Case Report

Fatal Intoxication Due to Brucine

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Abstract

A sensitive method for identifying and quantifying brucine by means of liquid chromatography–tandem mass spectrometry is presented in this article. Based on a solid-phase extraction for human serum, the validation indicated limits of detection and quantification of 0.12 and 0.23 ng/mL, respectively. In one case of lethal suicidal brucine monointoxication, brucine concentrations of 1.51 µg/mL, 1.69 µg/mL, 9.94 µg/mL, 16.4 µg/g, 0.99 µg/g, 0.75 µg/g, and 1.95 mg/g were determined in femoral blood, urine, bile collected from the gallbladder, liver tissue, cerebellum, cerebrum, and stomach contents, respectively.

Introduction

Brucine (2,3-dimethoxystrychnine) is an alkaloid of the strychnine type. It is found together with strychnine and other indole alkaloids in various plants of the Strychnine family (1). The literature describes high concentrations especially in the seeds of the Strychnine tree (*Strychnos nux-vomica* L.), of approximately 1.5% strychnine and brucine. It has been alleged that the bark of *Strychnos nux-vomica* L. contains no strychnine, but about 2–3% brucine (2,3).

Strychnose alkaloids have been used for therapeutic purposes for some time now; in smaller doses, they should have a central stimulation effect and improve circulation and muscle tone (4,5). Strychnine itself has been used as a doping agent and is still on the current World Anti-Doping Agency (WADA) list of banned substances (6). Both agents are constituents of herbal preparations, as, for example, in traditional Chinese medicines (7,8). In addition, brucine has significance in diastereomer separation (9) and as a bitter constituent (10). Studies in the past also report on the anti-inflammatory properties and pain modulation (11) and apoptotic effects (12) of brucine and other strychnose derivatives.

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On the other hand, the strychnose alkaloids have long been identified as toxic substances (13,14); these properties led to their use in pesticides, etc. (15,16). Like strychnine, brucine also functions as antagonist at the glycine receptor and paralyzes the inhibitory neurons (17,18). Among other effects, modulation or blocking of other receptors by brucine in animal models has been described (19,20). Typical intoxication systems of strychnine poisoning in humans include initial restlessness, fear, nausea, vomiting, and stiffness of the jaw and neck muscles, leading to severe cramping of the entire musculature, including the respiratory muscles. This is accompanied by metabolic alkalosis, rhabdomyolysis and sometimes acute renal failure, which ultimately lead to death through suffocation and exhaustion (5,16,21).

Whereas a lethal dose of 30–120 mg (in adults) is assumed for strychnine (1,21), brucine has only 1/10–1/50 of its effectiveness in humans, so a 1000-mg dose (22) has been specified as lethal.

In strychnine preparations, strychnine and brucine are present simultaneously. Because of its lesser toxicity, the presence of brucine is of less relevance, and it is therefore only seldom determined in addition to strychnine (23). In contrast, pure brucine intoxication occurs very rarely. In Pubmed-listed articles, we could only research one clinical instance (3) that assumed an ingestion of brucine, yet did not substantiate this with the corresponding laboratory investigation.

The literature presents electrochemical methods (24–26), capillary electrophoresis (7,27–29) and especially chromatographic techniques as various methods to determine the presence of brucine. Chromatographic determinations were conducted with thin-layer chromatography (8), gas chromatography (GC) with nitrogen-phosphorus detection (30,31), GC–mass spectrometry (MS) (23,30,32–35), and liquid chromatography (LC) with UV or diode-array detection (DAD) (36–39). However, in recent years, LC–MS has come to dominate the literature (40–44); with it, the limits of quantification (LOQ) in bodily fluids can be reduced to < 1 ng/mL.

In the present work, a validated method to detect brucine is presented and applied to a case of monointoxication. This yields investigative results for brucine concentration in bodily fluids and tissue specimens subsequent to a suicidal ingestion.

Case History

Together with his spouse, a 60-year-old male was found dead in their common apartment. A farewell letter raised financial problems as motive for this extended suicide. According to autopsy results, the spouse's death was due to suffocation by soft covering in combination with the effects of chloroform and heavy alcohol intoxication (blood alcohol concentration 1.91 g/kg). Next to the deceased male, who had been employed in a chemical laboratory, a plastic container bearing the inscription "Brucine" was found, along with a glass containing watery residues and substance traces. The autopsy was performed on the day of the event, approximately 12 h postmortem.

