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Simultaneous determination of six *Aconitum* alkaloids in proprietary Chinese medicines by high-performance liquid chromatography

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Abstract

By optimizing the extraction, separation and analytical conditions, a reliable and accurate high-performance liquid chromatography (HPLC) method coupled with photodiode array detector (DAD) was developed for simultaneous quantitative determination of six *Aconitum* alkaloids, i.e., aconitine, mesaconitine, hypaconitine, benzoylaconine, benzoylmesaconine and benzoylhypaconine, in Chinese medicinal herbs, aconite roots, and 12 proprietary Chinese medicines containing processed aconite roots. The separation of these *Aconitum* alkaloids was achieved on an ODS column with gradient elution using solvents of acetonitrile and ammonium bicarbonate buffer (pH 10.0 ± 0.2). Intra-assay and inter-assay precision of the analytes were less than 2.97%, and the average recovery rates obtained were in the range of 90–103% for all with RSDs below 3.28%. Good linear relationships were showed with correlation coefficients for the analytes exceeded 0.999. Quantitative analysis of the six *Aconitum* alkaloids in the unprocessed and processed aconite roots and in twelve proprietary Chinese medicines containing processed aconite roots showed that the contents of the alkaloids varied significantly. This method and quantitation results can provide a scientific and technical platform to the products manufacturers for setting up a quality control standard as well as to the public for quality and safety assurance of the proprietary Chinese medicines and other herbal preparations containing aconite roots.

Keywords: *Aconitum* alkaloids; Proprietary Chinese medicines; Aconite roots; Quantitative analysis; High-performance liquid chromatography (HPLC)

1. Introduction

The *Aconitum* alkaloids comprise three kinds of highly toxic diester-diterpene *Aconitum* alkaloids, namely, aconitine (AC), mesaconitine (MA) and hypaconitine (HA), and their respective hydrolyzed analogs called monoester alkaloids, i.e., benzoylaconine (BAC), benzoylmesaconine (BMA) and benzoylhypaconine (BHA) (Fig. 1). These six *Aconitum* alkaloids are mainly contained in the aconite roots, known as Fuzi, Chuanwu and Chaowu in Chinese (derived from roots of certain *Aconitum* species, Family *Ranunculaceae*). Toxic side effects and even death have been reported in China and Japan recently, because of the highly toxic diester-diterpene alkaloids in the herb [1,2]. Studies on the molecular mechanism of the toxic effects of the three alkaloids showed that they could presumably induce arrhythmias by increasing ectopic impulse formation, making representative triggered activities, due to early as well as delayed after-depolarization [3]. Although the aconite roots are highly toxic, but they are frequently used as an important ingredient of many prescriptions used in traditional Chinese medicine to treat joint pain, arthritic and rheumatic diseases for over 2000 years by Chinese and Japanese doctors. Because the toxic diester-type alkaloids also have marked pharmacological effects, such as antinociceptive, anti-inflammatory in optimal low dosages [4].

In the literature records of Chinese medicine, this herb must be properly processed with heating, steaming and soaking, so as to reduce its toxicity before being used in clin-

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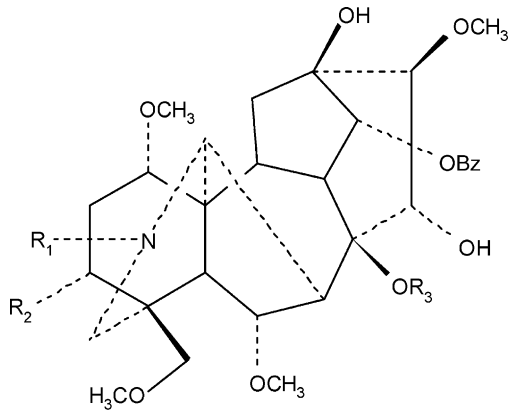

Name	R_1	R_2	R_3
Benzoylaconine	C_2H_5	OH	H
Aconitine	C_2H_5	OH	acetyl
Benzoylmesaconine	CH_3	OH	H
Mesaconitine	CH_3	OH	acetyl
Benzoylhypaconine	CH_3	H	H
Hypaconitine	CH_3	H	acetyl

Fig. 1. Chemical structures of benzoylaconine, aconitine, benzoylmesaconine, mesaconitine, benzoylhypaconine and hypaconitine.

ics. Phytochemical analysis demonstrated that those three diester-type *Aconitum* alkaloids undergo chemical degradation, especially through hydrolysis of the ester bonds during the course of processing. The acetyl group at the 8-postion in the diester-type alkaloids is easily hydrolyzed into monoester ones [5]. Ohta et al. successfully obtained the alkaloids of benzoylaconine, benzoylmesaconine and benzoylhypaconine from the diester-type alkaloids by hydrolysis [5]. Toxicological studies demonstrated that the toxicity of aconitine, hypaconitine and mesaconitine were almost the same with LD50 values for mice per injection about 0.15 mg/kg body weight, while the three hydrolyzed analogs, i.e., benzoylaconine, benzoylmesaconine and benzoylhypaconine, showed a much lower toxicity than that of the diester-type alkaloids [6–8]. However, the pharmacological studies showed that the decoction of the processed aconite roots as well as the hydrolyzed analog of benzoylmesaconine have significant anti-inflammatory and analgesic actions that would be the desired effects in clinical treatment using the aconite roots [9,10].

