

Pharmacokinetic Differences of Three Aconitum Alkaloids from *Aconiti lateralis* Radix Praeparata and Compatibility with *Pinellia* in Rats

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ABSTRACT

Aim: This study aims to develop a highly sensitive and specific UPLC-MS/MS method to explore the pharmacokinetic properties of three representative active alkaloids (benzoylhypaconine, benzoylmesaconine, benzoylaconine) after administration of extracts of *Aconiti lateralis Radix* Praeparata and compatibility with *Pinellia*, compare the influence of *Pinellia* on pharmacokinetic characterization of three aconitum alkaloids.

Methods: Chromatographic separation were performed on a C₁₈ column under the Multiple reaction monitoring (MRM) in the positive Electrospray ionization (ESI) mode. The pharmacokinetic parameters were evaluated by software DAS 3.0. **Results:** The specificity, precision and accuracy, matrix effect and extraction recovery rate and stability all meet the requirements. The validation of the method was achieved in accordance to the FDA guidelines. After oral administration of the extract, similar tendency was found in the mean concentration of the three analytes in the curves, illustrating that three analytes exhibited a similar absorption and metabolic route. Compared the pharmacokinetic characteristics, the AUC_{0-1h}, AUC_{0-∞} and C_{max} values of BMA and BHA in Fuzi group were lower than those in Fuzi-Banxia group. **Conclusion:** This finding indicated the significant statistical decrease ($p < 0.05$) in the absorption of these components. After Banxia in combination with Fuzi, the results showed that faster absorption and higher exposure of BMA and BHA in rat plasma.

Key words: Pharmacokinetic, *Aconiti lateralis Radix*, *Pinellia*, Aconitum alkaloids, UPLC-MS/MS.

INTRODUCTION

Most herbal medicines were prescribed in combination based on the theory of traditional Chinese herbal medicine to obtain synergistic effects or diminish the possible adverse reaction,¹ but meanwhile, there also have a special rule for TCM named Eighteen Incompatible Pairs. Incompatible pairs means two herbs are not suitable for clinical application together, otherwise there will be detrimental to therapeutic efficacy, causing toxicity to the body.² As an empirical summary obtained from long-term TCM clinical practice, the incompatibility theory is closely related to safe medication and has gained increasing concerns for modernization

research of TCMs.³ As recorded in Chinese Pharmacopeia, co-administration of *Pinellia* Banxia) and *Aconiti lateralis Radix* Praeparata (Fuzi) was prohibited.⁴ It has always been disputed, because there are many traditional Chinese herbal prescriptions including incompatible pairs, such as Wufu Jingfang, containing *Aconiti lateralis Radix* Praeparata and *Pinellia*.

In order to resolve the mystery of the ancient rule of TCM incompatibility, scientific experiments based on chemistry and pharmacology were carried out recently.^{5,6} Nevertheless, these methods are not sensitive enough for trace detection in a pharmacokinetic study.

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The powerful and reliable UPLC–MS/MS method and Multiple reaction monitoring technology has been widely used in pharmacokinetic studies because it can provide high sensitivity for quantification. The use of MRM technology improves the analytical selectivity and sensitivity and eliminate obvious endogenous interference from plasma. Fuzi, derived from the dried daughter root of *Aconitum carmichaelii*, three monoester alkaloids (MDAs) were considered to be the effective as well as the toxic components. In particular, the monoester-diterpene alkaloids have a narrow therapeutic index.⁷ As a highly toxic drug, there is little information available on the pharmacokinetic studies of the alkaloid constituents after compatibility with Banxia. In this study, a rapid, sensitive and robust UPLC-MS/MS method for simultaneous determination of benzoylaconine, benzoylmesaconine and benzoylhypaconine in plasma was developed and validated. Pharmacokinetic study was performed to explore the *in vivo* differences of the three alkaloids after oral administration of Fuzi-Banxia and each single herb extracts.

MATERIALS AND METHODS

Chemical and reagents

Aconiti lateralis Radix Praeparata and *Pinellia*, were purchased from the shan ke shu Medical Material Company (Harbin, China) and authenticated by Professor Yu-bin JI (Harbin University of Commerce, Harbin, China). The voucher specimens were deposited in the laboratory of Research Center on Life Sciences and Environmental Sciences (Harbin University of Commerce). Benzoylaconine, benzoylmesaconine, benzoylhypaconine and pentoxifylline (internal standard, IS) were all purchased from the National Institutes for Food and Drug Control (Beijing, China); Methanol, acetonitrile and formic acid (HPLC grade) was purchased from Sigma (St. Louis, MO, USA); Deionized water was purified on a Milli-Q system (Millipore, Milford, MA, USA).

