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Determination of *Aconitum* alkaloids in blood and urine samples. I. High-performance liquid chromatographic separation, solid-phase extraction and mass spectrometric confirmation

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Abstract

Determination of four toxic *Aconitum* alkaloids, aconitine, mesaconitine, hypaconitine and jesaconitine, in blood and urine samples has been established using high-performance liquid chromatography (HPLC) combined with ultraviolet absorbance detection, solid-phase extraction and mass spectrometry (MS). These alkaloids were hydrolyzed rapidly in alkaline solution (half lives ($t_{1/2}$)<one day), were stable in solutions of acetonitrile, tetrahydrofuran and diluted hydrochloric acid ($t_{1/2}$>five months) and were unstable in solutions of methanol and ethanol ($t_{1/2}$<one month). These alkaloids were separated on an octadecylsilica column with isocratic elution using a solvent mixture of tetrahydrofuran and 0.2% trifluoroacetic acid (14:86, v/v), which was found to be the optimal solvent of the elution systems examined. Calibration curves with UV detection were linear on injection of amounts ranging from 2.5 to 500 ng, and the limit of detection was 1 ng ($S/N=3$). These four alkaloids in aqueous solution were recovered almost totally by solid-phase extraction using the styrene polymer resin, Sep-Pak Plus PS-1, and were eluted using a mixture of acetonitrile and hydrochloric acid. These *Aconitum* alkaloids were confirmed by HPLC coupled with fast atom bombardment MS, giving their protonated molecular ions as base peaks. These alkaloids were detected by HPLC with UV detection from blood samples spiked with more than 50 ng ml^{-1} of alkaloids, but were not detectable from urine samples spiked with 5 μg ml^{-1} of alkaloids because of severe sample interference.

Keywords: Alkaloids; *Aconitum* alkaloids

1. Introduction

Aconite plants produce various kinds of C_{19} norditerpenoids and C_{20} diterpenoids [1]. Among them, some 8-acetyl-14-aroyl diester C_{19} alkaloids, such as aconitine, are highly toxic to man and animals [2]. The alkaloid components and amounts vary with species, place of origin, time of harvest and the method of processing [3]. Aconite roots and their processed products have been used as important ingredients in Chinese medical preparations for their pharmaceutical properties of anti-inflammatory, analgesic and cardiotronic effects [4].

There have been many cases of accidental poisoning by *Aconitum* alkaloids when aconites were eaten accidentally [5,6] and decoctions prepared from herbal prescriptions containing aconites were ingested [7]. In addition, aconite roots have been used for suicidal and homicidal purposes [8,9]. In such poisoning cases, it is toxicologically important to

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detect these alkaloids in body fluids taken from patients or victims. High-performance liquid chromatographic (HPLC) techniques have been used for the analysis of plant samples [3,10–12]. However, there have been few reports on the analysis of body fluids. Only the use of gas chromatography–mass spectrometry (GC–MS) with derivatization has been reported as a method used to detect trace levels of alkaloids in blood and urine [13]. In this paper, methods have been developed for the determination of *Aconitum* alkaloids in blood and urine samples, using HPLC combined with solid-phase extraction and mass spectrometry (MS).

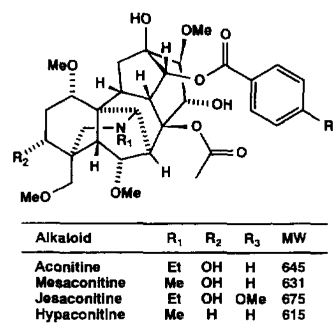
2. Experimental

2.1. Reagents

Aconitine was purchased from Sigma (St. Louis, MO, USA). Sep-Pak Plus PS-1 and C_{18} cartridges were obtained from Waters (Milford, MA, USA). Tetrahydrofuran (THF, HPLC-grade), acetonitrile, glycerin and trifluoroacetic acid (TFA) were from Tokyo Chemical Industry (Tokyo, Japan). The other chemicals were of analytical reagent grade.

