Simultaneous Determination of Three *Aconitum* Alkaloids in Six Herbal Medicines by High-Performance Liquid Chromatography

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Abstract

To simultaneously determine three components of aconitine, mesaconitine, and hypaconitine in six species of *Aconitum* genus, an extraction condition for the total alkaloids was specifically optimized and a simple analytical method of reversed-phased high-performance liquid chromatography (HPLC) was developed. The extraction rate of total alkaloids in *A. szechenyianum* Gay was 98.3% for repeated extracting three times with an acidic alcohol solution (alcohol: pH 3.0 HAc = 85:15, v/v). The chromatography was carried out on a Phenomenex Luna C_{18} column by gradient elution with a mobile phase of 0.03 mol/mL ammonium bicarbonate (pH = 9.50) –acetonitrile at a flow rate of 1.0 mL/min. The method for all three alkaloids had good linear relationships (r > 0.999) in the concentration range of 1.0–200.0 µg/mL. The average recoveries were 96.6–103.1%, and the LOQ and LOD were in the range of 25–37 ng/mL and 9–12 ng/mL, respectively. The quantitative results indicated that contents of the three alkaloids varied significantly among crude aconite roots, so quality control of traditional Chinese medicines containing aconite roots should be taken into account.

Introduction

Traditional Chinese medicine (TCM) plays an important role as a kind of natural drug in the prevention and treatment of diseases. Though it has been widely used in many countries for hundreds of years, the worldwide use of Chinese herbal medicine is blocked to some extent because the quality of TCM is significantly affected by many factors such as the inherent characteristics in crude herds, the time of their harvest, the soil and climate where the herbs grow, and so on. As Chinese medicines are becoming more popular, providing a safe application of those medicines to patients is necessary, and a method for standardization of those medicines is in demand. In other words, the quality control of traditional Chinese medicine is an urgent problem to be solved (1), especially for herbs that contains high toxic compounds.

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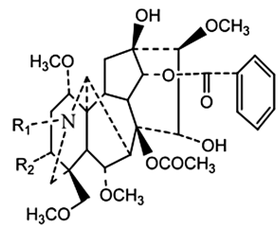
Aconitum plants (Ranunculaceae family) are widely distributed in the mountains and cooler regions of the Northern hemisphere. There are 167 species of *Aconitum* growing in China and among them 44 kinds have been used in folk medicine to treat rheumatic pain, paralysis due to stroke, and carbuncle and furuncle (2,3). *Aconitum* roots contain many diterpenoid alkaloids, including mainly aconitine, mesaconitine, hypaconitine (Figure 1). These alkaloids perform well in treatment of pain, neuronal disorders, and inflammation (4), but they have high toxicity. The LD_{50} of aconitine for mice is 1.8 mg/mL (orally) and 0.308 mg/kg (intraperitoneally) (5,6). Hypaconitine and mesaconitine have a similar toxicity (7).

As the main active and toxic constituents in *Aconitum*, the diterpenoid alkaloids provide an important index in quality evaluation of these crude drugs. In previous papers, researchers had explored different methods, such as thin-layer chromatography, high-performance liquid chromatography (HPLC) (3,8–11), and capillary electrophoresis (12,13) for quantifying these *Aconitum* alkaloids in a few of the aconite roots and proprietary Chinese medicines. However none of these methods investigated the extraction condition of alkaloids from *Aconitum* herbals before determination, and some were unable to baseline separate these alkaloids. As a result, the



Name	R_1	R_2
Aconitine	C_2H_5	OH
Mesacinitine	CH_3	OH
Hypaconitine	CH_3	H

Figure 1. Chemical structure of aconitine, mesaconitine, and hypaconitine.

quantification was short of accuracy. In the current work, extraction processes were optimized by $L_{16}(4^5)$ orthogonal test and univariant methods in turn, and a simple HPLC– photodiode array (PDA) method was developed for simultaneous quantitative determination of the three alkaloids in six herbals most often used in Chinese folk medicine.

