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Determination of α- and β-amanitin in clinical urine samples by Capillary Zone Electrophoresis

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ABSTRACT

Amanitins are toxins found in species of the mushroom genera *Amanita*, *Lepiota* and *Galerina*. Intoxication after ingestion of these mushrooms can be fatal with an estimated 20% of mortality rate. An early diagnosis is necessary in order to avoid invasive and expensive therapy and to improve patient's prognosis. In this paper, a Capillary Zone Electrophoresis method was developed and validated to determine α- and β-amanitin in urine in less than 7 min using 5 mM, pH 10 borate buffer as background electrolyte. The separation conditions were: capillary: 75 μm I.D., 41 cm effective length, 48 cm total length, 25 °C, 20 KV and PDA detection at 214 nm. Sample treatment for analysis only required urine dilution in background electrolyte. The method was validated following established criteria and was found to be selective, linear in the range 5–100 ng/ml. Intra- and inter-day precision and accuracy were within required limits. Limit of detection (LOD) and limit of quantification (LOQ) were 1.5 and 5 ng/ml, respectively. Eight urine samples from suspected cases of intoxication with amanitins were analyzed after 2 years of storage at −20 °C, and β-amanitin was determined in two samples with concentrations of 53 and 65 ng/ml, respectively. The method here described includes the use of non-aggressive reagents to the capillary or the system and is the first Capillary Electrophoresis method used to determine amanitins in clinical samples.

1. Introduction

α- and β-amanitin are fungal toxins occurring in some species of the genera *Amanita*, *Galerina* and *Lepiota* that are responsible for the most severe cases of poisoning by ingestion of the fruiting bodies of these mushrooms, with mortality rates up to 20% [1]. Amanitins are bicylcic octapeptides that induce deficient protein synthesis due to specific inhibition to RNA polymerase II. The target organs are intestinal mucosa, liver and kidney [2,3]. Intoxication produces cholera-like symptoms that generally appear after 12 h of ingestion. According to Iliev et al. [4] patients take an average of 32 h to receive first medical care in a medical institution. At this time, irreversible liver damage can occur and patient survival rate is low. An early detection and determination of amanitins is important in order to avoid invasive and expensive therapy such as liver transplantation [4], which is nearly impossible in Mexico due to lack of sufficient donors. An early diagnosis could contribute to eliminate the poison and to protect the liver from further damage [3,5–7].

Urine represents a valuable sample material for amanitin determination since patients in Mexico usually arrive at the hospital up to 4 days after ingestion. It is well known that after 12 h amanitins have already been eliminated from plasma but they remain to be detectable in urine until day 4 [8].

Several analytical methodologies have been applied to the analysis of α- and β-amanitin. Formerly, detection of amanitins was made by RIA [9–11] using ^{125}I-labeling, which has a short life and is not available in all hospitals. Haines et al. [12] used TLC to detect α- and γ-amanitin in body fluids from a patient that became unintentionally intoxicated with *Lepiota josserandii*. This TLC results were confirmed by RIA. Abuknesha and Maragkou [13] used com-

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petitive ELISA to measure β-amanitin in human serum and urine. The assay had a detection limit of 80 pg/ml, a dynamic range of 80–2000 pg/ml and a cross reactivity of 22% with α-amanitin. Butera et al. [1] also used ELISA to measure amanitins in urine as a diagnostic tool. Their cutoff values were 1.5, 5.0 and 10 ng/ml. Jaeger et al. [14] used HPLC to analyze toxins in plasma, urine, gastroduodenal fluid, feces and tissues. Maurer et al. [15] developed a liquid chromatographic–mass spectrometric assay for the determination of α- and β-amanitin in urine after immunoaffinity extraction. Enjalbert et al. [16] described a RP-HPLC that allows the simultaneous determination of up to eight amatoxins and phallotoxins including α- and β-amanitin. The system used included gradient elution with simultaneous monitoring at 214 and 295 nm. Levels of detection were in the range of 10 ng/ml and the system was applied to the analysis of a crude extract of *Amanita falloides*. Rieck and Platt [17] developed a RP-HPLC method to determine α-amanitin and phalloidin in human plasma using the column-switching technique. They established a quantitation limit of 10 ng/ml of plasma. Defendenti et al. [18] developed and validated a LC method with electrochemical detection to measure α-amanitin in urine samples after their pretreatment with double mechanism (reversed phase/cation exchange) solid phase extraction cartridges. Their limit of quantification (LOQ) was 10 ng/ml, a dynamic range of 10–200 ng/ml with an average recovery of α-amanitin of 78%. Validation parameters were obtained using spiked urine. Bruggemann et al. [9] reported a method to determine α- and β-amanitin in mushroom fruiting body extract and spiked urine by CZE. They used phosphate buffer and achieved the separation of both amanitins in 20 min with a detection limit of 1 μg/ml. This limit of detection (LOD) lies outside the clinical range which is ten to hundred times lower.

