Original article

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DETERMINATION OF MUSHROOM TOXIN ALPHA-AMANITIN IN SERUM BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY AFTER SOLID-PHASE EXTRACTION

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Alpha-amanitin is a cyclic peptide which belongs to a large group of mushroom toxins known as amatoxins. Being responsible for the majority of fatal mushroom poisonings, they require rapid detection and excretion from the body fluids. In accordance with these requirements, a simple and an accurate method was developed for successful identification and quantification of alpha-amanitin in serum with electrospray liquid chromatography–mass spectrometry (LC-ESI-MS) after collision-induced dissociation. The method conforms to the established International Conference on Harmonization Q2A/Q2B 1996 guidelines on the validation of analytical methods. Linearity, precision, extraction recovery and stability test on blank serum spiked with alpha-amanitin and stored in different conditions met the acceptance criteria. The obtained calibration curve was linear over the concentration range 5-100 ng/mL with a lower limit of quantification (LOQ) of 5 ng/mL and limit of detection (LOD) of 2.5 ng/mL. The mean intra- and inter-day precision and accuracy were 6.05% and less than ±15% of nominal values, respectively. The neutral solid phase extraction with copolymer hydrophilic–lipophilic balance cartridges was found optimal for sample preparation with the mean recovery of 91.94%. The proposed method demonstrated high sensitivity and selectivity which can be useful both for clinical and forensic toxicology analysis of alpha-amanitin at low concentrations. *Acta Medica Medianae 2015;54(1): 12-19.*

Key words: *alpha-amanitin, liquid chromatography-mass spectrometry, solid phase extraction, serum*

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Introduction

Alpha-amanitin belongs to a large group of protoplasmic mushroom toxins known as amatoxins; alpha-, beta-, gamma-, epsilon-amanitin, amanin and amanullin. Structurally, alpha-amanitin represents bicycle octapeptides with substantial toxicity effect (Figure 1) (1,2). Mush-room species like Amanita (A. phalloides, A. verna, A. virosa, A. bisporigera, A. ocreata, A. tenuifolia), also Galerina and Conocybe filaris, produce alpha-amanitin in amount sufficient to poison an adult person with liver damage and fatal outcome (LD50, p.o. humans; 0.1mg/kg) (3). Amatoxins are potent inhibitors of RNA polymerase II, essential enzyme for the synthesis of proteins, especially in liver cells (4). Several studies have shown that alpha-amanitin also exhibits prooxidant and antioxidant properties in vitro and induce disruptions of antioxidant enzyme defense. Free radical formation might contribute to increased levels of lipid peroxidation and severe amatoxin hepatotoxicity (5).

As the first symptoms of poisoning appear after a long lapse of time about 6-24h, alpha-amanitin is one of the most insidious poisons responsible for 80-90% of fatal mushroom poisoning (6). Amanita intoxication has been reported to be lethal in up to 25% of the cases with mortality rate in children under ten years of age above 50 % (7). Therefore, rapid identification of alpha-amanitin in serum within 6h, when symptoms lag, and the liver and kidney damage has not occurred, is the key of successful detoxification (8). At that time, the level of amatoxins is too low for identification in urine samples utilized routine detection methods such as an immunoassay (7). Early diagnosis of poisoning via serum allows the application of appropriate therapy, avoiding expensive and invasive treatments (e.g. hemo-

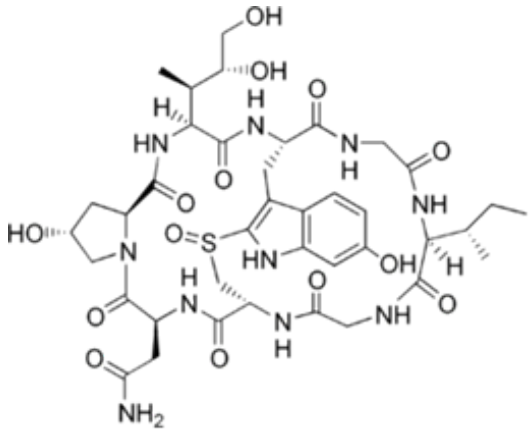

Figure 1. Structural representation of alpha-amanitin

dialysis, plasmapheresis or liver transplantation) and reduces the risk of death (9).