According to the regulations and the practice of Chinese medicine stipulated by the State Food and Drug Administration of China, only the processed aconite roots are allowed to use orally, in clinical herbal prescriptions, in herbal decoction, and in proprietary Chinese medicines. While processing significantly reduces the toxicity of these three alkaloids, large variation in alkaloid contents nevertheless remains—with significant impact on the safety and efficacy of medicines. This is because there are no standardized procedures and methods for processing different raw materials of the herb that came from various sources and cultivation places [11]. Our previous measurements on the contents of the three high toxic diester-type alkaloids in the unprocessed and the processed aconite roots showed significant differences, i.e., the unprocessed aconite roots contained much more aconitine, mesaconitine and hypaconitine than those in the processed ones. Moreover, the contents of those three alkaloids in different batches of the processed aconite roots showed also large variation [12]. Regarding the proprietary Chinese medicines containing processed aconite roots, many factors influence the contents of those six *Aconitum* alkaloids, such as the different manufacturing procedures as well as different raw materials used. Unfortunately, no investigation has been conducted in this aspect until now even though the proprietary Chinese medicines containing aconite roots are being widely used in daily life and commercially available. In our current studies, we collected twelve commonly used proprietary Chinese medicines from drug stores in mainland of China and developed a rapid, reliable and precise method for simultaneous determination of six *Aconitum* alkaloids in these medicines. This method can provide a scientific and technical platform to the products manufacturers for setting up a quality control standard as well as to the public for quality and safety assurance of proprietary Chinese medicines and other herbal preparations containing aconite roots.

Since 1981, researchers have been exploring different means for quantifying *Aconitum* alkaloids, aconitine, mesaconitine and hypaconitine, in the aconite roots because of their high toxicity, such as high-performance liquid chromatography (HPLC) [13–15], capillary electrophoresis [16], GC–MS [17], etc. Some groups have described GC–MS and LC–MS methods for analysis of *Aconitum* alkaloids in body fluids and tissues [18,19]. The methods of GC–MS and LC–MS were sensitive, but were based on time-consuming sample preparation, such as pre-column derivation and solid phase extraction (SPE). Acidic aqueous buffer, such as phosphate buffer with organic phase tetrahydrofuran (>10%) were used in most HPLC method with addition of sodium hexanesulfonate [13] or $CHCl_3$ [15]. These methods suffer from the drawback of incomplete resolution and HPLC column short lifetime, and, moreover, they are very difficult to satisfy the separation of complex samples, particularly the proprietary Chinese medicines. In recent years, with the development of column technology, alkaline buffer, such as ammonium bicarbonate were able to be used for separation of alkaloids, which have showed better resolution ability [20]. But, there are little reports about the effects of alkaline buffer on the chromatographic behavior of alkaloids, especially the six *Aconitum* alkaloids. In the current work, we investigated the effects of different alkaline buffer concentrations and pH values, and developed a modified HPLC analytical method successfully for simultaneous quantitative determination of the six *Aconitum* alkaloids in the proprietary Chinese medicines. Furthermore, we investigated the extraction methods, peak confirmation, mobile phase buffer and gradient elution program regarding to the complexity of proprietary Chinese medicines.

2. Experimental

2.1. The sources of the raw materials of aconite roots and its proprietary Chinese medicines

The processed aconitine roots were *Radix Aconiti Lateralis Preparata* (Processed Fuzi) and *Radix Aconiti Preparata* (Processed Chuanwu), from the medicinal markets of Sichuan and Guangdong Provinces of China, respectively. The unprocessed aconite roots were *Radix Aconiti Lateralis* (Crude Fuzi) and *Radix Aconiti* (Crude Chuanwu), which were obtained from Sichuan Province of China.

The proprietary Chinese medicines used in this study were Gui-Fu-Li-Zhong-Wan (GFLZW, from Fo Shan Feng Liao Xiang Pharmaceutical Co. Ltd., Guangdong Province, China), Gui-Fu-Di-Huang-Wan (GFDHW, from Wu Fu Zhang Hen Chun Pharmaceutical Co. Ltd., Zhejiang Province, China), concentrated Gui-Fu-Di-Huang-Wan (CGFDHW, from Lan Zhou Fu Chi Pharmaceutical Co. Ltd., Ganshu Province, China), San-Qi-Sang-Yao Tablet (SQSYT, from Chang Chun Ying Nuo Ke Pharmaceutical Co. Ltd., Jilin Province, China), San-Qi-Sang-Yao Capsule (SQSYC, from Guang Xi Yu Lin Pharmaceutical Co. Ltd., Guangxi Province, China), Da-Huo-Luo-Wan (DHLW, from Tong Ren Tang Pharmaceutical Factory, Beijing, China), Xiao-Huo-Luo-Wan (XHLW, from Lan Zhou Fu Chi Pharmaceutical Co. Ltd., Ganshu Province, China), Hai-Ma-Bu-Shen-Wan (HMBSW, from Tian Jin Zhing Xin Pharmaceutical Co. Ltd., Tianjing, China), Yao-Zui-Bi-Shen-Wan (YZBTW, from Guang Dong Hua Tian Bao Pharmaceutical Co. Ltd., Guangdong Province, China), Jin-Gui-Shen-Qi-Wan (JGSQW, Tong Ren Tang Pharmaceutical Factory, Beijing, China), Xiao-Jin-Dan (XJD, from Chuan Xi Pharmaceutical Co. Ltd., Shichuan Province, China) and Mu-Gua-Wan (MGW, from Hu Bei Guan Ren Medicament Co. Ltd., Hubei Province, China). These proprietary Chinese medicines were produced in China and are available in the drug stores. The main therapeutic indications of those medicines are muscular disorders, joint pain and arthritis.

