Biological activities and pharmacokinetics of aconitine, benzoylaconine, and aconine after oral administration in rats

Hai Zhang, a,b† Sen Sun, a† Wen Zhang, a Xiangqun Xie, c Zhenyu Zhu, b Yifeng Chai b* and Guoqing Zhang a*

Introduction

The Aconitum tuber has long been used as a traditional Chinese herbal medicine due to its analgesic, anti-inflammatory, local anaesthetic, and anti-epileptic activities.1–4 The main components of the Aconitum tuber are various forms of alkaloids, including diester-diterpenoid alkaloids (DDAs), monoester-diterpenoid alkaloids (MDAs), and alkylamino-diterpenoid alkaloids (ADAs).5–7 Aconitine (AC), benzoylaconine (BAC), and aconine (ACN) are three representative alkaloids. AC is a DDA with highly cardiotoxic and neurotoxic activities.8,9 The LD50 of AC is approximately 1.8 mg/kg in mice, and 1–2 mg/kg in humans. The toxicity of BAC is much lower than that of AC, the LD50 being 1500 mg/kg in mice. The LD50 of ACN is approximately 1/2000 of AC.10–12 Therefore, improper use of the Aconitum tuber may cause severe toxicity or death.13–15

The three forms of alkaloids (DDAs, MDAs, and ADAs) possess different pharmacological and toxicological activities. The toxicity of DDAs is much higher than that of MDAs and ADAs, and its efficacy is also stronger than that of the other two.16,17 In the decoction process of the Aconitum tuber, DDAs may transform to MDAs, and MDAs transform to ADAs.18–20

Although many pharmacological studies on Aconitum alkaloids have been reported,21–25 there are few systematic studies available on the pharmacokinetic characteristics and disposal process of the three forms of Aconitum alkaloids. The aim of this study was to determine the biological activities of three forms of Aconitum alkaloids in rat plasma, urine, faeces, and different tissues after oral administration by liquid chromatography-tandem mass spectrometry (LC-MS/MS), investigate their disposal process in vivo, and clarify their pharmacokinetic parameters, absorption and metabolic stability by using a Caco-2 cell monolayer model and rat liver microsome incubation system in vitro, hoping that this study would provide references for rational clinical use of the Aconitum tuber, and for further research on the pharmacology or toxicology of this medicinal plant.

Keywords: absorption; alkaloids; LC-MS/MS; metabolic rate; pharmacokinetics
Materials and methods

Chemicals and reagents

Standards of AC (purity > 98%), BAC (purity > 98%), ACN (purity > 98%) and isoliquiritigenin (purity > 98%) were all obtained from China’s National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Their molecular structures are shown in Figure 1. Lucifer yellow, non-essential amino acid, β-NADPH and alkaline phosphatase assay kit were obtained from Sigma-Aldrich (St Louis, MO, USA). Male rat liver microsomes were purchased from BD Gentest TM (Becton Dickinson, Franklin Lakes, NJ, USA). The Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Minimum Essential Media (MEM) and fetal bovine serum (FBS) were provided by Gibco (Grand Island, NY, USA). Acetonitrile, methanol and formic acid of HPLC grade were purchased from Burdick & Jackson (Honeywell, Ulsan, Korea). Ultrapure water was prepared by Milli-Q System (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

Instrumentation and conditions

Chromatographic analysis was performed by using an Agilent 1290 series liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA), including a binary pump, an on-line vacuum degasser, a surveyor autosampling system and a column temperature controller. The sample was separated on Waters Xbridge C18 column (100 × 3.0 mm, i.d.; 3.0 μm, Milford, Massachusetts, USA) and eluted with an isocratic mobile phase: solvent A (H2O: HCOOH, 100: 0.1, v/v) – solvent B (acetonitrile) (70: 30, v/v) for AC and BAC, and gradient elution of 5–20% B between 0 and 6 min for ACN. Guard column: Agilent XDB-C18 (12.5 mm × 4.6 mm, 5 μm, Agilent Technologies, Palo Alto, CA, USA). During the analysis of AC and BAC, these two compounds were mutually internal standard. The column temperature was set at 25°C, the flow rate at 0.4 mL/min and the injection volume at 5 μL. Mass spectrometric detection was carried out on an Agilent 6460 triple-quadruple mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with turbo ion spray, which is connected to the liquid chromatography system. The mass scan mode was MRM positive. The precursor ion and product ion were m/z 646.4 → 586.4 for AC, m/z 604.2 → 554.1 for BAC, m/z 500.1 → 450.3 for ACN, and m/z 255.0 → 212.2 for isoliquiritigenin. The collision energy for AC, BAC, CAN, and isoliquiritigenin was 30, 35, 35, and 30 eV, respectively. The MS/MS conditions were optimized as follows: fragmentor, 110 V; capillary voltage, 3.5 kV; nozzle voltage, 500 V; nebulizer gas pressure (N2), 40 psig; drying gas flow (N2), 10 L/min; gas temperature, 350°C; sheath gas temperature, 400°C; sheath gas flow, 11 L/min. Agilent MassHunter B 4.0 software was used for the control of the equipment and data acquisition. Agilent Quantitative analysis software was used for data analysis.