The findings for the male indicated pronounced, massive pulmonary edema and exclusively liquid cadaveric blood with evident hyperemia of the inner organs, among other effects.

Hyaline protein cylinders in tubules and the collecting duct system, together with vacuolar degeneration of the tubules, provided histological evidence of kidney damage.

Experimental

Chemicals and standards

High-performance liquid chromatography (HPLC)-grade methanol, water, 2-propanol, and acetic acid were supplied by J.T. Baker (Deventer, The Netherlands). Dichloromethane, ammonia, sodium dihydrogen phosphate monohydrate, and ammonium acetate were supplied by Merck (Darmstadt, Germany). Brucine and strychnine were obtained from LGC Standards (Wesel, Germany).

Apparatus and conditions

An Applied Biosystems (Darmstadt, Germany) API 2000 tandem MS equipped with electrospray ionization (ESI), a Shimadzu high-pressure gradient system with two solvent delivery units (LC-10Advp), system controller (SCL-10Avp), autoinjector (SIL-10ADvp), column oven (CTO-10Asvp), and degasser (DGU-14A) were used for LC–MS–MS analysis. The data were processed using Analyst 1.4 software.

Chromatographic separation was performed on a Phenomenex (Aschaffenburg, Germany) Luna 5µ phenyl-hexyl (50 × 4.6 mm) column. Both eluents contained 10 mM ammonium acetate and 0.1% acetic acid (pH 3.2) and were composed of 95:5 water/methanol (v/v, eluent A) or 5:95 water/methanol (v/v, eluent B), respectively. The mobile phase was pumped at a flow rate of 0.3 mL/min, programmed as follows: 35% B initially, increasing to 70% B from 0 to 4.5 min, increasing to 100% B from 4.5 to 5 min, hold for 1.5 min, ramping to 35% B from 6.5 to 7.5 min, hold for 1 min for column re-equilibration.

Positive ESI-MS–MS was performed in multiple reaction monitoring (MRM) mode with the following settings: source

Table I. Optimized MS–MS Parameters

Analyte	M1* (*m/z*)	M2 (*m/z*)	DP (V)	FP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Brucine	395	324	71	340	11.5	16	45	6
	395	244	71	340	11.5	16	49	10
Strychnine	335	184	81	370	12	14	51	8
	335	156	81	370	12	14	61	6

* Abbreviations: M1, precursor ion; M2, production ion; DP, declustering potential; FP, focusing potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; and CXP, collision cell exit potential.

