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SHORT COMMUNICATION

Comparison of a specific HPLC determination of toxic aconite alkaloids in processed Radix aconiti with a titration method of total alkaloids

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Abstract

Context: In traditional Chinese medicine, *Aconitum* (Ranunculaceae) roots are only applied after processing. Nevertheless, several cases of poisoning by improperly processed aconite roots have been reported.

Objective: The aim of this study was to develop a reliable analytical method to assess the amount of toxic aconite alkaloids in commercial aconite roots, and to compare this method with the commonly used total alkaloid content determination by titration.

Materials and methods: The content of mesaconitine, aconitine, and hypaconitine in 16 commercial samples of processed aconite roots was determined by an HPLC method and the total alkaloid content by indirect titration. Five samples were selected for *in vivo* toxicological investigation.

Results: In most of the commercial samples, toxic alkaloids were not detectable, or only traces were found. In four samples, we could detect >0.04% toxic aconite alkaloids, the highest with a content of 0.16%. The results of HPLC analysis were compared with the results obtained by titration, and no correlation was found between the two methods. The *in vivo* results reassured the validity of the HPLC determination.

Discussion and conclusion: Samples with mesaconitine, aconitine, and hypaconitine content below the HPLC detection limit still contained up to 0.2% alkaloids determined by titration. Since titration of alkaloids gives no information selectively on the aconitine-type alkaloid content and toxicity of aconite roots this method is not appropriate for safety assessment. The HPLC method developed by us provides a quick and reliable assessment of toxicity and should be considered as a purity test in pharmacopoeia monographs.

Keywords: Processed aconite root, toxic aconite alkaloids, HPLC determination, titration method, *Aconitum carmichaeli*, *Aconitum kusnezoffii*

Introduction

Aconitum carmichaeli Debx. and *Aconitum kusnezoffii* Reichb. (Ranunculaceae) have been widely used in the system of traditional Chinese medicine (TCM), primarily for analgesic purposes. Radix aconiti (dried mother root tuber of *A. carmichaeli* Debx., Chinese name: Chuanwu) and its processed form (Zhichuanwu), Radix aconiti lateralis praeparata (processed daughter root

of *A. carmichaeli* Debx., Chinese name: Fuzi), Radix aconiti kusnezoffii (dried root tuber of *A. kusnezoffii* Reichb., Chinese name: Caowu), and its processed form (Zhicaowu), and Folium aconiti kusnezoffii (Caowuye) are listed in the Pharmacopoeia of the People's Republic of China (Chinese Pharmacopoeia Commission, 2005). Unprocessed aconite roots are very toxic due to the presence of diterpene-diester alkaloids (Xiao et al., 2006);

therefore, *Radix aconiti* and *Radix aconiti kusnezoffii* are allowed to be applied only after processing.

The main toxic compounds of *A. kusnezoffii* and *A. carmichaelii* are the aconitine-type diterpene alkaloids aconitine, mesaconitine, and hypaconitine (Figure 1). These compounds possess high affinity to the site II of Na^+ channels, the ability to enhance $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$, a strong arrhythmogenic action that starts at about 30 nM, and high acute toxicity (LD_{50} values about 0.15 mg/kg) (Gutser et al., 1998). The main causes of death are refractory ventricular arrhythmias and asystole and the overall in-hospital mortality is 5.5%. In Asia, aconite poisoning is much more common because of the continued use of aconite roots in traditional medicine. However, with easy access to Asian herbal medicines in western societies, aconite poisoning can occur anywhere in the world (Chan, 2009).

Processing reduces toxicity because of hydrolysis of the ester groups of aconitine-type alkaloids (Chan, 2009). First, the acetyl group is hydrolyzed and in the second step the benzoyl group is hydrolyzed (Figure 2). Since the benzoyl ester is more stable, in the properly processed plant material the concentration of benzoylaconine, benzoylmesaconine, and benzoylhypaconine is remarkable. The LD_{50} values of aconitine in mice are 1.80 mg/kg p.o., 0.31 mg/kg i.p., and 0.12 mg/kg i.v., of benzoylaconine 1500 mg/kg p.o., 70 mg/kg i.p., and 23 mg/kg i.v., and of aconine 120 mg/kg i.v. (Wada et al., 2006). *Aconitum* roots also contain the so-called lipo-alkaloids, which are compounds esterified with fatty acids in the C-8 position. Lipo-alkaloids are more stable than aconitine-type alkaloids and can be detected

both in unprocessed and in processed roots (Wang et al., 2002, 2003). Lipo-alkaloids are substantially less toxic than aconitine-type compounds as evidenced by the difference in the lethal intravenous single doses (>10 mg/kg for lipo-alkaloids) (Csupor et al., 2009). Due to insufficient control, several cases of poisoning have been reported (Singhuber et al., 2009), that may be the result of diverse alkaloid content of the starting material, improper processing, and not least the lack of appropriate quality control.

