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Determination of aconitine in body fluids by LC-MS-MS

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Abstract A very sensitive and specific method was developed for the determination of aconitine, the main toxic alkaloid from plants of the genus *Aconitum L.*, in biological samples. The method comprised solid-phase extraction using mixed-mode C_8 cation exchange columns followed by liquid chromatography-tandem mass spectrometry (LC-MS-MS). Chromatographic separation was achieved with a RP_8 column. Detection of aconitine was achieved using electrospray in the positive ionisation mode and quantification was performed using multiple reaction monitoring with m/z 646.4 as precursor ion, i.e. $[M+H]^+$ of aconitine and m/z 586.5, m/z 526.4 and m/z 368.4 as product ions after collision-induced dissociation. The method was fully validated for the analysis of blood samples: the limit of detection and the limit of quantitation were 0.1 ng/g and 0.5 ng/g, respectively. Within the linear calibration range of 0.5–25 ng/g, analytical recovery was 79.9%. In two fatal cases with suspected aconite intoxication, aconitine could be detected in blood samples at concentrations of 10.0 and 12.1 ng/g. In one case, aconitine could also be detected in the stomach content (3 ng/g) and in the other in the urine (180 ng/ml).

Keywords Aconitum · Aconitine · Body fluids · Solid-phase extraction · LC-MS-MS · MRM

Introduction

Plants of the genus *Aconitum L.* (family Ranunculaceae) are known to be among the most toxic plants of the northern hemisphere. They are widespread across Europe, northern Asia and North America. Two plants from this genus are of particular importance: the blue-flowered *Aconitum napellus L.* (monkshood) which is cultivated as an ornamental plant in Europe and the yellow-flowered *Aconitum vulparia Reichb.* (wolfsbane), which is mainly used in the traditional Asian herbal medicine [1, 2].

Predominantly in traditional Chinese and Japanese medical preparations, aconite tubers (*Tubera aconiti*) and their processed products are used for their pharmaceutical properties including anti-inflammatory, analgesic and cardiotonic effects [3, 4, 5, 6, 7]. These effects can be attributed to the presence of C20 diterpene and C19 norditerpene alkaloids which are found in all of the tissues of the plant, but especially in the tubers. The main alkaloids are aconitine, mesaconitine, hypaconitine and jesaconitine. The composition and amount of these alkaloids varies depending on the species, place of origin and the time of harvest [8].

The physiological effects of the Aconitum alkaloids are due to the action on nerve potential resulting from their affinity to the voltage-sensitive sodium channels in the axons. Aconitine is known to suppress the inactivation of voltage-dependent sodium channels by binding to the neurotoxin binding site 2 of the channel protein [9, 10, 11].

The first symptoms of aconitine poisoning appear approximately 20 min to 2 hr after oral intake and include paraesthesia, sweating and nausea. This leads to severe vomiting, colicky diarrhoea, intense pain and then paralysis of the skeletal muscles. Following the onset of life-threatening arrhythmia, including ventricular tachycardia and ventricular fibrillation, death finally occurs as a result of respiratory paralysis or cardiac arrest [2, 12, 13, 14].

The use of Aconitum alkaloids as a homicidal agent has been known in Europe and Asia for more than 2000 years. In the western hemisphere, intoxication by aconite is rare. However, in traditional Chinese medicine, the use of aconite-based preparations are common and poisoning has been reported, not only during clinical use but also as consequence of accidental ingestion, e.g. by eating plant

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material or by the uptake of Aconitum preparations. The suicidal and homicidal use of aconite tubers has also been reported [12, 13, 14, 15, 16, 17, 18, 19, 20, 21].

In forensic cases, and particularly if an intoxication is suspected, the determination of uncommon substances is often requested within a very short time [22, 23]. In the past, liquid chromatography-mass spectrometry (LC-MS) has been successfully applied for the analysis of other toxic plant ingredients [24]. For the determination of aconitine-type alkaloids in particular, a variety of analytical techniques have been used. LC-MS analysis, using an ion trap mass spectrometer, was performed to characterise the alkaloids of *Aconitum kusnezoffii* and to study the ester exchange reactions of the diester-diterpenoid alkaloids in decoctions of aconite tubers [25, 26, 27].

For the analysis of the Aconitum alkaloids in biological specimens such as blood, serum and urine, several GC-MS methods have been described [12, 18, 21, 28, 29]. These employ a variety of extraction procedures followed by derivatisation to their TMS derivatives.

Hayaschida and co-workers developed a LC-MS method which utilised electrospray ionisation and detection in the selected ion monitoring mode for the determination of several Aconitum alkaloids in serum samples [30].

