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Simultaneous identification and characterization of amanita toxins using liquid chromatography-photodiode array detection-ion trap and time-of-flight mass spectrometry and its applications

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ABSTRACT

Rapid and accurate identification of multiple toxins for clinical diagnosis and treatment of mushroom poisoning cases is still a challenge, especially with the lack of authentic references. In this study, we developed an effective method for simultaneous identification of amanita peptide toxins by liquid chromatography coupled with photodiode array detection and ion trap time-of-flight mass spectrometry. The accuracy and selectivity of the methodology were validated through similar multiple fragmentation patterns and characteristic ions of standard α- and β-amanitin. The developed method could successfully separate and identify major toxic constituents in *Amanita* mushrooms. Two amatoxins and three phallotoxins were confirmed in a single run through their fragmentation patterns and characteristic ions, which can be used as diagnostic fragment ions to identify mushroom toxins in complex samples. Furthermore, the performance of the developed method was verified by using real biological samples, including plasma and urine samples collected from rats after intraperitoneal administration of toxins. Thus, the development methodology could be crucial for the accurate detection of mushroom toxins without standard references.

1. Introduction

Toxic *Amanita* mushrooms, which mainly contain three kinds of toxic components, namely, amatoxins, phallotoxins, and virtoxins, are very notorious for their lethal toxicity (Wieland, 1986). Following accidental ingestion of these mushrooms, some sudden and violent gastrointestinal symptoms may appear in 6–24 h, including abdominal pain, vomiting, and aqueous diarrhea (Karlson-Stiber et al., 2003). These symptoms can easily be misdiagnosed as classic gastrointestinal disorders resulting from common food poisoning if no convincing evidences of the toxins are uncovered. Death can eventually occur after several days of fulminant hepatic failure and renal damage (Karlson-Stiber and Persson, 2003). Amatoxins produced by *Amanita* spp., especially α- and β-amanitin, are the major lethal toxins. In addition, phallotoxins can cause gastrointestinal symptoms initially after ingestion (Santi et al., 2012). As various toxins produce evidently severe symptoms and death, their timely and accurate identification is essential, critical, as well as valuable for subsequent clinical diagnosis and treatment (Gonmori et al., 2011; Garcia et al., 2015).

A number of analytical methods have been developed so far to detect amanita toxins. Among them, liquid chromatography tandem mass spectrometry (LC–MS/MS) methods, such as LC coupled with multistage linear ion trap mass spectrometry (Filigenzi et al., 2007), LC coupled with triple quadrupole mass spectrometry (Xu et al., 2017; Zhang et al., 2016), LC coupled with time-of-flight mass spectrometry (Tomkova et al., 2015; Walid et al., 2010), and LC coupled with MALDI time-of-flight mass spectrometry (Kunio et al., 2012), are known to be powerful and reliable tools to detect amanita toxins owing to their high selectivity and sensitivity. In these LC–MS/MS methods, α- and β-amanitin have been used as references to analyze different samples, and their molecular ions have often been utilized to perform qualitative and quantitative analyses. However, other amanita toxins and their characteristic ions have not yet been investigated owing to the lack of references and other reasons, thus limiting the identification of mushroom poisoning cases. Furthermore, detection of only α- and β-amanitin in mushroom poisoning cases restricts the diagnosis because various toxins other than α- and β-amanitin may also be involved. Therefore, detection and identification of multiple toxins in samples can

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provide more convincible evidences to accomplish correct clinical diagnosis, especially with the current lack of standard toxins for the diagnosis of mushroom poisoning cases.

Liquid chromatography coupled with photodiode array detection and ion trap time-of-flight mass spectrometry (LC-PDA-IT-TOF-MS) is a promising tool for simultaneous detection of multiple toxins both quantitatively and qualitatively (Liang et al., 2010; Zhao, 2012). This technique has demonstrated adequate sensitivity to meet the qualitative analytical requirements (Lech et al., 2015; Xing et al., 2014). The most prominent application of IT-TOF-MS is qualitative analysis of unknown compounds in a complex system, elucidating their formulas and structures through the multistage fragments obtained by MS^n and determining their masses accurately by TOF, although sufficient authentic references for comparison are limited (Chen et al., 2011; Ogura et al., 2013). In this study, LC-IT-TOF-MS was used to analyze multiple amanita toxins in biological samples without authentic references.

Currently, there are only a few commercially available standard toxins as references, and their market price is expensive. This shortage has been a bottleneck for simultaneous detection of multiple toxins and clinical diagnosis of increasing cases of mushroom poisoning. The aim of this study was to develop a simple analytical strategy to simultaneously identify amanita toxins using LC-PDA-IT-TOF-MS. A total of five amanita toxins were separated and identified in a single run according to their chromatograms and common multistage fragment ions. In addition, the developed method was also successfully applied to detect toxins in real samples.

2. Materials and methods

2.1. Chemicals and reagents

Both α- and β-amanitin standards were supplied by Alexis Corporation (San Diego, CA, USA). Acetonitrile and methanol (HPLC grade) were obtained from Tedia Company (USA). Formic acid and ammonium acetate (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was obtained using a Milli-Q integral water purification system (Millipore, USA). Dried *Amanita exitialis* were provided by Prof. Zuohong Chen (School of Life Sciences, Hunan Normal University, China) and kept in a dark, cool, and dry place.

