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ORIGINAL ARTICLE

GC/MS determination of ibotenic acid and muscimol in the urine of patients intoxicated with *Amanita pantherina*

Jan Stříbrný · Miloš Sokol · Barbora Merová · Peter Ondra

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Abstract Ibotenic acid and muscimol are substances which mostly participate in psychotropic properties of *Amanita pantherina* and *Amanita muscaria*. They are rapidly absorbed from the gastrointestinal tract and readily excreted in urine. The poisoning with *A. pantherina* is in the majority of cases accidental because it can be easily mistaken for the edible species (*Amanita rubescens*, *Amanita spissa* and *Macrolepiota procera*). Intoxication with *A. muscaria* is mostly intentional for recreational purposes. Prognosis of the poisoning is generally good; lethal cases are rare. Mushroom poisoning is often proved by microscopic examination of spores in the stomach or intestinal content. Authors of this article introduce an instrumental method of proving *A. pantherina* or *A. muscaria* poisoning. The article describes the isolation of ibotenic acid and muscimol from urine, the derivatization step and the determination of these compounds by gas chromatography/mass spectrometry. Isolation of these alkaloids from urine was performed on a strong cation exchanger (Dowex® 50W X8), and the elution and derivatization of the alkaloids were made in one step with ethyl chloroformate in aqueous solution of sodium hydroxide with the addition of ethanol and pyridine. Cycloserine was used as internal standard. By this method, concentrations of ibotenic acid and muscimol in the urine of four persons intoxicated with *A. pantherina* were determined. In this study, mass spectra of derivatized ibotenic acid and muscimol are shown, and validation of the method is described.

Keywords Ibotenic acid · Muscimol · *Amanita muscaria* · *Amanita pantherina* · Intoxication · GC/MS

J. Stříbrný (✉) · M. Sokol
Military Institute of Forensic Medicine,
Central Military Hospital Prague,
Prague, Czech Republic
e-mail: jan.stribrny@uvn.cz

J. Stříbrný
Department of Toxicology, Faculty of Military Health Sciences,
University of Defence,
Hradec Králové, Czech Republic

B. Merová
Department of Medical Chemistry and Biochemistry,
Palacký University,
Olomouc, Czech Republic

P. Ondra
Faculty Hospital, Institute of Forensic Medicine and Medical Law,
Palacký University,
Olomouc, Czech Republic

Introduction

Amanita pantherina and *Amanita muscaria* are poisonous mushrooms with psychotropic properties. *A. pantherina* grows in Europe, Asia, North America and also in South Africa. *A. muscaria* is a cosmopolitan species. Ritual intoxication with these mushrooms, chiefly with *A. muscaria*, probably goes with man from the ancient time [1, 2]. The tradition of ritual, therapeutic and recreational use of *A. muscaria* was still alive in the last century in Siberia and Northeast Asia [3, 4]. At present, the intoxication with *A. muscaria* is mostly intentional for recreational purposes, while intoxication with *A. pantherina* is usually accidental due to mistaking this mushroom for edible species (e.g. *Amanita rubescens*, *Amanita spissa* and *Macrolepiota procera*).

Both *Amanitas* contain ibotenic acid and muscimol which mostly participate in psychotropic properties of these mushrooms. Their isolation and structure determination were performed by several research teams almost at the

same time in 1964 and 1965 [5–8]. Ibotenic acid and muscimol are 3-hydroxyisoxazole-derived alkaloids. Psychoactive properties of these compounds are interpreted by their structural similarity with the endogenous neurotransmitter glutamic acid and gamma-aminobutyric acid (GABA). Both compounds act probably as false neurotransmitters, ibotenic acid is an agonist of *N*-methyl-D-aspartate glutamate receptors, while muscimol is a potent $GABA_A$ agonist [9–12]. The psychoactive dose of ibotenic acid is about 30–60 mg and of muscimol is about 6 mg [13]. Other author estimated the psychoactive dose at 50–90 mg of ibotenic acid and 7.5–10 mg of muscimol [14]. Sufficient amount of alkaloids could be contained in one cap of *Amanita* [15, 16]. Besides ibotenic acid and muscimol, these mushrooms contain other active compounds, e.g. muscazone with minor pharmacological activity or highly toxic muscarine which, however, is not present in a significant amount [9].