2.2. Preparation of Aconitum alkaloids

Aconitum alkaloids were extracted from "UZU", processed roots of *Aconitum deflexum* Nakai and *Aconitum carmicaeli* Debaux (Uchida Wakanyaku, Tokyo, Japan) with a 1 *M* hydrochloric acid (HCl) solution, and the resulting aqueous layer was alkalized with concentrated ammonia to pH 10. The crude alkaloid fraction was extracted with chloroform and the resulting chloroform layer was evaporated to dryness over anhydrous sodium sulfate. Alkaloids were separated from each other by thin-layer chromatography (TLC) using silica-gel TLC plates (Merck) with a developing solvent of diethylether–ethylacetate (20:1, v/v; saturated with concentrated ammonia). R_f values after TLC were 0.31 for aconitine, 0.17 for mesaconitine, 0.75 for hypaconitine and 0.20 for jesaconitine. Each alkaloid was confirmed by 1D- and 2D- ^{1}H- and ^{13}C-NMR. The structures of *Aconitum* alkaloids are shown in Fig. 1.



Alkaloid	R_1	R_2	R_3	MW
Aconitine	Et	OH	H	645
Mesaconitine	Me	OH	H	631
Jesaconitine	Et	OH	OMe	675
Hypaconitine	Me	H	H	615

Fig. 1. Structures of *Aconitum* alkaloids.

2.3. High-performance liquid chromatography

The instrument used was an 800-series LC system (JASCO, Tokyo, Japan) consisting of 880-PU pumps, an 880-50 degasser, an 870-UV–Vis detector and a Rheodyne 7125 injector (Cotati, CA, USA). L-Column ODS (150×4.6 mm I.D., Chemical Inspection and Testing Institute, Tokyo, Japan) was used as the stationary phase. The mobile-phase consisted of a mixture of organic solvent and diluted acidic solution and the flow-rate was 1 ml min^{-1}. The column was maintained at 40°C in an 860-CO column oven. A 10-μl volume of the sample was injected. The wavelengths selected for UV detection were 260 nm for jesaconitine and 235 nm for the other alkaloids.

The HPLC–MS system used was a JMS-LX2000 double-focusing mass spectrometer (JEOL, Tokyo, Japan). The ionization mode used was fast atom bombardment (FAB) with xenon atoms and was operated at 3 kV. A model MDS-520E multidimensional LC system (GL Sciences, Tokyo, Japan) was used as the HPLC system. Inertsil ODS-2 (150×4.6 mm I.D., GL Sciences) was used as the stationary phase. The mobile-phase consisted of a mixture of THF, 0.3% TFA and glycerin (19:81:0.3, v/v/v) and the flow-rate was 1 ml min^{-1}. The column was maintained at 40°C. After splitting, 1 μl min^{-1} of HPLC eluate was introduced into the FAB–MS interface.

2.4. Solid-phase extraction

Outdated transfusion blood was used as the blood samples. Urine samples were obtained from normal

volunteers. Blood samples (2 ml) were combined with four volumes of acetonitrile–3.0% perchloric acid (4:96, v/v) and the supernatant was obtained by centrifugation (2000 *g*, 5 min). Urine samples (5 ml) were filtrated through cellulose membrane filters (pore size, 0.45 μm). Deproteinized blood and filtered urine samples were applied to Sep-Pak cartridges, which had been equilibrated with distilled water. After washing the cartridge with 5 ml of 1 *M* HCl, alkaloids were eluted using a series of acetonitrile–HCl solutions (5 ml each). The eluate was gently evaporated to dryness and dissolved in 10 μl of methanol and diluted with 90 μl of distilled water.

3. Results

3.1. Preparation of *Aconitum* alkaloids from aconite roots

Crude alkaloids (913 mg) were obtained from 105 g of "UZU" by extraction with HCl and then chloroform. By further preparative TLC, 35 mg of aconitine, 52 mg of mesaconitine, 99 mg of hypaconitine and 145 mg of jesaconitine were isolated. Each alkaloid was confirmed by NMR [14]. The aconitine sample purified from "UZU" was identical to the commercial product on NMR analysis.