Preparation of the calibration, quality control and internal standards

Stock solutions were prepared by dissolving AC (1.30 mg·mL−1), BAC (1.22 mg·mL−1), ACN (1.24 mg·mL−1) and isoliquiritigenin (1.20 mg·mL−1) in acetonitrile. Working solutions and IS solutions (20 ng·mL−1) were achieved by diluting the stock solutions with acetonitrile. All solutions were stored at 4°C.

The calibration standard was prepared by adding an appropriate amount of the standard solution in plasma (100 μL) to yield a final concentration of 0.65–65 ng·mL−1 for AC, 0.61–61 ng·mL−1 for BAC, and 0.62–62 ng·mL−1 for ACN. Three batches of quality control (QC) samples were prepared in plasma for AC (1.3, 6.5, and 32.5 ng·mL−1), BAC (1.22, 6.1, 30.5 ng·mL−1), and ACN (1.24, 6.2, 31 ng·mL−1). The plasma samples (standard and QC samples) were extracted on each analytical batch along with the unknown samples. The calibration standards of tissue, urine, faeces, caco-2 cell transport media and microsomes samples were prepared in a way similar to that for the plasma samples, and the only difference was that blank urine, faeces, or tissue controls were added to yield the final concentrations.

Sample pretreatment

Before homogenizing the tissue and faeces samples, 5 volumes of normal saline solution was added, and then tissue and faeces samples were homogenized by using a motor-driven Teflon pestle. Plasma, urine, tissue, and faeces homogenates (100 μL) were pipette transferred into plastic microtubes, and then added with 20 μL acetonitrile and 180 μL IS solution (20 ng·mL−1). After 30-s vortexing, samples were centrifuged at 1500×g for 10 min. An aliquot of 5 μL supernatant was injected into the LC-MS/MS system for analysis.

Method validation

The method validation assay was performed according to the United States Food and Drug Administration (FDA) guidelines. Selectivity was investigated by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma to monitor interference of endogenous substances and metabolites. To obtain the calibration curve, seven concentrations of the calibration standard were processed and determined as described above. The linearity of AC, BAC and ACN calibration curves was constructed by plotting peak area ratios (y) of analytes with weighted (1/x2) least square linear regression. The lower limit of detection (LOD) and lower limit of quantification (LLOQ) were determined as the concentration of the analytes with a signal-to-noise ratio at 3 and 10, respectively. The intra-day precision and accuracy of the method were confirmed by determining QC samples at three different concentrations five times on a single day, and the inter-day precision and accuracy were assessed by determining the QC samples over three consecutive days. For each concentration, five

Figure 1. The molecular structures of AC (a), BAC (b), ACN (c), and IS (d).
Eighteen rats were equally randomized to three groups: AC, BAC, fasted with free access to water for 12 h prior to each experiment. Animals were acclimatized to the facilities for 5 days, and then administered at an oral dose of 1 mg·kg⁻¹. Blood samples (300 μL) were allowed at 22 ± 2°C and 50 ± 10% relative humidity. Water and food (laboratory rodent chow, Shanghai, China) were allowed ad libitum. The Ethics Committee of the Second Military Medical University (Shanghai, China) approved the animal experimental protocol. Male Sprague-Dawley (SD) rats weighing 220 ± 10 g were used. Male Sprague-Dawley (SD) rats weighing 220 ± 10 g were used. The rats were maintained in an air-conditioned animal quarter at 22 ± 2°C and 50 ± 10% relative humidity. Water and food (laboratory rodent chow, Shanghai, China) were allowed ad libitum. The animals were acclimatized to the facilities for 5 days, and then fasted with free access to water for 12 h prior to each experiment. Eighteen rats were equally randomized to three groups: AC, BAC, and ACN. The three monomers were dissolved in water at the concentration of 1 mg·mL⁻¹. The animals in the three groups were administered at an oral dose of 1 mg·kg⁻¹. Blood samples (300 μL) were collected into heparinized tubes via the ocular choioideae vein before drug administration and at 0.083, 0.167, 0.333, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h after oral administration of the drugs. After centrifugation at 4000 rpm for 10 min, plasma samples were obtained and frozen at -80°C until analysis. The pharmacokinetic parameters were calculated by using DAS version 3.0 (BioGuider Co., Shanghai, China).

