Detecting Mushroom Peptide Toxins in Body Fluids by Capillary Electrophoresis

Deadly poisonous mushrooms of the genus *Amanita* produce cyclic oligopeptide toxins. The author extracted these toxins from human body fluids with organic solvents and determined their presence by a capillary electrophoresis method. He separated the toxins with pH 6.8 phosphate buffer at room temperature. The efficiency of his *Amanita* toxins isolation technique was 70–75%, and the detection limit was 40–50 ng of the toxins in the sample.

The Death Cap mushroom (*Amanita phalloides*) causes more than 50% of all mushroom poisonings. The *Amanita* genus accounts for 95% of related fatalities. *A. phalloides, A. virosa, and A. verna* are the predominant European poisonous mushrooms. Several other *Amanita* species — *A. brunnescens, A. ocreata, and A. bisporigera* — are more prevalent in North America than *A. phalloides. A. bisporigera* appears to be the most toxic American species (1). Mushrooms of genus *Amanita* produce three classes of toxic oligopeptides: amatoxins (the group of nine similar in structure to bicyclic octapeptides), phallotoxins (the group of six bicyclic heptapeptides), and virotoxins (the recently discovered five individual substances of the monocyclic heptapeptides group) (2).

The amatoxins are slow-acting poisons, in that their lethal interval is at least 15 h; however, they are 10–20 times more toxic than phallotoxins and virotoxins. The doses at which the probability of causing death is 50% (LD_{50}) are in the range of 0.15–0.5 mg/kg in mice. Amatoxins cause cell necrosis, especially in the liver and the kidneys, which leads to death in humans in 5–7 days. Because all symptoms described for lethal poisonings with *A. phalloides* mushrooms likewise can be produced by pure amatoxins, researchers have concluded that amatoxins are the sole cause of fatal human poisonings. These toxins are treacherous because the first symptoms of intoxication arise after irreversible conjugation of toxins with receptors. In that situation, the treatment of victims is ineffective (3).

The phallotoxins act relatively quickly — lethal intervals occur within 1–2 h and LD_{50}s are in the range of 1.5–2.5 mg/kg in mice. The phallotoxins bind strongly to F-actin, especially in liver cells, which results in high stability of the conjugate against depolymerization and degradation (3).

The recently discovered virotoxins, which were observed only in *A. virosa,* are somewhat related to the phallotoxins in chemical structure and are very similar to them in toxicity and biological action (3).

Some analytical assays were applied for the detection and determination of the *Amanita* toxins. Highly sensitive and very specific analytical methods such as ribonucleic acid (RNA)–polymerase competition binding assay (4) and radioimmunoassay (5) are used for amanitin analysis. Researchers also have described the application of high performance liquid chromatography for *Amanita* toxins analysis (6–8).

One of the modern, highly sensitive, and very specific analytical methods that allow users to separate compound mixtures and simultaneously identify each component is capillary electrophoresis (CE). During the past few years, CE has become the method of choice for this kind of analysis. Some researchers already investigate of *Amanita* toxins in urine and in mushroom bodies (9).

Yuri Bidnychenko
Department of Toxicological and Analytical Chemistry, Lviv State Medical University, Pekarska Street, 69, Lviv 79010, Ukraine, e-mail bidnyuri@meduniv.lviv.ua

Early on, I studied the electrophoretic properties of *Amanita* toxins using electrophoresis in polyacrylamide gel (10). In my research, I applied electrophoresis separation to *Amanita* toxins. The aim of my research was to establish methods of toxicological and forensic chemical examination of poisonings by genus *Amanita* mushrooms.

In my work, I studied the possibility of isolating *Amanita* toxins from human body fluids and developed a CE method for the identification and quantitative determination of these toxins in the internal fluids of poisoned persons. My goal was to establish a reliable diagnostic method of clinical investigations to be used in the Ukraine. (In the Ukraine, 1067 poisoning cases with 129 deaths, including 43 children, occurred in 2000.)

Experimental

I worked out a technique of isolating *Amanita* toxins from the blood and urine of persons poisoned with the genus *Amanita* mushrooms. To imitate a food poisoning case, I also infused the samples of blood and urine with mixtures of extracts from genus *Amanita* mushrooms. All researched mushrooms were collected during 1995–1998 in the woods of western Ukraine.

I prepared the extracts from the *A. phalloides* and *A. verna* mushrooms by homogenizing 50 g of air-dried mushrooms with an equal amount of water in a mixer. After centrifugation, I washed the pellet with the same amount of water and added the washings to the extract. I used the supernatant to represent artificial poisoning of the biological material.

