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Short communication

Quantitative determination of diterpenoid alkaloids in four species of *Aconitum* by HPLC

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Abstract

A high performance liquid chromatography (HPLC) method has been developed for the determination of five principal alkaloids (benzoylmesaconine, mesaconitine, aconitine, hypaconitine and deoxyaconitine) found in four species of genus *Aconitum*. The five alkaloids were analyzed simultaneously with an XTerra^{TM}RP18 column by gradient elution using 0.03 M ammonium hydrogen carbonate–acetonitrile as mobile phase. The recovery of the method was 94.6–101.9%, and all the alkaloids showed good linearity ($\gamma = 0.9999$) in a relatively wide concentration range. The results indicated that contents of alkaloids in *Aconitum* varied significantly from species to species; hence quality control of *Aconitum* drugs is very necessary.

Keywords: Benzoylmesaconine; Mesaconitine; Aconitine; Hypaconitine; Deoxyaconitine; High performance liquid chromatography

1. Introduction

Plants of genus *Aconitum* (Ranunculaceae) are widely distributed across North Asia and North America. It is stated that there are 167 species of *Aconitum* grown in China and among them 44 kinds have been used in folk medicine to treat rheumatic pain, paralysis due to stroke, carbuncle and furuncle [1,2]. *Aconitum* roots contains many diterpenoid alkaloids which have analgesic, antipyretic and local anaesthetic properties [3], but they are highly toxic and there is a narrow margin of safety between a therapeutic and toxic dose. Fatalities from ingestion of *Aconitum* are frequent and numerous in eastern Asia. In China alone, 878 cases were reported from 1977 to 1985 [4].

Since diterpenoid alkaloids are the main active and toxic constituents in *Aconitum*, their amounts could be an important index in quality evaluation of these crude drugs. In previous paper, several high performance liquid chromatography (HPLC) methods and capillary electrophoresis methods have been used for determination of aconitine, mesaconitine and hypaconitine in herbs and Chinese medicinal preparations [5–8]. None of these methods, however, are able to baseline separate these alkaloids, so the quantitative results are not accurate. Until now, it has not been possible to simultaneously determine and quantity of benzoylmesaconine, mesaconitine, aconitine, hypaconitine and deoxyaconitine in *Aconitum* spp. A simple HPLC method is presented for quantification of the five major alkaloids in four species of *Aconitum*, which are mostly often used in Chinese folk medicine.

2. Experiment

2.1. Materials

The standard samples of mesaconitine (2), aconitine (3) and hypaconitine (4) were provided by National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, PR China). Benzoylmesaconine (1) and deoxyaconitine (5) were prepared from *Aconitum carmichaeli* Debx in our laboratory (by extraction, chromatographic fraction and crystallization, 99.5% purity), (see Fig. 1). Crude drug samples were collected by the authors and identified by Professor Hubiao Chen (School of Pharmaceutical Sciences, Peking University).

Solid phase extraction (SPE) column (Oasis®MCX) was obtained from Waters (Milford, MA, USA). Acetonitrile and

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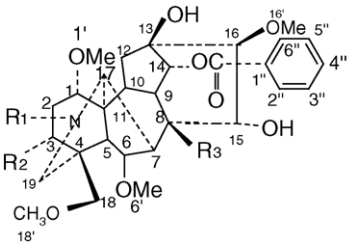

	R_1	R_2	R_3
1. Benzoylmesaconine	Me	OH	OH
2. Mesaconitine	Me	OH	OAc
3. Aconitine	Et	OH	OAc
4 .Hypaconitine	Me	H	OAc
5. Deoxyaconitine	Et	H	OAc

Fig. 1. Chemical structures of 1–5.

methanol were of HPLC grade from Fisher Scientific (Pittsburgh, PA, USA), other solvents and chemicals were purchased from Beijing Chemical Engineering Factory (Beijing, China). Deionized water was prepared from Millipore water purification system (Millipore, Milford, MA, USA). Solvents were filtered through a Millipore filter (0.45 μm) before use.