2.2. Chemicals and reagents

Acetonitrile was of HPLC grade (International Laboratory, USA). Twenty-eight percent ammonia solution (AJAX CHEMICALS, Australia), 95% ethanol (UNI-CHEM, Hong Kong, China), chloroform (TEDIA, USA), ethyl acetate, diethyl ether anhydrous (International Laboratory, USA) and hydrochloric acid (MERCK, German) were of GR grade. Ammonium bicarbonate was of analytical grade (MERCK, German). Deionized water was prepared using a Millipore (Billerica, MA, USA) water purification system. All solvents and solutions were filtered through a Millipore filter (0.2 μm) before use.

Reference standards of aconitine, mesaconitine and hypaconitine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. Their chemical structures were further identified by LC–MS in our laboratory.

Benzoylmesaconine, benzoylaconine and benzoylhypaconine were prepared from mesaconitine, aconitine and hypaconitine, respectively, in our laboratory, by heating at 100 °C in dioxane–water for 6 h, and then purified by column chromatography and crystallization. The identity and purity (≥95%) of benzoylmesaconine, benzoylaconine and benzoylhypaconine were confirmed by chromatographic analysis including TLC and HPLC methods and then by comparing with the published spectral data (^{1}H NMR, and MS) [21].

2.3. Chromatographic system

An Agilent 1100 series LC system (Hewlett-Packard, CA, USA) consisting of a G1311A Quaternary Pumps, a G1322A degasser, a G1315A Diode-Array Detector and a G1313A Autosampler was employed in this research.

The measurements of the *Aconitum* alkaloids were carried out on an Alltima^{TM} RP18 (250 mm × 4.6 mm I.D.; particle size 5 μm; Alltech Associates, USA) protected by an Alltima^{TM} RP18 guard column (7.5 mm × 4.6 mm I.D.) at room temperature. The solvents used for HPLC separation of the six alkaloids in samples were carried out with an acetonitrile (A) and buffer solution (containing 10 mM ammonium bicarbonate, adjusted with 28% ammonia solution to pH 10.0 ± 0.2) (B) at a flow-rate of 1.0 ml/min. The gradient elution of mobile phase was 20–25% (A) in 0–10 min, 25–34% (A) in 10–30 min, 34–45% (A) in 30–67 min, 45–60% (A) in 67–75 min. Detection was carried out at 240 nm with the reference wavelength of 550 nm.

2.4. Preparation of sample solutions

The processed and unprocessed aconite roots were pulverized into powder, passed through a 0.45 mm sieve, accurately weighed to approximately 0.5 g. Proprietary Chinese medicines in the dosage forms of pills and tablets were pulverized in a mortar and pestle, mixed well, accurately weighed to 1.0 g and put into 50 ml centrifuge tubes; the honey pill (DHLW) was cut into little pieces. The hard gelatin capsules were emptied and the contents were weighed (1.0 g) directly. Each weighted sample was extracted with 10 ml HCl solution (0.05 M) by sonication for 60 min (Branson 5210 ultrasonicator) at room temperature, and then extracted with ethyl acetate for three times (10 ml each time) to remove non-alkaloid components. Then the acidic aqueous solution was basified with 28% ammonia solution to pH 10 and further extracted with chloroform three times (10 ml each time) by vortex-mixing for 2 min each. The resulting mixtures were centrifuged at 3000 rpm for 5 min and the combined supernatants were evaporated to dryness under air stream. The residue was further dissolved with 1.0 ml HCl solution (0.01 M) by sonication for 30 min.

All final resulting solutions were filtered through a 0.45-μm filter membrane, and 50-μl filtrate was injected into the HPLC system for quantitation.

2.5. Preparation of standard solutions

The standard chemicals of six alkaloids were accurately weighed and then dissolved with 0.01 M HCl to produce stock standard solutions. These stock solutions were used for preparation of standard solutions, which were stored at 4 °C and remained stable for at least one month (verified by re-assaying the standard solutions). Calibration curves were established based on seven concentrations with a range of 0.8–250 μg/ml for each alkaloid by diluting the stocking solution with 0.01 M HCl solution in appropriate quantities. For the recovery test, the standard solutions were also prepared at concentrations of 28.59, 3.10, 5.30, 2.42, 2.25 and 2.11 μg/ml for benzoylmesaconine, benzoylaconine, benzoylhypaconine, mesaconitine, aconitine and hypaconitine, respectively.

2.6. Validation of the chromatographic method

2.6.1. Precision

Precision was evaluated by HPLC analysis with a standard mixture solution of the six alkaloids under the selected optimal conditions five times in one day for intra-day variation and twice a day on three consecutive days for inter-day variation, expressed as relative standard deviation (RSD).

