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Short communication

Simultaneous determination of three aconitum alkaloids in urine by LC-MS-MS

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Abstract

A sensitive method has been developed for the identification and quantification of the toxic alkaloids yunaconitine, crassicauline A, and foresaconitine in urine specimens. After solid-phase extraction using Oasis® MCX cartridges, the extracts were analyzed by LC-MS-MS. The limit of detection is 0.03 ng/mL urine for yunaconitine and 0.05 ng/mL urine for crassicauline A and foresaconitine; the limit of quantitation is 0.15 ng/mL urine for yunaconitine and 0.20 ng/mL urine for crassicauline A and foresaconitine. The method was employed in the analysis of the urine of a 55-year-old male who died after ingestion of herbal medicine powder made from the roots of aconite. Yunaconitine, crassicauline A and foresaconitine were identified in the urine. Crassicauline A and foresaconitine were thus identified in a biological specimen for the first time.

Keywords: Yunaconitine; Crassicauline A; Foresaconitine; SPE; LC-MS-MS

1. Introduction

Plants of the genus *Aconitum* (Ranunculaceae) are widely distributed across northern Asia and North America. Aconite roots have been used in China to treat rheumatic pain, paralysis due to stroke, carbuncle, and furuncle [1]. Since it contains the highly toxic diesterditerpene type aconitum alkaloids, there is a narrow margin of safety between a therapeutic dose and a toxic dose. Fatalities from the ingestion of aconitum, therefore, are frequent and numerous in different parts of the world. In China alone, for example, 878 cases were reported from 1977 to 1985 [2]. The toxicology of aconitum alkaloids is due to activate the sodium channel of excitable cell membranes and leads to rapid paralysis of cardiac, neural, and muscular tissues [3]. Typical symptoms of intoxication include the rapid onset of facial and extremity paresthesias, nausea, generalized weakness, chest discomfort, hypotension and arrhythmias. In severe poisoning; ventricular tachycardia could lead to death [4]. Therefore, identification and quantification of these toxic alkaloids in biological specimens is necessary for forensic medicine and therapeutic drug monitoring.

GC/MS [5–8], HPLC [9–10] and LC/MS [11–14] methods have been used previously for the determination of aconitum alkaloids in biological samples. Most of these methods, however, have focused on the detection of aconitum alkaloids which contain the 8-*O*-acetyl-14-benzoyl functional group, such as aconitine, mesaconitine, hypaconitine, deoxyaconitine and their semi-hydrolyzed derivatives; yunaconitine was detected from a urine sample only in one of these methods [14]. Importantly, yunaconitine together with crassicauline A and foresaconitine which contain the 8-*O*-acetyl-14-anisoyl functional group, are the main active and toxic constituents in many species of *Aconitum*, such as *Aconitum vilmorinianum*, *Aconitum delavayi* and *Aconitum transsectum* which are often used as fork medicine in China. Animal studies ranked yunaconitine as highly toxic as aconitine and crassicauline A showed moderate toxicity, comparable with deoxyaconitine [15]. This study, therefore, explored a new LC-MS-MS method combined with SPE (Solide Phase Extraction). It enables a very sensitive and specific detection of yunaconitine, together with crassicauline A and foresaconitine in urine.

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2. Experimental

2.1. Chemicals and reagents

Yunaconitine, crassicauline A, and foresaconitine were prepared from *Aconitum vilmorinianum* Kom. in our laboratory (by extraction, chromatographic fractionation and crystallization, purity > 99.5%). Extraction columns (Oasis® MCX) were purchased from Waters (Milford, MA, USA). Ammonium bicarbonate was purchased from Fluka Co. (Fluka, Switzerland). Acetonitrile was HPLC grade and purchased from Buridick and Jackson Co. (Muskegon, USA). Deionized water was prepared from Millipore water purification system. All other reagents and solvents were of analytical grade and obtained from Beijing Chemical Co. (Beijing, China).

2.2. Standard solutions

Stock solutions of yunaconitine, crassicauline A, and foresaconitine were prepared in acetonitrile at concentrations 100.0 μg mL^{-1} and were stored at 4 °C and renewed every 6 months. Working standards at a concentration of 1.0 μg mL^{-1} were prepared fresh daily in acetonitrile for preparation of calibration curves.

2.3. Liquid chromatography

An Finnigan Surveyor LC system was used (Thermo Fisher Scientific). These components were directly controlled by an LCQ data system. The mobile phase consisted of acetonitrile: 10 mM ammonium bicarbonate (adjust to pH 9.5 with concentrated ammonium hydroxide) (50:50, v/v). It was pumped at a flow rate of 0.2 mL min^{-1}. The column employed was a XTerra® RP18, 3.5 μm-particle size (100 × 2.1 mm) with guard column XTerra® RP18 (10 × 2.1 mm) (Milford, MA, USA) and was operated at 30 °C.