3.2. High-performance liquid chromatographic separation of *Aconitum* alkaloids

HPLC separation of *Aconitum* alkaloids on a reversed-phase column was optimized by increasing the number of theoretical plates by optimizing the solvent systems used. Methanol, acetonitrile and THF were examined as the organic solvent, and TFA, perchloric acid and phosphoric acid were examined as the acid. A non-acidic solvent system did not elute alkaloids from the column. Of the solvent systems examined, the solvent containing THF and 0.2% TFA (14:86, v/v) separated the four alkaloid peaks from each other and gave the highest number of theoretical plates (4500–4900). Under the optimized HPLC conditions, calibration curves of *Aconitum* alkaloids with UV detection were linear on injection of amounts ranging from 2.5 to 500 ng. The detection limit was 1 ng per injection ($S/N=3$) for all four alkaloids. Coefficients of variation ($n=7$) for HPLC detection of 50 ng of aconitine, mesaconitine, hypaconitine and jesaconitine per injection were 8.5, 3.3, 4.2 and 5.3%, respectively.

The deproteinized blood and filtered urine samples were applied to this optimized HPLC system with UV detection, however, severe interfering peaks were observed around the elution position of *Aconitum* alkaloids, making detection of sub-μ*M* levels of alkaloids in these samples impossible (data not shown).

3.3. Stability of *Aconitum* alkaloids in various solvents

Aconitum alkaloids contain ester moieties in their molecules and are easily hydrolyzed to form more polar compounds. The stability of *Aconitum* alkaloids in solution has been investigated in order to select a suitable solvent for dissolving the standard stock solution in and also to act as the HPLC eluting solvent. The solutions spiked with 20 μg ml^{-1} of aconitine, mesaconitine and hypaconitine were stored in various kinds of solvent in the dark at room temperature, and the alkaloid concentrations were quantitated by HPLC at various time intervals over several months. As shown in Table 1, these alkaloids were degraded rapidly in alkaline solution and were not very stable in methanol or ethanol (protic organic solvents). These alkaloids are very stable in acetonitrile or THF (aprotic organic solvents) and are quite stable in acidic aqueous solution.

Table 1
Half-lives of *Aconitum* alkaloids in organic and aqueous solutions

Solvent	Aconitine	Mesaconitine	Hypaconitine
Ammonia (pH 10)	4 h	19 h	10 h
Methanol	16 days	21 days	14 days
Ethanol	16 days	16 days	20 days
1 *M* HCl	5 months	6 months	5 months
Acetonitrile (dry)	>1 year	>1 year	>1 year
Tetrahydrofuran (dry)	>1 year	>1 year	>1 year

The half-lives were calculated from the time course curves of the alkaloids remaining in a solution that was spiked originally with 20 μg/ml alkaloids and was subsequently stored at 20°C over several months.

3.4. Solid-phase extraction of Aconitum alkaloids

Solid-phase extraction was performed to isolate these alkaloids from interfering compounds in blood and urine, using two hydrophobic phases, Sep-Pak C_{18} and Sep-Pak Plus PS-1 cartridges. These alkaloids were completely adsorbed on both the cartridges under acidic aqueous conditions. *Aconitum* alkaloids were not eluted completely from Sep-Pak C_{18} cartridges using an elution solvent composed of acetonitrile and HCl; a proportion (more than 25%) of the applied alkaloids were strongly retained in the cartridge (data not shown). These alkaloids were also eluted using methanol and HCl as the solvent, but the recoveries (less than 80%) were not satisfactory. In contrast, all the alkaloids retained on Sep-Pak Plus PS-1 under acidic solutions were eluted completely with an acidic acetonitrile solution, leading to almost complete recoveries (Fig. 2).