Experiments

The sources of the raw materials of *aconite* roots

The raw materials of *Aconitum carmichaeli* Debx. (Chuanwu), *Aconitum Kusnezoffii* Reichb. (Caowu), and *Aconitum pendulum* Bush were obtained from the Xi'an medical market of Shaanxi province (China). *Aconitum taipeicum* Hand-Mazz, *Aconitum szechenyianum* Gay (a), and *Aconitum szechenyianum* Gay (b) were harvested on Taibai Mountain (Shaanxi province of China). All plants were identified by Professor Yi Ren (School of Biological Science, Shaanxi Normal University).

Chemicals and reagents

Acetonitrile was of HPLC grade (Fisher Scientific, Hampton, NH). Deionized water was prepared using a Millipore water purification system (Waters, Milford, MA), which was used after filtering through a 0.45-µm filter. All other reagents were analytical grade.

Reference standards of aconitine, mesaconitine, and hypaconitine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Chromatographic system

A Waters series HPLC system consisting of a model 1525 pump, a model 2996 PDA detector, a model 7725i sample injector equipped with a 10-µL loop, and an Empower software package for data collection and treatment, was employed in this research. A Thermo Orion 868 (Waltham, MA) was used to control the pH of the mobile phase.

The separation of the *Aconitum* alkaloids was carried out on a Phenomenex Luna C_{18} column (250 mm × 4.6 mm, particle size 5 µm) at room temperature (Torrance, CA). The eluents were (A) acetonitrile and (B) ammonium bicarbonate buffer solution (0.03 mol/L, adjusted with concentrated ammonia solution to pH 9.50 ± 0.02). All solvents for HPLC were degassed before use. The gradient elution of mobile phase was 35% (A) in 0–30 min, 35–45% (A) in 30–38 min, and 45% (A) in 38–70 min at a flow rate of 1.0 mL/min. Detection was carried out at 231 nm, and the absorption spectra of the compounds were recorded from 200 to 400 nm.

Preparation of standard solutions and quality control samples

The standard chemicals of three alkaloids were accurately weighed and then dissolved with acetonitrile to prepare the stock solutions (400.0 µg/mL). All stock solution was stored at 4°C before use.

A series of different concentrations of working solutions were prepared by further diluting the stock solution with acetonitrile. Quality control samples at three levels (low, medium, and high) were also independently prepared in the same way. The working solutions and quality control samples were freshly prepared before use.

Preparation of sample solutions

The aconite roots were pulverized into powder and passed through a 0.45-mm sieve, then accurately weighed to approximately 1.0 g. Each weighted sample was refluxed in a 6 mL acidic alcohol solution (alcohol: pH 3.0 HAc = 85:15, v/v) for 1 h and repeated extraction three times. The liquid phase was filtered and combined, and then alcohol was evaporated under reduced pressure. The acidic aqueous solution was basified with concentrated ammonia solution to pH 10.0 and further extracted with chloroform three times (20, 15, 15 mL, respectively). The chloroform was evaporated in a vacuum to dryness, and the solid residue was redissolved with 2.00 mL acetonitrile.

All final resulting solutions were filtered through a 0.45-µm filter membrane, and a 10-µL filtrate was injected into the HPLC system for quantitation.

Calibration

The calibration curve was established on nine data points covering a concentration range of 1.0–200.0 µg/mL for each of the analyzed compounds. Aliquots (10 µL) were used for the HPLC injection. The peak area of each marker was plotted against the corresponding concentration. Linear regression was performed in order to estimate the slope, intercept, and correlation coefficient.

Validation of the chromatographic method

Peak purity. The values of purity angle and purity threshold were used to estimate the spectrum homogeneity and the peak purity. If the value of the purity angle is less than that of the purity threshold, the peak is spectrally homogeneous and the peak purity is good. The values of purity tngle and purity threshold were obtained by treating the spectra with Empower Pro software package (Waters).

Precision. The intra-day precision of the method was researched by determining the high, middle, and low concentration levels of the quality control samples under the selected optimal conditions five times in one day, and the inter-day precision was measured on five consecutive days, expressed as relative standard deviation (RSD).