Quality control criteria of pharmacopoeias are not always sufficient to warrant safety. In case of *Radix aconiti* and *Radix aconiti kusnezoffii*, the diterpene alkaloid content is not limited in the Chinese Pharmacopoeia and the warning of the Chinese Pharmacopoeia ("be cautious about the unprocessed root taken orally") is not commensurable to the danger of toxicity. In the *Radix aconiti praeparata* and *Radix aconiti kusnezoffii praeparata* monographs, a rather complicated colorimetric assay is used for the determination of diester alkaloids (required content level should not be $>0.15\%$), and a titrimetric assay for the determination of the total alkaloid content (required level should not be $<0.20\%$ of alkaloids, calculated as aconitine). The dosage of these two herbal drugs is 1.5–3 g, which may contain as much as 4.5 mg diester-type alkaloids (Chinese Pharmacopoeia Commission, 2005). Moreover, for *Radix aconiti lateralis praeparata*, the dose of which is 3–15 g, only a quantitatively not too reliable TLC analysis of aconitine is specified (Chinese Pharmacopoeia Commission, 2005). Taking into account that the minimum lethal dose of aconitine is 3–6 mg (Caravati et al., 2004), it is obvious that only careful

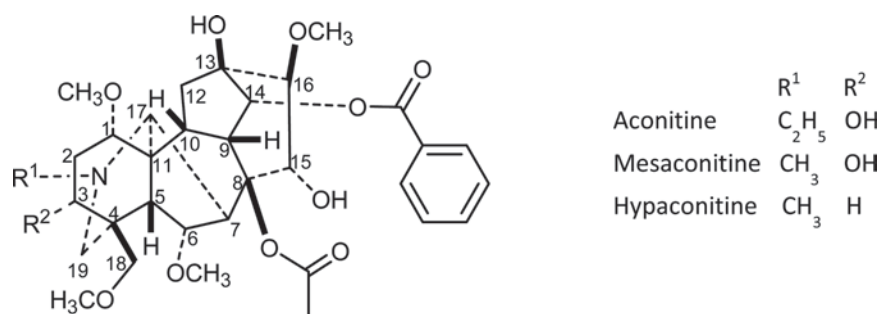


Figure 1. The main toxic *Aconitum* diterpene alkaloids.

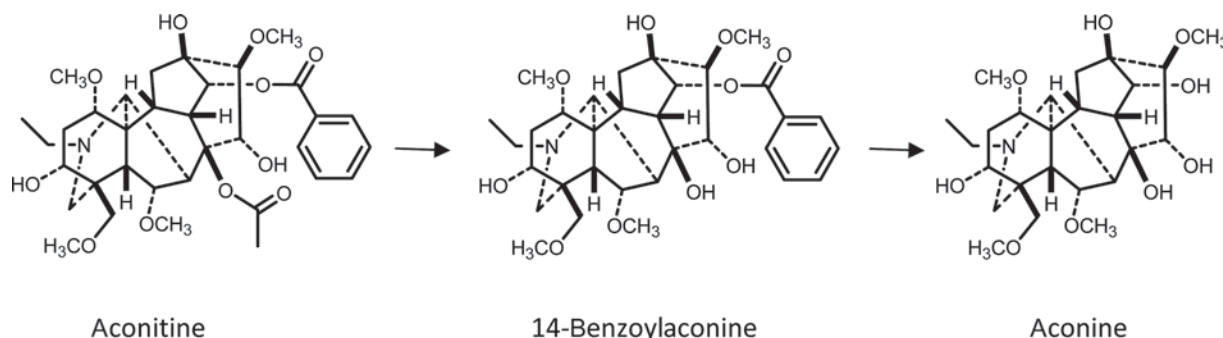


Figure 2. Decomposition of aconitine during processing of *Aconitum* drugs.

processing and quality control may warrant the safety of aconite containing medicinal products.