2.4. Mass spectrometry

The high-performance liquid chromatography analyses were performed using an LCQ Advantage MAX mass spectrometer (Thermo Fisher Scientific) with an ESI source. The scanning mode was in the positive ion mode. The capillary temperature was 275 °C, and the spray voltage 4.5 kv. Nitrogen was used as sheath gas at a flow rate of 35 arbitray units, and helium was used to induce dissocication for acquisition of MS/MS data. The same tune file was created for each standard in continuous flow mode to determine the optimum capillary voltages, lens settings, collision energies, isolation widths and fragment ions, which are listed in Table 1. Full scan and full scan MS/MS data were collected simultaneously. The acquisition was divided into three time segments, each with two scan events, including a full scan and a dependent full MS/MS scan.

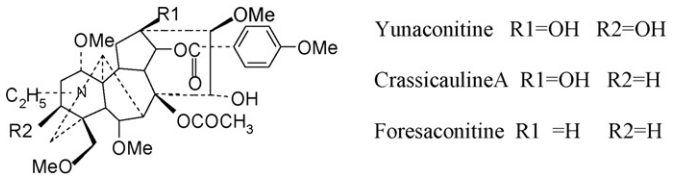

Fig. 1. Chemical structures of yunaconitine, crassicauline A and foresaconitine. Yunaconitine R1 = OH R2 = OH. Crassicauline A R1 = OH R2 = H. Foresaconitine R1 = H R2 = H.

2.5. Sample preparation

Urine samples obtained from three normal volunteers were collected. Standard solutions were spiked into urine. The spiked urine samples (2 mL) were acidified with 50 μL 5 M HCl, and filtered through cellulose membrane filter (0.45 μm). The urine samples were applied to Oasis® MCX cartridge, which had been conditioned and equilibrated with methanol followed by deionized water. After washing the cartridge with 0.1 M HCl and methanol, successively, the alkaloids were eluted by using 5% ammonium hydroxide in 70% methanol. The eluate was evaporated to dryness under 40 °C and dissolved in 100 μL of mobile phase. Ten microliters of the sample was injected into the LC-MS system.

3. Results and discussion

Under the experimental conditions, yunaconitine, crassicauline A, and foresaconitine were detected in full scan, predominantly as a precursor ion $[M+H]^+$ at *m/z* 660, 644 and 628, respectively. The major product ion at *m/z* 600, 584, and 568, corresponding to the cleavage of acetyl group [M-CH_3CO_2] was used to monitor these alkaloids (Fig. 1).

3.1. Specifity

Two healthy female volunteers and one healthy male of comparable age (30–35 years) participated in this study. The volunteers were asked to stop taking any medicines at least 1 week before the study began. After overnight fasting, urine samples were collected from each of the three and analyzed for the presence of interfering peaks at the retention times of yunaconi-

Table 1
Summary of the retention times, MS/MS parameters, and precursor and product ions observed for each of the target compounds

Compound	Retention time (min)	Precursor ion	Product ion	Optimal collision energy (%)	Capillary voltage (V)	Lens setting (V)	Isolation width
Yunaconitine	4.8	660	600	30	42	15	1.0
Crassicauline A	9.5	644	584	30	42	15	1.0
Foresaconitine	12.2	628	568	30	42	15	1.0

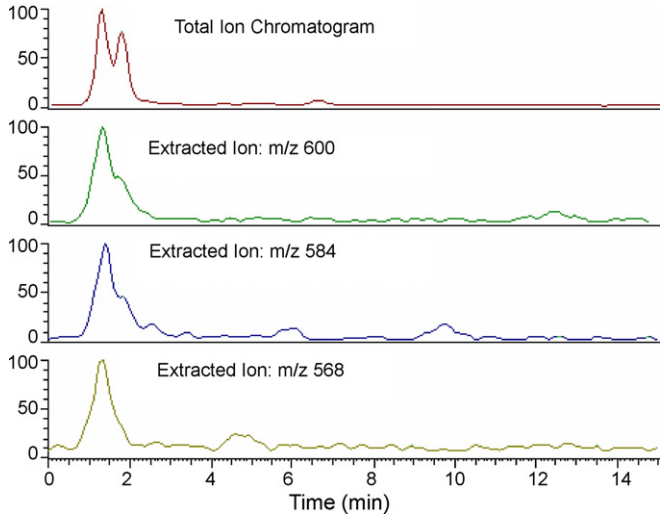

Fig. 2. Application of the LC-MS-MS method to the blank human urine.