The content of isoxazole alkaloids in *A. muscaria* and *A. pantherina* was examined by, e.g. Benedict et al. [17], Chilton and Ott [18], Repke et al. [19], Lund [20], Gore and Jordan [21] and Tsujikawa et al. [22, 23]. The method of isolation and identification of *Amanitas* toxins in human urine was described by Eugster et al. [7] and Merová et al. [24].

Mushroom poisoning is often proved by microscopic examinations of spores in the stomach or intestinal content. Authors of this article introduce an instrumental method of proving *A. pantherina* or *A. muscaria* poisoning. The objective of the present study was to prove and quantify muscimol and ibotenic acid in the urine of intoxicated persons by gas chromatography/mass spectrometry (GC/MS). The article describes the isolation and determination of ibotenic acid and muscimol from the urine of four persons intoxicated with *A. pantherina*.

Case reports

Case 1

A woman, 28 years old, ingested by mistake food with admixture of *A. pantherina*. Ninety minutes after ingestion, she suffered from hallucinations and vomiting. She was admitted to hospital. Gastric lavage was made and Carbosorb (activated charcoal) was instilled. It was necessary to give her artificial respiration. The condition of the patient was improving, and the next day she was feeling well.

Case 2

A man, 66 years old, mistook *A. pantherina* for edible *A. rubescens* and ingested food prepared from it. After eating he began to suffer from dizziness. Without assistance he visited hospital. Gastric lavage was made and Carbosorb administered. The patient was conscious, undisturbed and cooperative. His respiration was efficient, blood pressure was 130/80, heartbeat was regular, sinus, 70 per min, pupils were isocoric and properly light responsive. After 2 h he left the hospital (on revers).

Case 3

A married couple, both 62 years old, ingested by mistake food prepared with an admixture of *A. pantherina*. After two and a half hours, they made an emergency call.

The woman was admitted to hospital. She suffered from nausea, vomiting and hallucinations. Lactulosum and Carbosorb were administered. Forced diuresis was carried out. At this time, she was able to communicate, her hallucinations were retreating, respiration was efficient and pupils were isocoric and properly light responsive. Her heartbeat was regular, sinus, 70 per min and blood pressure normal. The condition of the patient was improving, and the next day she was feeling well.

The man visited hospital 6 h after intoxication, and he brought the rest of the food with him. He was talkative, agitated, slightly incoherent, eupnoeic and his pupils were isocoric and properly light responsive. He had one time diarrhoea and no vomit. His heartbeat was regular, sinus, 70 per min and blood pressure was 150/80. His subjective feeling was as if he was drunk. He left the hospital after medical examination (on revers). In all three cases, *A. pantherina* poisoning was proved by a microscopic examination of spores in the gastric contents and in case 3 also in the rest of the food.

Materials and methods

Chemicals and reagents

Ibotenic acid monohydrate from Amanita sp. (Fluka, >99%), muscimol hydrobromide (Sigma, >98%), D-cycloserine (Fluka, >96%), exchanger Dowex 50W X8 (Fluka, p.a.) and ethyl chloroformate (Fluka, purum) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pyridine (p.a.) and dichloromethane (p.a.) were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (p.a.), ethanol (p.a.) and sodium hydroxide (p.a.) were obtained from Penta (Prague, Czech Republic). Saline (for injection) was obtained from Fresenius Kabi (Bad Homburg, Germany). Water was distilled in our laboratory.

Biosamples

Blank urine samples were collected from healthy volunteers. Authentic urine samples of patients intoxicated

with amanita mushrooms were submitted to our lab for diagnosis.

Preparation of standard solutions and suspension of exchanger

A solution of ibotenic acid, muscimol and cycloserine was prepared in distilled water in concentration of 1 mg/ml. The solutions were stored at −20°C.

A suspension of exchanger (Dowex 50W X8) was prepared by adding 1 g of exchanger to 2 ml of 0.1 N HCl. The mixture was shaken and the suspension which was ready to use was stored in the refrigerator.

Calibrators

Blank urine was spiked with the solution of cycloserine (internal standard) to reach a final concentration of 5 μg/ml. Further blank urine was spiked with the solution of muscimol and ibotenic acid to reach concentrations 1.0, 2.5, 5.0, 7.5, 10.0 and 15.0 μg/ml for both compounds.

Analysis of creatinine

Determination of the concentration of creatinine in urine was performed photometrically at 505 nm using Jaffe reaction.