A blood sample spiked with *Aconitum* alkaloids (250 ng ml^{-1} of each) was deproteinized and the resulting supernatant was subjected to solid-phase extraction using Sep-Pak Plus PS-1. As shown in Fig. 3, these alkaloids were detected on HPLC with UV detection. Calibration curves of *Aconitum* alkaloids with UV detection were linear over the range 100 to 10 000 ng ml^{-1} of blood. Coefficients of variation ($n=7$) for the HPLC detection of blood spiked with 1000 ng of aconitine, mesaconitine, hypaconitine and jesaconitine were 14.2, 10.7, 11.7 and 12.9%, respectively. The detection limit for alkaloids was 50 ng ml^{-1} in blood ($S/N=3$). In contrast, a solid-phase extract of a urine sample, even when spiked with high levels of these alkaloids (5 μg ml^{-1} each), did not lead to detection of the alkaloids on HPLC (data not shown).

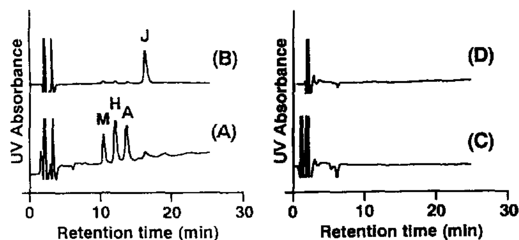


Fig. 3. High-performance liquid chromatograms of blood samples spiked with *Aconitum* alkaloids. A 2-ml volume of blood spiked with *Aconitum* alkaloids [500 ng each of aconitine (A), mesaconitine (M), hypaconitine (H) and jesaconitine (J)] was deproteinized and the resulting supernatant was subjected to solid-phase extraction using a Sep-Pak Plus PS-1 cartridge. The alkaloid fraction was applied to a HPLC system (A, B). A blank test (extract without spiked alkaloids) was also performed (C, D). Column, L-column ODS (150×4.6 mm); mobile-phase, THF–0.2% TFA (14:86, v/v); flow-rate, 1 ml min^{-1}; UV detection wavelength, 235 nm (A, C) and 260 nm (B, D); column temperature, 40°C.

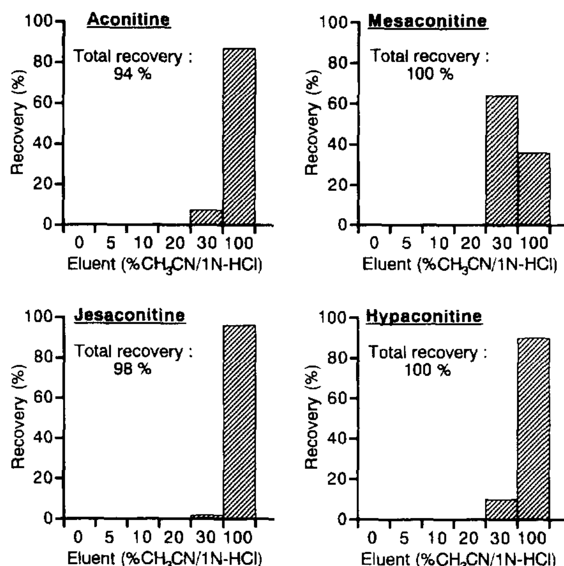


Fig. 2. Solid-phase extraction of *Aconitum* alkaloids. An aqueous solution containing *Aconitum* alkaloids (100 ng of each) was applied to a Sep-Pak Plus PS-1 cartridge that had been equilibrated with distilled water and the solution was eluted with 5 ml of a mixture containing acetonitrile and HCl. The percentage acetonitrile in the mixture is designated in the abscissa. The alkaloid content of each eluate fraction was measured by HPLC.