Animals

Male Sprague-Dawley rats (200±20 g) were supplied by the Laboratory Animal Center of Harbin Medical University (Harbin, China). Eighteen healthy male SD rats were randomly divided into three groups (n=6) and were given Fuzi, Banxia or Fuzi-Banxia extracts by gastric perfusion, respectively (20 g/kg, the single herb/body weight). The study was approved by Animal Ethic Committee of the Harbin University of Commerce (No. EA_20160107) and the experiments with rats were in full compliance with the European Communities

Council Directive of 24 November 1986 (86/609/EEC) and with the Guidelines laid down by the NIH in the US.

Preparation of Fuzi-Banxia extracts

Fuzi, Banxia and Fuzi-Banxia herb pair (at the ratio of 1:1) were weighed respectively, soaked in water (10 folds) for 1h, then refluxed for 0.5h, 3 times altogether. After filtering with 3 layers of gauze, the filtrates were merged and concentrated to a concentration of 1.0 mg/mL (equivalent to the single herb). The aqueous extracts were stored at 4°C until administration.

Preparation of standard solutions, calibration and quality control (QC) samples

Benzoylaconine, benzoylmesaconine, benzoylhypaconine were accurately weighed and dissolved in a water–methanol (50:50, v/v) solution. Working solutions were obtained by serially diluting the stock solutions in water–methanol (50:50, v/v) solution to produce the final concentrations of 0.5-500 ng/mL for benzoylmesaconine, 0.25-250 ng/mL for benzoylhypaconine and 0.25-250 ng/mL for benzoylaconine. The concentration of IS was 25 ng/mL in methanol. Before use, all of the solutions were stored at 4°C. Calibration samples were prepared by spiking 5 µL of working solutions at the corresponding concentrations into 45 µL of blank rat plasma. The Lower Limit of Quantification (LLOQ), low, medium and high quality control (QC) samples were prepared in the same way. All solutions were stored in a refrigerator (-40°C) until analysis.

Instrumentation and LC-MS/MS analytical conditions

An Agilent 1290 series UPLC system (Agilent Technologies, Santa Clara, CA, USA) consisted of a binary pump, an online degasser, an auto plate sampler and a thermostatically-controlled compartment. Separation of analytes was performed on a Waters Acquity UPLC C₁₈ column (100 mm × 2.1 mm, 1.7 µM, Waters, Milford, MA, USA). The mobile phase was composed of 0.1% formic acid (A) and acetonitrile (B) using a gradient elution of 95–80% A at 0–2 min, 80–70% A at 2–3 min, 70–50% A at 3–4 min, 50% A at 4–4.4 min and the re-equilibration time of gradient elution was 0.6 min. The injection volume was 5 µL and the flow rate was 0.35 mL/min. The MRM transitions included m/z 590.3→105.1 for benzoylmesaconine, with a collision energy of 49 eV; m/z 574.3→105.1 for benzoylhypaconine, with a collision energy of 50 eV; m/z 604.1→105.1 for benzoylaconine, with a collision energy of 49 eV; m/z 278.3→180.1 for pentoxifylline, with a collision energy of 49 eV. Other parameters were as follows: carrier gas (N₂) flow rate, 11.0 L/min.

Sample preparation

Blood samples (100 μL) were collected from the sub-orbital venous plexus at 5, 10, 15, 30, 45, 60, 90, 120, 240, 360, 480, 720 and 1440 min after administration. Collected blood samples were placed into heparinized tubes and centrifuged at 4500 r/min for 15 min and the plasma was stored at -40°C before analysis. 50 μL plasma sample and 200 μL IS (25.0 ng/mL) vortexed for 3 min. The mixture was centrifuged at 13 500 r/min for 15 min. The organic layer was separated, transferred and dried under nitrogen at 40°C . Mobile phase (50 μL) was applied to reconstitute the residue and then the mixture was vortexed for 3 min, followed by centrifugation at 13500 r/min for 5 min. For analysis, 5 μL of the supernatant was injected.

Method validation

Method validation was performed to evaluate selectivity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability according to the Guidance for Industry-Bioanalytical Method Validation recommended by the US Food and Drug Administration (FDA, 2014).