Himmelman et al. [19] reported a lethal *Amanita phalloides* intoxication case from stored mushrooms in the freezer for 7–8 months, which reveals the stability of amanitins under such conditions. Maurer et al. [15] measured the stability of amatoxins in frozen urine samples for up to 6 months.

There is only one published CE method to determine amanitins in urine of patients suffering from amanitin poisoning [9]. However, due to its lack of sensitivity, amanitins could not be quantified.

Although there are several methods that can be used to determine amanitins in biological samples each one have drawbacks. LC–MS instrumentations are not available in all laboratories and not all methods fulfill the requirements of sensitivity, accuracy, specificity, simplicity of performance and rapidity; some methods proposed to determine amanitins use toxic substances such as ethidium bromide [20]. In this paper, a CZE method is developed to measure α- and β-amanitins in urine samples of suspected cases of amanitin intoxication. In this method an extraction step is not required since the amanitins can be detected without the interference of urine compounds; it only requires sample dilution in the background electrolyte (BGE). The method was validated according to established criteria [21]. The urine of patients was preserved for over 2 years at −20 °C and, β-amanitin was detected in some samples at an average concentration of 60 ng/ml.

2. Experimental

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Potassium phosphate monobasic, sodium tetraborate, phosphoric acid (85%), hydrochloric acid (37%) and sodium hydroxide were supplied by JT Baker (Phillipsburg, NJ, USA). α- and β-amanitin were purchased from Sigma (St. Louis, MO, USA).

2.2. CE analysis

Borate buffer solutions covering the pH range 8–10 were prepared at the following concentrations: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mM. pH was adjusted by adding 1 M NaOH or 1 M HCl. Phosphate buffer solutions at pH 2.4 (100 mM) and at pH 7 (5, 30, 50 mM) were also prepared. Stock solutions of each amanitin were prepared independently in methanol at 500 μg/ml.

2.3. Biosamples

Blank urine samples were collected from healthy volunteers. Authentic urine samples from suspected intoxication cases were provided by the laboratory of the Children's Hospital "Eva Samano" Morelia, Michoacan, Mexico. All urine samples were filtered with an Acrodisk of 0.45 μm (Pall Gelman No. 4556B), prior to dilution in BGE, and a 10 μl aliquot was diluted in 200 μl of BGE.

2.4. Instrument

The experiment was performed on a Beckman P/ACE MDQ Glycoprotein System (Beckman Instruments, Fullerton, CA, USA). A 48 cm × 75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was inserted in a capillary cartridge, thermostated at 25 °C (±0.1 °C). Standard solutions and samples were injected hydrodynamically at 35 mbar for 5 s. Experiments were conducted under normal polarity, applying a voltage of 20 KV during the electrophoretic separations. The detection window was located at 41 cm from the inlet of the capillary. A photodiode array detector was set at 214 nm. All data were recorded and analyzed by the Karat 32 software from Beckman (Beckman Instruments). New capillaries were preconditioned by consecutively flushing methanol for 5 min, deionized water for 2 min, 1 M HCl for 5 min, deionized water for 2 min, 1% NaOH for 10 min, deionized water for 2 min and BGE for 5 min, all at 1400 mbar. After each run the capillary was rinsed with 1% NaOH for 3 min, deionized water for 2 min and BGE for 5 min. A 5 s plug of BGE was used after each sample injection.

Buffer vials were replenished after each run to prevent changes in buffer composition and electrophoretic behavior.

2.5. Assay validation for urine analysis

The CE method was validated for the determination of α- and β-amanitin in urine according to established criteria [20].

2.5.1. Preparation of analytical standards, calibration standards and control samples

Portions of the stock solutions were diluted with BGE to prepare the analytical standard solutions that were used to spike urine preparations for the calibration standards and quality controls. Calibration standards (10–100 ng/ml) and quality control samples (10–100 ng/ml) of α- and β-amanitin were prepared using pooled blank urine from independently prepared analytical standard solutions. All solutions were stored at 4 °C.