There are no *Aconitum* monographs neither in the U.S. Pharmacopoeia (USP) nor in the European Pharmacopoeia (Ph. Eur.). However, *Aconitum napellus* is included in the Swiss Pharmacopoeia (*Pharmacopoeia Helvetica*, 1988) and the monographs for *Aconiti carmichaeli* radix, *Aconiti radix praeparata*, *Aconiti radix lateralis praeparata*, *Aconiti kusnezoffii* radix praeparata, and *Aconitum napellus* and praeparationes homeopathicas are under preparation according to the on-line database of Ph. Eur. (EDQM homepage). Since *A. napellus* has been applied in homeopathy, several homeopathic pharmacopoeias contain its monograph. However, the essence of these methods as demonstrated in the most prominent homeopathic pharmacopoeia, the *German Homeopathic Pharmacopoeia* (2005), is the titrimetric determination of total alkaloid content (*German Homeopathic Pharmacopoeia*, 2005).

Since the titrimetric determination provides no direct information on the toxic diester-type alkaloid content of aconite roots, more reliable methods are required for the quality control of processed plant material. The goal of our work was to develop a quick and simple HPLC method for the quality control of aconite roots with comparable or better reliability to those of the previously published methods (Jiang et al., 2005; Xie et al., 2005; Wang et al., 2006; Kang et al., 2010), to compare the results of titration method with HPLC analysis of the toxic alkaloids, and to assess the reliability of these methods using an *in vivo* toxicity test.

Methods

The content of mesaconitine, aconitine, and hyaconitine has been determined in 16 commercial samples of processed aconite roots by HPLC and alkaloid titration.

Processed *Aconitum* samples were obtained from different suppliers (see Table 1). Unprocessed *A. carmichaelii* roots (one sample) were obtained from a pharmacy in China. Aconitine, mesaconitine, and hyaconitine were purchased from PhytoLab GmbH, Vestenbergsgreuth, Germany.

Acetonitrile (Merck) was of HPLC grade. All other chemicals were purchased from Spektrum-3D Ltd., Hungary. HPLC analysis was carried on a Waters HPLC-DAD system equipped with Waters 2998 Photodiode Array Detector.

For HPLC, an improved version of a recently published method (Csupor et al., 2009) has been applied. In brief, 1 g roughly powdered drug was ground, passed through a 0.315 mm sieve, extracted with 5 mL of 1% HCl solution for 20 min at room temperature in an ultrasonic bath. After centrifugation (10 min at 4500 rpm), the filtered (0.45 µm filter membrane) supernatant was analyzed by HPLC (column: Phenomenex Gemini 5 µm, 0.1 m × 4.6 mm; mobile phase: A: 10 mM aqueous NH₄HCO₃ buffer pH 10 ± 0.2 with 28% ammonia solution, B: acetonitrile; 0 min: 70%

Table 1. Origin of investigated aconite roots.

Code	Simplified code for HPLC analyses
Zhicaowu— <i>Aconiti kusnezoffii</i> praeparata	
37951	B3 (EDQM)
37747	B4 (EDQM)
—	B6 (Hong Kong Baptist University)
Zhichuanwu (Shanxi)— <i>Aconiti</i> praeparata (radix)	
32969	D1 (EDQM)
—	D2 (Hong Kong Baptist University)
<i>Aconiti carmichaeli</i> radix praeparata/ <i>Aconiti</i> radix praeparata	
28479	E1 (EDQM)
36890	E2 (EDQM)
Shanghai market— <i>Aconiti</i> radix praeparata/ <i>Aconiti</i> radix lateralis praeparata	
37271	F2—2003
37270	F3—2004
37269	F4—2005
37275	F5—2008
37272	F6—2009
German market— <i>Aconiti</i> radix praeparata/ <i>Aconiti</i> radix lateralis praeparata	
37276	G1—2004
37265	G2—2004
37263	G4—2007
37266	G5—2007

A, 4 min: 60% A, 20 min: 55% A, 30 min: 20% A, 35 min: 70% A; flow rate: 1.1 mL/min, detection: 233 nm, injected sample volume: 20 µL). Peaks of mesaconitine, aconitine, and hyaconitine were identified by comparison of the HPLC-DAD chromatograms of the extracts of aconite roots with the HPLC-DAD chromatograms of reference solutions. Alkaloid content was calculated by comparison of the sum of the areas under curves (AUC) of mesaconitine, aconitine, and hyaconitine on the basis of the calibration curve established for aconitine. Calibration was established for aconitine based on five concentrations (with a range of 0.05–1.625 µg).

