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Determination of *Aconitum* alkaloids in blood and urine samples II. Capillary liquid chromatographic–frit fast atom bombardment mass spectrometric analysis

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Abstract

Determination of fourteen alkaloids, toxic *Aconitum* alkaloids, aconitine, mesaconitine, jesaconitine, hypaconitine and deoxyaconitine, and their hydrolysis products, benzoylaconines and aconines, have been established using capillary liquid chromatography (LC) fast atom bombardment mass spectrometry (FAB-MS) with a frit interface. Protonated molecular ions were observed as base peaks in the FAB-MS for these fourteen alkaloids. All the alkaloids were simultaneously quantified with linear gradient LC elution by solvent mixture of acetonitrile and 0.3% trifluoroacetic acid using selected ion monitoring of the protonated molecular ions. The calibration curves of these alkaloids were linear in injection amounts ranging from 5 to 500 pg, and their detection limits were 1 pg per injection ($S/N=3$). Solid-phase extraction using Sep-Pak Plus PS-1 was also investigated to clean-up and concentrate alkaloids in blood and urine samples, and showed satisfactory recoveries. This capillary LC–frit-FAB-MS method enables determination of low levels of *Aconitum* alkaloids in blood and urine samples, coupled with solid-phase extraction.

Keywords: *Aconitum* alkaloids

1. Introduction

Some *Aconitum* alkaloids, such as aconitine, are highly toxic to man and animals [1]. In both accidental and intentional poisoning cases, it is important to detect these alkaloids in body fluids drawn from patients or victims. In the previous paper, a method has been developed for the determination of toxic *Aconitum* alkaloids in blood and urine samples, using high-performance liquid chromatographic (LC) technique and solid-phase extraction [2]. However, it is difficult to detect lower than toxic blood levels of these alkaloids. Many compounds found in biological samples interfered with the detection of these alkaloids in LC monitoring by ultraviolet (UV) absorption. Also, these alkaloids are subject to biological degradation, especially through hydrolysis of ester bonds. Acetyl function at the 8-position in the parent *Aconitum* alkaloids, aconitines, is easily hydrolyzed to produce benzoylaconines, and then,

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the benzoyl or anisoyl function at the 14-position is hydrolyzed to produce the final degradation products, aconines. The structures of the hydrolysis products are shown in Fig. 1 along with the parent *Aconitum* alkaloids. It is difficult to detect the hydrolysis products using LC monitoring by UV absorbance, except for benzoylaconines. The sample matrix interference and low detectability of the hydrolysis products can be overcome by specific detection such as mass spectrometry (MS) coupled with LC. LC–MS techniques have been used as a sensitive and selective detection methods for various kinds of hydrophilic and labile polar compounds [3].

LC–frit-fast atom bombardment (FAB) MS reported in the previous paper [2] provided confirmation and sensitive detection (detection limit 100 pg in selected ion-monitoring mode (SIM) of protonated molecular ions) of *Aconitum* alkaloids. However, because of the limitation of LC flow into the frit-FAB interface, better sensitivity could not be expected and so the previous method could not show the detection of therapeutic blood levels of alkaloids. In this paper, hydrolysis products were prepared from toxic *Aconitum* alkaloids with alkaline treatment. By using a micro LC system with a fused-silica capillary packed column and SIM of the protonated molecular ions, simultaneous determination of low levels of *Aconitum* alkaloids has been achieved. Solid-phase extraction has also been investigated for purification of the alkaloids from blood and urine samples.

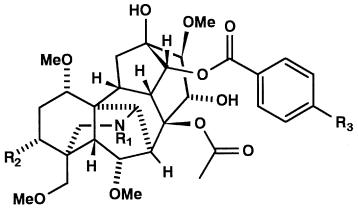
2. Experimental

2.1. Reagents

Sep-Pak Plus PS-1 cartridges were obtained from Waters (Milford, MA, USA). Acetonitrile, glycerin and trifluoroacetic acid (TFA) were from Tokyo Chemical Industry (Tokyo, Japan). The other chemicals used were of analytical reagent grade.