Limit of quantitation and detection. The limit of quantitation (LOQ) and detection (LOD) were defined as the concentrations of each compound that produce analytical signals equal to ten times and three times the noise signals, respectively.

Repeatability. Three measurements were taken for *Aconitum kusnezoffii* Reichb extract. The relative standard variation of measurements was calculated as the evaluation of the repeatability.

Recovery. For the estimation of recovery, the quality control samples of three alkaloids were added to *A. kusenzoffii* Reichb. extracts whose contents were known. The recoveries were

determined by comparing the found values with the added value of the QC samples. The found values were obtained by subtracting the values of *A. kusenzoffii* Reichb. extracts from those of the samples prepared with the added standards. Mean values with RSD were reported.

Results and Discussion

Optimization of separation conditions

To obtain chromatograms with better separation of adjacent peaks and shorter analysis time, a series of experiments about the influence of different mobile phases and the mobile phase pH values were performed.

In the beginning, we attempted to separate the three alkaloids from each other using a mobile phase of acetonitrile–0.2% acetic acid (adjusted to pH 6.25 with triethylamine). The result showed that these alkaloids exhibited low retention time and did not separate from each other. Then mobile phases at higher pH value were considered. Acetronitrile–phosphate buffer (pH = 8.67), acetronitrile–ammonium bicarbonate buffer (pH = 8.50, 9.00), and methanol–ammonium bicarbonate buffer (pH = 8.50, 9.00) were traced. The acetronitrile–ammonium bicarbonate buffer was chosen as the mobile phase because it produced a sharper and symmetrical chromatographic peak.

