ORIGINAL ARTICLE

Isolation and identification of the Amanita muscaria and Amanita pantherina toxins in human urine

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Abstract

OBJECTIVES: Ibotenic acid, muscimol and muscarine were recognized as responsible for psychotropic effects of *A. muscaria* and *A. pantherina*. Demand for their specific and sensitive identification and quantitation in biological material lead to effort to develop reliable analytical method, but satisfactory solution is still lacking. Presented article describes liquid chromatography-mass spectrometry method suitable for isolation and identification of principal toxins of *A. muscaria* and *A. pantherina* in urine.

METHODS AND RESULTS: Dedicated liquid chromatography-mass spectrometry method is reported. Technique consists of an extraction of analytes on Strata X-CW and Discovery SCX SPE cartridges and separation is achieved using a Gemini C18 column (150 mm × 2.0 mm, 5 micron) and 8 mM heptafluorobutyric acid as mobile phase. Detection at *m/z* 159 for ibotenic acid, *m/z* 115 for muscimol and *m/z* 174 for muscarine was used. Retention times and LODs are 2.6 min and 50 ng.ml^{-1} for ibotenic acid, 4.6 min and 40 ng.ml^{-1} for muscimol and 14.2 min and 3 ng.ml^{-1} for muscarine.

CONCLUSION: A sensitive and specific liquid chromatography-mass spectrometry assay was developed for analysis of principal toxins of *A. muscaria* and *A. pantherina* in urine. Method was found to be sufficiently sensitive and specific for analysis of urine of intoxicated patients.

Abbreviations

LC-MS	- liquid chromatography-mass spectrometry	SPE	- solid-phase extraction
GC-MS	- gas chromatography-mass spectrometry	LOD	- limit of detection
HPLC	- high performance liquid chromatography	m/z	- mass/charge
HILIC	- hydrophilic interaction liquid chromatography	t_R	- retention time
ESI	- electrospray ionization		

INTRODUCTION

Amanita muscaria [1–4] and *Amanita pantherina* [4–5] are psychotropic mushrooms growing in Europe, North America, Africa and Asia. Over the past years, experimenting with these mushrooms as well as with other natural psychotropic substances [6], especially among young drug addicts, has been brought to the foreground of interest again.

The principal toxins (Figure 1) are ibotenic acid [7,8], muscimol [9–12] (the decarboxylation product of ibotenic acid) and muscarine [13–15] together with other less significant toxicological compounds [1–4,16,17].

These toxins are very similar to neurotransmitters in the brain and they can cause hallucinogenic symptoms, together with dizziness, confusion, blurred vision, muscle spasm, tiredness, drowsiness and deep sleep [18–20]. Their toxicity was tested on animals: muscarine has a mouse LD_{50} (i.v.) of 0.23 mg.kg^{-1}, ibotenic acid has a rat LD_{50} (i.v.) of 42 mg.kg^{-1} and LD_{50} (p.o.) of 129 mg.kg^{-1}, resp. muscimol has a rat LD_{50} (p.o.) of 45 mg.kg^{-1} [21]. The toxic concentrations of these toxins in human serum and urine have not been published yet.

Although intoxications by these mushrooms are rarely lethal, it is important to determine them soon and initiate a medical treatment. The problem of today's clinical and forensic toxicology is the absence of an objective analytical method for identification and determination of the mushrooms toxins from blood, urine and gastric content. The isolation of these analytes from urine or blood is a complicated problem due to their chemical structures and complexity of biological matrices. At present, the diagnosis of poisoning is almost entirely determined by microscopic examinations.

Only a few reports dealing with determination of ibotenic acid, muscimol and muscarine in *Amanita muscaria* and *Amanita pantherina* have been published so far. For determination of these toxins in mushrooms performance using high-performance liquid chromatography (HPLC) [22], gas chromatography-mass spectrometry (GC-MS) [23, 24] and liquid chromatography-mass spectrometry (LC-MS) [25] was used. The determination of muscarine in mushrooms was performed using hydrophilic interaction liquid chromatography (HILIC) tandem mass spectrometry [26].

This article describes the analytical method available for the isolation and identification of muscarine, ibotenic acid and muscimol in human urine.