2.2. Preparation of standard solutions and samples

The stock solutions (100 μg/mL) were prepared by dissolving α- and β-amanitin in methanol. A total of 10 μg/mL of working standard solution were prepared by diluting stock solutions with methanol. All the solutions were stored at −20 °C in the dark until use.

The crude extract of *Amanita* mushrooms was obtained using reflux extraction method. The dried mushrooms were homogenized into powder for 10 s using a high speed universal grinder (FW100, Tianjin Tester Instrument Co. Ltd., China). Subsequently, 5 *g* of the powder were added into a 100-mL two-necked flask containing 50 mL of water, boiled, and extracted for 3 h. The water decoction was centrifuged (10,000 × *g*, 15 min, 4 °C) and filtered through a microporous membrane filter (0.45 μm). Then, tenfold dilution of the filtrate was injected into LC-PDA-IT-TOF-MS for analysis. The rest of the supernatant was freeze-dried into powder and stored at −20 °C for subsequent experiments.

Animal studies were performed in accordance with the guidelines of the Institutional Animal Care Committee of Xi'an Jiaotong University, China. Sprague-Dawley rats (200 ± 15 *g*, 6–8 weeks), obtained from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China), were housed in an environmentally controlled breeding room with a temperature of 26 °C, fixed 12-h light-dark cycle, and free access to standard laboratory rodent chow. The animals were acclimatized for at least 3 days before the experiments. After intraperitoneal administration of crude extract of *Amanita* mushrooms (dissolved in normal saline) at a dose of 0.5 mg/kg (body weight, based on α-amanitin which its content in dried mushrooms was 1287.26 μg/g calculated by the external standard method), 500 μL of blood were sampled via orbital veins under anesthesia at 15 and 120 min and transferred into heparinized tubes. Then, the blood samples were centrifuged at 5000 × *g* (4 °C) for 15 min to obtain plasma. Besides, urine sample was collected through metabolism cage for 4 h. A total of 200 μL of plasma and urine samples were respectively mixed with 800 μL of acetonitrile. Each of the sample was sonicated for 30 min at 4 °C and centrifuged for 15 min at 12,000 × *g* and 4 °C. The supernatant was evaporated to dryness using a gentle stream of nitrogen at room temperature, and the residue was redissolved in 200 μL of methanol by vortexing. The final solutions were filtered through a Millipore filter (0.22 μm) and subjected to LC-PDA-IT-TOF-MS analysis.

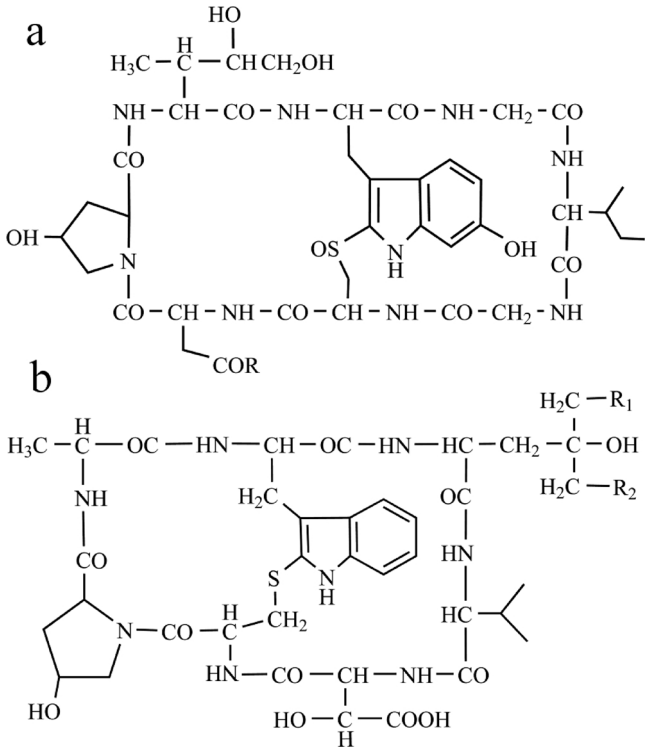

Fig. 1. Structures of amatoxins (a: R = NH_2, α-amanitin; R = OH, β-amanitin) and phallotoxins (b: R_1 = H, R_2 = H, phallacin; R_1 = OH, R_2 = H, phallacidin; R_1 = OH, R_2 = OH, phallisacin).

2.3. Instrumentation conditions

The LC-PDA-IT-TOF (Shimadzu Corporation, Japan) was equipped with a SPD-M20 A photodiode array detector, two LC-20AD binary pumps, an online DGU-20A3 degasser unit, a SIL-20ACXR autosampler, a CTO-20AC column oven, and a hybrid ion trap and time-of-flight mass spectrometer. Chromatographic separation was performed on an Inertsil ODS-3 column (100 × 2.1 mm i.d., 3-μm particle size, Shimadzu Corporation, Japan) with a column oven temperature of 40 °C. The mobile phase was delivered at a total flow rate of 0.2 mL/min with the constituents of 20 mM ammonium acetate containing 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The gradient elution program was as follows: 10% B for the first 5 min, 10%–20% B from 5 to 15 min, 20%–50% B from 15 to 20 min, 50%–90% B from 20 to 25 min and held for 3 min, and 90%–10% B from 28 to 28.5 min and held for 3.5 min. After each run, the column was reequilibrated for 5 min before next injection. The PDA spectra were acquired over a scan range of 190–400 nm, and the injection volume was set at 10 μL.