We have developed a simple and very sensitive liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for the determination of aconitine in various body fluids. The method comprises solid-phase extraction (SPE) followed by LC-MS-MS. Chromatographic separation was carried out on a RP_8 column. Detection of aconitine was achieved using electrospray in the positive ionisation mode and quantification was performed using multiple reaction monitoring (MRM). The method was fully validated for the determination of aconitine from whole blood samples and applied in two cases of fatal poisoning.

Materials and methods

Reagents and chemicals

Aconitine base was purchased from Sigma (Deisenhofen, Germany) and a stock solution of 1 mg/ml was initially prepared in acetonitrile. This stock solution was further diluted, using HPLC mobile phase, to assess the linearity of response and using water for the preparation of spiked blood calibrators. Acetonitrile, methanol, dichloromethane and 2-propanol of picograde quality were obtained from Promochem (Wesel, Germany), purified water was from Baker (Griesheim, Germany) and ammonium acetate from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

For solid-phase extraction (SPE), mixed-mode columns Clean-Screen ZSDAU 020, 200 mg Restek (Sulzbach, Germany) were used. Phosphate buffer 0.15 M pH 6.0 for sample preparation consisted of a mixture of 444.5 ml of 9.07 g KH_2PO_4/l and 55.5 ml of a solution of 11.87 g $Na_2HPO_4.2H_2O$/L. The elution solvent for SPE was a mixture of 80 ml dichloromethane, 20 ml 2-propanol and 2 ml ammonia (25% v/v in water) which was prepared fresh directly prior to the elution.

The HPLC mobile phase consisted of 0.1 % ammonium acetate, adjusted to pH 6.0 with 1 M acetic acid, and 50% methanol.

Spiked blood calibrators

In order to evaluate specificity, recovery, precision and accuracy of the method and to record the calibration curve, blank blood samples obtained from six healthy volunteers, were spiked using aqueous aconitine solutions.

Standard addition experiments

The influence of ion suppression was studied by the analysis of the case blood samples after standard addition. To 0.5 g of authentic blood samples 5 ng, 10 ng and 15 ng aconitine were added. The samples were analysed in duplicate as described below.

Sample preparation and SPE procedure

Prior to extraction, blood samples and tissue samples (each 0.5 g) were mixed with 3 ml of 0.15 M phosphate buffer pH 6.0, homogenised and centrifuged at 5,000 *g* for 10 min.

The supernatants were decanted and transferred to the SPE columns which were conditioned with 3 ml methanol, 3 ml water and 1 ml 0.15 M phosphate buffer pH 6.0. The samples were passed through under atmospheric pressure and then washed with 3 ml of water, 1 ml of 0.01 M HCl and dried under vacuum for 5 min. A second washing step followed with 2 ml of dichloromethane and drying for 2 min. A third washing step was performed with 2 ml methanol, followed by drying for 10 min. Aconitine was eluted with two aliquots of 1.5 ml elution solvent each. Pooled eluents were evaporated to dryness under a stream of nitrogen at 40°C and reconstituted with 100 µl HPLC mobile phase.

HPLC conditions and MS settings

LC was performed using a Waters Alliance 2695 series HPLC pump with autosampler. Chromatographic separation was achieved with a Waters XTerra RP_8 pre-column (2.1×10 mm, 3.5 µm) and a XTerra RP_8 analytical column (2.1×150 mm, 3.5 µm) at a flow rate of 200 µl/min and a column temperature of 40°C. The injection volume was 10 µl with a total run time of 10 min. All aspects of system operation and data acquisition were controlled using MassLynx NT 4.0 software (Waters).

A Micromass Quattro micro-tandem mass spectrometer equipped with a *Z*-spray ion interface was used for all

analyses. Ionisation was achieved using electrospray in the positive ionisation mode. The following conditions were found to be optimal for the analysis of aconitine: capillary voltage 1 kV, cone voltage 40 V, source temperature 120°C and desolvation gas (nitrogen) was heated to 350°C and delivered at a flow rate of 510 l/h. Data were recorded in the multiple reaction monitoring (MRM) mode using m/z 646.4 as precursor ion of aconitine and m/z 586.5 after collision-induced dissociation at 35 eV as the main product ion. Two secondary transitions were recorded in order to confirm the substance identity (m/z 646.4>m/z 526.4 at 45 eV and m/z 646.4>m/z 368.4 at 48 eV) Collision gas (argon) pressure was maintained at 6×10^{-3} mbar.

Quantification was performed by integration of the area under the specific MRM chromatogram (m/z 646.4>m/z 586.5). Automated data processing was performed using QuanLynx (Waters).

Results and discussion

The method was developed for the determination of aconitine in biological samples collected during the autopsy in cases of suspected intoxication with aconite. Prior to the analysis of forensic samples, the assay was validated for the determination of aconitine in whole blood samples.