2.5.2. Peak purity and selectivity

Ten blank urine samples from healthy volunteers were analyzed for peaks that could interfere with the detection of analytes.

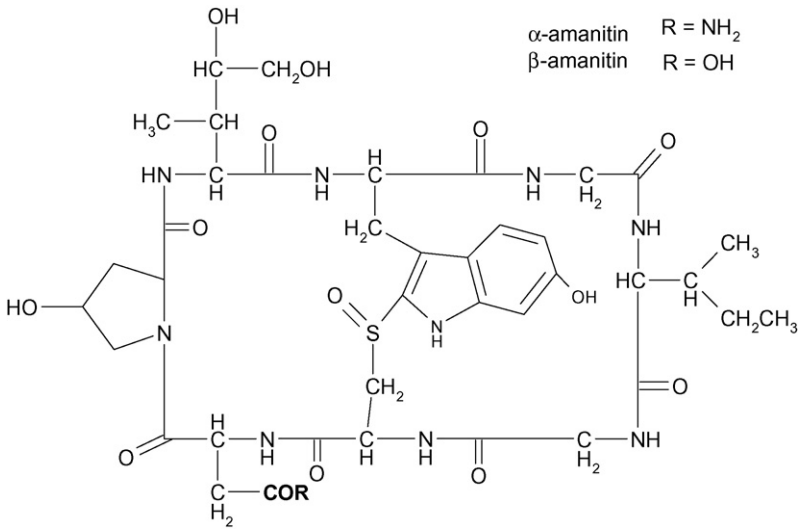

Fig. 1. Chemical structure of α- and β-amanitin.

2.5.3. Linearity of calibration

Calibration standards with concentrations of 5, 10, 25, 50, 75 or 100 ng/ml of α- and β-amanitin were assayed directly without prior extraction ($n = 5$).

2.5.4. Repeatability

Control samples of α- and β-amanitin were independently prepared, five at low concentrations (10 ng/ml, LOW) and five at high concentrations (75 ng/ml, HIGH), which were combined and directly analyzed. Each solution was injected ten times randomly.

2.5.5. Accuracy and precision

Spiked control samples of α- and β-amanitin ($n = 5$) at each of the three concentrations (10, 50, 75 ng/ml) were assayed against a calibration curve to determine the intra-day accuracy and precision. The analyte concentrations were calculated by using a linear regression analysis and these concentrations were compared to the nominal concentrations. The calculated values at each concentration were averaged and the percentage bias calculated to estimate accuracy. The relative standard deviation (R.S.D.) was calculated to estimate precision. The inter-day accuracy and precision of the method was assessed from the comparison of the analysis of control samples over 5 consecutive days with the above-mentioned method.

2.5.6. Limits

Quality control samples with 2.5 and 5 ng/ml of α- and β-amanitin were assayed ($n = 5$) for the determination of the limit of detection (signal-to-noise ratio 3:1) and of the limit of quantification (signal-to-noise ratio 10:1).

2.5.7. Proof of applicability

Eight urine samples from suspected cases of amanitins intoxication were assayed using the method here described.

3. Results and discussion

3.1. Optimization of the principal parameters

Bruggeman et al. [9] published a CZE method using phosphate buffer of pH 2.4 as the BGE. In this work, the use of a low pH buffer caused the capillary ends inserted in the acidic BGE to become brittle and break due to the corrosion of the polyimide coating. Besides, α- and β-amanitin peaks were not sharp enough. Based on their chemical structures (Fig. 1), it was instead decided to use a basic pH. A tetraborate buffer was chosen since it gave better results without causing current changes or falls. The only structural difference between α- and β-amanitin is the R substituent (see Fig. 1); therefore, α-amanitin is neutral whereas β-amanitin is acidic. Consequently, at basic pH conditions β-amanitin is negatively charged and migrates slower than α-amanitin. The buffer concentration was varied from 5 to 60 mM and the pH from 8 to 10. The peak resolution values obtained were larger than using a 5 mM borate buffer, pH 10. The migration time increased as the buffer concentration increased and the opposite effect could be observed for the electrophoretic mobility (Fig. 2). The results of the analyses were obtained in less than 5 min, enough time to observe both analytes and any possible interference from the urine matrix. The effect of buffer pH and concentration on the reproducibility of the migration time, peak area and peak height was measured. It was found that the R.S.D. was <0.5% for migration time, <2% for peak area and <1.5% for peak height, for both amanitins. In all cases, $n = 50$. Thus the background electrolyte used for validation and analysis was 5 mM borate pH 10 solution since it produced better peak shapes and resolution. Fig. 3 shows the separation of α- and β-amanitin in a spiked urine sample as well as a blank urine sample.