Extraction and derivatization

Two hundred microlitres of the suspension of the exchanger was added to 500 μl of urine (or calibrator) with internal standard and mildly shaken. After 1 min of shaking, the mixture was centrifuged and the top aqueous layer was discarded. The exchanger was rinsed with 0.5 ml of the solution of 0.1 N HCl and ethanol (2:1). The mixture was again mildly shaken for 30 s and centrifuged. The top aqueous layer was discarded. To the rinsed exchanger were added 280 μl of ethanol, 500 μl of 1% solution of NaOH in saline and 670 μl of 8% solution of ethyl chloroformate in dichloromethane. The mixture was intensively shaken for 10 s, and then it was left standing 1 min. Then, 70 μl of pyridine was added and the mixture was intensively shaken for 10 s again. Then the mixture was left standing 1 min, centrifuged and the top aqueous layer was discarded. The organic layer was rinsed by adding of 1 ml of 1 N HCl. The mixture was intensively shaken for 10 s and then centrifuged. The bottom organic layer was transferred to another vial and evaporated by a stream of nitrogen at room temperature. The dry residue was dissolved with 50 μl of ethyl acetate, and 1 μl of solution was injected to GC/MS.

GC/MS conditions and settings

GC/MS analysis was carried out with the Thermo Trace DSQ system operated in full scan (FS, *m/z* 40–400) together with selected ions monitoring mode (*m/z* 115 for cycloserine, *m/z* 113 for muscimol and *m/z* 257 for ibotenic acid). The injection (1 μl) was splitless for 30 s at 220°C (injector temperature). The temperature of transfer line was set at 250° C and temperature of ion source at 200°C. A fused-silica capillary column HP-5MS UI 15 m/0.25 mm I.D./0.25 μm from HPST (Prague, Czech Republic) with the carrier gas (helium) set at 1.5 ml/min (constant flow) was used. The oven programme was as follows: initial temperature 70°C, held for 1 min, ramp 10°C/min to 170°C, then finally ramp 30°C/min to 300°C, held for 4.67 min. The total run time was 20 min.

Validation and calibration curves

The recovery of the extraction procedure was 80% for muscimol and 74% for ibotenic acid. The recovery was calculated from the peak area ratios of standard aqueous solutions without extraction procedure and spiked urine samples with extraction procedure (five replicas of standard aqueous solutions and spiked urines at concentration of 10 μg/ml for both alkaloids were used).

The limit of detection (LOD) 1 μg/ml for both alkaloids was estimated empirically by dilution of spiked urine. At LOD concentration, the difference of relative ions intensities were still less than 20% compared with relative ions intensities of the standard at concentration of 10 μg/ml. Also the imprecision at this concentration was within the limits of acceptability for the limit of quantification.

The calibration curves obtained for ibotenic acid and muscimol were linear across the range 1–15 μg/ml for both alkaloids. If the concentration of the alkaloids in the sample was higher than the concentration of the highest calibration standard, the sample was diluted with saline. The correlation coefficient (R^2) of the calibration curves was >0.99. To determine the imprecision and bias, nine replicate analyses were performed with spiked urine

Table 1 Variation data of the determination of muscimol and ibotenic acid in urine (CV (%), bias (%))

	At level 1 μg/ml		At level 10 μg/ml	
	CV (%)	Bias (%)	CV (%)	Bias (%)
Muscimol	14.6	13.1	11.5	−0.7
Ibotenic acid	14.9	15.9	12.0	16.5