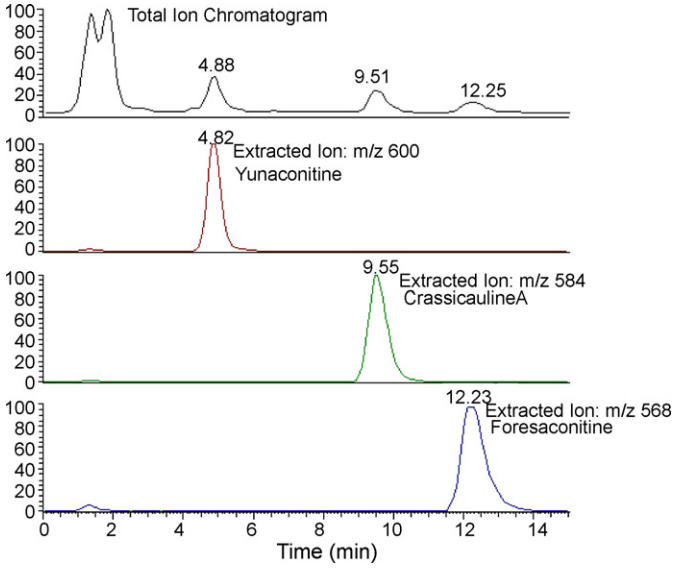

Fig. 3. Application of the LC-MS-MS method to the extracted human urine spiked with 50 ng/mL yunaconitine, crassicauline A and foresaconitine.

tine, crassicauline A, and foresaconitine measured at the above molecular mass ions. No interference was detected in any of the three cases, which confirmed the high specificity of the alkaloids in urine (Figs. 2 and 3).

Table 3
Recovery of the alkaloids (mean ± S.D.)

Concentration (ng/mL)	Yunaconitine (%)	Crassicauline A (%)	Foresaconitine (%)
0.5	75.2 ± 7.3	72.3 ± 3.3	70.1 ± 8.4
50.0	91.4 ± 5.3	88.5 ± 2.3	81.6 ± 3.1
500.0	99.7 ± 0.5	96.0 ± 1.2	89.7 ± 1.6

3.2. Validation of the methods

Linear regression analysis for each of the three alkaloids was performed by the external standard method. The standard curves were prepared by spiking blank urine with known quantities of yunaconitine, crassicauline A, and foresaconitine. The calculated results are given in Table 2, where a, b and γ were the coefficients of the regression equation $y = ax + b$, x referred to the concentration of the alkaloid (ng mL^{-1}), y the peak area, and γ the correlation coefficient of equation. All the alkaloids showed good linearity ranging from 0.2 to 500.0 ng mL^{-1} (Table 2). The limits of detection (LOD) ranged from 0.03 to 0.05 ng mL^{-1} and the limits of quantification (LOQ) ranged from 0.15 to 0.20 ng mL^{-1}.

Absolute recoveries of the analyte were determined in triplicate in normal urine by extracting blank urine samples spiked with yunaconitine, crassicauline A, and foresaconitine. Recoveries were calculated by comparison of the analyte peak areas of the extracted samples with those of the unextracted response standard mixtures representing 100% recovery. The recoveries were calculated using the response standard, since no difference in ionization between extracted samples and pure solutions was observed (Table 3).

Precision and accuracy were assessed based on within and between run analysis. For the within run analysis, five replicates were analyzed at each concentration level. For the between run analysis, three replicates were analyzed at each concentration level. The results are shown in Table 4. The coefficients of variance (CV%) and mean percentage differences from nominal (M%D) for all the analysis were below 15%.

3.3. Forensic application

A 55-year-old man with a history of arthritis was prescribed an herbal remedy, in which aconites were prescribed together with five other nontoxic herbs. Thirty minutes after ingestion of the herbal decoction, he developed extremity paresthesias, gen-

Table 2
Linear regression equation and linear ranges ($n = 5$)

Compound	Regression equation	γ	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Yunaconitine	$y = 0.0125x - 0.0607$	0.998	0.2–500.0	0.03	0.15
Crassicauline A	$y = 0.0823x - 0.0910$	0.998	0.2–500.0	0.05	0.20
Foresaconitine	$y = 0.0375x - 0.0212$	0.999	0.2–500.0	0.05	0.20

In the regression equation $y = ax + b$, x refers to the concentration of the alkaloids (ng mL^{-1}), y the peak area, γ the correlation coefficient of the equation and LOD is the limit of detection which was defined as the concentration corresponding to signal at the y-intercept plus three times its standard deviation, LOQ is the limit of quantification which was defined as the concentration corresponding to signal at the y-intercept plus 10 times its standard deviation.