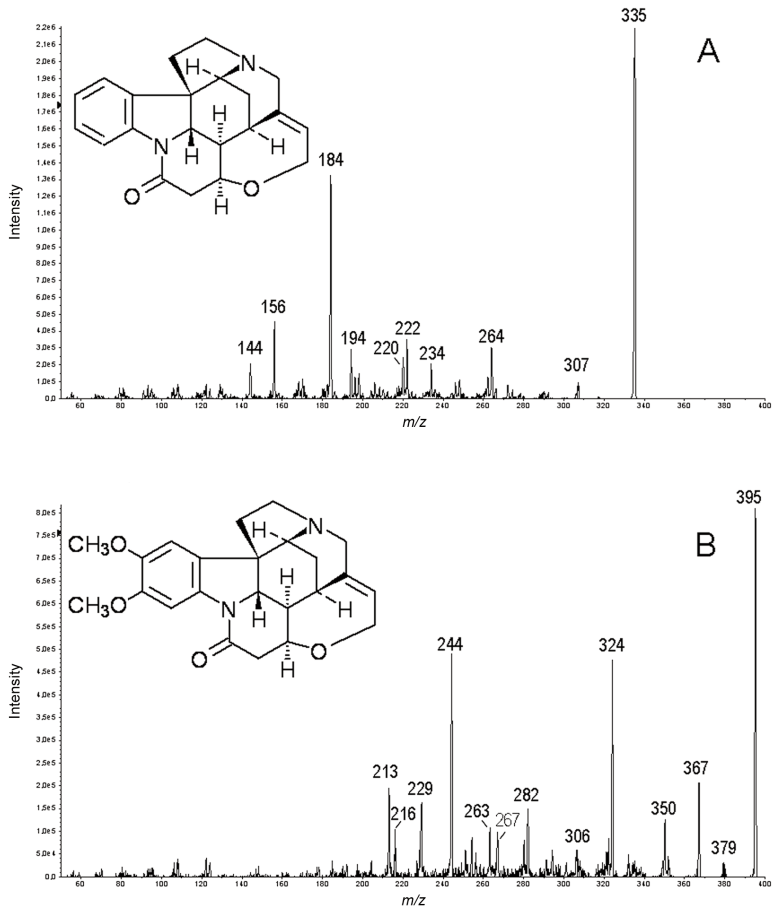

Figure 1. Chemical structure and product ion spectrum of strychnine (A) and brucine (B).

temperature, 400°C; ion spray needle voltage, +2000 V; curtain gas, 25 psi (nitrogen); nebulizer gas, 40 psi (nitrogen); auxiliary gas, 60 psi (nitrogen); collision gas set on 4 (nitrogen); dwell time, 200 ms; and pause between mass ranges, 5 ms. Further analyte-dependent MS–MS parameters (Table I) were optimized by continuous infusion of standards dissolved in eluent B (1 µg/mL) at a flow rate of 10 µL/min. Figure 1 presents the structural formula and production spectra of brucine and strychnine.

Sample preparation

In a 10-mL glass tube, 0.1 mL blood or body fluids, 7 mL of phosphate buffer (pH 6), and 20 µL of the internal standard solution (strychnine 0.5 ng/µL) were collected and mixed for 20 s on a vortex mixer. The samples were transferred onto a solid-phase extraction cartridge (Bond Elut LRC Certify 130 mg, Varian, Palo Alto, CA) previously conditioned with 2 mL methanol followed by 7 mL phosphate buffer (pH 6), and then the cartridge with sample was washed with 2 mL acetic acid (0.1 M) followed by 3 mL methanol. Vacuum was applied for 5 min to remove the washing liquids. The analytes were eluated with 1.5 mL dichlormethane/2-propanole/25% ammonia (v/v) (70:30:40). The eluate was evaporated to dryness at 36°C under a gentle stream of nitrogen. The residue was reconstituted in 200 µL of mobile phase, and a 25-µL aliquot was injected onto the LC–MS–MS system.

Tissue and stomach contents were homogenized using an Ultra-Turrax (5 g substrate + 20 g phosphate buffer pH 6); 0.5 g of the homogenate in a corresponding dilution was treated as described (internal standards were 50 ng/g).

Method validation

The method was validated according to the proposals recently published using the reduced design for single case analyses (45). This procedure included linearity, selectivity, accuracy, precision, limit of detection (LOD), LOQ, and matrix effects. Stock solutions were prepared in methanol containing 1 mg/mL of brucine or strychnine. The calibration samples were obtained by adding 100 µL of drug-free human serum, 100 µL of fresh diluted stock solution of brucine, and 20 µL of fresh diluted stock solution of strychnine (internal standard). Control samples were prepared in the same way, but separately, because no certified reference samples were available.

Linear regression analysis was used to generate calibration curves with duplicate measurements per level. Drug-free serum samples were spiked with 0, 50, 100, 200, 300, 400, and 500 ng/mL brucine and 100 ng/mL of the internal standard. The calibration range was selected with consideration of fatal cases. For the purposes of selectivity check, six different serum samples were extracted and screened for interfering peaks.

To evaluate bias and repeatability, accuracy and precision experiments were carried out using five replicates of each of the two different concentration levels (50 and 400 ng/mL brucine).

The LOD and the lower LOQ were estimated by means of spiked serum samples containing 5.0, 1.0, and 0.5 ng/mL brucine. The values for the LOD and the LOQ were calculated solely from the signal-to-noise values of the 0.5 ng/mL sample.

Ion suppression/enhancement and matrix effects experiments were conducted using standard solutions diluted in HPLC eluent (batch 1), five different lots of human serum spiked with 100 ng/mL brucine and strychnine after extraction (batch 2), and human serum spiked before extraction (batch 3). Method described by Matuszewski et al. (46) was used to determine matrix effect, recovery of the extraction procedure and process efficiency.