2.2. HPLC apparatus and conditions

A Waters HPLC system, consisting of a 600E pumps, an AF in-line degasser, a 717 plus autosampler and a PDA 996 detector coupled with Millennium^{32} workstation.

Separation was carried out with a XTerra^{TM}RP18 column (250 mm × 4.6 mm i.d., 5 μm) protected by a guard column (20 mm × 3.0 mm i.d.) containing the same packing. The eluents were A (aqueous 0.03 M ammonium hydrogen carbonate, adjusted to pH 9.5 with concentrated ammonia) and B (100% acetonitrile). The mobile phase was linearly gradient of A–B (0 min 70:30, 40 min 62:38, 60 min 55:45, v/v). The flow rate was 1 ml min^{-1}, the analysis was monitored at 233 nm and absorption spectra of the compounds were recorded between 200 and 300 nm. The column temperature was 35 °C and the sample injection volume was 10 μl. The compounds were identified by comparing their retention time and UV spectra with those of standards.

2.3. Sample preparation

Powdered samples (100 mesh, 0.1 g) were extracted three times with 0.2 M HCl (2 ml) with ultrasonic batch at room temperature for 30 min. The liquid phase was filtered and the residues were washed twice with 0.2 M HCl (1 ml). The liquid phase was combined and loaded onto Oasis®MCX extraction cartridge previously conditioned with 3 ml methanol and 3 ml water. After washing the cartridge with 3 ml 0.1 M HCl, 1 ml methanol and 3 ml 1% NH_4OH successively, the alkaloids were eluted with a 3 ml solution containing 5% triethylamine in methanol. The eluate was dried under nitrogen at 35 °C and the residues were constituted with 1 ml 0.1 M HCl. The obtained solution was filtered through a membrane filter (0.45 μm) prior to injection.

2.4. Linearity studies

For standard solutions, reference compounds 1 (5.3 mg), 2 (4.7 mg), 3 (4.9 mg), 4 (6.5 mg) and 5 (5.1 mg) were dissolved in acctonitrile and diluted with 0.1 M HCl to give five different concentrations. Each concentration was analyzed five times using the same HPLC condition as described above.

3. Results and discussion

3.1. Optimization of separation conditions

The choice of experimental conditions was guided by the need to obtain chromatograms with better resolution of adjacent peaks within short analysis time, especially when numerous similar samples were to be analyzed.

In the beginning, various mixtures of methanol–water–chloroform–triethylamine, 0.1% trifluoroacetic acid–tetrahydrofuran and methanol–water–acetonitrile were used as mobile phase but separation was not satisfactory. Acetonitrile and ammonium hydrogen carbonate buffer was then chosen as mobile phase because the alkaloids could be separated much better in this condition. It was also suggested that separation was improved when column temperature was increased to 35 °C.

Although the five alkaloids could be separated with isocratic HPLC elution (A:B, 50:50, v/v), according to UV absorption spectra, besides the five known compounds most of other peaks on the chromatograms are also *Aconitum* alkaloids; gradient elution was carried out so as to get a good chromatogram for all alkaloids contained in the crude drugs and we could use the same experimental conditions to further study *Aconitum* alkaloids fingerprints.

3.2. Regression equations

Linear regression analysis for each of the five alkaloids was performed by the external standard method. The calculated results are given in Table 1, where a, b and γ were the coefficients of the regression equation $y = ax + b$, x referred to the concentration of the alkaloid (μg ml^{-1}), y the peak area and γ the correlation coefficient of equation. All the alkaloids showed good linearity ($\gamma = 0.9999$) in a relatively wide concentration range. The limits of detection (LOD) ranged from 15 to 30 ng ml^{-1} detected at 233 nm.