Methods currently available in the literature use either sophisticated sample preparations in order to obtain amanitins for analysis,

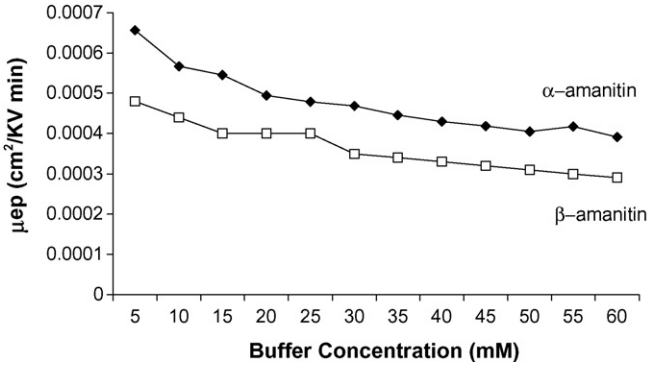

Fig. 2. Effect of borate buffer concentration on the electrophoretic mobility of α- and β-amanitins. Borate buffer pH 10. Capillary 48 cm (41 cm to detector) × 75 μm I.D. Applied voltage: 20 KV. $T = 25 \pm 0.5$ °C. PDA detection at 214 nm.

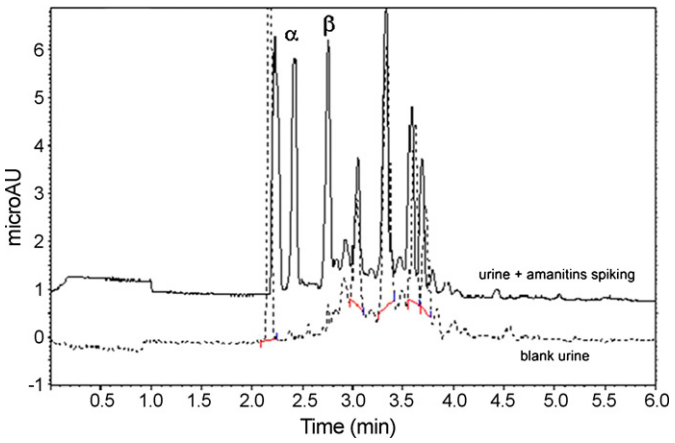

Fig. 3. Electropherograms of a blank urine sample and a urine sample spiked with 5 μg of α- and β-amanitins. Running electrolyte: 5 mM borate buffer pH 10. Other conditions as in Fig. 2.

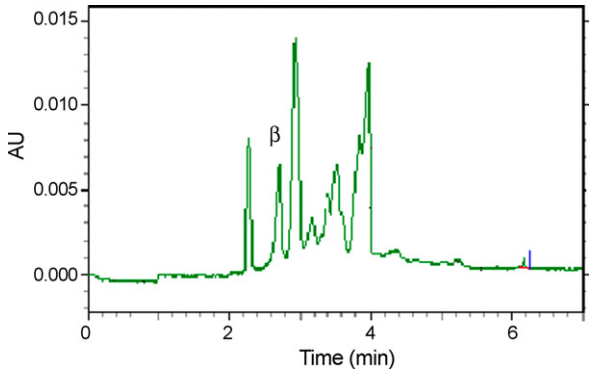

Fig. 4. Electropherogram of a clinical sample containing 53 ng/ml of β-amanitin.

or, detectors that are expensive and are not available in all laboratories [15,18,22,23]. By the method presented here, urine dilution in the BGE was the only necessary step for sample preparation, and detection was performed by a PDA; conditions which confer an advantage to the CZE method over previously reported procedures.