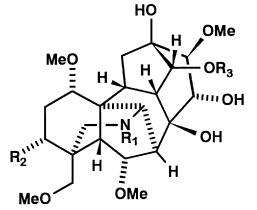
2.2. Preparation of hydrolysis products of *Aconitum* alkaloids

Aconitine, mesaconitine, jesaconitine and hypaconitine were prepared as described before [2]. Deoxyaconitine was also prepared by the same manner, and its R_F value in a thin layer chromatography (TLC) system using silica-gel TLC plates (Merck, Darmstadt, Germany) with developing solvent of diethyl ether–ethyl acetate (20:1, saturated with concentrated ammonia) was 0.87. The partial hydrolysis products, benzoylaconine, benzoylmesaconine, anisoylaconine, benzoylhypaconine and benzoyldeoxyaconine were prepared from the parent alkaloids through mild hydrolysis conditions



Aconitum Alkaloids

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
Deoxyaconitine	Et	H	H	629



Hydrolysis Products of *Aconitum* Alkaloids

Alkaloid	R_1	R_2	R_3	MW
Benzoylaconine	Et	OH	benzoyl	603
Benzoylmesaconine	Me	OH	benzoyl	589
Anisoylaconine	Et	OH	anisoyl	633
Benzoylhypaconine	Me	H	benzoyl	573
Benzoyldeoxyaconine	Et	H	benzoyl	587
Aconine	Et	OH	H	499
Mesaconine	Me	OH	H	485
Hypaconine	Me	H	H	469
Deoxyaconine	Et	H	H	483

Fig. 1. Structures of *Aconitum* alkaloids and their hydrolysis products.

i.e. refluxing in water for 2 h. The final decomposition products, aconine, mesaconine, hypaconine and deoxyaconine were prepared through treatment with 0.5 *M* potassium hydroxide solution for 8 h. The prepared hydrolysis products were deionized by solid-phase extraction using Sep-Pak Plus PS-1 (washing with water and elution with acetonitrile) and purified by the above mentioned TLC system. Each alkaloid was confirmed by 1D- and 2D-^{1}H- and ^{13}C-NMR and electron impact ionization-MS.

2.3. Capillary LC/frit-FAB-MS

The LC system consisted of an Ultra Plus HPLC (Micro-Tech Scientific, Sunnyvale, CA, USA). The stationary phase was a capillary Inertsil ODS-2 (150×0.32 mm I.D., Micro-Tech Scientific). Injection volume was set to 0.5 μl with a Model 7520 Rheodyne injector (Coati, CA, USA). Mobile phase was a mixture of acetonitrile and 0.3% TFA solution with linear gradient elution from 0:100 to 80:20 (v/v) for 40 mm, and flow-rate was 5 μl/min. The column was maintained at 40°C. LX-2000 double-focusing mass spectrometer (JEOL, Tokyo, Japan) was used for FAB-MS using xenon atoms with 3 kV energy. Glycerin was premixed in the mobile phase (0.8%, v/v) as a matrix for FAB ionization. The entire column eluate was introduced into the FAB ion source without splitting.

2.4. Sample treatment using solid-phase extraction

Blood samples were obtained as outdated transfusion blood. Urine samples were obtained from normal volunteers. Two ml of blood sample was combined with 8 ml of 3% perchloric acid with sonication and shaking for 3 min. The resulting precipitates were re-extracted three times with the same solution, and the resulting supernatant fractions were mixed and applied to a Sep-Pak Plus PS-1 cartridge preequilibrated with water. The cartridge was washed with 5 ml of distilled water and the alkaloids were eluted with 5 ml of acetonitrile. Five ml of urine samples were filtered through cellulose membrane (0.45 μm), and applied directly to the cartridge, washed with 5 ml of distilled water and the retained alkaloids were eluted with 5 ml of acetonitrile. The eluate was evaporated to dryness gently (up to 40°C) and dissolved in 100 μl of methanol and diluted with 400 μl of distilled water.