**Tissue distribution study in rats**

Fifty-four rats were equally randomized to three groups: AC, BAC, and ACN. Rats under ether anesthesia were sacrificed by bleeding the abdominal aorta at 0.5, 1, 2, 4, 6 and 24 h (3 rats per time point) after oral administration of AC (1 mg/kg), BAC (1 mg/kg), or ACN (1 mg/kg). Heart, liver, spleen, lung, kidney, brain, testes, and muscle samples were dissected, weighed, snap-frozen and ground with mortar and pestle in liquid nitrogen, and then stored at -80°C until analysis.

**Excretion study in rats**

To collect urine and faeces, 18 rats were placed in metabolic cages and equally randomized to AC, BAC and ACN groups. After administration of an oral dose of AC 1 mg/kg, BAC 1 mg/kg, and ACN 1 mg/kg, urine and faeces were collected in tubes at 2, 4, 6, 8, 10, and 24 h and stored at -80°C to keep the samples stable.

**Determination of the metabolic rate of the three forms of alkaloids in rat liver microsome system in vitro**

The rat liver microsome incubation experiment was performed according to the method previously reported. In brief, except for NADPH-generating system (10 mM G-6-P, 1 mM NADP⁺, 4 mM magnesium chloride, 1 unit·mL⁻¹ of G-6-PDH), 10 μL rat liver microsome (20 mg·mL⁻¹), 4 μL drug solution (100 μM) and 366 μL PBS buffer (0.1 M, pH 7.4) were added to the centrifuge tubes on ice. The sample at t = 0 was achieved by removing the 95 μL incubation mixture, addition 5 μL PBS buffer and mixing with 300 μL acetonitrile. There was a 3-min pre-incubation step at 37°C before initiating the reaction by adding NADPH-generating system into the microsomal suspension. A 30 μL reaction sample was collected at 5, 10, 15, 20, 30, 45, and 60 min, mixed with 90 μL acetonitrile in a 1.5 mL centrifuge tube, vortexed for 30 seconds, and centrifuged at approximately 10500×g for 10 min. An aliquot of 100 μL supernatant was transferred into an injection vial for LC-MS/MS analysis. All depletion data were fitted to the monoeponential decay model described in equation: C (t) = C₀e⁻ᵏᵗ.

In vitro half-life (T₁/₂) was obtained using equation

\[ T_{1/2} = 0.693/k \]  

**Cell culture and transportation study in the Caco-2 cell monolayer model**

The Caco-2 cell transportation experiment was performed according to the method previously reported. In brief, Caco-2 cells were cultured in MEM medium containing 15% FBS, 1% NEAA and 100 U·mL⁻¹ penicillin and streptomycin, and maintained at 37°C in a humidified, 5% CO₂ incubator. For transportation study, cells at passage 30–40 were seeded on transwell polycarbonate insert filters (1.12 cm² surface, 0.4 μm pore size, 12 mm diameter) in 12-well plates at a density of 1 × 10⁵ cells·cm⁻². After cells were allowed to grow for about 21 days, the transepithelial electrical resistance (TEER) of monolayer cells was measured by using Millipore ERS-2 electrical resistance system, and qualified monolayers with TEER > 400 Ω·cm² were used for transportation study. After pre-incubation, cell monolayers were incubated with the three forms of alkaloids in fresh incubation medium from either the apical or basolateral side for the designated times at 37°C. The volume of incubation medium on the apical and basolateral sides was 0.5 mL and 1.5 mL, respectively, and a 100 μL aliquot of the incubation solution was withdrawn at the designated time points from the receiver compartment and replaced with the same volume of fresh pre-warmed HBSS buffer. The dilution was taken into consideration during the flux calculation. After incubation for 30, 60, 90, and 120 min at 37°C, the permeability of the three forms of alkaloids (2 μM) was measured bi-directionally either from the apical (AP) to basolateral (BL) side or from the BL to AP side under all the above conditions.

The apparent permeability coefficients (Papp) were calculated by using the equation of Artursson and Karlsson: 

\[ P_{app} = \frac{1}{Q} \times \frac{ΔC}{Δt} \times \frac{A}{C₀} \]

where Papp is the apparent permeability coefficient (cm/s), ΔC/Δt (μmol·s⁻¹) is the rate at which the compound appears in the receiver chamber, C₀ (μM) is the initial concentration of the compound in the donor chamber, and A (cm²) represents the surface area of cell monolayer.