Alkaloid titration was carried out according the method of the *German Homeopathic Pharmacopoeia* (2005). To 2 g of the powdered drug, 8 mL of 1% HCl solution was added and extracted for 20 min at room temperature in an ultrasonic bath. After centrifugation, the supernatant was filtered and washed with 1 mL of 1% HCl solution. Diluted ammonia was added to the filtrate until the mixture became alkaline. CHCl₃ (60 g) was added to the extract and shaken for 15 min, then 2 g of freshly powdered tragacanth was added, shaken again, and filtered into a dry flask. The filtrate was concentrated to a few milliliters; 10 mL of 0.01 M HCl and 5 mL of H₂O were added; and the remainder of the CHCl₃ was evaporated. This solution was titrated with 0.01 M NaOH using methyl red as indicator.

To assess the relation of alkaloid content and toxicity, after the quantitative analysis five samples were selected for *in vivo* toxicological investigation. The extracts of these drugs were dissolved in physiological saline containing 5% dimethyl sulfoxide and intraperitoneally

administered to male CFLP mice (24–30 g). Two of the tested extracts (E2, F5) were administered at three doses (equivalent to 240, 800, and 2400 mg of drug/kg), whereas the others (B3, D, G2) were given only at the highest dose for 5–5 animals. Mice were observed 5 min, 2 h, and 6 h after administration as well as once a day for five additional days. The experimental protocol satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the University of Szeged.

Results and discussion

The developed HPLC method provides good separation of the main toxic alkaloids (Figure 3) and therefore serves as basis for reliable quantitative analysis. For quantitative determination, linear regression analysis for aconitine was performed by the external standard method. The regression equation for aconitine was $y = 2\,999\,256\,695.1674x + 66\,930.4245$ (x stands for the amount of injected alkaloid in mg, y denotes the area under the curve). The correlation coefficient (R^2) was 0.9994. Using the HPLC method, in most of the samples no toxic alkaloids or only traces could be detected (Table 2). However, in four samples (D1, E2, F5, G4), >0.04% of aconitine, hypaconitine, and mesaconitine, the highest with a content of 0.162% (E2), were quantified. The alkaloid content of these samples is high enough to question their safety.

The alkaloid content determined by titration was considerably higher than determined by HPLC. Samples, in which toxic alkaloids were not detectable by HPLC, still contained up to 0.2% alkaloids according to titration. Careful processing obviously degrades the toxic alkaloids but does not remove all alkaloids. Total alkaloid titration determines not only the toxic diester alkaloids but also the monoester, unesterified, and lipo-alkaloids. There was no correlation between the low values (<0.02% HPLC), but there was a correlation in high values of alkaloid contents determined by the two methods (Figure 4). When taking into consideration only samples with measurable

alkaloid content by HPLC, a correlation coefficient of 0.91 was found between the results of the two methods.

Acute toxicity of batches (E2, F5) high in diterpene alkaloids has been confirmed in CFLP mice. Extract of E2 proved to be the most toxic batch resulting in death of two out of five mice at the highest dose. These fatal cases were preceded by intensive skeletal muscle cramps and shortness of breath. Similar excitatory skeletal symptoms were characteristic for all groups except for G2. An intensive defecation was observed on mice treated with extracts E2 and F5. The next day and later on, all surviving animals were in good condition exhibiting no obvious toxic symptoms.

Although the pharmacological characteristics of *Aconitum* diterpene alkaloids have been extensively studied (Ameri, 1998), the exact mode of action of traditionally applied aconite drugs has not been elucidated so

Table 2. Content of alkaloids determined by titration and by HPLC analysis of toxic alkaloids (sum of mesaconitine, aconitine, and hypaconitine).

Code	HPLC (%)	Titration (%)
Radix aconiti kusnezoffii praeparata		
B3	Not detectable	0.065
B4	0.007	0.013
B6	Not detectable	0.194
Radix aconiti carmichaeli praeparata/Radix aconiti lateralis praeparata		
D1	0.054	0.207
D2	Not detectable	0.120
E1	0.013	0.062
E2	0.162	0.433
F2	Not detectable	0.045
F3	Not detectable	0.103
F4	Not detectable	0.123
F5	0.125	0.342
F6	Not detectable	0.070
G1	0.003	0.113
G2	0.026	0.129
G4	0.043	0.097
G5	0.011	0.116