For modeling a poisoning, I added 0.1 mL of a mushroom extract to 10 mL of the biological fluids. To compare the biological fluids, I also mixed the samples with individual toxin standards. These mixtures were stored for one day and investigated as described below.

Isolation of toxins from urine: I placed 10 mL of urine in a separatory funnel and added 20% sulfuric acid solution to adjust the pH level to pH 2. I added 10 mL of *n*-butanol to the acidified urine and shook the mixture for 5 min. After phase division, I poured a butanolic extract into a porcelain cup and either dried the sample in a boiling water bath or evaporated it in a vacuum. I dissolved the residue in pH 6.8 phosphate buffer (≈ 5 mL) and analyzed it by CE.

Isolation of toxins from blood: I shook 5 mL of blood for 10 min with 5 mL of acetonitrile that was acidified by a 20% solution of sulfuric acid to pH 2 and then centrifuged the mixture. The supernatant was evaporated in a vacuum, and the residue was dissolved and adjusted to a volume of 5 mL in phosphate buffer (pH 6.8). Then, I analyzed the blood sample by CE.

CE analysis: The CE analysis was performed using a Beckman Coulter P/ACE 2000 system (Fullerton, California). I developed a CE method for protein analysis that used simultaneous filtration and cleanup with a high-ionic-strength 0.05 M phosphate running buffer (pH 6.8). I used a 57 cm × 75 μm capillary (Beckman Coulter). I used UV-absorbance detection at 280 nm for compatibility with the UV-absorption maxima of the researched substances. The applied voltage was 5 kV, and the sample temperature was 25 °C. The injection volume for all samples was 5 μL.

The calibration curve of peak area versus injected *Amanita* toxin quantity was constructed for five repeated injections of five standard solutions of the toxins. The curve was linear in the 0.01–0.2 μg (10–200 ng) range.

The 0.1-mg/mL stock solutions were prepared by dissolving 1.0000 mg of suitable toxin, exactly weighed, in phosphate buffer (pH 6.8) and diluted to volume in a 10.0-mL volumetric flask. Convenient working standards were prepared by diluting this solution with phosphate buffer (pH 6.8) to the required volumes. I obtained a standard curve by using five phosphate buffer (pH 6.8) dilutions of the standard substances: α-amanitin, β-amanitin, and phalloidin (all from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany); phalloin (extracted in-house from *A. phalloides* using the technique described in reference 11); and viroidin and viroisin (extracted in-house from *A. virosa* mushroom bodies using the technique described in reference 12).

The method sensitivity and isolation efficiency were determined by artificially poisoning biological fluids with the standard solutions of the appropriate toxins. I added 0.05, 0.1, or 0.2 μg of the toxins to 10 mL of samples. I processed additional samples as described above. Figure 1 shows a mixture of equal amounts of the convenient standards.

Samples of blood and urine were collected from people poisoned with *Amanita* mushroom during 15–32 h after their poisoning. I prepared these samples and tested them as described above. I was able to detect the presence of the toxins and even identify the species of the poisoning mushroom.

Mushrooms of the species *A. phalloides* produce amatoxins and phallotoxins. Figure 2 shows a blood sample of a person poisoned with *A. phalloides*. *A. virosa* also contains virotoxins, which are biochemical predecessors of phallotoxins. Therefore, I detected the virotoxins present in fluid samples of people poisoned with *A. virosa* (Figure 3). To confirm my supposition, I investigated the donor's urine simultaneously poisoned with extract from *A. virosa* mushroom body (Figure 4). I identified acidic phallotoxins and virotoxins in both samples.

I used two quantitative techniques — a calibration plot and a standard additions method — to analyze real samples of blood and urine, collected during tests of poisoning victims. Those methods allowed me to judge the accuracy of determination and avoid incorrect toxin identification.

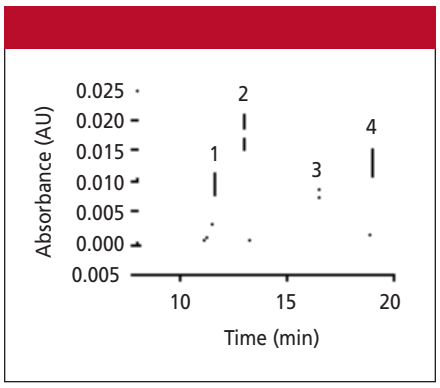

Figure 1: Separation of amanitins and phallotoxins. Capillary: 57 cm × 75 μm; buffer: 0.05 M phosphate (pH 6.8); separation voltage: 5 kV; temperature: 25 °C; detection wavelength: 280 nm; analyte amount: 40 ng each. Peaks: 1 = β-amanitin, 2 = phalloin, 3 = α-amanitin, 4 = phalloidin.