Results

Linearity and selectivity

Calibration curves were linear over the range investigated. The equation for the quantifier transition was $y = 0.00346x + 0.03644$ ($r = 0.998$) and for the qualifier transition was $y = 0.00338x + 0.03839$ ($r = 0.998$). No interference was observed in blank samples at the elution time for detection of brucine and strychnine.

Accuracy and precision

The intraday precision (expressed as %RSD) and accuracy (expressed as %bias) were summarized in Table II. The RSD values were between 1.4 and 5.3%, and the accuracy value were found to be between 0.72 and 1.3%. All values met the acceptance criterion of less than 15%.

Limits

The LOD is the lowest amount of brucine for identification criteria to be met. The LOD was determined from three times the noise value of the qualifier transition (*m/z* 395/244) and equalled 0.12 ng/mL.

The LOQ is the lowest concentration of brucine at which quantitative results can be reported with a high degree of confidence. The LOQ was calculated from 10 times the noise value of the quantifier transition (*m/z* 395/324) and equalled 0.23

Table II. Accuracy, Precision, Matrix Effects, Recovery, and Process Efficiency

Accuracy and Precision				Matrix Effect		Recovery		Process Efficiency	
Concentration added (ng/mL)	Average concentration found (ng/mL)	RSD (%)	Bias (%)	Brucine (%)	Strychnine (%)	Brucine (%)	Strychnine (%)	Brucine (%)	Strychnine (%)
50	49.4	5.3	–1.3	75.4	73.6	98.9	102.0	74.6	75.1
400	397.1	1.4	–0.72						

ng/mL. However, for determining the case samples, the LOQ was set at 50 ng/mL in accordance with the lowest calibration point used.

Matrix effects

Matrix effect, recovery of the extraction procedure, and process efficiency were determined by comparing the averaged absolute peak areas according the following formula: matrix effect = average area batch 2/average area batch 1 × 100%, recovery = average area batch 3/average area batch 2 × 100%, and process efficiency = average area batch 3/average area batch 1 × 100%. The data obtained are listed in Table II.

The results (Table II) indicate that ion suppression was significant for brucine and strychnine. However, both the target analyte brucine and the internal standard are equally affected, showing almost identical properties. Consequently, strychnine is a suitable internal standard and represents the substance of choice where determination of brucine alone is involved, so long as deuterated analogues remain commercially unavailable. Cases of mixed strychnose alkaloid intoxication must naturally resort to other compounds. In this regard, the literature mentions papaverine (33–35), chloroquine (38), and others.

Table III. Analytical Findings

Specimen	Brucine Concentration
Femoral blood	1510 ng/mL
Heart blood	4470 ng/mL
Urine	1685 ng/mL
Bile	9936 ng/mL
Liver	16443 ng/g
Cerebellum	988 ng/g
Cerebrum	746 ng/g
Gastric contents*	1949 µg/g

* Stomach contents 310 g = 604 mg brucine.

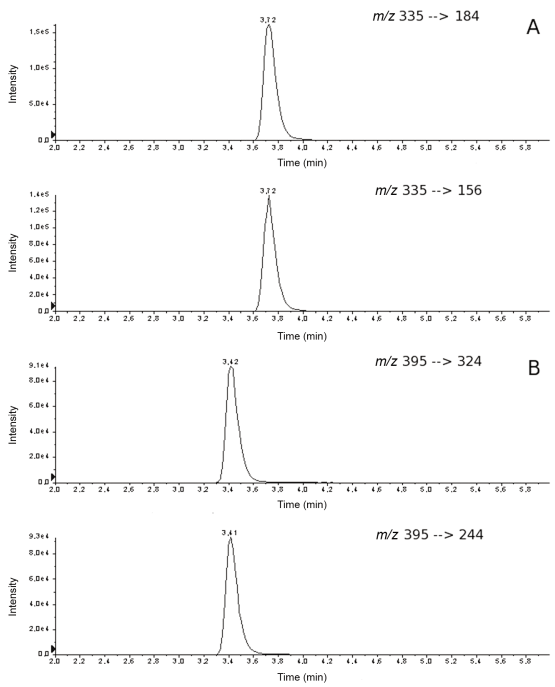

Figure 2. MRM chromatograms obtained from the femoral blood (dilution 1:10) containing strychnine (internal standard) (A) and 1.5 µg/mL brucine (B).