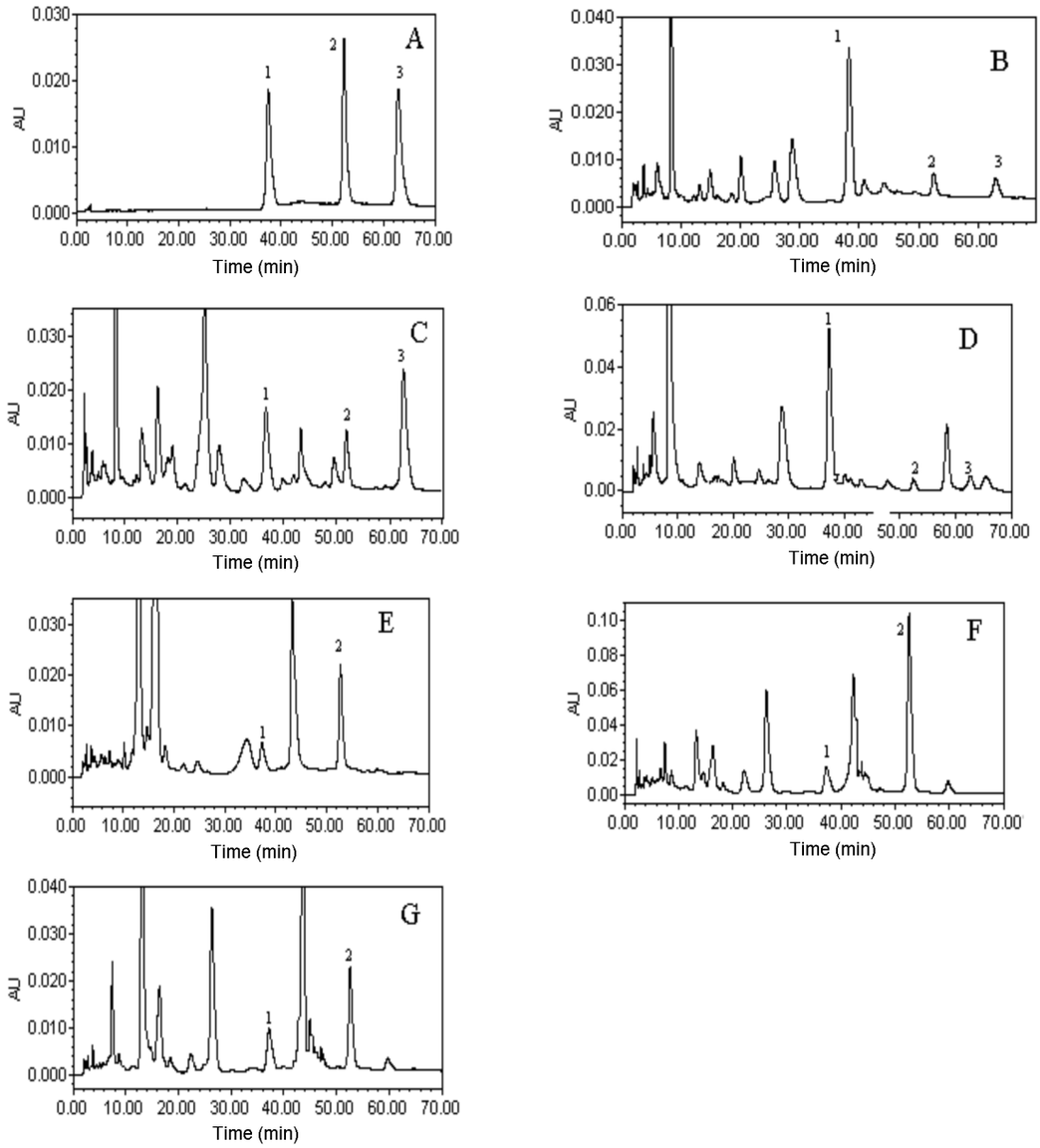


Figure 2. HPLC chromatogram of (A) standard mixture, (B) A. *Kusnezoffii Reichb.*, (C) *A. carmichaeli* Debx., (D) *A. taipeicum* Hand-Mazz., (E) *A. pendulum* Bush., (F) *A. szechenyianum* Gay harvested in October, and (G) *A. szechenyianum* Gay harvested in June. Mesaconitine, 1; Aconitine, 2; and Hypaconitine, 3. Mobile phase: acetonitrile (A)–0.03 mol/L ammomium hydrogen carbonate (B); 0–30 min, 35% (A); 30–38 min, 35–45% (A); 38 min, 45% (A); detection wavelength: 231 nm; injection volume: 10 µL.

The pH dependence of separation efficiency was further investigated with a series of acetronitrile–ammonium bicarbonate buffer (pH = 8.00–10.00). The experimental results showed that the elution times of the markers were prolonged and that interference among different compounds was decreased as the pH value of the buffer increased. At the pH value of 9.5, three alkaloids could be baseline separated from each other. So, the chromatographic analytic condition for the study was carried out at a pH of 9.50 ± 0.02.

Usually speaking, compounds can be eluted using either isocratic elution mode or gradient elution mode in HPLC. The isocratic mode has the advantage of operating easy. The gradient mode is useful for a complex sample, which contains many different polar compounds to be separated. Satisfactory separation was achieved with a mode of isocratic elution for the quality control samples and some real samples. Unfortunately, none of the isocratic elution programs resulted in a good separation of those alkaloids and other unknown compounds in all the samples. Through changing the proportion of acetronitrile in acetronitrile–ammonium bicarbonate buffer (pH = 9.50), a better gradient elution program was obtained for the simultaneous determination of the three alkaloids in six species of *Aconitum* genus.

Figure 2 is an illustration of the separation of alkaloids (1–3) in a standard mixture (A) and each sample extract (B, C, D, E, F, and G) under the condition. All the peaks corresponding to the alkaloids (1–3) are symmetrical.

Extraction condition optimization

In order to obtain the best extraction efficiency of total alkaloids from *Aconitum szechenyianum* Gay extraction processes were optimized by $L_{16}(4^5)$ orthogonal test and univariant methods in turn.