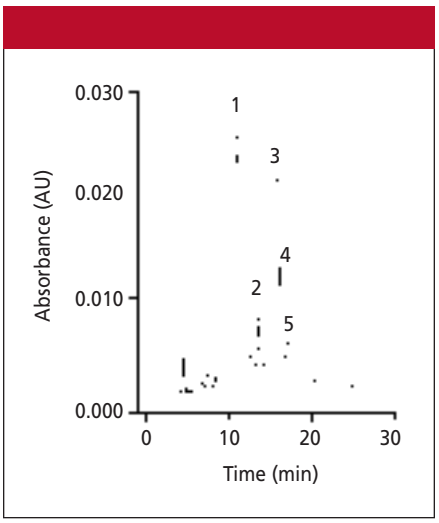

Figure 2: CE separation of a blood sample from a person poisoned with *Amanita phalloides*. Conditions were the same as in Figure 1. Peaks: 1 = β-amanitin, 2 = phalloin, 3 = α-amanitin, 4 = phallolisin, 5 = phalloidin.

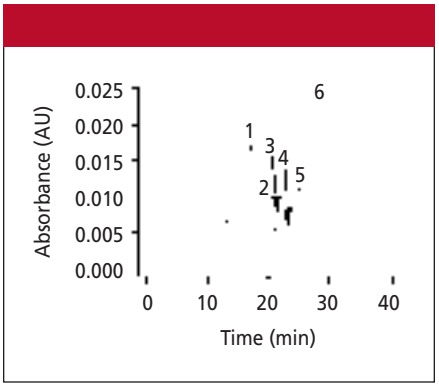

Figure 3: Electropherogram of a urine sample from a person poisoned with *Amanita virosa*. Conditions were the same as in Figure 1. Peaks: 1 = phalloin, 2 = α-amanitin, 3 = phalloidin, 4 = phallacidin, 5 = viroisin, 6 = viroidin.

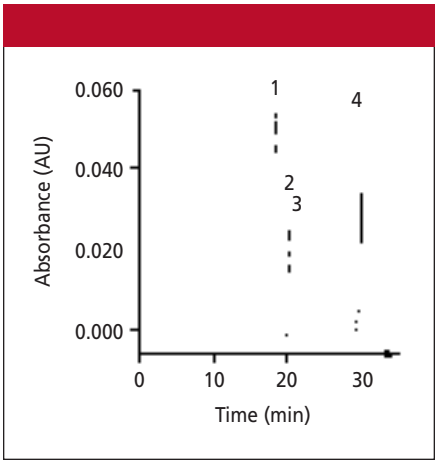

Figure 4: CE separation of a urine sample artificially poisoned with *Amanita virosa* mushroom extract. Conditions were the same as in Figure 1. Peaks: 1 = phalloin, 2 = α-amanitin, 3 = phalloidin, 4 = viroidin.

Results and Discussion

In my studies, I developed a simple method of isolating *Amanita* mushroom toxins from biological liquids such as blood and urine. Using CE, I was able to identify the toxins of *Amanita* genus mushrooms — *A. phalloides* and *A. virosa* — in examined samples. This isolation method enabled me to recover as much as 70–75% and 78–82% of the *Amanita* toxins, respectively, from the artificially poisoned blood and urine samples.

The in-sample sensitivity of the CE method was 15 ng for phallotoxins (phalloidin and phalloin) and virotoxins (viroidin and viroisin) and 10 ng for amanitins (α-amanitin and β-amanitin). The limit of detection for *Amanita* toxins in 5 mL of blood was 50–60 ng, and the limit of detection for *Amanita* toxins in 10 mL of urine was 40–45 ng. The linear range of measurement for toxins in a sample was 10–130 ng. The error of *Amanita* toxins determination was 2.3% for standard solutions and 7.6% for simulated poisoning samples.

Conclusion

I used a high-efficiency technique to isolate toxins of the genus *Amanita* mushrooms from human body liquids. The isolated cyclopeptide toxins could be qualitatively and quantitatively determined in blood and urine by CE assay. The sensitivity and accuracy of this determination technique were sufficient for toxicological and forensic investigation of poisoning cases.

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