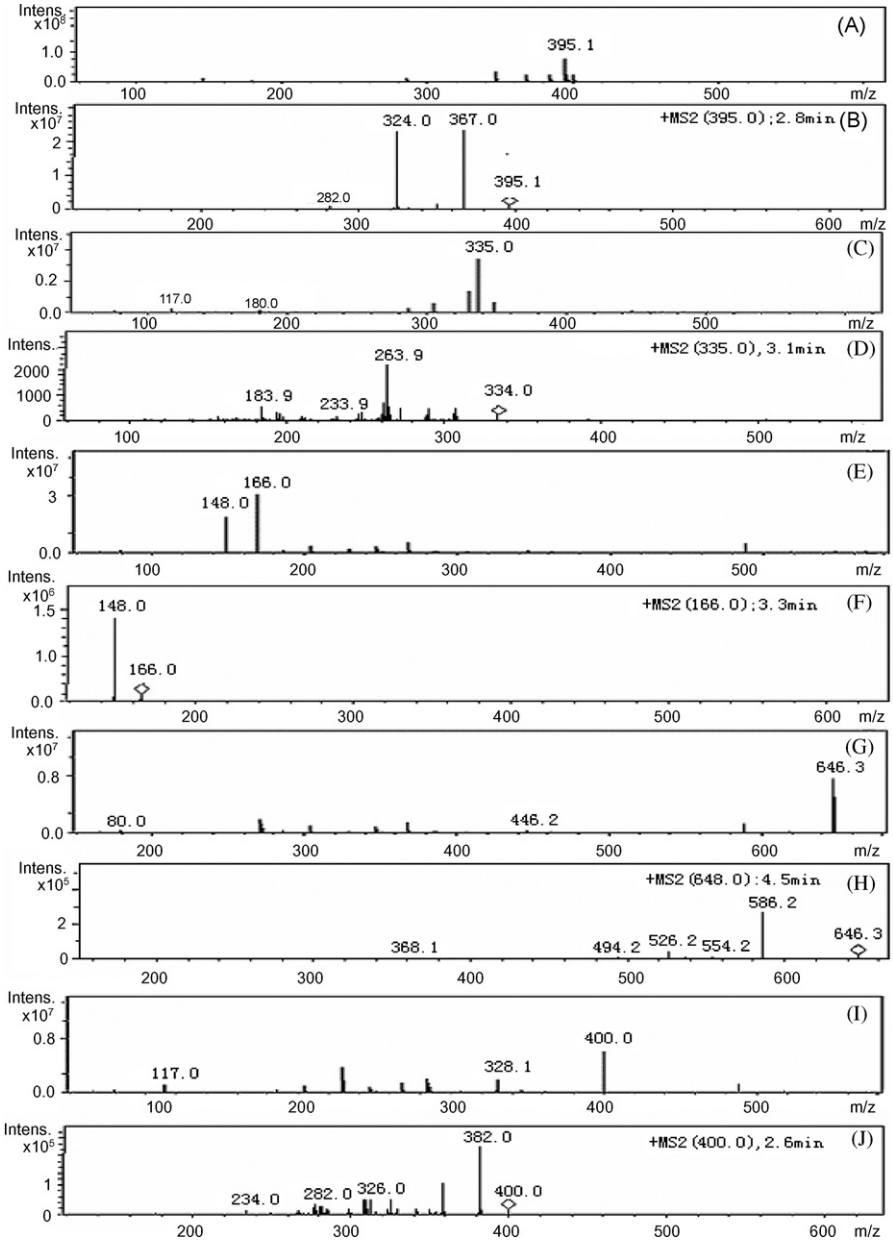

Fig. 2. The MS spectra for (A) Brucine, (C) Strychnine, (E) Ephedrine, (G) Aconitine, (I) Colchicine and MS/MS spectra for (B) Brucine, (D) Strychnine, (F) Ephedrine, (H) Aconitine, (J) Colchicine.

optimized flow rate of 0.5 mL min^{-1} was applied, the column temperature was maintained at 35 °C. The elution was started with 100% A and changed linearly to 40% A at 3.5 min and was further changed to 80% A at 7.5 min. DAD detection wavelength was set at 235 nm.