CV coefficient of variation

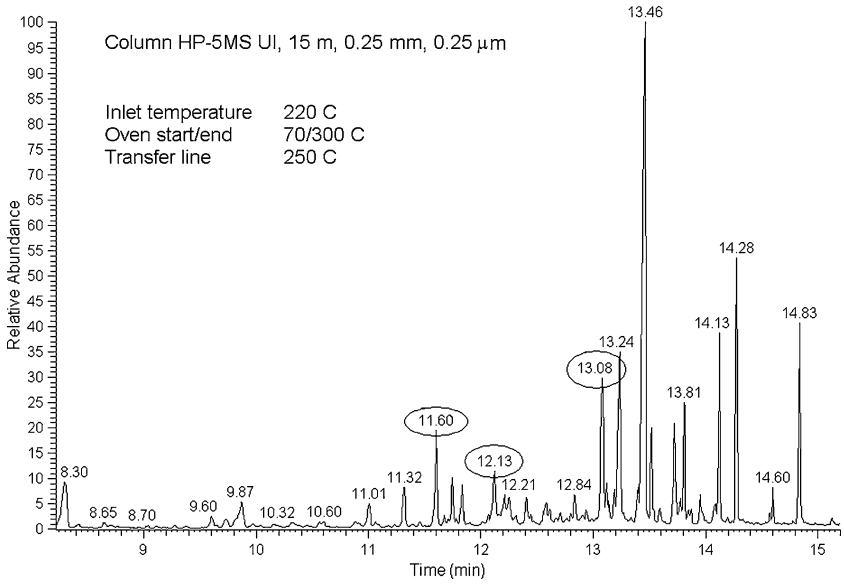

Fig. 1 Chromatogram of derivatized extract of urine recorded in the full scan mode. Retention time of internal standard (cycloserine) was 11:60 min, muscimol 12:13 min and ibotenic acid 13:08 min

samples at 1.0 and 10 µg/ml of muscimol and ibotenic acid. The coefficient of variation and bias are in Table 1.

The specificity was tested with urine of 15 healthy volunteers. The urine was measured and no interfering signals were found.

Results and discussion

Isolation of muscimol and ibotenic acid from the urine was performed on a strong cation exchanger. The elution and derivatization of the alkaloids were made in one step with a mixture of aqueous solution of sodium hydroxide with the addition of ethanol and pyridine and the solution of ethyl chloroformate in dichloromethane. In aqueous alkaline media, ethyl chloroformate reacts with amino group of muscimol and ibotenic acid, and the resulting carbamate derivative has satisfactory GC properties. The carboxylic group of ibotenic acid is esterified with ethyl chloroformate to ethyl ester. Pyridine is used as esterification catalyst. Derivatized alkaloids were extracted into dichloromethane. This method of derivatization is (in modifications) used in the analysis of amino acids, organic acids, amines and many other types of compounds [25–29].

For its structural similarity with isoxazole alkaloids, cycloserine was used as an internal standard (deuterated standards of ibotenic acid and muscimol were not available). The proof of the presence of ibotenic acid and muscimol in the tested samples was carried out by comparing the mass spectra and retention times (RT) of

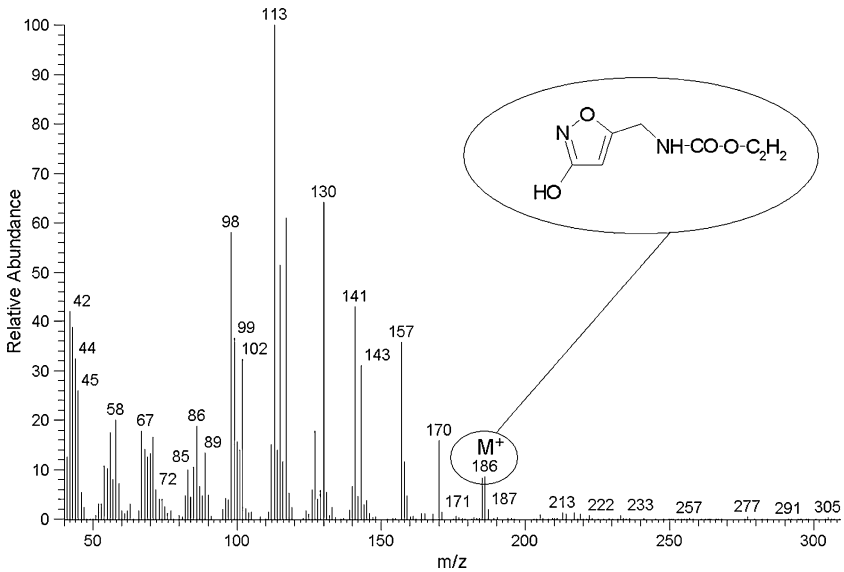

Fig. 2 Mass spectrum, molecular ion *m/z* 186 and chemical formula of derivatized muscimol