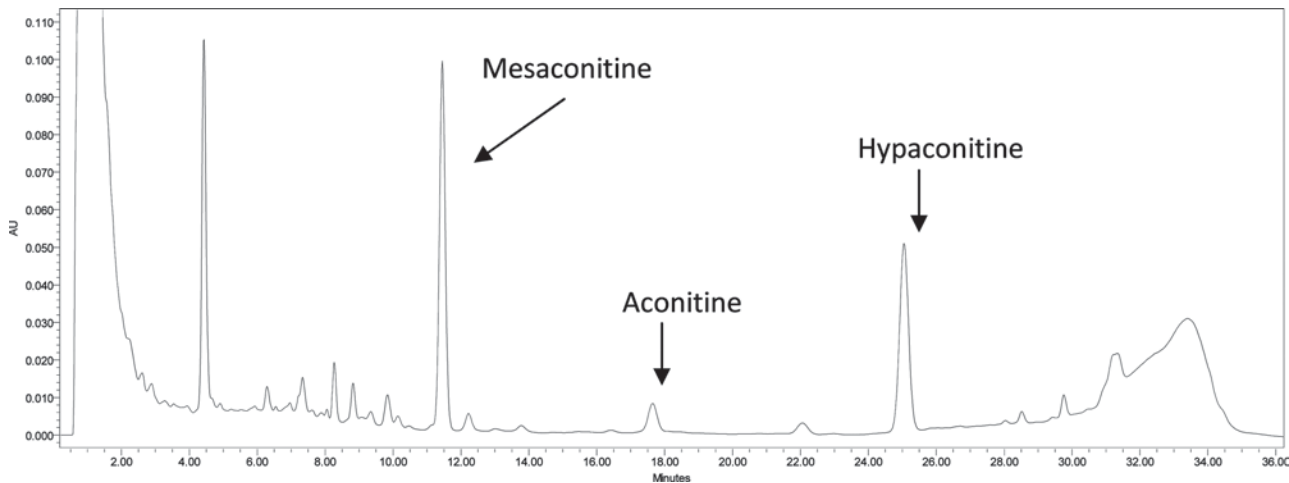


Figure 3. HPLC chromatogram of the extract prepared from a crude aconite root sample.

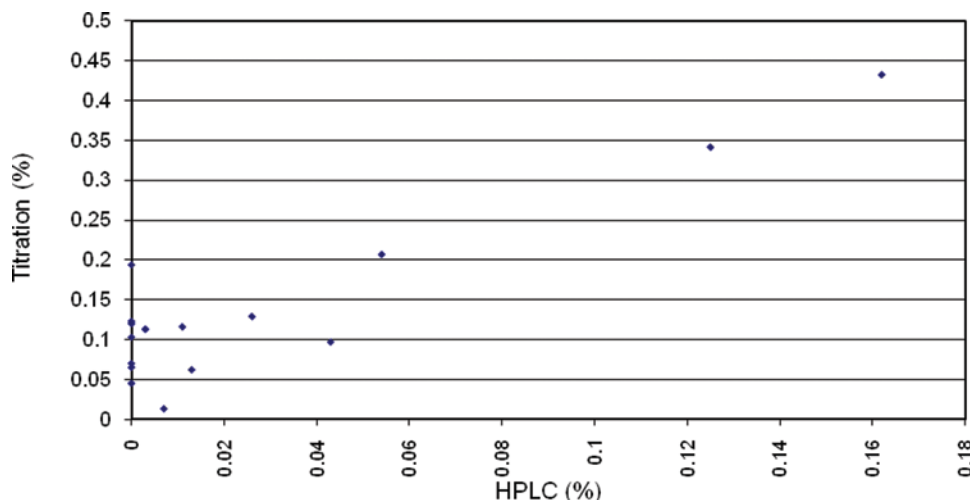


Figure 4. Correlation of the alkaloid contents by HPLC quantification and titration.

far; therefore, it is not possible to standardize these plant materials to any active agent.

Conclusions

Alkaloid titration, a method applied in the quality control of homeopathic aconite tinctures and aconite drugs of the Chinese Pharmacopoeia, may give information on total content of alkaloids, but allows no conclusion on the content of toxic alkaloids. Therefore, quantification of diester-type diterpene alkaloids is essential to ensure the safety of medicines based on these herbal drugs.

The sample preparation and HPLC analysis method developed by us offers a quick and reliable possibility to determine the quantity of the most important toxic alkaloids of *A. carmichaeli* and *A. kusnezoffii* and should now be considered as a purity test in pharmacopoeias. The relevance of our method was confirmed in *in vivo* toxicity studies, which exhibited an apparent correlation between the alkaloid content and the severity of toxic symptoms.

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Declaration of interest

The authors report no declarations of interest.

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