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Simultaneous Determination of Three Diterpenoid Alkaloids by HPLC in Unprocessed and Processed *Fuzi*

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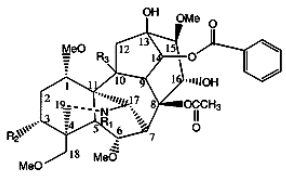
An HPLC method was developed for the simultaneous determination of three diterpenoid alkaloids (mesaconitine, aconitine and hypaconitine) in unprocessed and processed *Fuzi*. The solid phase was Gemini C18 column with isocratic elution using 0.04 M ammonium acetate-acetonitrile as the mobile phase. The results of methodology all fit the analytical requirements and the recoveries were 95.8-103.4 %. Quantitative analysis of the three alkaloids showed that the contents of the alkaloids varied significantly. It was the first report that figured out the alkaloids quantitative differences in unprocessed and processed *Fuzi* and could provide a scientific and technical platform for setting up a quality control standard.

Key Words: *Fuzi*, Diterpenoid alkaloid, HPLC.

INTRODUCTION

Aconite (*Aconitum carmichaeli*) is widely distributed over the southwest provinces of China. Its lateral roots and those of other same-genus species, which are used as a traditional chinese medicine in China, Korea and Japan, *etc.*, share the common name *Fuzi*. Modern pharmacological researches showed that aconitine-type alkaloids from *Fuzi* possessed various bioactivities such as analgesia, diuresis, antiinflammatory and cardiotonic actions[1-3]. These aconitine-type alkaloids shared a common C19-norditerpenoid skeleton and traditionally were divided into three major types according to the substitute at the C8, namely diester-diterpenoid alkaloids (DDAs), mono-ester-diterpenoid alkaloids (MDAs) and lipo-alkaloids (LPAs)[4-6]. The structures of the known alkaloids are summarized in Fig. 1. Nevertheless, these alkaloids were also significantly toxic, the raw lateral roots of aconite, namely unprocessed *Fuzi* (shengfuzi), therefore could not be used directly because of their high contents of aconitine-type alkaloids[7-9]. The over 2000-year historical clinical experience suggested that proper processing of aconite herbs with heating, steaming and soaking could significantly reduce the toxicity. Nowadays, there are three major kinds of pretreated *Fuzi* on the market: Baifupian, Heishunpian and Yanfuzi, which all have their own characteristic refining processes[10].



Aconitines	$[M+H]^+$	R_1	R_2	R_3
Hypaconitine	616	CH_3	H	H
Deoxyaconitine	630	C_2H_5	H	H
Mesaconitine	632	CH_3	OH	H
Aconitine	646	C_2H_5	OH	H
10-OH-mesaconite	648	CH_3	OH	OH
10-OH-aconitine	662	C_2H_5	OH	OH

Fig. 1. Structures of some aconitine-type alkaloids in *Aconitum*

Since Shengfuzi had significant toxicity and must be treated to Baishunpian, Heishunpian or Yanfuzi before clinical use, it would be of great meaning to determine whether the main aconitine-type alkaloids ever changed after the processing procedures. Some methods had been developed for Aconitum alkaloids analysis[11-13], while to the best of our knowledge, the simultaneous determination of the three main toxic ingredients (mesaconitine, aconitine and hypaconitine) and the analysis of the difference between processed and unprocessed *Fuzi* had

not been reported. The aim of this study is to develop a simple HPLC method for the simultaneous determination of the three major toxic diester-diterpenoid alkaloids both in unprocessed and processed *Fuzi* for further safety and efficiency in clinical use.

EXPERIMENTAL

Reference compounds and chemicals: Standards of mesaconitine, aconitine and hypaconitine were supplied by Zhejiang Institute for Food and Drug Control (Hangzhou, Zhejiang Province, China). Unprocessed and processed *Fuzi* were purchased from Sichuan, China. Methanol and acetonitrile were HPLC grade and other reagents used were analytical grade. Deionized water was prepared using a Millipore water purification system.