Selectivity

The selectivity of the method was evaluated by analyzing blank plasma samples from six different rats, plasma samples spiked with benzoyleaconine, benzoylmesaconine, benzoylhypaconine and IS were analyzed and compared to assess the selectivity of the method, as shown in figure 1.

Linearity and low limits of quantification (LLOQ)

A $1/x^2$ weighted least square linear regression model was used to construct calibration curves by plotting the peak-area ratios of each analyte to IS versus plasma concentrations. LLOQ was determined based on the two criteria: (1) the analyte response at the LLOQ would be at least 10 times that of the blank; and (2) the analyte peak would be discrete and reproducible with a precision within 20% and accuracy of 80%–120% (Table 1).

Precision and accuracy

The inter-day precision and accuracy were determined on three consecutive days and the intra-day precision was evaluated on the same day by determination of QC samples. Analysis ($n=6$) of QC samples at different concentrations (low, middle and high) and one calibration curve was included in each run. Relative standard deviation (RSD) was used to express the precision and relative error (RE) was applied to express the accuracy.

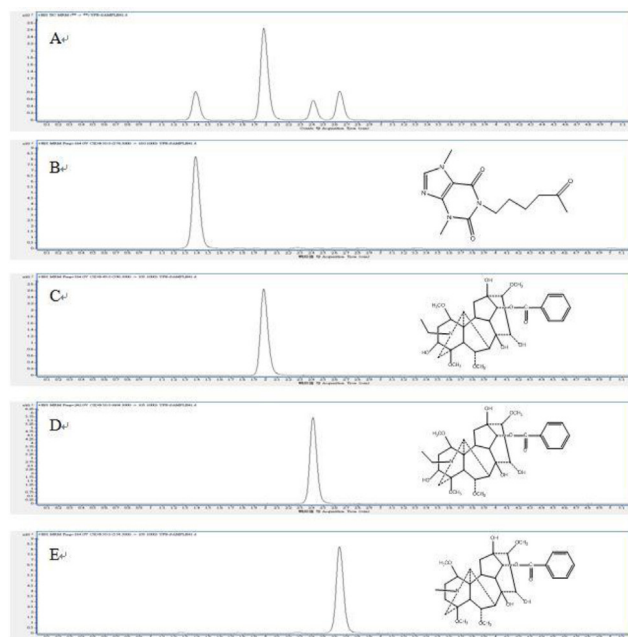


Figure 1: A Chromatograms of blank plasma samples with benzoylmesaconine $T_r=2.05\text{min}$, benzoyleaconine $T_r=2.42\text{min}$, benzoylhypaconine $T_r=2.65\text{min}$, pentoxifylline (IS) $T_r=1.39\text{min}$; B. Extract chromatograms of plasma sample of 30 min after administration of extracts pentoxifylline (IS) $T_r=1.39\text{min}$, C. Extract chromatograms of plasma sample of 30 min after administration of extracts benzoylmesaconine $T_r=2.05\text{min}$, D. Extract chromatograms of plasma sample of 30 min after administration of extracts benzoyleaconine $T_r=2.42\text{min}$, E. Extract chromatograms of plasma sample of 30 min after administration of extracts benzoylhypaconine $T_r=2.65\text{min}$

Extraction recovery and the matrix effect

Extraction recovery experiments were performed by comparing the mean peak area of extracted samples (spiked before extraction) to the peak area of unextracted samples (QC working solutions spiked in extracted plasma) at LQC, MQC and HQC levels. The matrix effect was assessed by comparing the peak area obtained from an amount of the analyte added in extracted blank plasma to the peak area obtained from equal concentration of the analyte in solvent at low, medium and high concentrations in Table 2.

Stability

QC samples at three concentrations under different sample storage conditions and processing procedures. Short-term stability (4 h at room temperature), long-term stability (15 d at -40°C), freeze-thaw stability (three cycles, from -40°C to room temperature as a cycle) and post-preparation stability (12 h at 4°C) were determined and summarized in Table 3.

Table 1: Linear regression, linear range of the analytes in rat plasma (n=6).