3.5. High-performance liquid chromatography–frit-fast atom bombardment mass spectrometric detection of Aconitum alkaloids

FAB–mass spectra of the alkaloids (Fig. 4) showed the protonated molecular ions as base peaks and also the degradation ions of either methoxyphenylcarbonyl cation (m/z, 135) or phenylcarbonyl cation (m/z, 105). The blood or urine samples spiked with these alkaloids were subjected to solid-phase extraction and applied to a HPLC–FAB–MS system using selected ion monitoring (SIM) of protonated molecular ions. As shown in Fig. 5, these alkaloids

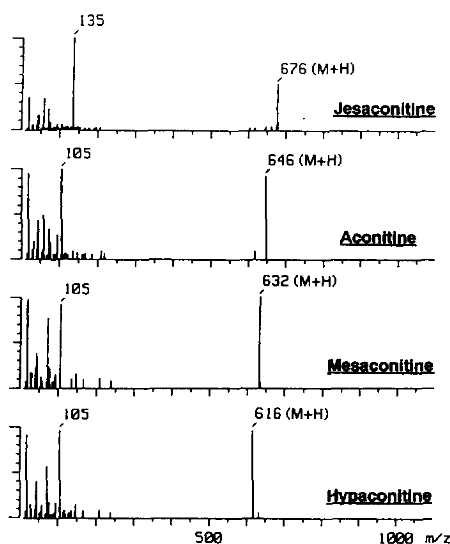


Fig. 4. Mass spectra of *Aconitum* alkaloids. An *Aconitum* alkaloid solution was applied to a HPLC–FAB–MS system and the spectra of the alkaloids (A, jesaconitine; B, aconitine; C, mesaconitine and D, hypaconitine) were measured. The HPLC–MS system was as follows: A JEOL JMS-LX2000 double-focusing mass spectrometer was used. The ionization mode was FAB using xenon atoms with an energy of 3 kV. Inertsil ODS-2 (150×4.6 mm I.D.) was used as the stationary phase. The mobile phase was a mixture of THF, 0.3% TFA and glycerin (19:81:0.3, v/v/v) and the flow-rate was 1 ml min^{-1}. The column was maintained at 40°C. The HPLC eluate was introduced into the FAB–MS system, after splitting, at a rate of 1 μl min^{-1}.

were simultaneously detected on HPLC–FAB–MS and no interfering peaks were observed. The detection limits were 2.5 ng in scan mode and 100 pg in SIM mode (S/N=3).

3.6. Detection of Aconitum alkaloids in a blood sample taken from a patient intoxicated by aconite roots

The method established in this paper using solid-phase extraction HPLC with UV detection was performed on a blood sample drawn from a patient suspected of accidentally eating food containing aconite roots. As shown in Fig. 6, aconitine, mesaconitine and jesaconitine were easily detected in the serum, and the serum levels were 80, 250 and 50 ng ml^{-1} for aconitine, jesaconitine and mesaconitine, respectively.

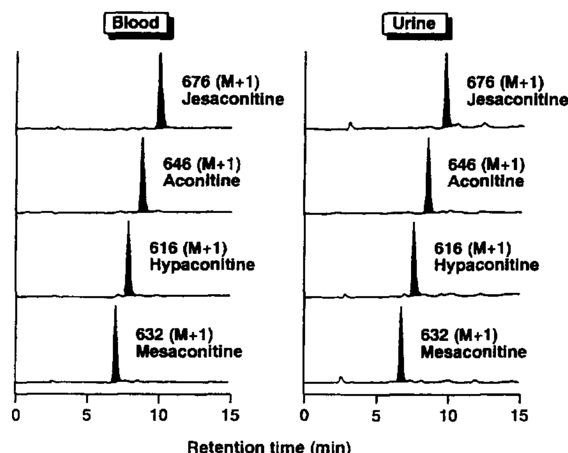


Fig. 5. Selected ion monitoring chromatograms of solid-phase extracts of blood and urine samples spiked with *Aconitum* alkaloids. Solid-phase extracts of blood (2 ml) and urine (5 ml) spiked with *Aconitum* alkaloids (100 ng of each) were applied to a HPLC–FAB–MS system, selectively monitoring the corresponding protonated molecular ions of m/z 676 (jesaconitine), 646 (aconitine), 616 (hypaconitine) and 632 (mesaconitine).