HPLC conditions: An Agilent 1200 series LC system was employed in this research, which consisted of a G1379B Quaternary Pumps, a G1376B degasser, a G1316A Diode-Array Detector, a G1376B Autosampler.

The analysis of the alkaloids were carried out on a Gemini 110A RP18 (250 × 4.6 mm I.D., 5 μm; Phenomenex, USA), protected by a SecurityGuardTM RP18 guard column (4 × 3.0 mm I.D., Phenomenex, USA).

The solvents used for HPLC separation of the three diester-diterpenoid alkaloids in samples were acetonitrile (A) and buffer solution (B, containing 40 mM ammonium acetic and its pH adjusted to 9.5 with concentrated ammonia) at a flow rate of 1.0 mL·min-1. The mobile phase was isocratic elution with A-B (46:54, v/v) and the analysis was monitored at 235 nm. The column temperature was 35 ºC and the sample injection volume was 10 μL.

Preparation of sample solutions: The unprocessed and processed *Fuzi* were pulverized into powder, then passed through a 0.45 mm sieve, accurately weighted to *ca.* 10 g. 10 mL 10 % ammonia solution and then 200 mL diethyl ether were added for ultrasonic batch at room temperature for 0.5 h. The extract was then evaporated to dryness at 40 ºC under a stream of nitrogen. The residue was dissolved to a 10 mL volumetric flask with methanol-0.5 % hydrochloric acid. The solution was ready for chromatographic analysis after passing through a 0.45 μm membrane filter.

Preparation of standard solutions: Three standard solutions, reference compounds mesaconitine (12.49 mg), aconitine (5.02 mg) and hypaconitine (12.55 mg) were dissolved with methanol-0.5 % hydrochloric and diluted to five different concentrations.

RESULTS AND DISCUSSION

Optimization of HPLC separation and mass conditions: Normally, the mobile phase would often be alkalified for better separation of peaks and better resolutions for the alkaloids-present samples. Under such conditions, the alkaloids would be in their free-base forms and bind with the stationary phase closely, then the longer retention time and better separation between the alkaloids would be achieved. In short, the choice of experimental conditions was guided by the need of obtaining chromatograms with better resolution of adjacent peaks within short analytical time, especially several analytes with similar skeleton to be analyzed.

The influence of pH to the peak symmetry and retention time was also investigated with a range of 9.0-10.5 pH values by adjusting concentrated ammonia solution as well as the ammonium acetic concentration was kept at 40 mM. The results showed that with the increase of the pH values, the peak shape was magnified and the retention times of the three diester-diterpenoid alkaloids also increased for the stronger interactions with the stationary phase. A pH value 9.5 was chosen finally for the comprehensive consideration of the retention time as well as the peak shape. Under this pH value, the three diester-diterpenoid alkaloids were baseline separated from each other with resolution values above 1.5.

Validation of the chromatographic method for three DDAs

Regression equations: Linear regression analysis for each of the three diester-diterpenoid alkaloids was performed by the external standard method. Calibration curves were established based on five points for mesaconitine with concentrations of 10, 200, 250, 300 and 350 μg mL^{-1}; five points for aconitine with concentrations of 1.00, 10.04, 20.08, 30.12, 40.16 μg mL^{-1}; six points for hypaconitine with concentrations of 10, 100, 150, 200, 250 and 300 μg mL^{-1}. The calculated results were given in Table-1. All the alkaloids showed good linearity in a relatively wide concentration range.

TABLE-1
LINEAR REGRESSION EQUATION AND LINEAR RANGES

Alkaloids	Regression equation	Correlation coefficient (R^2)	Linear range (μg mL^{-1})
Mesaconitine	Y = 120.9 X + 66. 47	0.9997	10.0~350.0
Aconitine	Y = 122.5 X + 0.014 81	1.0000	1.00~40.16
Hypaconitine	Y = 122.2 X + 53.62	0.9995	10.0~300.0

X denoted the concentrations and Y denoted the peak areas.