MATERIAL AND METHODS

Reagents and chemicals

Ibotenic acid and heptafluorobutyric acid were obtained from Fluka (Buchs SG, Schweiz), muscimol hydrobromide and muscarine chloride were obtained from Sigma-Aldrich (St. Louis, USA). High purity solvents were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade from Lach-Ner, Neratovice, CR.

Biological material

Urine is the most frequently collected material for toxicological analyses. In this study negative urine spiked with muscarine, ibotenic acid and muscimol as well as urine of intoxicated patients was used. Both negative urine from individual donors and collected urine samples show no interferences.

Instrumentation

LC-MS analysis was performed with a LCMS-2010A (Shimadzu, Japan) with an electrospray ionization (ESI) interface in the positive mode. Chromatographic separation was performed on a Gemini C18 column (150 mm × 2.0 mm, 5 micron) maintained at 30 °C. The mobile phase was 8 mM heptafluorobutyric acid with a constant flow rate of 0.2 ml.min^{-1}.

Standards and working solutions

Stock standard solutions (1.0 mg.ml^{-1}) were prepared by dissolving of ibotenic acid, muscimol and muscarine in water, and were stored at –20 °C.

Working standard solutions were prepared by dilution of stock solution with water to concentration of 100 µg.ml^{-1}. The working standard solutions were stored at 4 °C.

Isolation of Amanita toxins by solid-phase extraction (SPE)

SPE cartridges Spec Dau, Strata X, Strata X-C, Strata X-CW, Discovery WCX, Discovery SCX, Discovery MCAX, Strata Screen A, Strata AW and Discovery SAX were used for isolation of ibotenic acid, muscimol and muscarine from human urine. SPE cartridges Strata X-CW, resp. Discovery SCX provided the best results concerning to isolation properties.

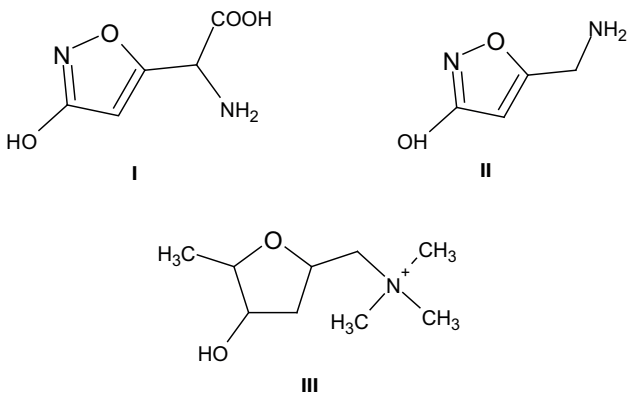

Figure 1. Chemical structures of ibotenic acid (I), muscimol (II) and muscarine (III)

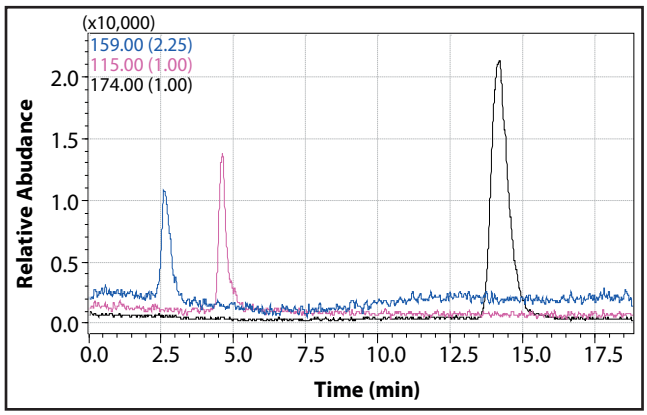

Figure 2. Chromatogram of a mixed standard of ibotenic acid (c= 2 μg.ml^{-1}, t_R 2.6 min), muscimol (c= 2 μg.ml^{-1}, t_R 4.6 min) and muscarine (c= 0.2 μg.ml^{-1}, t_R 14.2 min)