An Agilent LC/MSD Trap SL mass spectrometer was used in positive mode with a capillary voltage of 1900 V, a capillary exit voltage of 250 V, a dry temperature of 350 °C, a flow rate of high purity dry nitrogen (99.999%) gas of 9.0 L min^{-1}, and a nitrogen nebulizer pressure of 35.0 psi. The mass spectrometer was operated in MRM mode to analyze fragmental ions: Brucine m/z: 395 → 324, Strychnine m/z: 335 → 264, Ephedrine m/z: 166 → 148, Aconitine m/z: 646 → 586 and Colchicine m/z: 400 → 382. The MRM parameters were shown in Table 1. The MS spectra and MRM chromatograms were shown in Figs. 2 and 3 respectively.

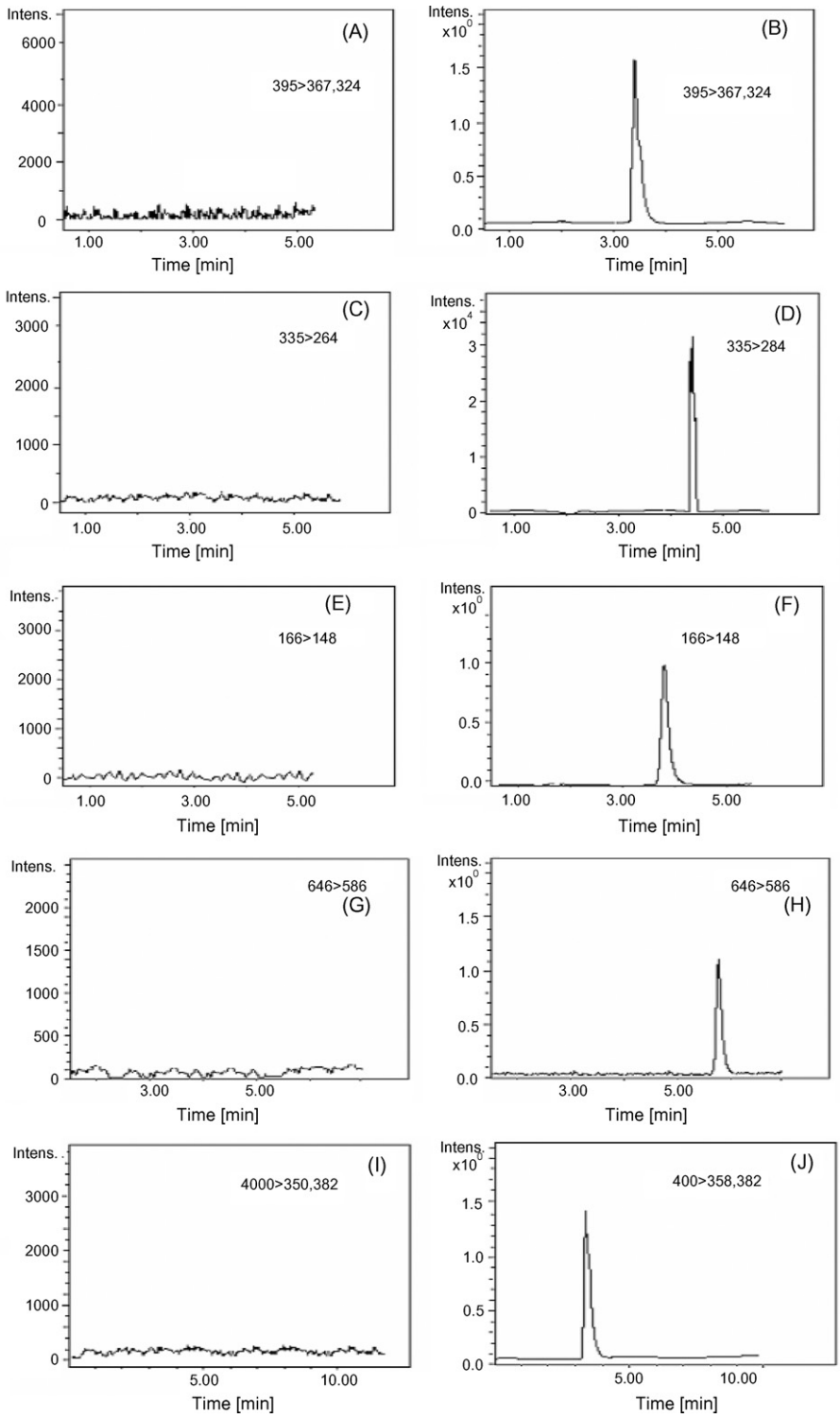

Fig. 3. The MRM chromatograms for blank blood (A) Brucine, (C) Strychnine, (E) Ephedrine, (G) Aconitine, (I) Colchicine, and for spiked with 20 ng/mL (B) Brucine, (D) Strychnine, (F) Ephedrine, (H) Aconitine, (J) Colchicine.