Table 4
Precision and accuracy of within and between run analyses for the alkaloids in human urine

Compound	Nominal (ng/mL)	Within run[a]		Between run[b]	
		Precision[c]	Accuracy[d]	Precision[c]	Accuracy[d]
Yunaconitine	0.5	10.3	108.3	12.2	110.5
	50.0	5.0	102.5	6.5	106.5
	500.0	2.3	99.3	5.6	100.6
Crassicauline A	0.5	9.8	109.3	11.6	107.6
	50.0	5.7	101.3	8.8	98.2
	500.0	1.2	103.5	6.3	95.2
Foresaconitine	0.5	11.3	109.9	13.2	112.3
	50.0	5.8	97.1	7.6	102.4
	500.0	3.1	99.1	2.3	97.8

[a] Five replicates at each level.
[b] Based on five runs, three replicates at each level in each run.
[c] Coefficient of variance in percentage (CV%).
[d] Mean percentage difference from nominal (M%D).

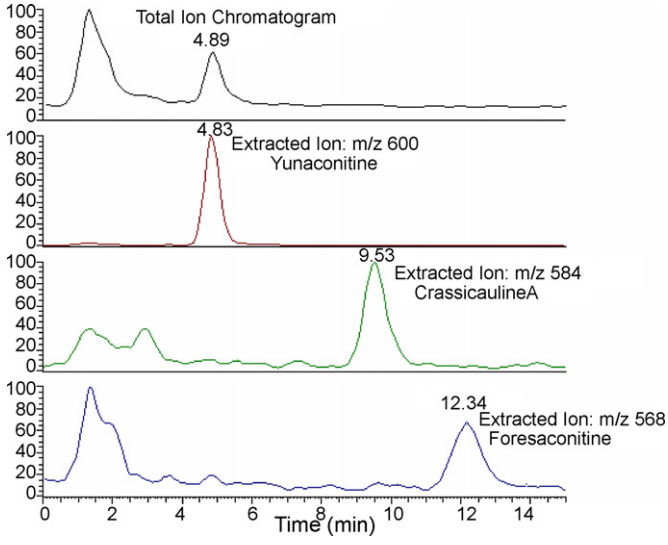

Fig. 4. Application of the LC-MS-MS method to the patient's urine.

eralized numbness, and vomiting. He died 6 h later. Urine was collected for laboratory analysis, using the described method. As a result, yunaconitine together with crassicauline A and foresaconitine were detected. The levels determined were 50.9, 8.9, and 1.2 ng mL^{-1} for yunaconitine, crassicauline A, and foresaconitine, respectively (Fig. 4).

4. Conclusions

The developed method of yunaconitine, crassicauline A and foresaconitine quantification in urine sample is characterized by high sensitivity and appropriate, validating parameters: linearity, precision and accuracy, which can be used in the case of intoxication with these alkaloids in forensic toxicology. The method can be also used in monitoring of these alkaloids in clinical therapy. The work demonstrates that the solid-phase extraction product is clearer and the LC-MS-MS method based on product ion is more sensitive than the previous method [14].

References

[1] Y.T. Tai, P.P. But, K. Young, C.P. Lau, Lancet 340 (1992) 1254–1256.
[2] Y. Gaillard, G. Pepin, J. Chromatogr. B 733 (1999) 181–229.
[3] N.G. Bisset, J. Ethnopharmacol. 4 (1981) 247–336.
[4] C.C. Lin, T.Y. Chan, J.F. Deng, Ann. Emerg. Med. 43 (2004) 574–579.
[5] N. Yoshioka, K. Gonmori, A. Tagashira, O. Boonhooi, M. Hayashi, Y. Saito, M. Mizugaki, Forensic Sci. Int. 81 (1996) 117–123.
[6] K. Ito, Y. Ohyama, Y. Konishi, S. Tanaka, M. Mizugaki, Planta Med. 63 (1997) 75–79.
[7] M. Mizugaki, K. Ito, Y. Ohyama, Y. Konishi, S. Tanaka, K. Kursawa, J. Anal. Toxicol. 22 (1998) 336–340.
[8] K. Ito, S. Tanak, M. Funayama, M. Mizugaki, J. Anal. Toxicol. 24 (2000) 348–352.
[9] H. Ohta, Y. Seto, N. Tsunoda, J. Chromatogr. B 691 (1997) 351–356.
[10] Z.H. Wang, D. Guo, Y. He, C.H. Hu, J.Z. Zhang, Phytochem. Anal. 15 (2004) 16–20.
[11] H. Ohta, Y. Seto, N. Tsunoda, Y. Takanashi, K. Matsuura, K. Ogasawara, J. Chromatogr. B 714 (1998) 215–221.
[12] S.P. Elliott, Sci. Justice 42 (2002) 111–115.
[13] J. Beike, L. Frommherz, M. Wood, B. Brinkmann, H. Köhler, Int. J. Legal Med. 118 (2004) 289–293.
[14] C.K. Lai, W.T. Poon, Y.W. Chan, J. Anal. Toxicol. 30 (2006) 426–433.
[15] D.Y. Zhu, D.L. Bai, X.C. Tang, Drug Dev. Res. 39 (1996) 147–157.