Analytes	Linear range (ng mL ⁻¹)	Regression equation	R ²	LLOQ
benzoylmesaconine	0.500~500	Y=0.00124x+0.0143	0.9961	0.500
benzoylaconine	0.250~250	Y=0.00116x-0.00104	0.9968	0.250
benzoylhypaconine	0.250~250	Y=0.00454x+0.00614	0.9959	0.250

Table 2: Precision and accuracy for the determination of three analytes in rat plasma (n = 6).

analytes	concentration (ng/mL)	Inter-day RSD (%)	Intra-day RSD (%)	Precision (%)	Extraction recovery (%)	Matrix effect (%)
benzoylmesaconine	1.00	5.8	3.8	96.5%	101.3±2.9	102.9±4.1
	20.0	6.5	5.4	100.3%	98.5±4.2	102.5±2.7
	400	4.5	4.7	97.1%	95.6±3.8	97.4±5.4
benzoylaconine	0.500	6.2	5.2	100.2%	97.8±3.9	103.8±2.4
	10.0	7.4	5.1	97.8%	96.5±4.1	98.4±4.5
	200	5.5	4.3	99.6%	97.1±2.6	97.1±3.2
benzoylhypaconine	0.500	5.3	6.4	102.4%	102.1±3.2	102.4±4.1
	10.0	7.2	4.9	103.6%	96.4±2.7	101.4±2.9
	200	5.1	6.3	97.8%	97.4±3.6	97.4±3.4

Table 3: Stability of three analytes in rat plasma (n = 6).

Analytes	Concentration ng/mL	25°C for 4 h		-40°C for 15 days		freeze-thaw cycles		4°C for 24 h	
		RSD (%)	Remaining (%)	RSD (%)	Remaining (%)	RSD (%)	Remaining (%)	RSD (%)	Remaining (%)
benzoylmesaconine	1.00	8.6	100.5	8.4	101.2	7.2	95.4	6.7	102.3
	20.0	5.3	101.6	7.6	103.5	7.8	100.1	8.0	98.9
	400	5.7	101.2	5.4	104.5	5.6	104.8	7.6	100.9
benzoylaconine	0.500	8.2	101.9	6.2	104.7	8.4	97.4	5.8	101.2
	10.0	5.4	102.6	5.9	102.6	5.6	101.4	6.1	103.2
	200	4.6	103.7	4.2	102.1	6.7	105.6	6.4	102.5
benzoylhypaconine	0.500	8.7	104.6	7.3	103.2	5.2	98.9	6.8	103.0
	10.0	5.2	103.4	6.7	104.8	7.4	101.4	7.6	97.5
	200	5.4	102.3	4.1	102.3	5.4	103.6	7.9	102.6

Table 4: Pharmacokinetic parameters of alkaloids after the oral administration of Wutou decoction in rats (n = 6).

analytes		T _{1/2z} (min)	C _{max} (ng/mL)	T _{max} (min)	AUC _{0-t} (ng*min/ml)	AUC _{0-∞} (ng*min/ml)	MRT _{0-t} (min)
benzoylmesaconine	Fuzil	661±75	43.2±15.6	30(30,45)	5019±1023	5954±803	564±31
	Fuzi-Banxia	654±42	86.2±7.14*	30(30,45)	9624±235*	10717±426*	537±25
benzoylaconine	Fuzil	493±36	25.4±14.3	30(30,45)	3046±386	4059±326	304±38
	Fuzi-Banxia	474±38	23.4±13.2	30(30,45)	2945±264	3859±214	345±26
benzoylhypaconine	Fuzil	452±47	29.8±11.4	30(30,45)	3319±156	4225±168	372±31
	Fuzi-Banxia	436±39	66.9±6.51*	30(30,45)	7045±39*	8025±134*	395±38

*Significantly from oral administration of extract in rats (p<0.05)

Pharmacokinetic study

Plasma concentration-time curves were plotted and pharmacokinetic parameters were calculated by using the DAS 3.0 pharmacokinetic analysis software. All the data were presented as mean \pm standard deviation (SD). Analysis of the data was executed by non-compartmental model and statistical moment calculation, from which pharmacokinetic parameters were obtained. The Student's test was used to test significant differences between Fuзи group and Fuзи-Banxia group and $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

LC-MS/MS condition

Positive ESI ion modes were employed to achieve the mass spectra of benzoylaconine, benzoylmesaconine, benzoylhypaconine and IS. The responses for each compound were higher in positive mode. Precursor ions and product ions were monitored by MRM to enhance selectivity and lower interference. The capillary and cone voltages as well as the source temperature and desolvation gas temperature/flow were selected to optimize the specificity and sensitivity of protonated analyte molecules. To obtain the maximum response of the analyte fragmentation, as well as IS, the collision energy of

Collision-Induced Decomposition (CID) was optimized.