2.6.2. Repeatability

Five measurements were taken of one of the proprietary Chinese medicines, concentrated-Gui-Fu-Di-Huang-Wan (CGFDHW). The processes of the measurements were in accordance with the "2.4. Preparation of sample solutions" in parallel. The variation (RSD) within the five measurements was calculated and used for evaluation of the repeatability.

2.6.3. Recovery

Accurately weighted 0.25 g of proprietary Chinese medicines (CGFDHW) which the contents of the six *Aconitum* alkaloids were known, and added with 10 ml HCl solution (0.05 M). Then the sample solution was spiked with 500 μl of the standard solution for recovery test. The prepared samples above ($n = 6$) were then processed in accordance with the "2.4. Preparation of sample solutions" in parallel and quantified in accordance with the selected method.

3. Results and discussion

3.1. Effects of pH value in the mobile phase buffer on the retention time of HPLC

Traditional HPLC applications on silica-based columns were performed at low or mid-range pH values in the mobile phase buffer. But the *Aconitum* alkaloids in the HPLC chromatograms exhibited low retention time in such mobile phase; therefore it was difficult to separate them from each other and from other compounds in the proprietary Chinese medicines. In this paper, an alternative method was employed to separate them by using a mobile-phase pH which was above their p*K*a. Under such conditions, they were in their free-base forms because their positive charges were diminished, resulting in the improvement of the peak shape. The pH dependence of the retention time was investigated with a range of 8.5–10.5 pH values by adjusting with 28% ammonia solution, in which the ammonium bicarbonate concentration reached at 10 mM. The results in Fig. 2(A) show that along with increase of the pH values in the buffer, the retention

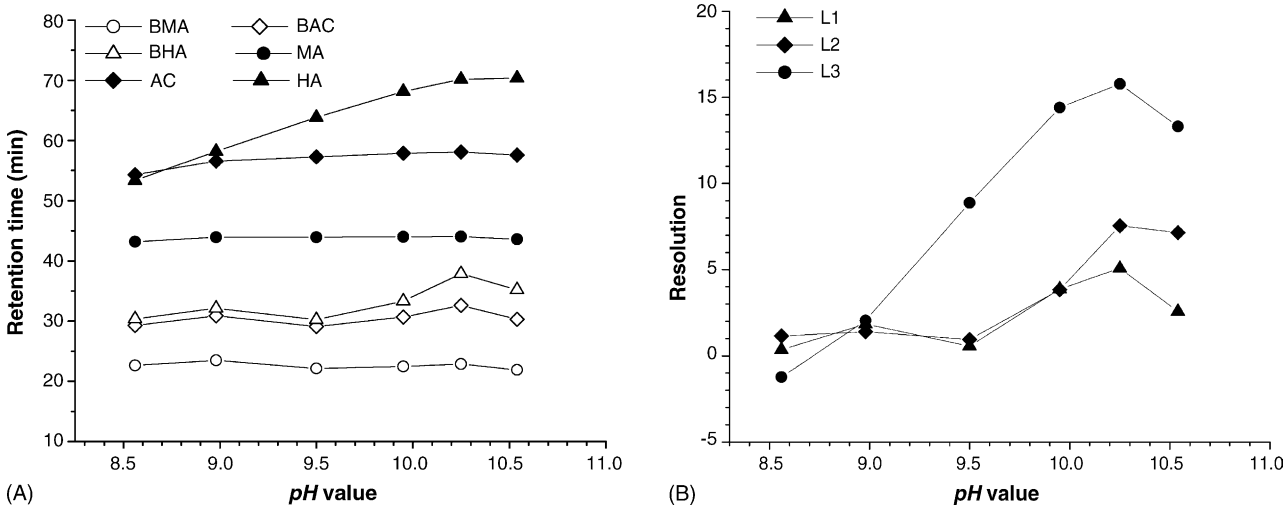

Fig. 2. Effects of the pH values on the retention time of the six *Aconitum* alkaloids (A) and on the resolution of the adjacent peaks (B). L1: resolution of unknown compound and benzoylaconine; L2: resolution of benzoylaconine and benzoylhypaconine; L3: resolution of aconitine and hypaconitine. For conditions see Section 2.

time of HPLC was increasing, due to the stronger interactions between this less-polar molecule and the hydrophobic bonded phase.

The results of chromatographic analysis indicated that the pH value has distinct effects, not only for the resolution of the six *Aconitum* alkaloids, but also for separation of the alkaloids and other unknown compounds in the samples in Fig. 2(B). At the pH value of 9.95, all the six alkaloids were baseline separated from each other with resolution values above 1.5. Considering the long elution time and drifting baseline at pH values above 10.5, the chromatographic analytic condition for the present studies was carried out at a pH range of 10.0 ± 0.2, where the buffer solution works effectively.

3.2. Effects of the mobile phase buffer concentration

To decide an optimal concentration of the mobile phase buffer, three concentrations (5, 10 and 20 mM) of the ammonia carbonate buffer solution with pH 10.0 were used together with acetonitrile as the mobile phase for the HPLC analysis.