The factors (levels) for $L_{16}(4^5)$ orthogonal test were the type of extracting solvent (pH 3.0 HCl, pH 3.0 acetic acid, water, and 85% alcohol solution), the time for refluxing (0.5, 1.0, 1.5, and 2.0 h), the time with ultrasonic (0, 15, 30, and 45 min), and the ratio of material and solvent (1:4, 1:6, 1:8, and 1:10, g/mL). The content of total alkaloids was measured for each single extraction from 10.0 g of *A. szechenyianum* Gay with a bromophenol blue colorimetry (14) and used as an index to evaluate the extraction condition. The variance analysis showed that extracting solvent and refluxing time were very important factors, that applying ultrasonic had a slight influence, and that the ratio of material and solvent had basically no influence. The extracting condition obtained from the orthogonal test was extracting solvent, 85% alcohol; solvent volume, 6 times of material; refluxing time, 1 h; and no use of ultrasonic.

The result of the orthogonal test suggested that extracting solvents of 85% alcohol and HAc (pH 3.0) both had good extracting capacity. In order to find out a better condition, extracting solvents in the ratio of ethanol to HAc (pH 3.0) of 100:0, 85:15, 60:40, 15:85, and 0:100 (v/v) were further researched. Figure 3 showed that the maximum yield of total

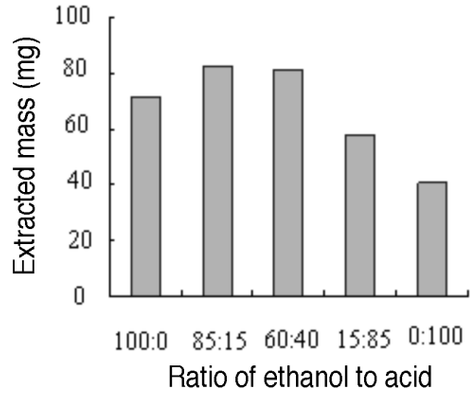


Figure 3. Effect of the ratio of ethanol to HAc (pH 3.0) on the yield of extraction.

Table I. RP C_{18} Column Performance in the Separation of Alkaloids from the Extract of Aconite Roots

Alkaloid	t_R (*n* = 3)	Number of theoretical plates (*n* = 5)	Linear regression equation (*n* = 9)	Range studies (µg/mL)	Correlation coefficient (*r*)
Mesaconitine	37.1	11676	$y = 10957x + 12874$	1.0–200.0	0.9997
Aconitine	52.1	37734	$y = 12208x + 15694$	1.0–200.0	0.9996
Hypaconitine	62.5	24613	$y = 12579x + 12863$	1.0–200.0	0.9997

Table II. Precision of the Intra-day and Inter-day Measurements, LOD, LOQ, and Repeatability of the Three Alkaloids

		Precision (RSD%, *n* = 5)				
Compound	Concentration (µg/mL)	Intra-day	Inter-day	LOD (ng/mL)	LOQ (ng/mL)	Repeatability (RSD%)*
Mesaconitine	160	3.98	5.26	9	25	1.25
	80.0	2.74	3.69			
	8.0	2.85	1.90			
Aconitine	160	2.67	4.81	12	37	1.27
	80.0	4.08	2.97			
	8.0	2.37	2.64			
Hypaconitine	160	3.79	5.48	9	27	0.41
	80.0	1.91	4.61			
	8.0	2.96	5.97			

* Determined with *A. kusnezoffii* Reichb (*n* = 3).

alkaloids was obtained when the ratio of ethanol to HAc (pH 3.0) was 85:15.

Then the influence of multiple extracting was explored with acidic alcohol solution (ethanol: pH 3.0 HAc = 85:15, v/v). The result showed that the sum extracts of total alkaloids increased with extracting times increasing up to five times. Apparently, the alkaloids had been extracted entirely after five extractions. Considering the cost of extraction and that the sum of extracts increased slightly after three extractions, three times extraction was chosen as the optimum condition at which the yield was 98.3% of that of five extractions.

Integrating the results of L_{16} $(4)^5$ orthogonal test and several univariant methods, the optimum extraction condition was obtained: refluxing the sample in acidic alcohol solution of six times the volume to sample for 1 h and being put through three extractions.

Calibration

The regression equations of calibration curves are presented in Table I. All of the alkaloids exhibited good linearity (r > 0.999) and obeyed Beer's law in the investigated concentration range of 1.0–200.0 µg/mL.