3.3. System suitability test

The recovery assays of the five alkaloids were carried out by adding the standards to the crude drug powder, which was treated

Table 1
Linear regression equation and linear ranges ($n = 5$)

Standard	Regression equation	γ	Linear range ($\mu g\,ml^{-1}$)	LOD ($ng\,ml^{-1}$)
1	$y = 1.09e + 004x - 7.28e + 003$	0.9999	0.53–530.00	15
2	$y = 1.27e + 004x - 1.43e + 004$	0.9999	0.47–470.00	20
3	$y = 9.86e + 003x - 1.81e + 004$	0.9999	0.49–490.00	24
4	$y = 7.74e + 003x - 1.62e + 004$	0.9999	0.65–650.00	27
5	$y = 1.07e + 004x - 1.43e + 004$	0.9999	0.51–510.00	30

In the regression equation $y = ax + b$, x refers to the concentration of the alkaloids ($\mu g\,ml^{-1}$), y the peak area, γ the correlation coefficient of the equation and LOD is the limit of detection. For chemical structure of the standards see Fig. 1.

according to the procedure described in Section 2.3. The average recovery for the alkaloids was 94.6–101.9% (see Table 2).

The precision test was carried out by injecting the same sample solution five times, and the results showed that relative standard deviation (R.S.D.) of peak area of each alkaloid was 0.14–0.67%.

For stability test, the same sample solution was analyzed every 12 h in 2 days, and the analytes were found to be rather stable within 48 h (R.S.D. < 0.98%).

3.4. Application

Ten microlitres of each sample solution were injected into the instrument. Peaks in the obtained chromatograms were identified by comparing the retention time and on-line UV spectra with those of the standards (see Fig. 2).

Retention parameters for 1, 2, 3, 4 and 5 were 10.23, 31.98, 46.97, 49.20 and 63.26 min, respectively. The contents of the five alkaloids in different samples were calculated and the results