Linearity

In order to assess method linearity in the absence of a biological matrix, a series of aconitine standard solutions (0, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 125, 150 and 200 ng/ml) were prepared and analysed in duplicate by the LC-MS-MS method. Standard response curves were generated using a least-squares linear regression and found to be linear over the range investigated, with a correlation coefficient of R^2=0.9996.

Specificity

Specificity was evaluated by comparison of the chromatograms obtained following the analysis of 12 aconitine-free human blood samples (from 6 individuals) with the chromatogram of a blood calibrator spiked with 1 ng of aconitine/g blood. No peaks were observed in the matrices which interfered with the detection of aconitine. This is certainly the result of the highly specific detection in the MRM mode. The protonated molecular species of aconitine (m/z 646.4) passes selectively through MS1 and is dissociated in the collision cell to the specific main fragment m/z 586.5 and the secondary fragments m/z 526.4 and m/z 368.4 (see Fig. 1 and [27]). These fragments pass selectively through MS2 and are detected.

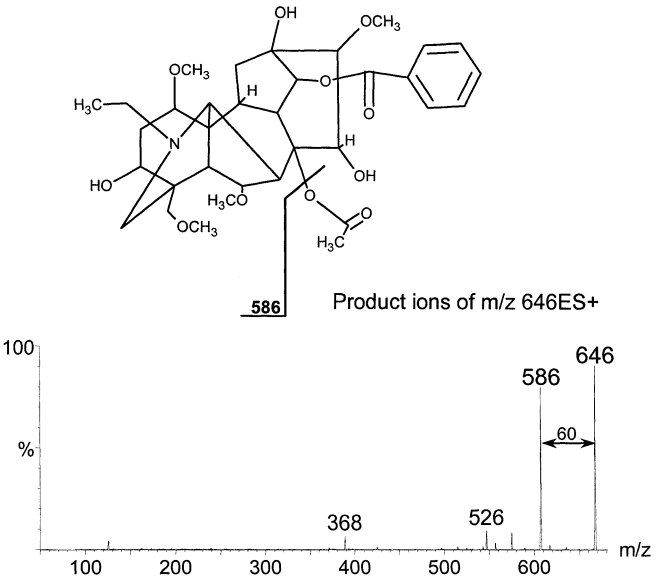

Fig. 1 The product ion spectrum of aconitine after collision-induced dissociation (collision energy 35 eV) shows in addition to the unfragmented precursor ion m/z 646 ($[M+H]^+$of aconitine), the main product ion m/z 586 and the secondary product ions m/z 526 and m/z 368. The fragment m/z 586 is attributed to the loss of an acetyl group with 60 amu [27]

Calibration

The calibration curve was recorded by the duplicate analysis of spiked blood calibrators in the range 0–25.0 ng aconitine/g. The linear regression of the calibration graph is described by the function y=1699x+60 with the correlation coefficient r^2=0.999. The graph was linear and of acceptable quality.

Recovery

Recovery was calculated from the ratio of the slope of the regression line obtained with standard aqueous solutions without extraction procedure and spiked blood samples after SPE. The recovery was found to be acceptable at 79.9% across the concentration range examined. An internal standard such as deuterated aconitine or a chemically related alkaloid was not used in the course of method development. The use of an appropriate internal standard could certainly improve the data for recovery because a loss of analyte could be compensated by the loss of the internal standard in the same range.

Precision and accuracy

Precision and accuracy were assessed by the analysis of blank blood samples spiked at 0.5, 10, and 20 ng aconitine/g blood. For the determination of precision, these samples were extracted and analysed 5 times within 1 day (intra-assay precision). This series was repeated 3 times each on 3 different days (interassay precision).

Coefficients of variation (% CV) ranged between 2.5 and 15.6% (Table 1), accuracy was between 90 and 116%.

Also in this respect, the use of an internal standard could certainly improve the results of precision and accuracy.

Limits of detection and quantitation

Analysis of a blood sample spiked with aconitine at a concentration of 0.1 ng/g resulted in a signal-to-noise (S:N) ratio of 14:1 (Fig. 2a). The mean intensities of the secondary transitions m/z 646.4 > m/z 526.4 and m/z 646.4 > m/z 368.4 were 39% and 37% of the main transition m/z 646.4 > m/z 586.5. Referring to the requirements for qualitative substance identification [31] a maximum tolerance of ±25% is acceptable for an unambiguous substance identification. The concentration of 0.1 ng aconitine/g blood was the lowest concentration that met these criteria. Lower concentrations up to 0.02 ng/g resulted in a distinct signal for the main transition but with unacceptable ratios for the secondary transitions. Therefore, the limit of detection (LOD) was 0.1 ng aconitine/g blood.