3. Results

3.1. Capillary liquid chromatography fit fast atom bombardment mass spectrometric determination of Aconitum alkaloids and their hydrolyzed products

It is known that Brönsted acids strengthen the intensity of protonated molecular ions in FAB-MS. Protic acids, perchloric acid and phosphoric acid, decreased the intensity of protonated molecular ions of each alkaloid 10-fold or less than TFA (data not shown). Acetic acid is a good protic acid for LC–FAB-MS, but was not used because acetic acid may cause acetylation of free hydroxyl groups of *Aconitum* alkaloids and their hydrolysis products. In the LC separation of *Aconitum* alkaloids, phosphate buffer or perchloric acid have been used as mobile phases, but in LC–MS use of these solvents was limited because of the requirement of volatility for use as an LC solvent. Nonvolatile buffers obstruct capillary tubing. Therefore, volatile TFA was introduced to the LC solvent. FAB mass spectra of all alkaloids presented their protonated molecular ions as base peaks. Aconitines except for jesaconitine and benzoylaconines except for anisoylaconine showed fragment ion peaks of phenylcarbonyl cation *(m/z* 105) generated from 14-benzoyloxy group in their background-subtracted mass spectra. The fragment ion peak of the methoxyphenylcarbonyl cation *(m/z* 135) coming from 14-anisoyloxy group was also observed in the mass spectra of jesaconitine and anisoylaconine. The other remarkable fragment ion peaks were not observed in all the spectra. In Table 1 the mass numbers of the detected ions in FAB-MS are shown. Representative results of FAB mass spectra of alkaloids (aconitine, benzoylaconine and aconine) are shown in Fig. 2.

Baseline separation of all fourteen alkaloids could not be achieved by the LC separation system (150× 4.6 mm I.D. ODS column, isocratic elution) reported previously [2]. Capillary LC column with gradient elution enabled direct separation of these alkaloids and whole introduction into MS, showing good resolution and high sensitivity. Among the solvents

Table 1
Detected ion peaks on FAB-MS and reproducibility in LC–MS determination of *Aconitum* alkaloids

Compound	Base peak (m/z)	Other peak (m/z)	C.V. (%)[a]
Aconitine	646 (M+H)	105	11.9
Mesaconitine	632 (M+H)	105	11.5
Jesaconitine	676 (M+H)	135	10.9
Hypaconitine	616 (M+H)	105	10.4
Deoxyaconitine	630 (M+H)	105	11.5
Benzoylaconine	604 (M+H)	105	8.39
Benzoylmesaconine	590 (M+H)	105	6.50
Anisoylaconine	634 (M+H)	135	8.86
Benzoylhypaconine	574 (M+H)	105	6.67
Benzoyldeoxyyaconine	588 (M+H)	105	7.90
Aconine	500 (M+H)	–	10.2
Mesaconine	486 (M+H)	–	27.0
Hypaconine	470 (M+H)	–	10.4
Deoxyaconine	484 (M+H)	–	5.97

C.V.=Coefficient of variation.
[a] Alkaloid solution (each 50 pg) was injected into LC–MS seven times, and the areas of alkaloid peaks were measured.