Table 2
The parameters of the method.

Compound	Regression equation	Correlation coefficient	Linear range (ng mL^{-1})	Limit of quantification for MRM (ng mL^{-1})
Brucine	$A^a = 1.25 \times 10^5 C^b + 3.25 \times 10^4$	0.9995	0.05–50.0	0.03
Strychnine	$A^a = 8.02 \times 10^4 C^b + 2.87 \times 10^2$	0.9992	0.1–50.0	0.05
Ephedrine	$A^a = 2.98 \times 10^5 C^b - 4.39 \times 10^3$	0.9997	0.1–50.0	0.20
Aconitine	$A^a = 3.89 \times 10^5 C^b - 1.87 \times 10^3$	0.9992	0.01–10.0	0.05
Colchicine	$A^a = 1.87 \times 10^5 C^b + 5.04 \times 10^3$	0.9996	0.01–10.0	0.01

[a] A represents the peak area.
[b] C represents the concentration of the analytes in blood sample (ng mL^{-1}).

2.6. Calibration curves

Blank blood and urine samples spiked with working standard solutions at five different concentrations (0.01, 0.05, 1.00, 10.0 and 50.0 ng mL^{-1}) were prepared, 20 μL of each final reconstitute solution after SPE was injected into the HPLC–MS/MS in the MRM mode for establishing calibration curves. Calibration curves were peak area versus concentration for each analyte.

3. Results and discussion

3.1. Optimization of chromatographic conditions

In order to optimize the chromatographic conditions for the target compounds in blood and urine, the compositions of mobile phase were studied thoroughly.

In recent years, alkaline buffer, such as ammonium bicarbonate was able to be used for separation of alkaloids, which have showed better resolution ability [24]. Since the values of pK_a of the five alkaloids are different, the effect of mobile phase containing three different pH was examined. They were (A) H_2O/MeCN solution containing 10 mmol L^{-1} of ammonium bicarbonate (pH 7.0); (B) H_2O/MeCN solution containing 10 mmol L^{-1} of ammonium bicarbonate (aqueous ammonia was used to adjust pH 10.5); (C) H_2O/MeCN solution containing 10 mmol L^{-1} of ammonium bicarbonate (aqueous ammonia was used to adjust pH 12.5) respectively. The results indicated that the peak shape and sensitivity have no distinction at different pH of mobile phases for the five alkaloids, but the differences of retention time of the five alkaloids were quite significant. In order to separate the five alkaloids within reasonable retention time and acceptable resolution within the same run, we chose the mobile phase (B) to optimize the separation. As shown in Fig. 4, the baseline of separation could be achieved with MeCN/H_2O (40/60) at initial time. The first peak was Colchicine with retention time of 3.2 min. Through gradient programming (changed linearly to 40% acetonitrile at 3.5 min and was further changed to 80% acetonitrile at 7.5 min) the five alkaloids could be well separated only within 7 min.

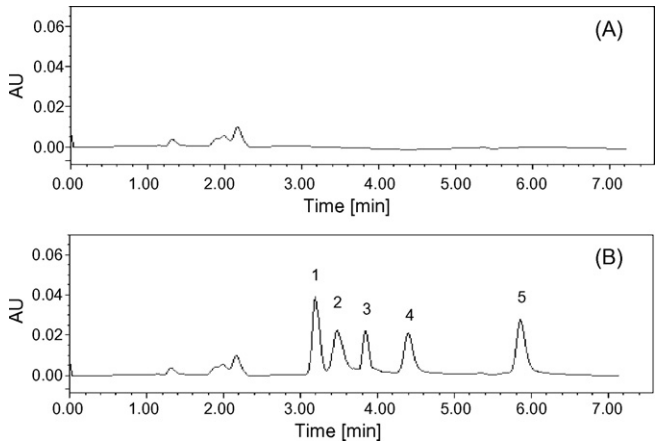

Fig. 4. UV chromatograms for blank blood (A) and spiked (B) with analytes at 0.25 mg L^{-1} each. Peak identity: (1) Colchicine; (2) Brucine; (3) Ephedrine; (4) Strychnine; (5) Aconitine.