**Results and discussion**

**Method development**

To develop a sensitive and accurate LC-MS/MS method for the determination of the three forms of alkaloids in rat plasma, quantitative analysis was performed by using MRM mode owing to its...
high selectivity and sensitivity. The precursor and product ions were \( m/z \, 646.4 \rightarrow 586.4 \) for AC, \( m/z \, 604.2 \rightarrow 554.1 \) for BAC, \( m/z \, 500.1 \rightarrow 450.3 \) for ACN, and \( m/z \, 255.0 \rightarrow 120.2 \) for isoliquiritigenin. The mass ion spectra of AC, BAC, ACN and isoliquiritigenin are shown in Figure 2. The MS/MS conditions were optimized to achieve better sensitivity and selectivity. To obtain the appropriate retention time and response, methanol, acetonitrile, water and formic acid were tested as mobile phases. After optimization, 0.1% formic acid was found to enhance the efficiency of ionization and obtain a better intensity than pure water for all compounds tested.

To achieve high recovery efficiency in sample preparation, the direct precipitation method was used for its convenience and low matrix effect. Then, the extraction recovery of the precipitation solvents (methanol and acetonitrile) was investigated. The extraction efficiency exceeded 90% by using acetonitrile as extraction solution, suggesting that it was an ideal precipitation agent.

**Method validation**

After comparing the matrix in different media, we found that plasma is the most complex matrix among all the samples (tissue, urine, faeces, Caco-2 cell transport media and microsomes samples) collected from various studies, and therefore, the method validation was conducted in rat plasma.

Figure 2 displays the LC-MS/MS chromatograms of AC, BAC, CAN, and isoliquiritigenin. The peaks obtained had good shapes and were separated well. AC, BAC, and ACN calibration curves were constructed and the least-squares linear calibration data are summarized in Table 1, showing a good linear correlation over the concentration range \( r > 0.99 \). The standard curves for the three forms of alkaloids in other matrix were all linear in their corresponding concentration range with correlation coefficient values > 0.99. In addition, LLOD and LLOQ were sensitive enough for pharmacokinetic study of rat plasma.

The intra- and inter-day precision of the method was assessed at three concentration levels of spiked analytes in triplicate, and verified by determining the ratios of the peak areas of these compounds to the internal standard with relative standard deviation (RSD) as listed in Table 2. The overall intra- and inter-day variation was less than 15%, indicating satisfactory precision of the instrumentation. The precision and accuracy in other matrix were all within an acceptable range of 15%.

As shown in Table 3, the extraction recovery rate was greater than 80% for AC, BAC, and ACN, and no significant difference was noticed between the three concentrations. In addition, the matrix effect of the analytes was all less than 10%, suggesting that the method was reliable with minimal matrix effect.

Analyte stability was assessed under various conditions. As shown in Table 3, the results indicated that AC, BAC and ACN under the three different conditions (room temperature for 24 h, -40°C for...
30 day, and freeze-thaw for 3 cycles) were all stable in plasma and there was no significant degradation (RE < 15%).

**Pharmacokinetic study**

The analytical method was successfully used to quantitate the three analytes in rat plasma samples obtained from 18 male SD rats orally administered AC, BAC and ACN, respectively. The mean plasma drug concentration-time curves (n = 6) of the three alkaloids are shown in Figure 3. The pharmacokinetic parameters calculated by using DAS 3.0 according to the non-compartmental pharmacokinetic model are shown in Table 4.

The maximum plasma concentration of AC, BAC and ACN was achieved within 1 h, indicating that the three alkaloids could be absorbed into blood circulation rapidly. The elimination half-life \( (t_{1/2}) \) of the three alkaloids was 1.41 ± 0.06, 9.49 ± 0.49, and 3.32 ± 1.52 h, respectively. These results indicate that AC can be eliminated more rapidly than BAC and ACN. The \( C_{\text{max}} \) of AC, BAC and ACN was 10.99 ± 1.74, 3.99 ± 1.20, and 4.29 ± 0.47 ng·mL\(^{-1}\) respectively, indicating that the extent of AC absorption was much higher than ACN and BAC. The \( C_{\text{max}} \) and \( AUC_{(0-t)} \) of BAC and ACN were much lower than AC, the results indicated that BAC and ACN was much difficult to be absorbed than AC.