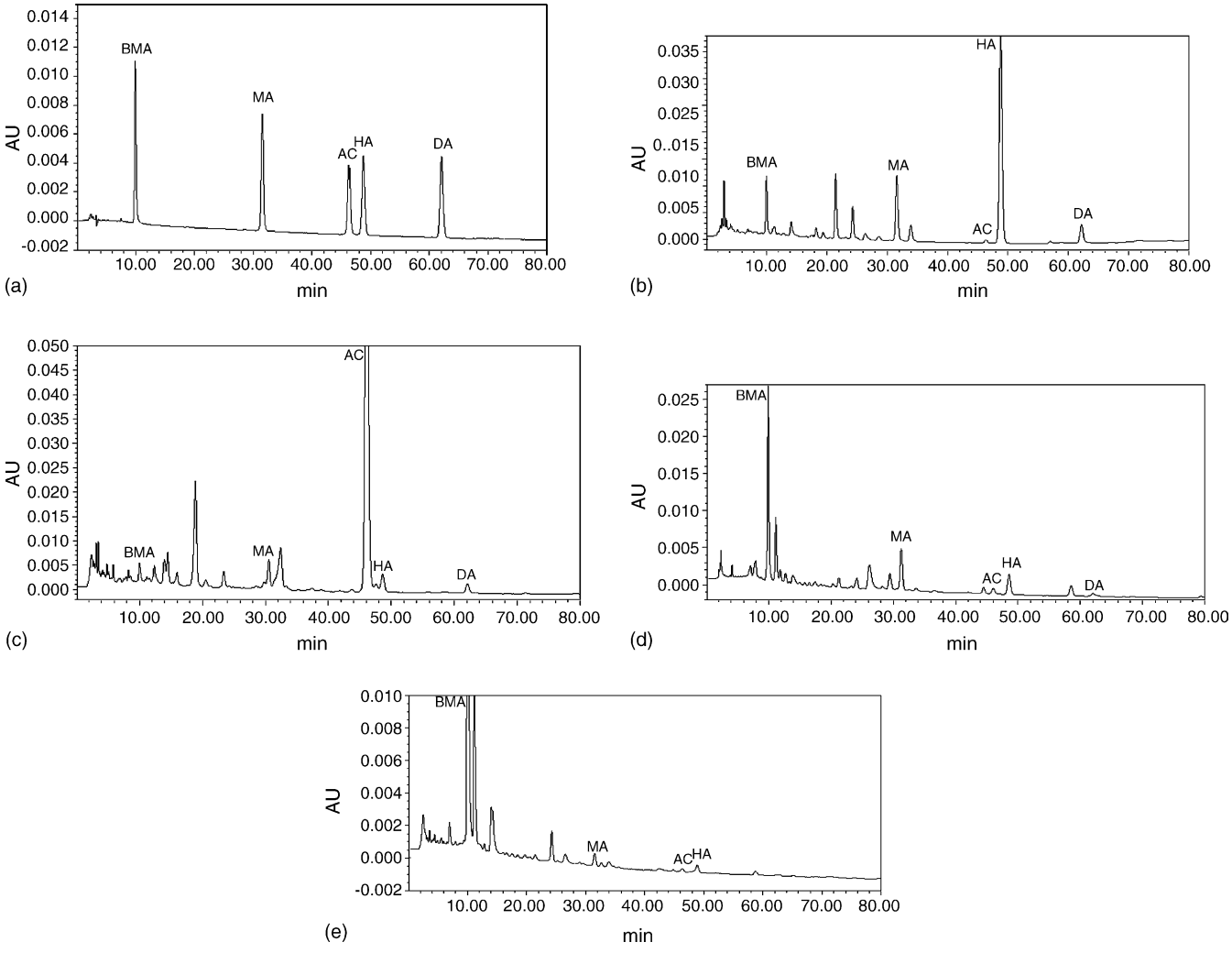

Fig. 2. HPLC chromatogram of (a) standard mixture, (b) *Aconitum carmichaeli* Debx, (c) *Aconitum pendulum* Bush, (d) *Aconitum hemsleyamun* Pritz and (e) *Aconitum transseltum* Diels. Benzoylmesaconine (BMA), mesaconitine (MA), aconitine (AC), hypaconitine (HA), deoxyaconitine (DA). Conditions: mobile phase, 0.03 M ammonium hydrogen carbonate–acetonitrile; flow rate, 1 ml min^{-1}; detection wavelength, 233 nm; column temperature, 35 °C and injection volume, 10 μl.

Table 2
Recovery of the alkaloids 1–5 ($n = 5$)

Compound	Initial amount (μg)	Added amount (μg)	Detected amount (μg)	Recovery (%)	R.S.D. (%)
1	17.43	5.30	21.51	94.65	2.84
2	27.15	4.70	30.93	97.12	1.83
3	3.20	4.90	8.25	101.90	2.51
4	179.00	13.00	192.63	100.33	2.32
5	13.01	5.10	17.93	99.00	2.10

For chemical structure of the compounds see Fig. 1.

Table 3
Contents of five alkaloids in different *Aconitum* samples (μg g^{-1}, $n = 3$)

Samples	Contents (μg g^{-1} crude drug)				
	BMA	MA	AC	HA	DA
A. carmichaeli	174.05	271.11	32.20	1790.53	130.15
A. pendulum	86.61	141.00	4846.75	206.16	77.23
A. hemsleyanum	41.12	134.21	38.37	141.42	21.10
A. transsectum	1136.29	28.17	17.09	37.00	n.d.

BMA, benzoylmesaconine; MA, mesaconitine; AC, aconitine; HA, hypaconitine; DA, deoxyaconitine; n.d., not detected.

are shown in Table 3 with the mean values of three replicate injections.

The data in Table 3 shows that contents of the five determined alkaloids in *Aconitum* varied considerably from species to species. Since alkaloids are important biological active secondary metabolites and the main toxic compounds of *Aconitum* spp., it is indicated that quality of these crude drugs can vary significantly. This may be one of the most predominant reasons why fatalities from ingestion of *Aconitum* crude drugs are frequent.

4. Conclusions

This was the first report of simultaneous determination of the five highly active and toxic alkaloids in different *Aconitum* species. A simple and accurate assay approach was presented. The HPLC profile and the data obtained suggested that contents of alkaloids in *Aconitum* spp. varied significantly. The HPLC method developed here presented an excellent technique for quality control of *Aconitum* crude drugs.

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