4. Discussion

A small amount of aconite root extract can cause pharmacological effects, when introduced into the human body. In contrast, a large amount causes neurotoxic and cardiotoxic effects and can lead to death resulting from ventricular arrhythmia or direct paralysis of the heart [4]. These toxic effects are based on the *Aconitum* alkaloids. The molecular mechanism of the toxicity is the permanent activation of the voltage-sensitive sodium channels of

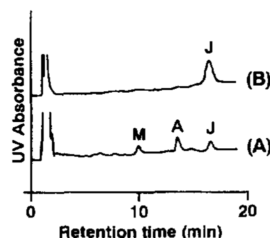


Fig. 6. High-performance liquid chromatogram of a serum sample extract. The serum, drawn from a patient suspected of having eaten food products containing aconite roots, was deproteinized and the resulting supernatant was subjected to solid-phase extraction using a Sep-Pak Plus PS-1 cartridge. The alkaloid fraction was subjected to HPLC analysis with UV detection. The UV detection wavelengths used were 235 nm (A) and 260 nm (B).

excitable membranes [15]. The LD_{50} value of aconitine for mice per oral injection was reported to be 1.8 mg/kg body weight [2]. Therefore, detection of low levels of toxic alkaloids in body fluids seems difficult. Mizugaki et al. [13] reported a GC–MS method for the detection of alkaloids using trimethylsilyl derivatization and they found the detection limit by SIM mode to be as low as 10 pg. In this paper, instead of adopting the GC–MS technique, which contains tedious extraction and derivatization procedures, we established a HPLC method. Because these alkaloids are water-soluble, polar and labile to hydrolysis, the HPLC method has been widely used for the analysis of plant samples and products in the field of pharmacognosy.

Acetyl function at the 8-position in the aconitine molecule is easily hydrolyzed and then the benzoyl or anisyl function at the 14-position is hydrolyzed. This degradation process can occur in various kinds of solvent. Moreover, in the preparation of alkaloids from UZU, especially in the alkaline extraction process, some fractions of alkaloids should be hydrolyzed. Although alkaloids were degraded considerably in alcohol solutions, they were very stable in acetonitrile or THF, indicating that these organic solvents may be useful as the stock solution solvent or as the HPLC eluting solvent (Table 1). In acidic aqueous solutions, the alkaloids were quite stable and there was no danger of them being degraded during the acidification process.

Mizugaki et al. [13] reported that the lethal blood level of aconitines was 100–470 ng ml^{-1}. Although the detection limit (1 ng) of the HPLC method used in this paper was less than this level, blood and urine samples contained compounds that interfered with the HPLC detection of trace levels of the alkaloids. Solid-phase extraction has been widely used in the analysis of drugs and other chemicals and for clean-up and concentration pretreatment. Although the ODS cartridge did not give good recovery of the alkaloids, the styrene polymer resin, Sep-Pak Plus PS-1, gave complete recovery. This difference may be due to non-specific adsorption onto the silica support. In concert with solid-phase extraction, the HPLC method showed high sensitivity (detection limit of 50 ng ml^{-1}), enabling detection of fatal levels of blood alkaloids, although sample interference disturbed detection of high levels of alkaloids in urine samples.

In the toxicological field, it is necessary to confirm poisons themselves, and the MS technique is widely used. HPLC–MS provides the possibility of simultaneously determining both polar and labile compounds, using a soft ionization technique. Frit-FAB is designed as a new ionization mode and is used for the analysis of polar chemicals [16]. *Aconitum* alkaloids were shown to give protonated molecular ions as base peaks and, in the SIM mode, the sensitivity of alkaloid detection was remarkable (detection limit of 100 pg). At present, we are establishing a more sensitive determination method using frit-FAB–MS coupled with HPLC, using a capillary column and the SIM mode, which will make it possible to detect therapeutic levels of alkaloids in blood and urine.

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