Precision: Precision was evaluated by HPLC analysis with a standard mixture solution of the three DDAs five times and the results showed that relative stand deviation (RSD) of peak area of each alkaloid was 0.8-2.0 %.

Repeatability: Five measurements were taken using Shengfuzi and the processes were in accordance with the established method in parallel. The results showed that RSD of peak area were 1.3-3.4 %.

Stability: For stability test, the same sample solution was analyzed in 24 h and found to be rather stable (RSD: 0.8-1.7 %).

Recovery test: The sample of which the alkaloid content had already been determined, was spiked with known amounts of the three standards. Then the spiked sample was extracted, processed and quantified in accordance with the established method. The average recovery for the alkaloids determined was 95.8-103.4 % (Table-2).

Application of the HPLC method for quantitation studies: 10 μL unprocessed and processed *Fuzi* solution were injected into the instrument. The representative HPLC chromatograms of them were shown in Fig. 2, respectively. Peaks in the obtained chromatograms were identified by comparing the retention time and on-line UV spectra with those of the standards.

TABLE-2
RECOVERY OF THE THREE DIESTER-DITERPENOID ALKALOIDS (n = 5)

Component	Contents in materials (mg)	Added (mg)	Found (mg)	Recovery (%)	Mean (%)	RSD (%)
Mesaconitine	1.0240	1.004	1.0020	99.8	101.5	1.3
	1.0180	1.004	1.0380	103.4		
	1.0090	1.004	1.0120	100.8		
	1.0350	1.004	1.0190	101.5		
	1.0110	1.004	1.0250	102.1		
Aconitine	0.1021	0.100	0.0959	95.9	96.7	1.1
	0.1083	0.100	0.0963	96.3		
	0.1104	0.100	0.0972	97.2		
	0.1095	0.100	0.0958	95.8		
	0.1142	0.100	0.0984	98.3		
Hypaconitine	1.2190	1.200	1.1980	99.9	100.2	0.8
	1.1890	1.200	1.2110	100.9		
	1.1950	1.200	1.1920	99.3		
	1.2420	1.200	1.2140	101.2		
	1.2530	1.200	1.1960	99.7		