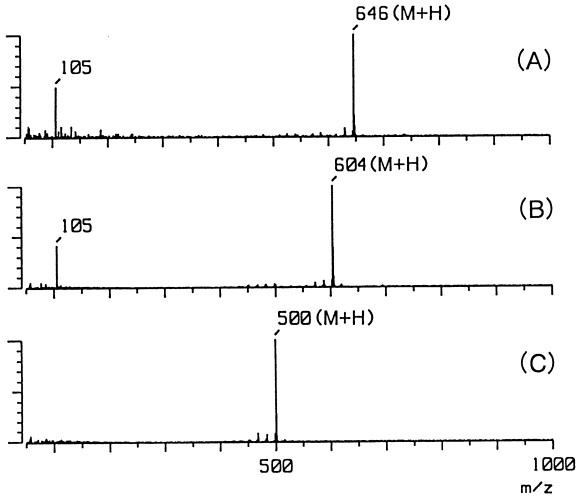


Fig. 2. Mass spectra of aconitine and its hydrolysis products. An aqueous solution containing aconitine (A), benzoylaconine (B) and aconine (C) was applied to LC–MS, and the spectra of alkaloid peaks were measured. LC–MS conditions were as follows: 0.5 μl of sample solution was injected into a capillary Inertsil ODS-2 (150×0.32 mm I.D., Micro-Tech Scientific), eluted with a mixture of acetonitrile and 0.3% TFA solution with a linear gradient from 0:100 to 80:20 (v/v) for 40 mm at a flow-rate of 5 μl/min at 40°C. All the eluates were introduced without splitting, after premixing with matrix glycerol (0.8%, v/v), into the FAB ion source of an LX-2000 double-focusing mass spectrometer (JEOL, Tokyo, Japan) using xenon atoms (3 kV energy).

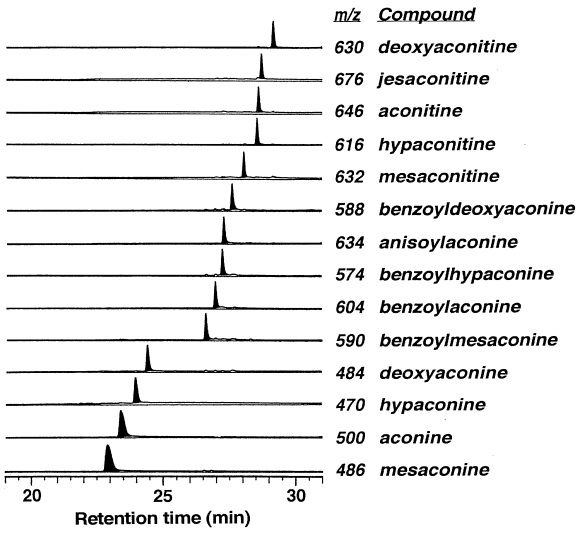


Fig. 3. Selected ion-monitored chromatograms of *Aconitum* alkaloids and their hydrolysis products. An aqueous solution containing 14 alkaloids (each 50 pg) was applied to LC–MS, selectively monitoring their protonated molecular ions.

examined, only a solvent mixture of acetonitrile and 0.3% TFA with gradient elution gave simultaneous separation of all alkaloids (Fig. 3).

Under the optimized LC–MS condition, calibration curves were linear for injection amounts ranging from 5 to 500 pg with a correlation coefficient better than 0.997. The coefficients of variance 50 pg samples were around 10% (Table 1). Detection limits were about 1 pg (S/N =3).

3.2. Solid-phase extraction of Aconitum alkaloids

Compounds in blood and urine interfere with the determination of alkaloids even in LC–MS systems. Therefore, solid-phase extraction was examined to clean up alkaloids from samples. Fourteen alkaloid mixture solutions (each 100 ng of toxic *Aconitum* alkaloids and their hydrolysis products) were spiked into 2 ml of blood or 5 ml of urine, alkaloids were extracted by solid-phase extraction and quantified by LC–MS system. The fourteen alkaloids were well detected on SIM chromatograms of solid-phase extract without interfering peaks (data not shown). As shown in Table 2, the recoveries of alkaloids (each 50 ng/ml) from blood sample were satisfactory (more than 70%) and the coefficients of variance