3.2. Clean-up optimization

Although mass spectrometry provides both high sensitivity and selectivity, due to the possible very low concentrations of five objective compounds in urine and blood, preconcentration of the samples before HPLC–MS/MS analysis was usually required. The solid-phase extraction (SPE) procedure using Oasis® MCX cartridge could efficiently reduce the high background resulting from the matrix and strengthen the abundance of each alkaloid. To separate and enrich target compounds, the solid-phase extraction column was washed with acid, base and organic solvent. Acidic methanol (containing hydrochloric acid) was used to remove protein, neutral, acidic impurity, and basic methanol (containing 5% ammonia) was used to remove basic impurity.

In order to establish the optimum conditions for the SPE procedure using Oasis® MCX cartridge, the composition of washing solutions and eluting solutions were investigated. Different proportions of the washing solutions (hydrochloric acid: methanol and 5% ammonia methanol) were evaluated. The results indicated that when the concentration of hydrochloric acid in methanol was 0.1 mol L^{-1} and methanol ratio with 5% ammonia was more than 50%, the objective compounds can be determined in washing solutions. Thus, 2 mL of methanol (containing 0.05 mol L^{-1} hydrochloric acid) and 2 mL of methanol: 5% aqueous ammonia (40/60, V/V), respectively, were chosen as the washing solutions without loss of analytes. Different proportions of the eluting solution (5% ammonia:methanol) in the following proportions 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 (V/V) were studied. When 5% ammonia: methanol was 70:30, the eluent reached 100%, therefore, 2 mL of 5% aqueous ammonia/methanol (20/80, V/V) was chosen as the eluting solution.

3.3. Linearity, reproducibility and limits of detection

The calibration experiments were based on five calibration curves. The assay was linear over a concentration range of 0.05–50.0 ng mL^{-1} for Brucine, 0.1–50.0 ng mL^{-1} for Strychnine and Ephedrine, 0.01–10.0 ng mL^{-1} for Aconitine and Colchicine (see Table 2). The linearity of the calibration curve was tested and evaluated using regression equation $A = kC + q$, where A is the peak area and C is the concentration of the analytes in blood sample (ng mL^{-1}), with the correlation coefficient of $r = 0.999$ (see Table 2).

The limits of quantification LOQ of five alkaloids ranged from 0.01 to 2.0 ng mL^{-1}.

Within-day precision was evaluated by continuously performing six repeated analyses for three spiked samples (added five alkaloids to blood and urine, at three different concentrations of

Table 3
The results of recovery, precision ($n = 6$, $\bar{x} \pm s$) and matrix effects.

Compd.	Added ($\mu g\,L^{-1}$)	Blood				Urine			
		ME (%)	RE (%)	RSD (%)		ME (%)	RE (%)	RSD (%)	
				Intra-day	Inter-day			Intra-day	Inter-day
Brucine	0.10	89.4	102.0	5.8	7.8	87.6	114.0	8.3	8.7
	2.00	87.5	108.0	5.7	7.6	83.8	103.0	7.3	6.4
	10.00	86.7	98.0	5.9	6.5	87.5	101.0	7.8	7.8
Strychnine	0.10	88.2	106.0	7.3	7.8	81.8	105.0	6.5	10.6
	2.00	90.4	110.0	4.2	4.8	88.2	108.0	4.9	6.6
	10.00	85.7	103.0	7.2	5.9	82.3	103.0	5.8	8.8
Ephedrine	0.10	91.8	112.0	4.8	7.5	89.8	98.0	7.0	8.6
	2.00	83.3	104.0	7.8	8.9	83.5	110.0	6.1	7.9
	10.00	88.2	94.0	7.9	5.2	85.8	104.0	7.6	7.9
Aconitine	0.10	88.4	111.0	6.1	4.9	88.9	105.0	6.7	4.5
	2.00	87.5	102.0	4.3	6.7	90.7	110.0	5.7	7.0
	10.00	87.7	105.0	5.7	7.7	87.5	98.0	5.2	5.8
Colchicine	0.10	84.9	113.0	7.4	9.0	90.2	106.0	8.2	8.1
	2.00	83.7	105.0	5.8	6.6	81.6	105.0	6.8	6.8
	10.00	86.1	95.0	6.3	8.6	84.6	96.0	7.8	7.7

0.1, 2.0 and 10.0 ng mL^{-1}, respectively). Within-day precision (RSD) on the basis of alkaloids content was less than 8.3% for all the five alkaloids (Table 3). Day-to-day precision was also evaluated by performing repeated analyses twice for three spiked samples as described above each day on 8 different days within a 2-week period. Day-to-day precision (RSD) on the basis of alkaloids content was less than 10.6% for all the five alkaloids (Table 3).