Pharmacokinetic study

The validated assay was applied for comparative pharmacokinetic study of the three alkaloids in Fuзи group and Fuзи-Banxia group after oral administration of extract. The mean plasma concentration-time curves (mean \pm SD) of the three analytes are presented in Figure 2-4 and the corresponding pharmacokinetic parameters are summarized in Table 4. After oral administration of the extract, similar tendency was found in the mean concentration of the three analytes in the curves, illustrating that three analytes exhibited a similar absorption and metabolic route. Compared the pharmacokinetic characteristics, the AUC_{0-} , $AUC_{0-\infty}$ and C_{max} values of BMA and BHA in Fuзи group were lower than those in Fuзи-Banxia group. This finding indicated the significant statistical decrease ($p < 0.05$) in the absorption of these components. After Banxia in combination with Fuзи, the results showed that faster absorption and higher exposure of BMA and BHA in rat plasma.

CONCLUSION

In this study, a rapid, sensitive and specific UPLC-MS/MS method was established and applied to a comparative pharmacokinetic study of three aconitine-type alkaloids after oral administration of Fuзи, Banxia and Fuзи-Banxia extracts. Combined administration of Fuзи and Banxia accelerated the absorption of BMA and BHA, this comparative pharmacokinetic study would be helpful for improving clinical therapeutic efficacy and further pharmacological studies of Fuзи and Banxia and their herb-herb interaction.

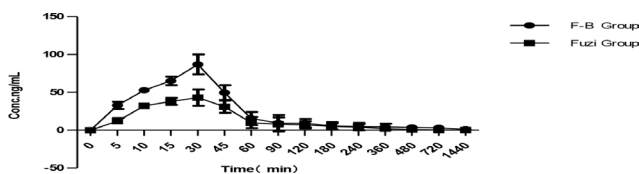


Figure 2: Plasma concentration-time curves of benzoylmesaconine after administration of extracts.

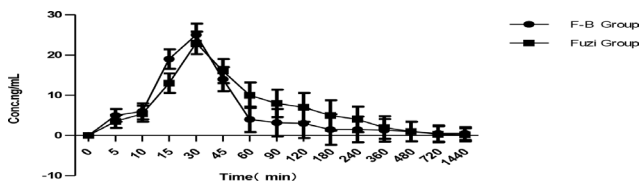


Figure 3: Plasma concentration-time curves of benzoylaconine after administration of extracts.

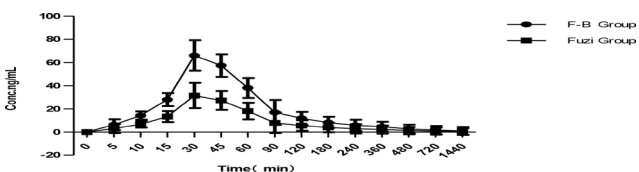


Figure 4: Plasma concentration-time curves of benzoylhypaconine after administration of extracts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BMA: Benzoylmesaconine; **BHA:** Benzoylhypaconine; **BAC:** Benzoylaconine; **UPLC-MS/MS:** Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry.

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PICTORIAL ABSTRACT



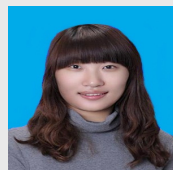
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SUMMARY

- Incompatible pairs means two herbs are not suitable for clinical application together, otherwise there will be detrimental to therapeutic efficacy, causing toxicity to the body. As recorded in Chinese Pharmacopoeia, co-administration of *Pinellia* (Banxia) and *Aconiti lateralis Radix* Praeparata (Fuji) was prohibited. It has always been disputed, because there are many traditional Chinese herbal prescriptions including incompatible pairs, such as Wufu Jingfang, containing *Aconiti lateralis Radix* Praeparata and *Pinellia*. In this study, a rapid, sensitive and robust UPLC-MS/MS method for simultaneous determination of benzoylaconine, benzoylmesaconine and benzoylhyaconine in plasma was developed and validated. Pharmacokinetic study was performed to explore the *in vivo* differences of the three alkaloids after oral administration of Fuji-Banxia and each single herb extracts. After Banxia in combination with Fuji, the results showed that faster absorption and higher exposure of BMA and BHA in rat plasma.

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