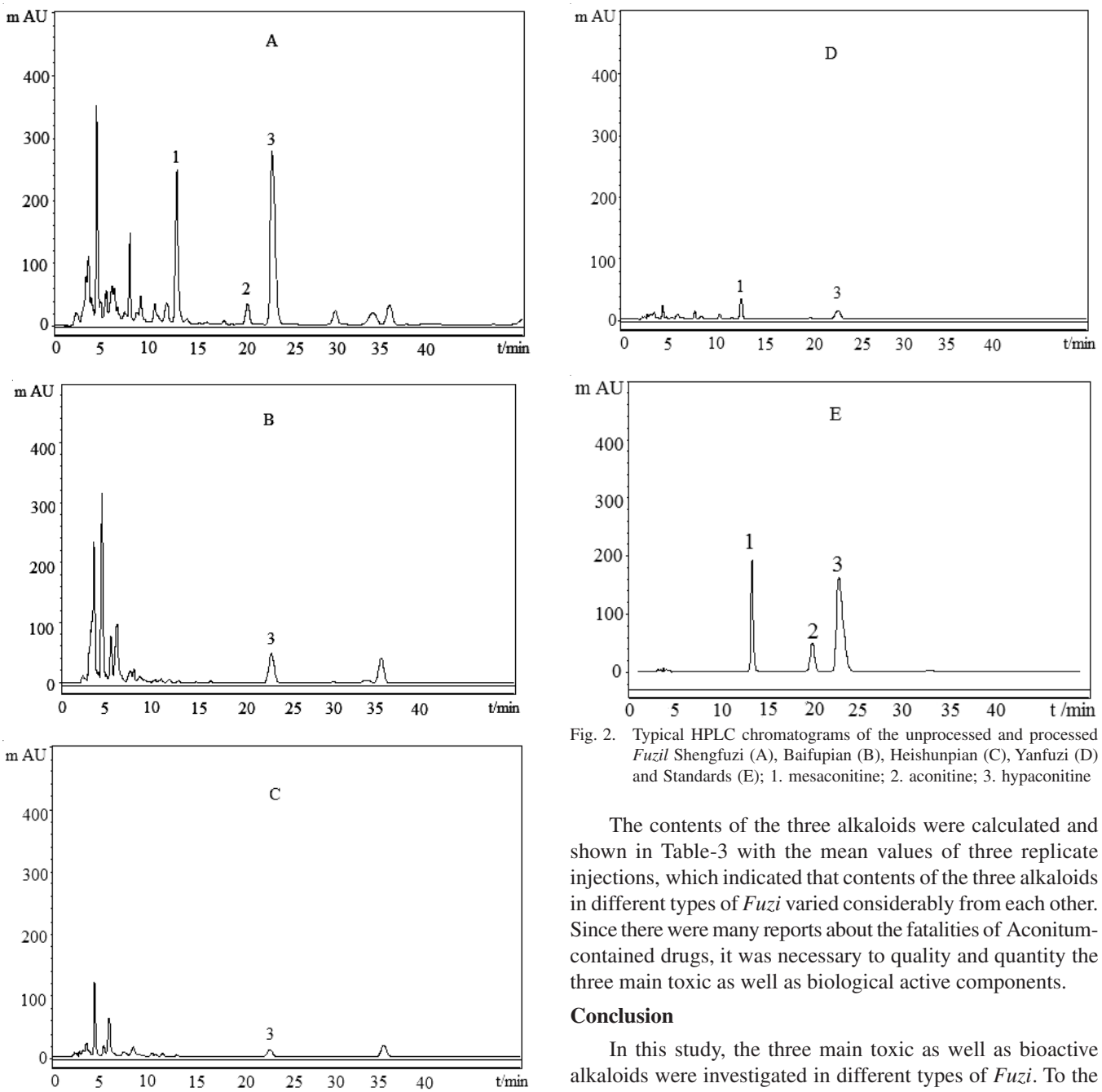


Fig. 2. Typical HPLC chromatograms of the unprocessed and processed *Fuzil* Shengfuzi (A), Baifupian (B), Heishunpian (C), Yanfuzi (D) and Standards (E); 1. mesaconitine; 2. aconitine; 3. hypaconitine

The contents of the three alkaloids were calculated and shown in Table-3 with the mean values of three replicate injections, which indicated that contents of the three alkaloids in different types of *Fuzi* varied considerably from each other. Since there were many reports about the fatalities of Aconitum-contained drugs, it was necessary to quality and quantity the three main toxic as well as biological active components.

Conclusion

In this study, the three main toxic as well as bioactive alkaloids were investigated in different types of *Fuzi*. To the

TABLE-3
CONTENTS OF THREE DIESTER-DITERPENOID ALKALOIDS IN UNPROCESSED AND PROCESSED *Fuzi* (n = 3)

Sample	Mesaconitine (mg)	Aconitne (mg)	Hypaconitine (mg)
Crude *fuzi*	2.011 (RSD = 1.8 %)	0.1588 (RSD = 2.3 %)	2.4550 (RSD = 1.5 %)
Bai fupian	–	–	0.2517 (RSD = 1.9 %)
Heishunpian	–	–	0.2126 (RSD = 2.2 %)
Yan fuzi	0.2528 (RSD = 2.7 %)	–	0.1754 (RSD = 2.5 %)

best of our knowledge, it was the first article which simultaneously determined the three main toxic alkaloids not only quantitatively but also qualitatively. The developed method had been clearly demonstrated to be able to distinguish the different samples and thus could be used for alkaloid profiling purposes. On the other hand, it could guide the manufacturer to monitor the effects of processing and preparation on the alkaloid contents, which would further provide a safe application and good manufacture practices.

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