Several reports have indicated that amatoxins are extreme toxic substances, and many non-chromatographic and chromatographic methods have been developed for the determination in plant, animal and biological material (10). Owing to their sensitivity, the first published thin-layer chromatography (TLC) methods were successfully applied only in the study of amanitin-containing fungi, not in the assay of samples from intoxication subjects. Later, radioimmunoassay (RIA) enables the detection of amatoxins in urine at ng/ml range, but it was not routinely accepted because of the need for dedicated areas, radio-labeled tracers and unavailability in every laboratory (11). Methods relying on conventional enzyme-linked immunosorbent assay (ELISA) also offered high sensitivity (0.22ng/ml) but throughput without harmful radiation. Therefore, they were extensively used for production of commercial kits as the BÜHLMANN Amanitin ELISA Test which enabled quantification of amanitins in urine within 6-60 hours after ingestion, i.e. at the time when clinical symptoms were present (11). However, in many cases ELISA may lead to false-negative or false-positive results owing to the lack of specificity. Because the antibodies against the analytes may cross-react with matrix in the samples, immunoassay may require confirmation through alternative techniques when legal validation is necessary. These limitations directed the further analytical investigations to the development of a high-performance liquid chromatographic methods (HPLC) for this purpose. Jehl at al. (12) and Nishizawa at al. (13) established high-performance liquid chromatography with ultraviolet (UV) and electrochemical (EC) methods for determining alpha-amanitin in urine, serum and crude extracts of mushrooms, respectively. HPLC appears as the technique that could separate a wide range of amatoxins with satisfactory sensitivity (2-20ng/ml); however, published methods were not ideal for urgent determinations in biological samples because they were time-consuming for sample preparation and long chromatographic run time. Likewise, they need a relatively large amount of sample to reach a low quantification limit (14-16).

Two new methods for the determination of the amanitins in urine using high-performance liquid chromatography-tandem mass spectrometry (LC-MS-MS) have been published; the first with time–consuming solid phase extraction (8) and the other with on-line extraction mode (17). Detection limits for these methods were ranged from 0.5-1 ng/ml. The tandem-mass spectrometry is widely recognized as a powerful analytical tool which can provide both qualitative and quantitative data that may not be readily available by other techniques, but expensive and thus unavailable for many laboratories.

As the compromise, high-performance liquid chromatography-mass spectrometry (LC-MS) methods have already been rapidly developed and are not matrix-specific in single ion monitoring mode, which makes a single assay potentially suitable for both clinical and forensic studies of alpha-amanitin in serum and urine (18). The obtained sensitivity of 2.5ng/ml showed satisfactory limits for this purpose, but developed methods were validated only for urine samples.

Sample pretreatment methods such as liquid-liquid extraction (LLE) (19), deproteinization (PPE) (20), solid-phase extraction (SPE) (12,8,16), and immunoaffinity extraction (IAE) (21) were reported for isolation of the amatoxins from biological fluids. While PPE and LLE showed an unacceptable purity for MS chromatography measurement, SPE and IAE achieved high recovery and purity, but long time-running. Therefore, SPE was improved to conform to MS requirements, achieving high recovery and throughput for a short time.

In the present study, a rapid, sensitive, and reliable LC-MS method for determination of alpha-amanitin in serum after solid phase extraction has been developed. An electrospray ionization (ESI) interface in positive single ion monitoring mode (SIM) and collision ion dissociation (CID) were used. The method was validated in terms of specificity, accuracy, precision, sensitivity, recovery and stability. The validated method was also characterized by short run time; satisfactory limit of detection (2.5 ng/mL) and high extraction recovery (>90%). Isolation of alpha-amanitin from serum was performed by simple, four-step solid phase extraction conducted with hydrophilic-lipophilic balance (HLB) cartridges at neutral pH. This high pure sample preparation procedure, followed by electrospray LC-MS measurement, has provided chromatographic results within 20min. The method was successfully applied at the two emergency clinical cases suspected for the mushroom poisoning. This validated method would also be useful for resolving cases of poisoning under unexplained and suspicious circumstances in forensic toxicology.