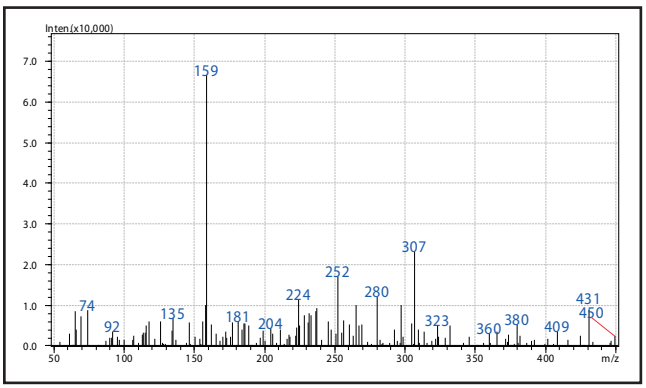

Figure 3. Mass spectrum of ibotenic acid

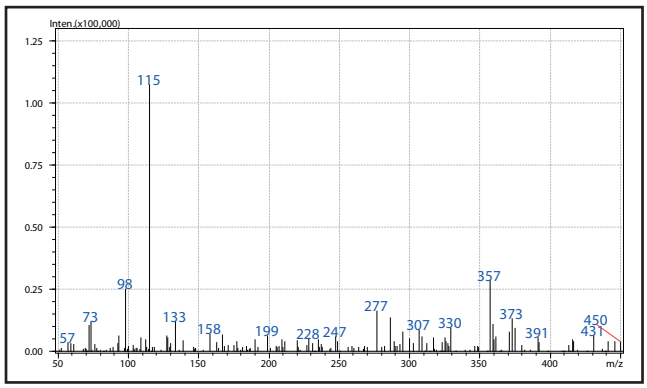

Figure 4. Mass spectrum of muscimol

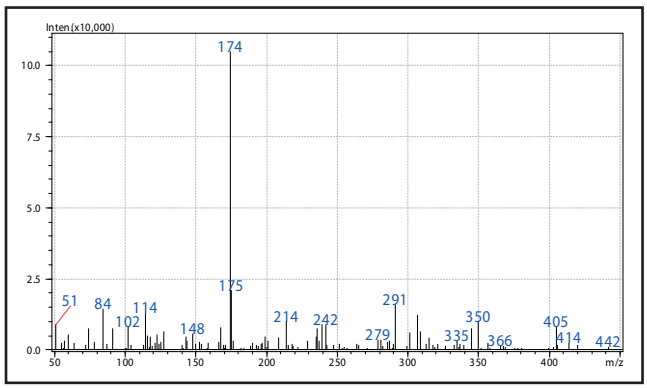

Figure 5. Mass spectrum of muscarine

Table 1. SPE cartridges and recoveries of ibotenic acid, muscimol and muscarine

	Recovery [%]		
SPE	**Muscarine**	**Ibotenic acid**	**Muscimol**
Spec Dau	0	0	0
Strata X	45	0	0
Strata X-C	40	0	0
Strata X-CW	90	0	0
Discovery WCX	55	0	0
Discovery SCX	0	15	22
Discovery MCAX	48	0	0
Strata Screen A	75	0	0
Strata AW	5	0	0
Discovery SAX	0	0	0

Table 2. LODs of ibotenic acid, muscimol and muscarine

Analytes	***m/z***	**LOD**
Ibotenic acid	159	50 ng.ml^{-1}
Muscimol	115	40 ng.ml^{-1}
Muscarine	174	3 ng.ml^{-1}