Table 2
Recovery of *Aconitum* alkaloids using solid-phase extraction

Alkaloid	Blood	Urine
Aconitine	84.5±6.0	99.7±7.0
Mesaconitine	95.8±6.4	93.3±6.2
Jesaconitine	77.1±7.0	98.5±8.4
Hypaconitine	81.0±6.6	77.4±8.2
Deoxyaconitine	76.2±9.1	88.3±11.6
Benzoylaconine	88.9±13.2	56.1±7.4
Benzoylmesacinine	78.1±9.4	73.8±8.8
Anisoylaconine	88.7±12.8	71.0±6.3
Benzoylhypaconine	89.1±8.7	56.8±8.6
Benzoyldeoxyyaconine	83.8±13.4	57.2±9.3
Aconine	73.4±12.5	50.5±8.6
Mesaconine	84.6±25.7	51.3±11.6
Hypaconine	76.3±14.7	54.1±10.6
Deoxyaconine	74.1±12.3	75.7±8.3

Alkaloids (each 100 ng) were spiked into 2 ml of control human blood or 5 ml of urine. After deproteinization or acidification, the alkaloids were extracted with Sep-Pak Plus PS-1, and the concentrations of eluates were determined by LC–MS.

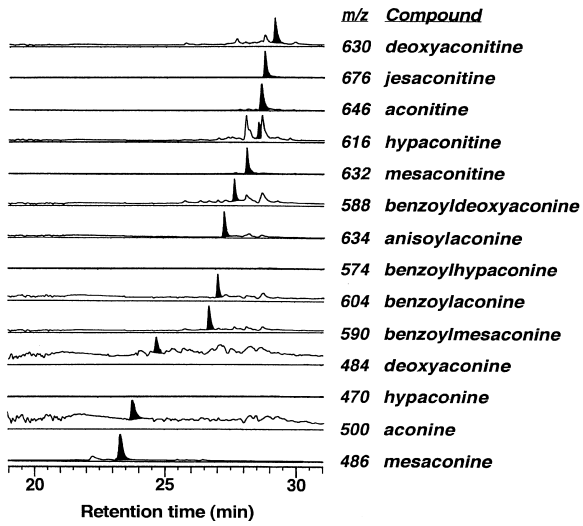


Fig. 4. Selected ion-monitored chromatograms of the extract of urine sample. Two ml of the urine collected from a patient suspected to have eaten food products containing aconite roots was filtered and subjected to Sep-Pak Plus PS-1, and an aliquot (0.5 μl) of the resulting concentrated extract (0.1 ml) was applied to LC–FAB-MS, selectively monitoring the protonated molecular ions.

were less than 20% except for mesaconine. The recoveries of toxic alkaloids (each 20 ng/ml) from urine samples were also satisfactory (more than 75%). In contrast, those of the hydrolysis products (each 20 ng/ml) from urine sample were not as high (between 50% and 75%). The coefficients of variance of recoveries of alkaloids from urine sample were less than 20%.

Calibration curves were linear for spiked amounts ranging from 10 to 100 ng (four points) of alkaloid in 2 ml of blood (5–50 ng/ml) and 5 ml of urine (2–20 ng/ml) samples with correlation coefficients better than 0.95. Detection limits ($S/N=3$) were about 1 ng/ml in blood and 0.5 ng/ml in urine, respectively.

3.3. Detection of Aconitum alkaloids in a urine sample from a patient intoxicated by aconite roots

The method established in this paper using capillary LC–frit-FAB-MS and solid-phase extraction was performed for the urine sample collected from a patient suspected to have eaten food containing aconite roots by mistake. As shown in Fig. 4, 11 alkaloids were detected in the urine. The alkaloid levels in the urine sample were 7.5, 8.8, 219 and 0.2 ng/ml for aconitine, mesaconitine, jesaconitine and deoxyaconitine, respectively. The levels of hydrolysis products were estimated as 0.2, 0.2, 0.4, 3.7, 6.6 and 0.2 ng/ml for benzoylaconine, aconine, benzoylmesaconine, mesaconine, anisoylaconine and benzoyldeoxyaconine, respectively. This result concerning the urine levels indicates that the patients was suspected to have eaten food products containing aconitine, jesaconitine and mesaconitine.