Material and methods

Chemicals

Alpha-amanitin standard was obtained from Sigma-Aldrich (Deisenhofen, Germany). Methanol, ammonium acetate and acetic acid (gradient grade

for LC) were purchased from Merck (Darmstadt, Germany); while hydrochloric acid, ammonium hydroxide and anhydrous sodium sulfate (analytical grade) were purchased from Sigma-Aldrich. Ultrapure water was obtained from a Milli-RO20 system (Millipore, Molscheim, France). Oasis® HLB (30mg, 1ml) and Sep-Pak Vac C-18ec (200mg, 3ml), solid phase extraction (SPE) cartridges were obtained from Waters (Milford, MA, USA). Human serum matrix for method development was acquired from Blood Transfusion Clinic, Military Medical Academy (Belgrade, Serbia) in accordance with the approved protocols.

Stock and working standard solutions, calibration curves and quality control samples preparation

Stock solution of alpha-amanitin was prepared at concentration of 1mg/ml in methanol and was serially diluted to get working standard solutions for the preparation of calibration curves (CC). The stock solution was stored at -20°C and used up to one month from preparation, while working dilutions were freshly made when needed.

For the preparation of calibrators and quality control samples (QCS) for calibration graph, standard solutions of alpha-amanitin were spiked to 1ml of blank serum in 10ml glass tube, within calibration limit. Calibrators for this method were prepared at the following concentrations 5-10-25-50-100ng/ml and quality control samples at levels of lower limit of quantitation (LLOQ): 5ng/ml, low (LQC): 10ng/ml, medium (MQC): 25ng/ml and high (HQC): 100ng/ml. All samples were stored at 4°C and were analyzed during the same day.

Instrumentation and chromatographic conditions

The serum alpha-amanitin concentrations were quantified with liquid chromatography-mass spectrometry system, single quadrupole Finnigan SSQ 7000 LC/MS (Thermo Fisher Scientific, USA) equipped with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interface used to generate ions in positive (+) or negative (-) mode. All determinations were carried out at room temperature. Optimal chromatographic efficiency was achieved at reversed-phase column (LiChroCART RP-18ec, 250mmx4mm i.d.x5µm particle size, Merck, Germany) with an isocratic mobile phase consisting of 30% methanol in water and 10mM ammonium acetate buffer, pH5 adjusted with acetic acid (3:7,v/v). Before use, mobile phase was filtered through 0.45µm membrane filter paper. A HPLC Tsp-Thermo Separation System (Thermo Fisher Scientific, USA) with binary pumps (Spectra SYSTEM P2000), degasser (Spectra SYSTEM SCM1000) and auto-sampler (Spectra SYSTEM AS 3000) was used to achieve isocratic mobile phase flow rate of 0.8ml/min.

Alpha-amanitin isolation from the serum sample

Sample preparation was examined with solid phase extraction (SPE) at different pH values. PH values of spiked serum samples were adjusted before appropriate extraction with ammonium solution (pH9), phosphate-buffer (pH7) or hydrochloric acid (pH3). The adjusted spiked serum samples were stored at -20°C and allowed to thaw at room temperature before processing. SP extractions were examined with Waters hydrophilic–lipophilic balance (HLB) cartridges and silica-based Sep-Pak Vac C-18ec cartridge with the same procedure. Summarily, after stirring with the vortex mixer, the spiked sample with adjusted pH values were loaded onto cartridges. The cartridges were previously conditioned with 2ml of water and then 2ml of methanol. Washing step was performed using 5% methanol in water, and analyte was eluted twice with 1ml of methanol. The eluent was then evaporated in the stream of nitrogen at room temperature. After filtration, this solution was reconstituted with 1ml of mobile phase and injected into LC-MS.