**Tissue distribution of the three forms of alkaloids**

After oral administration of the three alkaloids, tissue samples including the heart, liver, spleen, lung, kidney, brain, testes, and muscle were dissected at indicated time points and determined by LC-MS/MS. After oral administration, BAC and ACN were seen distributing throughout all the tissues, while AC was not detectable in the brain, indicating that the absorption of AC into the brain may be prevented by the blood-brain barrier. The content of AC was...
much higher in the muscle than that in any other organs. While the content of BAC and ACN was higher in the heart and kidneys. These results indicate that AC is primarily accumulated in the muscle, while BAC and ACN are primarily accumulated in the heart and kidneys. As shown in Figure 4, AC levels in liver, kidney, and muscle were reached highest at 4 h, and while for spleen, lung, heart, and testes, the highest level were reached at 2 h after oral administration, and decreased thereafter. Similarly, the greatest tissue concentration of BAC and ACN were all reached highest within 4 h. These data show that ACN was quickly distributed throughout the rat body after oral administration.

**Excretion of the three forms of alkaloids**

As shown in Figure 5, AC and ACN were excreted primarily through urine, but the content of ACN was much higher than that of AC in urine, indicating that most ACN absorbed was not metabolized in vivo but excreted in prototype in urine. The content of BAC in rat faeces was much higher than AC and ACN after oral administration, combined with the previous pharmacokinetics data, that the $C_{\text{max}}$ and $AUC_{[0-t]}$ of BAC was much lower than AC, indicating that BAC was difficult to be absorbed into blood circulation and mainly excreted through faeces.

**Metabolism of the three forms of alkaloids in rat liver microsome incubation system**

We also investigated metabolic stability of AC, BAC, and ACN at 1 $\mu$M in rat liver microsomes. The results indicated that incubation in rat liver microsomes showed that AC metabolism occurred at $t_{1/2}=57$ min, but no significant metabolism of BAC and ACN was observed.
observed, indicating that BAC and ACN were difficult to be metabolized in rat liver microsomes.

Transportation of the three forms of alkaloids in the Caco-2 cell monolayer model

MTT showed no significant cytotoxicity with the three alkaloids at 2 μM, for 98% cells remained viable. Transportation of AC, BAC, and ACN at 2 μM across the Caco-2 cell monolayer from the apical to basolateral side (A to B) or from the basolateral to apical side (B to A) was investigated. In the equilibrium state, the \( P_{app} \) values of AC, BAC and ACN from A to B were 5.84 ± 1.4 × 10^{-7} cm/s, 4.73 ± 1.1 × 10^{-7} cm/s, and 9.76 ± 2.2 × 10^{-7} cm/s, respectively. In the opposite direction, the \( P_{app} \) values of AC, BAC and ACN were 175 ± 13.84 × 10^{-7} cm/s, 32.45 ± 5.32 × 10^{-7} cm/s, and 12.44 ± 3.61 × 10^{-7} cm/s, respectively. The \( P_{app} \) values of AC and BAC from B to A were significantly (\( P < 0.05 \)) higher than those from A to B, and the efflux ratio was 29.97 ± 5.46 and 6.86 ± 2.42. The \( P_{app} \) values of ACN from B to A were not significantly different from those from A to B. These results show that the efflux transporters were involved in the transportation of AC and BAC. AC is known as a type of diester-diterpenoid alkaloid, and its toxicity is much higher than that of BAC and ACN, and therefore the efflux transporters exert a high-efflux effect towards AC to limit its absorption. The effects of \( P-gp \) inhibitor verapamil on the transportation of the three alkaloids were also investigated. In the presence of 50 μM verapamil, the efflux of AC and BAC was totally inhibited and their efflux ratio decreased to 1.08 ± 0.23 and 2.11 ± 0.74, respectively, while no significant difference in ACN was observed in the presence of verapamil. These results indicate that \( P-gp \) mediated the transportation of AC and BAC. Knowing that the therapeutic range of AC and BAC is quite narrow, drugs or dietary supplements may influence \( P-gp \) when they are co-administered with foods, and consequently affect the pharmacokinetics of AC and BAC, resulting in undesirable toxicity.

Conclusions

In this study, we have developed and validated an LC-MS/MS method for determination of the three alkaloids (AC, BAC, and ACN) in different matrices. Using this method, we systematically investigated their absorption, distribution, metabolism and excretion in rats, and clarified the mechanisms of absorption and metabolism by using the Caco-2 cell monolayer absorption model and rat liver microsome incubation system in vitro, thus providing a better understanding about the disposal process of the three alkaloids, which may help further reveal their pharmacological and toxicological mechanisms in future studies.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (81273472) and the Scientific Foundation of Shanghai (124019000802, 13401900107).

Conflict of interest

The authors have declared no conflict of interest.

References