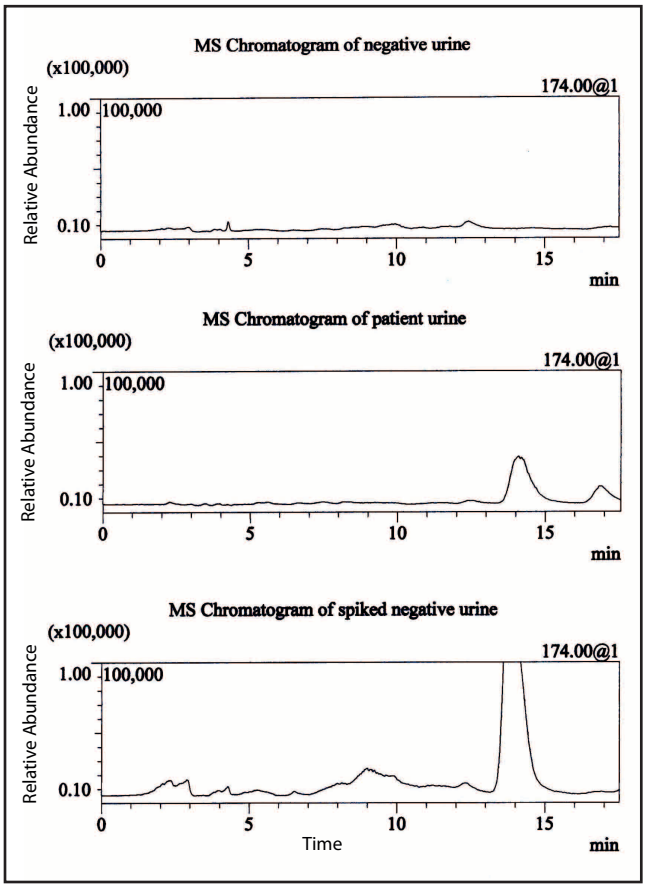

Figure 6. Chromatogram after extraction of a negative human urine, a patient urine and a negative human urine fortified with 0.2 μg.ml^{-1} muscarine

Isolation of muscarine by solid-phase extraction (SPE)

Human urine (1.0 ml) spiked with suitable amounts of a muscarine solution was poured into 1.5 ml tubes and acidified with 20 µl of phosphoric acid. After vigorous vortex-mixing for 1 min and centrifugation at 3 000 g for 10 min, the supernatant was transferred into Strata X-CW cation-exchange SPE cartridge (60 mg, 3 ml) preconditioned with 2 ml of methanol and 2 ml of 0.1 M hydrochloric acid. After loading the urine sample, the cartridge was washed with 2 ml of acetate buffer (pH 4.5). Then, muscarine was eluted into 4 ml tubes with 2 ml of 5% ammonium hydroxide solution in methanol. The eluate was evaporated under nitrogen at the room temperature. The dry residue was dissolved in 0.1 ml of water and 5 µl were injected onto LC-MS system.

Isolation of ibotenic acid and muscimol by solid-phase extraction (SPE)

Human urine (1.0 ml) spiked with suitable amounts of ibotenic acid and muscimol solutions was poured into 6 ml tubes and 4 ml 0.5% formic acid was added. After vigorous vortex-mixing for 1 min and centrifugation at 3000 g for 10 min, the supernatant was transferred into Discovery SCX cation-exchange SPE cartridge (500 mg, 3 ml) preconditioned with 2 ml of methanol and 2 ml of 0.5% formic acid. After loading the urine sample, the cartridge was washed with 2 ml of 0.1 M hydrochloric acid and ibotenic acid and muscimol were eluted into 4 ml tubes with 2 ml of 7.5% formic acid in methanol. The eluate was evaporated under nitrogen at room temperature. The dry residue was dissolved in 0.1 ml of water and 5 µl were injected onto LC-MS system.

RESULTS AND DISCUSSION

Isolation step was found to be crucial for successful LC-MS analysis. Variety of SPE cartridges were used for isolation of ibotenic acid, muscimol and muscarine from human urine (Table 1). Solid phase extraction procedure on modified silica was elaborated and proved to give satisfactory results with regard to recoveries (Table 1) and extract purity. Due to different structures of substances of interest, two isolation procedures had to be developed. The first procedure was optimized to the isolation of muscarine. SPE on Strata X-CW cartridge yielded the best results. For isolation of ibotenic acid and muscimol the best results were obtained on Discovery SCX cartridge. Both procedures afforded pure extracts with sufficient recoveries of analytes (Table 1). Subsequent LC-MS analysis was performed on C-18 column with 8 mM heptafluorobutyric acid as mobile phase.

A typical chromatogram of the analytes is shown in Figure 2. Retention times of the peaks of ibotenic acid, muscimol and muscarine were 2.6 min, 4.6 min and 14.2 min, respectively. Individual peaks were well separated with no interfering peaks. Corresponding mass spectra are shown in Figure 3, 4 and 5. The base peaks of the mass spectra (ESI scan mode) were *m/z* 159 for ibotenic acid, *m/z* 115 for muscimol and *m/z* 174 for muscarine. Table 2 shows the respective *m/z* of ions, according to which the concentrations of the corresponding analytes were calculated for their recoveries and detection limits (LOD).

Reported method was used for analysis of 55-year-old man's urine, who consumed unknown amount of *Amanita muscaria*. In the man's urine, only muscarine was identified (Figure 6).

Spectra of human urine and urine spiked with muscarine are also presented (Figure 6). Method was proved to be sufficiently sensitive and specific for intended purpose.

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