It was found that the retention times of these six alkaloids decreased with increasing concentrations of ammonium bicarbonate in the buffer solution. The resolutions of benzoylhypaconine and benzoylaconine were markedly affected by ammonium bicarbonate concentration, while others were baseline separated from each other. The action of buffer concentration might result from the interactions between the alkaloids molecules and solid phase influenced by the concentration of ammonium bicarbonate in the buffer solution. As the retention time of hypaconitine and benzoylhypaconine decreased greater than that of other alkaloids, it seems that the difference of the substitution group at the R_2 position (Fig. 1) has influenced on the chromatographic behavior of *Aconitum* alkaloids. Since the best separation efficiency and resolution for the alkaloids were achieved below the concentration of 20 mM and the background signal noise became larger in the 5 mM concentration of the buffer, it was, therefore, that the 10 mM buffer solution was finally chosen as the HPLC mobile phase for subsequent analysis.

3.3. Extraction conditions

The 0.05 M HCl solution was chosen for dissolving the six alkaloids which were reported to be stable in acidic solution [19]. Ethyl acetate was used to remove the interferences in the acidic aqueous solution before the final extraction of the alkaloids by organic solvent after alkalization with 28% ammonia solution up to pH value of 10. The influences of the extraction solvents on the yields of the six *Aconitum* alkaloids in three different proprietary Chinese medicines were investigated after the sample solution were basified. Considering the properties of the *Aconitum* alkaloids, diethyl ether, chloroform and ethyl acetate were employed as the extraction solutions. The results showed that ethyl acetate is better than other two solvents for the alkaloids extraction because more alkaloids can be extracted (Table 1). The results of the recovery test also demonstrated that the extraction method was adequate and appropriate for the analysis.

3.4. Validation of HPLC assay

A gradient elution program of HPLC was developed for separating and quantifying the six *Aconitum* alkaloids. They were successfully determined by a single run of HPLC. Benzoylmesaconine, benzoylaconine, benzoylhypaconine, mesaconitine, aconitine and hypaconitine were well resolved in HPLC chromatogram and eluted at 22.4, 30.2, 32.8, 44.3, 58.5 and 68.5 min retention time, respectively.

Six *Aconitum* alkaloids in the aconitine roots as well as in their medicines were identified by comparing both their retention times and the UV spectra with those of reference standards. The peak purity was confirmed by studying the photodiode array detector (DAD) data with peaks of six alkaloids in which no indication for impurities could be found.

Table 1
The influences of different solvents on the extraction rates of the six *Aconitum* alkaloids in three proprietary Chinese medicines

Medicines [a]	Solvents	Concentrations (μg/g)[b]					
		BMA	BAC	BHA	MA	AC	HA
MGW	Et_2O	87.5 ± 5.0	10.0 ± 0.6	18.4 ± 0.6	0.848 ± 0.015	–	3.37 ± 0.17
	$CHCl_3$	130 ± 6	12.1 ± 0.7	20.9 ± 1.1	1.80 ± 0.12	–	3.45 ± 0.32
	EtOAc	141 ± 4	13.3 ± 0.3	22.1 ± 0.7	1.30 ± 0.02	0.751 ± 0.035	4.86 ± 0.20
SQSYC	Et_2O	20.7 ± 0.0	20.6 ± 0.1	9.38 ± 0.07	–	1.04 ± 0.00	3.15 ± 0.07
	$CHCl_3$	216 ± 2	104 ± 1	43.3 ± 0.1	–	5.62 ± 0.1	3.06 ± 0.10
	EtOAc	231 ± 4	109 ± 1	50.1 ± 1.5	–	8.05 ± 0.4	3.77 ± 0.06
CGFDHW	Et_2O	36.5 ± 0.7	6.28 ± 0.05	6.44 ± 0.34	3.15 ± 0.03	1.59 ± 0.13	3.94 ± 0.01
	$CHCl_3$	48.4 ± 0.4	6.76 ± 0.04	5.72 ± 0.09	3.04 ± 0.11	1.10 ± 0.06	2.81 ± 0.22
	EtOAc	51.2 ± 1.1	8.02 ± 0.10	7.59 ± 0.14	3.63 ± 0.10	2.00 ± 0.04	5.03 ± 0.06

–: under detection limit.

[a] MGW is the short for the proprietary Chinese medicine Mu-Gua-Wan, SQSYC for San-Qi-Sang-Yao Capsule and CGFDHW for concentrated Gui-Fu-Di-Huang-Wan.

[b] BMA is the short for benzoylmesaconine, BA for benzolyaconine, BHA for benzoylhypaconine, AC for aconitine, MA for mesaconitine and HA for hypaconitine.

Table 2
Regression equations and their correlation coefficients of six *Aconitum* alkaloids

Alkaloids	Linear range (μg/ml)	Regression equation[a]	Correlation coefficient (R^2)
Benzoylaconine	2.48–248	$Y = 47.590X + 9.819$	0.9995
Benzoylmesaconine	0.817–81.7	$Y = 40.005X + 12.324$	0.9998
Benzoylhypaconine	2.12–212	$Y = 44.183X - 13.012$	0.9993
Aconitine	1.13–135	$Y = 51.788X - 13.469$	0.9999
Mesaconitine	1.21–146	$Y = 53.454X - 10.103$	0.9999
Hypaconitine	1.05–126	$Y = 53.041X - 19.527$	0.9999

[a] X denotes the concentrations and Y denotes the peak areas.