The limit of quantification (LOQ) was defined as the lowest concentration included in the calibration series and was 0.5 ng aconitine/g blood. Both intra-assay and interassay precision of the method were within a 20% CV limit at LOQ and within a 15% CV limit in the upper concentration range and thus met the limits of acceptability for the validation of bioanalytical assays [32].

Due to the simple and quick extraction procedure this new method is perfectly applicable to routine laboratory work.

Table 1 Interassay and intra-assay precision and accuracy for the determination of aconitine in blood samples

	Aconitine concentration added (ng/g blood)		
	0.5	10.0	20.0
Determined aconitine concentration (ng/g blood)			
Series 1 (n=5)	0.58	9.9	22.6
Series 2	0.48	9.0	21.2
Series 3	0.46	10.1	21.0
Mean value	0.51	9.7	21.6
Series 1–3 (n=15)			
Intra-assay precision (CV %) arithmetic mean value			
Series 1 (n=5)	12.9	4.4	3.4
Series 2	15.6	2.5	3.7
Series 3	10.6	4.0	2.9
Interassay precision (CV %) arithmetic mean value			
Series 1–3	10.4	4.9	3.3
Intra-assay accuracy (%) arithmetic mean value			
Series 1 (n=5)	116	99	113
Series 2	96	90	106
Series 3	92	101	105
Interassay accuracy (%) arithmetic mean value			
Series 1–3	101	97	108

Table 2 Concentrations of aconitine in autopsy samples from two cases of fatal aconite intoxication

	Aconitine concentration		
	Blood (ng/g)	Stomach content (ng/g)	Urine (ng/ml)
Case no. 1	10.0 (10.6[a])	3	not available
Case no. 2	12.1 (12.8[a])	not available	180

[a]Results in brackets refer to the standard addition method.

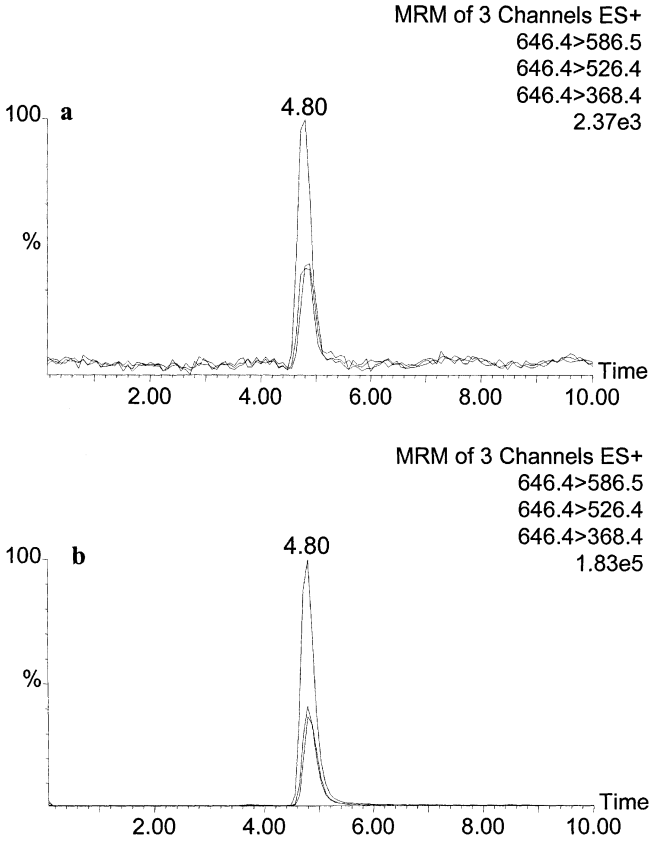

Fig. 2a,b MRM chromatogram of **a** a blood sample spiked with 0.1 ng aconitrine/g which represents the LOD of aconitrine in blood (S:N of 14:1 peak to peak). **b** MRM chromatogram of the blood sample from the victim in case 2 with 12.1 ng aconitrine/g which shows no interference although the body was in an advanced state of putrefaction

In two forensic cases with suspected aconite intoxication, aconitine was detected in the blood samples and also in the stomach contents of the deceased (Table 2). To study the influence of ion suppression on the results of the blood samples, standard addition experiments were carried out (Table 2, results in brackets). The good agreement of the results obtained with these different methods demonstrates the negligible influence of ion suppression in these cases. This might be due to the elaborated and efficient clean-up procedure.

The method is highly sensitive and no matrix peaks interfered with the detection of aconitine for authentic samples. In Fig. 2b the chromatogram of the blood sample of an aconite victim (case no. 2, Table 2) is shown. At the

time of autopsy the body was already in an advanced state of putrefaction. Despite these difficult circumstances, the chromatogram shows pure signals for both the main and the secondary transitions.

In conclusion, the validation data and the results of the forensic cases clearly demonstrate the utility of this method for the precise detection of aconitine in whole blood and other body fluids up to very low concentrations.

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