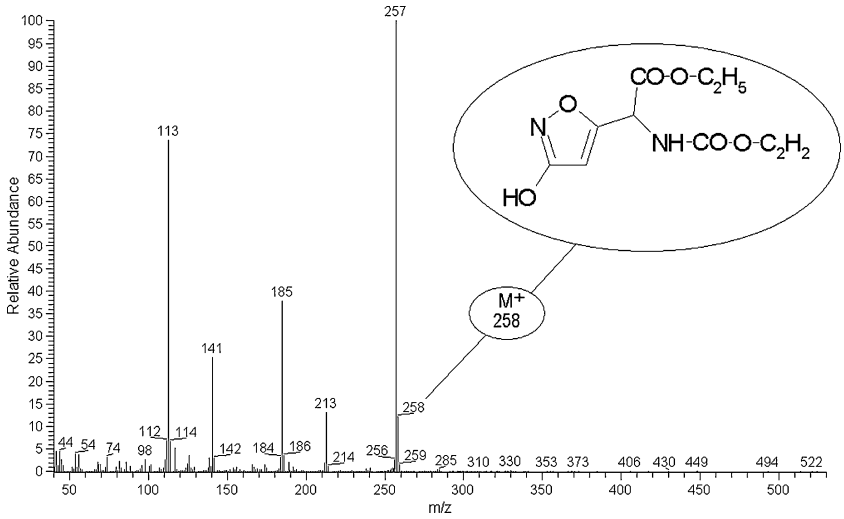

Fig. 3 Mass spectrum, molecular ion *m/z* 258 and chemical formula of derivatized ibotenic acid

the relevant peaks with the mass spectra and RT of the standards. GC/MS chromatogram of derivatized extract of urine recorded in the full scan mode is shown in Fig. 1 (RT of cycloserine was 11:60 min, muscimol 12:13 min and ibotenic acid 13:08 min). Mass spectra of derivatized muscimol and ibotenic acid are shown in Figs. 2 and 3.

By this method, ibotenic acid and muscimol were proved and determined in the urine of four people intoxicated with *A. pantherina*. Alkaloids were not proved in the serum of the cases. Nevertheless, it is probable that at the time of sample taking when the intoxicated people still felt the effects of *Amanita*, isoxazole alkaloids were present in the blood but their concentrations were under the limit of detection of the method. The results are summarized in Table 2.

The first effects of intoxication with *A. pantherina* and *A. muscaria* appear 30–120 min after the mushroom ingestion. The intoxication can resemble alcohol intoxication. Its symptoms are unsteadiness, dizziness, confusion, nausea, diarrhoea, hallucinations, disorientation in place and time, euphoria or depression, anxiety and mystical experiences. Severe intoxication can progress to coma; life-threatening respiratory and circulatory disorders may occur [30]. Fatal poisoning is rare. In most cases, recovery is complete after 24 h [9], similarly as in our cases.

Toxins responsible for the effects of *Amanita* are rapidly absorbed from the gastrointestinal tract and readily excreted in urine where they can be detected within 1 h after consumption [31]. In our cases, the found concentrations of isoxazole alkaloids in urine are significantly high. It seems that the found levels of isoxazole alkaloids do not correlate with the state of intoxication. For example, the woman in case 3 suffered from nausea, vomiting and hallucinations; nevertheless, the level of isoxazole alkaloids in her urine was lower than the level of alkaloids in the urine of her husband whose subjective feeling resembled alcoholic ebriety. After relating the found levels of isoxazole alkaloids to the concentration of creatinine, it seems that the results could correlate. But it should be kept in mind that the time between the ingestion and the sample taking was not the same in all cases.

Conclusion

By the present GC/MS method, ibotenic acid and muscimol were proved and determined in the urine of four people intoxicated with *A. pantherina*. This study is the first one (as far as we know) to quantify the amount of muscimol and

Table 2 Results of the authentic cases—concentration of IBO, MUS and CREA in urine and concentration of IBO and MUS in relation to creatinine concentration

Case report	IBO (μg/ml)	MUS (μg/ml)	CREA (mmol/l)	IBO/CREA	MUS/CREA	Sample was taken
Case 1	47.4	9.9	4.4	10.8	2.25	4 h after ingestion
Case 2	32.2	6.0	8.1	4.0	0.74	8 h after ingestion
Case 3—woman	37.3	7.6	5.9	6.3	1.3	3 h after ingestion
Case 3—man	55.2	7.4	9.4	5.9	0.8	6 h after ingestion

Also the time when urine was taken is reported

IBO ibotenic acid, *MUS* muscimol, *CREA* creatinine

ibotenic acid in the urine of intoxicated persons. The method is applicable to a diagnostic examination of intoxication with mushrooms containing muscimol and ibotenic acid. Its results can contribute to a more efficient treatment of patients and be useful in crime investigation.

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