Table 3
Precision of the intra-day and inter-day measurements of the analytical method for six *Aconitum* alkaloids

Alkaloids	Intra-day[a]		Inter-day[b]	
	Peak areas ($\bar{X}$ + SD)	RSD (%)	Peak areas ($\bar{X}$ + SD)	RSD (%)
Benzoylaconine	157.3 ± 2.6	1.67	157.4 ± 4.7	2.97
Benzoylmesaconine	1148.9 ± 5.8	0.50	1157.5 ± 16.4	1.41
Benzoylhypaconine	229.8 ± 2.8	1.22	228.6 ± 2.0	0.86
Aconitine	116.1 ± 1.3	1.12	116.6 ± 1.1	0.90
Mesaconitine	144.6 ± 1.9	1.33	145.6 ± 2.1	1.44
Hypaconitine	118.9 ± 2.0	1.72	120.6 ± 1.4	1.18

[a] The sample was analyzed five times during one day.
[b] The sample was analyzed over three consecutive days.

Furthermore, since the absorbance intensity changed in proportion to alteration of UV wavelength, the peak purity of the six alkaloids were further confirmed by comparing the chromatograms of 240 and 280 nm. The absorbance ratios of benzoylmesaconine, benzoylaconine, benzoylhypaconine, mesaconitine, aconitine and hypaconitine at 240 and 280 nm were 14, 10, 15, 12, 11, 12, respectively, i.e., they were consistent with the six alkaloids peak area ratios in the two chromatograms of the sample solutions of the proprietary Chinese medicines. If any component has interfered with the peaks of alkaloids in various sample solutions, the peak area ratio would have been altered.

The linearity of the plot concentrations (X, μg/ml) for each *Aconitum* alkaloid versus peak areas (Y) was investigated; the results are expressed as the values of the correlation coefficient (R^2) in Table 2.

The limit of detection, defined as the amount of the compounds needed to produce a signal at least three times larger than noise (S/N > 3), was determined to be 15 ng for all of the six *Aconitum* alkaloids.

The analytical precision from the data of the intra-daily (five times per day) and inter-daily (twice a day for three consecutive days) determinations was indicated by the relative standard deviations which were less than 2.97% for all six alkaloids (Table 3). The results also implied that these alkaloids are stable in acidic aqueous solution (0.01 M HCl solutions).

The RSDs of the repeatability test were less than 2.79% for all six alkaloids (Table 4). The average recovery rates obtained were in the range of 90–103% for all with RSDs below 3.28% (Table 4). Therefore, these chromatographic systems for quantitative determination of six *Aconitum* alkaloids were appropriate for aconite roots and their medicines.

3.5. Application of the HPLC method for quantitation studies

The contents of six *Aconitum* alkaloids in twelve proprietary Chinese medicines containing processed aconite roots, two kinds of processed aconite roots and two kinds of unprocessed aconite roots were determined with the HPLC method developed as described above. The representative HPLC chromatograms of the medicines, the processed aconite roots, and the unprocessed roots, are shown in Figs. 3–5, respectively. It can be seen that constituents of *Aconitum* alkaloids in proprietary Chinese medicines were same as those in the

Table 4
Repeatability and recovery tests of the analytic method for six *Aconitum* alkaloids[a]

Alkaloids	Contents ($\bar{X}$ + SD (μg/g))	RSD (%)	Recovery rates ($\bar{X}$ + SD (%))	RSD (%)
Benzoylaconine	8.02 ± 0.10	1.27	90.2 ± 1.8	2.01
Benzoylmesaconine	51.2 ± 1.1	2.11	91.4 ± 1.0	1.10
Benzoylhypaconine	7.59 ± 0.14	1.90	96.2 ± 1.4	1.47
Aconitine	2.00 ± 0.04	1.93	94.1 ± 3.1	3.28
Mesaconitine	3.62 ± 0.10	2.79	103 ± 2	1.65
Hypaconitine	5.03 ± 0.06	1.23	99.9 ± 1.8	1.75

[a] Proprietary Chinese medicines concentrated Gui-Fu-Di-Huang-Wan was used in this experiment.

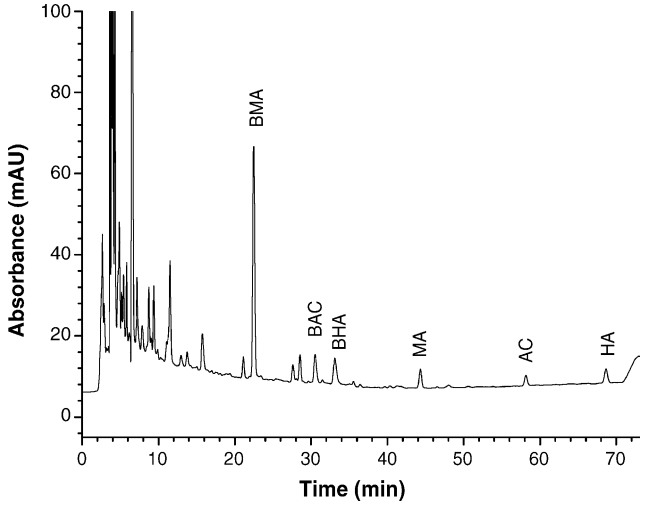

Fig. 3. Typical HPLC chromatogram of the six alkaloids in a proprietary Chinese medicines, concentrated Gui-Fu-Di-Huang-Wan (CGFDHW). For conditions see Section 2.

processed aconite roots, with a character of that benzoylmesaconine has a higher content than the others. Aconitine, mesaconitine and hypaconitine have higher contents in the unprocessed aconite roots, which is different from that of the proprietary Chinese medicines and processed ones. The contents of *Aconitum* alkaloids were calculated with the regression equations obtained from their calibration curves, and the results are shown in Table 5.