Brucine investigation

In the presented case, quantification of brucine in body fluids was performed using serum calibration curves. Thus, the tissue specimens were correspondingly diluted. As well, processing specimens without an internal standard determined that there were no traces of strychnine in any of the investigated substances. Quantification of tissues and stomach contents was performed by means of standard addition. Results of LC–MS–MS investigations for brucine are given in Table III and Figure 2.

Additional investigations

The systematic toxicological analysis comprised an immunochemical drug screening (cannabinoids, opiates, cocaine, amphetamines, methamphetamines, benzodiazepines, methadone, and barbiturates), GC–MS and HPLC–DAD analyses of blood and urine for organically extractable foreign substances (e.g., organic drugs, poisons, and their metabolites), and a forensic blood alcohol determination. Other than detection of brucine, these corollary investigations revealed no additional relevant findings.

Discussion

Among its other results, this validation indicates that the methods described here can provide a precise and sensitive determination of brucine. The literature contains scant examples of validation data in blood or serum. The LODs or LOQs described, naturally, are method dependent. New studies, based on LC–MS and/or LC–MS–MS as are those presented here, describe LODs of 0.05 ng/mL (43) or 0.1 ng/mL (44). For HPLC–UV or HPLC–DAD, comparable LODs of 0.5 ng/mL (23) to 60 ng/mL (38) are described. Methods based on GC–MS, although for strychnine, yield values of 30 ng/mL (33,34) to 100 ng/mL (35).

In the case presented here, LC–MS–MS detected brucine in the investigated bodily fluids and tissue specimens. In accordance with the findings presented, the case was classified as brucine monointoxication.

Pure brucine intoxication occurs extremely rarely. As explained, our search of extant literature only found one documented case (3) in which a 24-year-old female ingested a decoction containing brucine, made from the bark of the Strychnine tree, which should not contain any strychnine. The symptoms described in this article corresponded to those determined for strychnine intoxication, such as muscle spasms, convulsions, rhabdomyolysis, and acute renal failure. Unfortunately, this report did not substantiate the observed brucine

intoxication with toxicological analyses, so comparison values for concentration in bodily fluids are also unavailable. For strychnine intoxication, however, the literature contains many documented cases of accidental or suicidal ingestion. Among others, Marques et al. (35), Sarvesvaran (2), Rosano et al. (30), and Wood et al. (31) present instances of survived and lethal strychnine intoxication, summarize the extant publications as well as concentration distributions in bodily fluids and tissue samples, and discuss measured concentrations. According to these reports, blood and serum concentrations of 0.33–61 µg/mL in cases of fatal strychnine intoxication have been determined. In the case of a combined intoxication, Wang et al. (23) detected blood levels of 2.09 µg/mL strychnine and 1.88 µg/mL brucine. Based on this information, and when the lesser toxicity of brucine is considered, measured values of 1.51 or 4.47 µg/mL brucine in femoral or cardiac blood in representative cases of brucine intoxication emerge as rather lower than expected. The higher concentration in the liver tissue 16.4 µg/g and the non-reabsorbed portion of about 600 mg indicate that death followed shortly after oral ingestion. Relevant brucine concentrations in central tissues were also evidenced. The ingested dose cannot be accurately specified. According to the non-absorbed portion in the stomach contents and the missing amount in the aforementioned brucine container, an amount of about 1 g is probable.

As stated, autopsy on the corpse resulted in non-specific findings. After lethal strychnine intoxication, severe pulmonary edema, alveolar hemorrhage (32), congestion and edema of the viscera (35), as well as other effects were detected; in particular, inconspicuous postmortem lesions should also have been present (17,35).

The investigations conducted in this case could identify no brucine decomposition products. Unfortunately, the amount of urine found in the bladder during the autopsy was so small that other than the brucine quantification, no systematic investigation for any metabolites could be performed.

The presence of metabolites corresponding to those detected for strychnine poisoning in animal models (36,47), especially oxidative products, was anticipated.

Conclusions

With the validated method presented here, brucine can be registered by tandem MS down to the trace level (ng/mL level), where no toxic effects are to be expected. Despite its rarity, lethal brucine monointoxication does occur. According to the case presented here, oral ingestion of brucine is sufficient to cause lethal intoxication. The ingested amount, however, could not be reliably determined.

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