4. Discussion

Aconitines possess two ester functions in their molecules. As hydrolysis proceeds, toxicity of alkaloids is lowered and their polarity increases. Aconitines are converted to benzoylaconines and further to aconines. These hydrolysis products of toxic *Aconitum* alkaloids are included in raw plants, produced during plant sample preservation and also through the course of processing or alkaloid preparation. When food containing these alkaloids is introduced into the body, various kinds of esterases in blood and liver participate in the biodegradation, leading to detoxification. The detailed mechanism of the metabolism of these alkaloids has scarcely been

reported. In addition, during sample storage alkaloids may be degraded to similar hydrolysis products as the above biological process.

The confirmation of poisoning requires the detection of the alkaloids in body fluids. In several cases it is difficult to detect the toxic compounds because of the low levels of alkaloids and rapid biodegradation. In such a case, detection of their metabolites provides indirect confirmation of poisoning by alkaloids. In this paper, hydrolysis products of toxic *Aconitum* alkaloids were prepared, which were expected to be present in body fluids as a consequence of alkaloid poisoning. The prepared hydrolysis products are more polar and the final degradation products, aconines, show weaker UV absorbance than the parent alkaloids. Therefore, it is difficult to detect these hydrolysis products on LC using ODS monitored by UV absorbance. However, specific detection by LC–MS techniques should provide determination of polar alkaloids.

Recently, LC–MS methods using capillary columns have been reported to represent more sensitive detection of certain polar compounds [3]. On FAB-MS, as with the parent alkaloids, the hydrolysis products showed the protonated molecular ions as base peaks. Therefore, FAB is an appropriate ionization method for *Aconitum* alkaloids, and the protonated molecular ions of these alkaloids can be used for SIM. In a previous paper [2], LC eluate was introduced into a MS interface after splitting, and its sensitivity was not so good. In MS analysis, a capillary LC system has been developed for obtaining more sensitive detection compared to the standard LC column usage [4,5]. Coupled with SIM mode and a capillary LC system, a detection limit of 1 pg can be achieved in LC–FAB-MS. This sensitivity was comparable with the derivatization-GC–MS method [6]. Capillary LC systems have also enabled simultaneous determination of fourteen alkaloids. An LC–MS method using atmospheric pressure chemical ionization has been reported for the analysis of plant samples for *Aconitum* alkaloids [7], whose sensitivity is lower than that of our method.

Solid-phase extraction of toxic *Aconitum* alkaloids from blood and urine samples did not show complete recoveries (Table 2), compared to extraction from aqueous solution without blood and urine products [2]. However, the recovery levels are satisfactory (more than 70%) except for those of hydrolysis products from urine samples (between 50 and 75%). This may be due to interference in the adsorption of the polar hydrolysis products onto the solid-phase by urine components. The precision of the recovery was satisfactory, verifying that this method is efficient for sample cleanup and concentration. Coupled with solid-phase extraction using Sep-Pak Plus PS-1 cartridge, the LC–FAB-MS method should show more sensitivity and detection of therapeutic blood levels of toxic alkaloids and their hydrolysis products.

Mizugaki et al., using a derivatization GC–MS technique, have determined the levels of aconitines and their hydrolysis products in blood and urine samples drawn from patients periodically during hospitalization and investigated the time course of alkaloid levels [8]. We tried to examine the alkaloid analysis of a urine sample from a patient (Fig. 4). Aconitine, mesaconitine, jesaconitine and deoxyaconitine and their hydrolysis products have been detected, except for hypaconitine and its hydrolysis products. The detailed information about clinical diagnosis of the patient are already reported [9], and their urine levels are similar to our values. Therefore, capillary LC–frit-FAB-MS method, when adopted for analysis of biological samples, should provide useful information in drug monitoring or confirmation of poisoning.

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