The results show that there were significant differences in alkaloid contents between the processed and unprocessed aconite roots, i.e., the processing appeared to have markedly reduced the contents of aconitine, mesaconitine and hypaconitine, which were consistent with the reports in our previous studies of the aconite roots [12]. In this paper, we further determined the contents of monoester alkaloids, and calculated the content ratios of each diester alkaloids and their

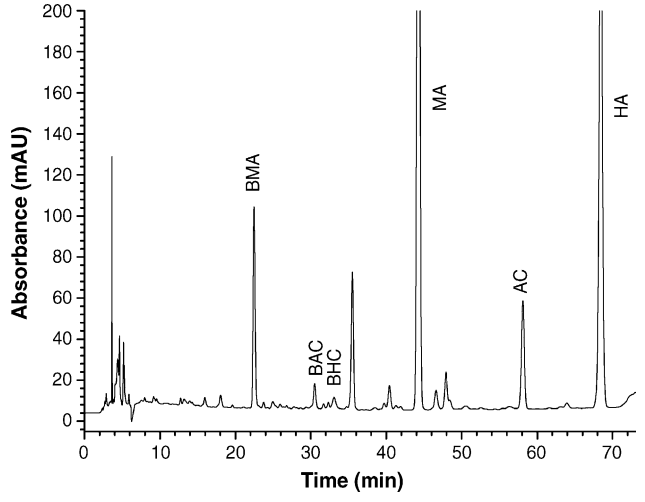

Fig. 4. Typical HPLC chromatogram of the six alkaloids in unprocessed aconite roots *Radix Aconiti Lateralis*. For conditions see Section 2.

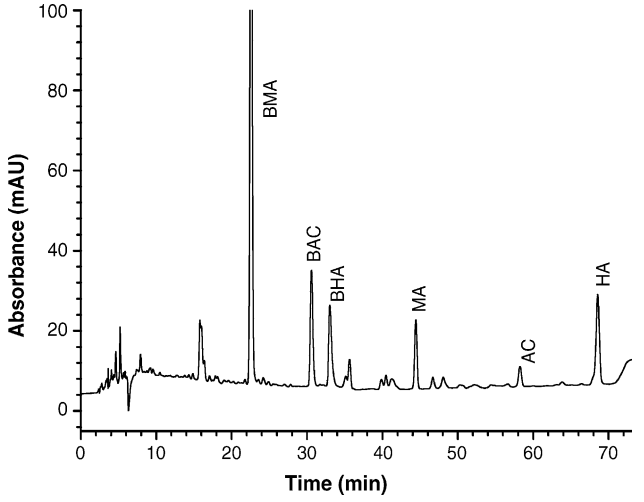

Fig. 5. Typical HPLC chromatogram of the six alkaloids in processed aconite roots *Radix Aconiti Lateralis Preparata.* For conditions see Section 2.

corresponding monoester alkaloids in each sample. It was found that a marked decrease of the content ratios in the processed aconite roots and the proprietary Chinese medicines (normally below 1%) in comparison with that in the unprocessed aconite roots (range from 2% to 38%). This indicates that those three toxic alkaloids were mostly hydrolyzed to monoester alkaloids during the processing of crude aconite roots. Moreover, there were large variations in the contents of the six *Aconitum* alkaloids among different kinds of proprietary Chinese medicines. These variations might be due to the manufacturing methods, different species and/or places of origin of the herbal materials, or to the possible effects of other compounds coming from different herbs in the formula.

Among six *Aconitum* alkaloids, the content of benzoylmesaconine is the highest in all of the twelve proprietary Chinese medicines and the processed aconite roots. It was reported that benzoylmesaconine was significantly effective in anti-inflammation and analgesia in animals [10,22], i.e., its (30 mg/kg, p.o.) analgesic potency was equivalent to that of processed aconite roots at 1000 mg/kg (p.o.), which were the demanded pharmacological effects for clinical treatment using the processed aconite roots as well as the proprietary Chinese medicines containing the herb. Thus, it is reasonable to select the benzoylmesaconine as the marker compound to assess the quality of the processed aconite roots and their proprietary Chinese medicines.