3.4. Recovery and matrix effect

Three sets (A, B and C) solutions were prepared for the evaluation of recovery and ionization suppression or enhancement. Set A was prepared to evaluate the MS–MS response of working standard solutions injected in mobile phase. Set B consisted of three blood and urine samples respectively spiked working standard solutions after SPE. Set C was composed of blood and urine samples spiked before SPE. Three replicates of each set were used for determination of extraction recovery and absolute matrix effect.

The matrix effect (ME) and the effect of the extraction recovery (RE) were evaluated by comparing results from analysis of three sets of samples as follows:

$$\text{ME}\ (\%) = \frac{B}{A} \times 100$$

A—mean peak area of Set A and B—mean peak area of Set B

$$\text{RE}\ (\%) = \frac{C}{B} \times 100$$

C—mean peak area of Set C

Results from study of extraction recovery and matrix effects are summarized in Table 3.

Despite a significant matrix effect (>15%), the sensitivity of the assay was still sufficient to detect the five alkaloids in the human blood and urine. Recovery results showed that matrix does not affect the precision of determination of the five alkaloids. The extraction recoveries for all the five alkaloids were achieved in the range of 96.0–114.0% in urine and 94.0–113.0% in blood (Table 3).

Table 4
Assessment of stability in human whole blood ($n = 3$).

Condition		Freeze–thaw stability (−4 °C)			Short-term stability (room temperature 25 °C)			
		Cycle 1	Cycle 2	Cycle 3	Time (h)			
					0.5	2.0	12.0	48.0
Ephedrine[a] (ng mL^{-1})	1.0	95.6	96.5	95.8	98.7	97.9	96.0	95.0
	15	97.7	98.8	97.8	96.1	97.0	95.9	98.1
	100	97.8	96.8	101.3	97.1	95.5	96.0	98.0
Aconitine[a] (ng mL^{-1})	1.0	95.0	97.9	98.0	98.6	96.0	95.7	97.9
	15	99.0	98.9	95.6	98.5	96.7	98.2	93.4
	100	96.6	96.3	95.3	95.7	96.5	95.5	95.9
Colchicine[a] (ng mL^{-1})	1.0	97.8	98.9	97.4	99.2	95.6	98.6	97.7
	15	96.7	96.8	97.5	98.9	97.3	97.4	94.7
	100	97.4	96.2	94.7	97.4	96.7	93.5	93.8
Brucine[a] (ng mL^{-1})	1.0	95.7	94.6	96.4	99.1	97.9	96.7	96.5
	15	97.5	95.8	95.4	95.7	97.0	99.4	96.8
	100	97.7	96.7	95.7	98.3	95.5	95.2	96.2
Strychnine[a] (ng mL^{-1})	1.0	93.7	93.0	96.0	97.8	97.3	96.7	95.3
	15	94.9	94.8	95.8	97.7	96.5	95.7	95.3
	100	96.9	94.1	92.2	95.7	97.7	96.8	98.4

[a] Expressed as the mean percentage change from time zero (nominal concentration).

3.5. Sample stability testing

The stabilities of QC samples at three different concentrations (1.0, 15.0 and 100.0 ng mL^{-1}) in the whole blood prepared according to the above-mentioned method were tested by freeze–thaw stability (−4 °C) and short-term stability (room temperature 25 °C) assays. Three replications for each concentration were performed. Table 4 shows that five alkaloids in the whole blood at different concentrations are highly stable at the testing conditions with the duration up to 48 h.

4. Conclusion

A rapid, accurate and sensitive method by HPLC–ESI-MRM mode has been developed and validated for the simultaneous determination of five alkaloids, consisting of Brucine, Strychnine, Ephedrine, Aconitine and Colchicine in biological matrix. The method of HPLC–ESI-MRM was successfully applied to the determination of the five alkaloids in blood and urine. Accordingly, the method provides a useful tool in toxicology and forensic science.

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