Validation of the chromatographic method

The analyte was usually identified by its retention time in the HPLC method. Use of the PDA detector has the advantage of identifying the analyte not only from its retention time but also from its UV spectrum. In this research, three alkaloids were identified by matching these ingredients with corresponding standards for retention time and UV spectrum and investigating the spectrum homogeneity and the peak purity (15). The retention times (Figure 2) and UV spectra (200–400 nm) of the three alkaloids in samples matched well with the corresponding standards. Purity angle values were 0.219, 2.057, and 1.236 respectively, for mesaconitine, aconitine and hypaconitine in *A. taipeicum* Hand-Mazz and less than the corresponding purity threshold values of 0.334, 2.550, and 1.297. All of these results confirmed that the three alkaloids were positively identified.

The results of the precision tests (Table II) demonstrated that the method was reliable with a relative standard deviation percentage of less than 6. The results of LOQ, LOD, and repeatability of the three alkaloids are also listed in Table II.

The average recoveries for mesaconitine, aconitine, and hypaconitine at three QC levels ranged from 99.49 to 101.9%, 101.2 to 103.1%, and 96.62 to 98.43%, respectively. The average recoveries and RSD of the three alkaloids are listed in Table III. From these data, it should be concluded that the proposed HPLC method was appropriate for the determination of the three alkaloids in *Aconitum* roots.

Table III. Recovery of the Three Major Alkaloids from the Extract of *A. kusnezoffii* Reichb

Compound	Added (µg/mL)	Found (µg/mL, n = 3)	Recovery (%)	RSD (%)
Mesaconitine	160.0	159.2	99.49	2.29
	80.00	81.52	101.9	1.74
	8.00	7.980	99.75	3.06
Aconitine	160.0	164.3	102.7	1.41
	80.00	82.50	103.1	1.03
	8.00	8.090	101.2	5.15
Hypaconitine	160.0	154.6	96.62	0.58
	80.00	78.75	98.43	3.77
	8.00	7.820	97.70	1.70

Table IV. Contents of Three Alkaloids in Different Aconitum Samples (µg/g, n = 3)

Samples	Contents (µg/g, n = 3)		
	Mesaconitine	Aconitine	Hypaconitine
A. carmichali Debx.	218.7	40.13	334.5
A. kusnezoffii Reichb.	646.7	69.65	77.80
A. taipeicum Hand-Mazz	1389	78.47	131.7
A. pendulum Busch	96.66	296.3	–†
A. szechenyianum Gay (a)*	345.0	1700	–†
A. szechenyianum Gay (b)*	194.9	331.7	–†

* *A. szechenyianum* Gay (a) was harvested in October, and *A. szechenyianum* Gay (b) was harvested in June.
† Not detected.

Quantitation of alkaloids in different aconite roots

The contents of the three alkaloids in six samples were determined by injecting 10 µL of each sample solutions. The results are presented in Table IV with the mean values of three replicate determinations.

The quantitation results suggested there were significant differences in alkaloids contents among species, even in the same species collected at different seasons, such as the contents of alkaloids in *Aconitum szechenyianum* Gay (a) and that in *Aconitum szechenyianum* Gay (b). Alkaloids are important biological active secondary metabolites, so their contents may vary with an herb's inherent species, where and how it is planted, when it is harvested, and other factors. This variability may be an important reason why fatalities from drinking decoctions of *Aconitum* crude drugs are frequent. Therefore, the contents of the three alkaloids must be determined in order to guarantee that medicines containing *Aconitum* roots are safe to administer.

Conclusion

A method for determination of three major active and toxic alkaloids in *Aconitum* roots was systematically established, including extraction from herbs, and separation and analysis on PHLC. Satisfactory chromatographic separation of three alkaloids was achieved from each other and from other compounds contained in herbs even though the three alkaloids have similar structure. The method developed herein can be applied to the routine quality analysis of TCM and preparations containing *Aconitum*.

Acknowledgments

This project was supported by the National Nature Science Foundation Committee of China (No. 20575039).

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Manuscript received March 10, 2007;
revision received May 18, 2007.