The contents of aconitine (lower than 5.7 ± 0.12 μg/g) in the samples of the processed aconite roots and all of the twelve proprietary Chinese medicines were lower than the upper limit of aconitine content stipulated in the Chinese Pharmacopoeia by TLC for the *Radix Aconiti Lateralis Preparata* (Edition 2000, volume 1), i.e., not more than 0.02%. However, the contents of hypaconitine and mesaconitine in the processed roots and some of the proprietary Chinese medicines were higher than the contents of aconitine. It is known that both hypaconitine and mesaconitine have toxic effect as aconitine [6–8]. Therefore, if the safety and

Table 5
Contents of the six *Aconitum* alkaloids in twelve proprietary Chinese medicines and aconite roots

Sample code[a]	Clinical dosages (g/day)	Concens ($\bar{X}$ + SD (μg/g))					
		BMA	BAC	BHA	MA	AC	HA
XHLW	2.2	95.8 ± 2.2	10.8 ± 0.0	21.5 ± 0.2	–	2.46 ± 0.04	2.57 ± 0.02
GFDHW	4.5	17.0 ± 0.6	3.03 ± 0.11	6.97 ± 1.61	–	–	0.818 ± 0.004
CGFDHW	4.5	51.2 ± 1.1	8.02 ± 0.10	7.59 ± 0.14	3.63 ± 0.10	2.00 ± 0.04	5.03 ± 0.06
YZBTW	6	10.7 ± 0.1	–	–	–	–	–
HMBSW	5.4	50.5 ± 0.3	6.18 ± 0.20	4.47 ± 0.10	7.53 ± 0.09	2.39 ± 0.08	4.90 ± 0.06
DHLW	14.4	6.94 ± 0.06	4.41 ± 0.12	–	–	–	1.29 ± 0.02
SQSYT	3.87	29.3 ± 0.5	25.0 ± 0.0	16.7 ± 0.1	–	3.36 ± 0.16	–
SQSYC	2.25	231 ± 4	109 ± 1	50.1 ± 1.5	–	8.05 ± 0.44	3.77 ± 0.06
MGW	16.8	141 ± 4	13.3 ± 0.3	22.1 ± 0.7	1.30 ± 0.02	0.751 ± 0.035	4.86 ± 0.20
GFLZW	18	6.75 ± 0.05	0.699 ± 0.000	5.65 ± 0.02	0.963 ± 0.211	–	0.681 ± 0.023
JGSQW	10	5.94 ± 0.06	1.16 ± 0.09	4.91 ± 0.02	–	–	1.58 ± 0.01
XJD	6	16.7 ± 2.0	0.871 ± 0.170	5.50 ± 0.04	–	3.37 ± 0.35	–
Fuzi[b]	/	198 ± 1	27.6 ± 0.2	26.8 ± 0.2	15.1 ± 0.2	5.71 ± 0.12	26.6 ± 0.2
Crude Fuzi	/	383 ± 4	43.6 ± 0.1	35.6 ± 1.3	(2.44 ± 0.05)×10^3	241 ± 4	(1.36 ± 0.02)×10^3
Chuanwu[b]	/	193 ± 1	29.6 ± 0.3	31.3 ± 0.5	4.47 ± 0.07	2.25 ± 0.05	19.7 ± 0.1
Crude Chuanwu	/	331 ± 1	31.2 ± 0.0	24.6 ± 1.0	801 ± 1	123 ± 1	453 ± 6

–: Under detection limit. [a]XHLW is the short for Xiao-Huo-Luo-Wan, GFDHW for Gui-Fu-Di-Huang-Wan, CGFDHW for concentrated Gui-Fu-Di-Huang-Wan, YZBTW for Yao-Zui-Bi-Shen-Wan, HMBSW for Hai-Ma-Bu-Shen-Wan, DHLW for Da-Huo-Luo-Wan, SQSYT for San-Qi-Sang-Yao Tablet, SQSYC for San-Qi-Sang-Yao Capsule, MGW for Mu-Gua-Wan, GFLZW for Gui-Fu-Li-Zhong-Wan, JGSQW for Jin-Gui-Shen-Qi-Wan and XJD for Xiao-Jin-Dan.
[b] Processed aconite roots.

efficacy of medicines containing aconitine roots in clinical use is to be guaranteed, the contents of all three alkaloids must be determined. Moreover, the clinical dosages of proprietary Chinese medicines were normally high (from 2 to 18 g/daily), i.e., about 116 μg content of total three toxic alkaloids were administrated daily if patients used Mu-Gua-Wan (MGW). If limits are placed on the quantity of total three toxic alkaloids in proprietary Chinese medicines, this would be helpful for safety treatment and also helpful for good manufacturing practices regarding quality control and assurance.

4. Conclusion

The six *Aconitum* alkaloids with similar structure were commonly co-exists in herbs which make them difficult to separate from each other and from other compounds consisted in proprietary Chinese medicines by using previously reported HPLC methods. By choosing an appropriate alkaline buffer solution (10 mM ammonium bicarbonate buffer, pH 10.0) as mobile phase with acetonitrile and suitable gradient elution program, satisfactory chromatographic separation of six *Aconitum* alkaloids with their adjacent peaks was achieved. The effects of ammonium bicarbonate buffer concentration and pH values on the chromatographic behavior of six alkaloids were investigated.

Since proprietary Chinese medicines containing aconite roots is increasingly becoming popular as a medicine used in the eastern Asia, method for standardization of those medicines are in demand. Method validation data indicate that the present method is a reliable, reproducible and accurate HPLC method for simultaneous determination of six *Aconitum* alkaloids in aconite roots and their proprietary Chinese medicines with optimizing extraction and separation conditions. The quantitation results showed that the contents of the six *Aconitum* alkaloids were significantly varied in twelve proprietary Chinese medicines, as well as the processed and the unprocessed aconite roots. So, it is highly recommended that the determination of those six alkaloids in the proprietary Chinese medicines containing the aconite roots must be done as a routine measurement, so as to provide a safe application to patients in clinics, and good manufacture practices.

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