3.2. Method validation

As shown in Fig. 2, no interference from endogenous compounds could be observed. Calibration curves for α- and β-amanitin were linear from 5 to 100 ng/ml with mean r^2 values of 0.9983 and 0.9963, respectively. The LOD (S/N 3:1) was 2.5 ng/ml and the LOQ (S/N 10:1) was 5 ng/ml. The latter is the lowest concentration used for the calibration curve. Both limits are similar to those established by other authors using LC–MS and are well below the limits obtained by Bruggemann et al. [9]. The repeatability was determined as described in the Experimental section; considering $n = 10$, the R.S.D. values for α- and β-amanitin are 6.5 and 7.2% for the LOW control, and 7.4 and 5.2% for the HIGH control. Tables 1 and 2 show the intra- and inter-day accuracy and precision for the quantification of the quality control samples.

3.3. Application

The analysis of eight authentic urine samples from suspected cases of amanitins intoxication showed peaks that could indicate the presence of amanitins. In order to confirm the suspected peak identities, urine samples were analyzed and the electropherograms recorded, then, samples were spiked with 50 ng of each α- and β-amanitin and their spectra compared both to the spectra produced by the pure standards and to those from the suspected peaks. No difference was observed between the three sets of peaks, concluding that only two samples were contaminated by β-amanitin (Fig. 4) with a toxin content that was calculated 53 and 65 ng/ml. These concentrations are in accordance with published data. The R.S.D. for migration time, peak area and peak height were 1.05, 4.07 and 3.7%, respectively, for the β-amanitin present in the samples. The R.S.D. values are smaller than those reported by Bruggeman et al. [9] for a dilute extract of *Amanita phalloides* (11.1 and 7.7% for peak area and peak height, respectively). It should be mentioned that patients were admitted to hospital and urine samples taken 4 days after mushrooms ingestion. Urine samples had been stored at −20 °C for over 2 years before CZE analysis and β-amanitin detection and quantification. Stability of amanitins was assessed in frozen urine [15] and in stored mushrooms [19]. In both cases no change was observed in amanitin content for up to 6–8 months. In this paper urine samples were not immediately analyzed and, despite having been stored for a long period of time, β-amanitin could still be

Table 1
Intra-day precision and accuracy of the CZE determination of α- and β-amanitin in urine

Intra-day ($n = 5$)	Nominal concentration (ng/ml)		Mean calculated concentration (ng/ml)		Precision (%)[a]		Accuracy (%)[b]	
	α	β	α	β	α	β	α	β
Low QC	10	10	10.35	10.5	2.5	3.1	3.5	5.0
Medium QC	50	50	49.68	50.89	2.2	2.7	−0.64	1.78
High QC	75	75	76.65	76.1	2.6	2.9	2.2	1.47

[a] R.S.D. = (S.D./Mean) × 100.
[b] ((Calculated concentration − Nominal concentration)/Nominal concentration) × 100.

Table 2
Inter-day precision and accuracy of the CZE determination of α- and β-amanitin in urine

Inter-day ($n = 5$)	Nominal concentration (ng/ml)		Mean calculated concentration (ng/ml)		Precision (%)[a]		Accuracy (%)[b]	
	α	β	α	β	α	β	α	β
Low QC	10	10	11.36	11.13	14.24	6.6	13.6	11.3
Medium QC	50	50	49.45	52.1	7.7	6.3	−1.1	4.2
High QC	75	75	80.42	79.24	4.5	3.0	7.23	5.65

[a] R.S.D. = (S.D./Mean) × 100.
[b] ((Calculated concentration − Nominal concentration)/Nominal concentration) × 100.

quantified. Since intoxicated patients in Mexico arrive at the hospital 3 or 4 days after mushroom ingestion, a time when it is difficult to measure amanitins in plasma, in these cases urine represents an ideal fluid for their determination and is easier to handle. Parant et al. [24] developed an ELISA method to determine α- and γ-amanitin in urine but their method is applicable only when the urine was collected within 36 h after amatoxin poisoning.

The method here presented is selective, repeatable, accurate and precise. It can be applied to diagnose amanitin intoxication within a few minutes since an extraction step is not required.

4. Conclusion

In this paper, a Capillary Zone Electrophoresis method was developed that is simple and applicable to the analysis of α- and β-amanitins in urine. The reagents and conditions used are non-aggressive to the capillary or system and non-contaminating solvents are used. The method met all the established validation criteria [21] and was applicable to the determination of β-amanitin in suspected cases of amanitin intoxication. Limits of detection here are below those established in other LC or even CE methods. Samples used to prove the applicability of the method are actual clinical samples and no spiking was necessary to determine the amanitin content.

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