Recovery of SPE

The absolute recovery of alpha-amanitin from serum by SPE was determined for five different standard concentrations by spiking the analyte into the blank serum. Extraction recovery was calculated by comparing the peak areas extracted from spiked samples with those of the same quantities added to the mobile phase. The recovery of five QCs concentrations in serum was repeated six times.

Method validation

The validation of the LC-MS method for the determination of alpha-amanitin in serum samples was performed in accordance with ICH Q2A/Q2B guidelines (23) and included the following parameters: sensitivity, linearity, accuracy and precision, selectivity and stability tests. The method was also tested by analysis of two urgent clinical samples suspected of having been exposed to amanitin mushrooms.

Statistical analysis

Statistical analysis was performed using Micfosot Office Excell 2013 software. Pearsons' correlation was used to evaluate the associations of peak area point to alpha-amanitin concentration. A probability of $p<0.005$ was considered to be significant.

Results and discussion

Optimization of the instrument parameters

The aim of this exemination was to develop a sensitive, rapid and simple method for identification and quantification of alpha-amanitin in serum. To achieve the goal, during the examination procedure, different options were evaluated to optimize

the sample extraction, chromatography conditions and detection parameters.

The LC chromatographic condition, especially the composition of mobile phase and the analytical column, were optimized to achieve symmetric peak shapes, good resolution and short run time. Two types of columns, C-8 and C-18, were tested and finally a reversed-phase LiChroCART C-18ec column (250mmx4mm i.d. x 5µm, Merck, Germany) was used for the chromatographic separation. Various concentrations of buffer solutions in methanol and acetonitrile were tested as a mobile phase. It was found that the mixture of 30% methanol in the water and 10 mM ammonium acetate buffer, pH 5 adjusted with acetic acid (3:7, v/v), could achieve our goal and was finally adopted as the mobile phase for chromatographic separation. All LC chromatographic conditions were considered similar to the literature data (8,21). The electrospray MS detection was performed in the scan and selected ion monitoring mode (SIM). The MS detecting conditions were operated according to the MS signal response of the target compound and the results showed that the positive mode of ionization was much more sensitive than the negative mode. In order to obtain the optimum parameters (lens voltages, multiplicator voltage, etc.) automated direct flow injection syringe pump was used at a flow rate of 30µL/min and standard solution concentration of 100mg/L. Achieved the MS conditions were as follow: electrospray needle voltage: 5kV; quad-rupole offset: -0.8V; octapol offset: -3.0V; lens 1-2: -24.9V; lens 1-1: -54.9V; tube lens: 159.6V; capillary: 42V; detector: 2.2kV; sheath gas pressure (N2): 30psi; auxiliary gas pressure (N2): 5psi; capillary temperature: 200°C. Following measurement of standard solution 100 mg/l, in full scan mode showed three characteristic fragment ions: m/z 919.3, m/z 920.4 and m/z 921.3, which were used for post quantification in single ion monitoring mode (SIM). Typical chromatogram of alpha-amanitin standard at 25ng/ml in the single ion monitoring mode was presented on the Figure 2. Same scan time: 5sec and scan wide: 0.3 were set at mode all measurements. Collision energy (CID) was optimized and applied at 40V, to achieve better specificity of the method with ions like m/z 746.06 and m/z 871.50.

Sample preparation

Sample preparation with high recovery and no matrix interferences is the key of successful MS determination of an analyte. One of the most common problems faced in the extraction of an analyte in biological matrices are solubility and natural organic substances in the samples that reduce extraction efficiency and hinder detection (22). A broad range of extraction techniques are currently used for this purpose and the most frequently exhaustive are the protein precipitation, PPE; liquid-liquid extraction, LLE; and solid-phase extraction, SPE. Since the alpha-amanitin is bicycle octapeptide compound with poor solubility in polar and non-polar organic solvents, the application of conventional PPE and LLE are limited by the low recovery and unsatisfactory purity for LCMS measurements. The reported analytical procedures of PPE and LLE of alpha-amanitin from serum showed unsatisfactory recovery, relatively high solvent consumption and long extraction time (19, 20).

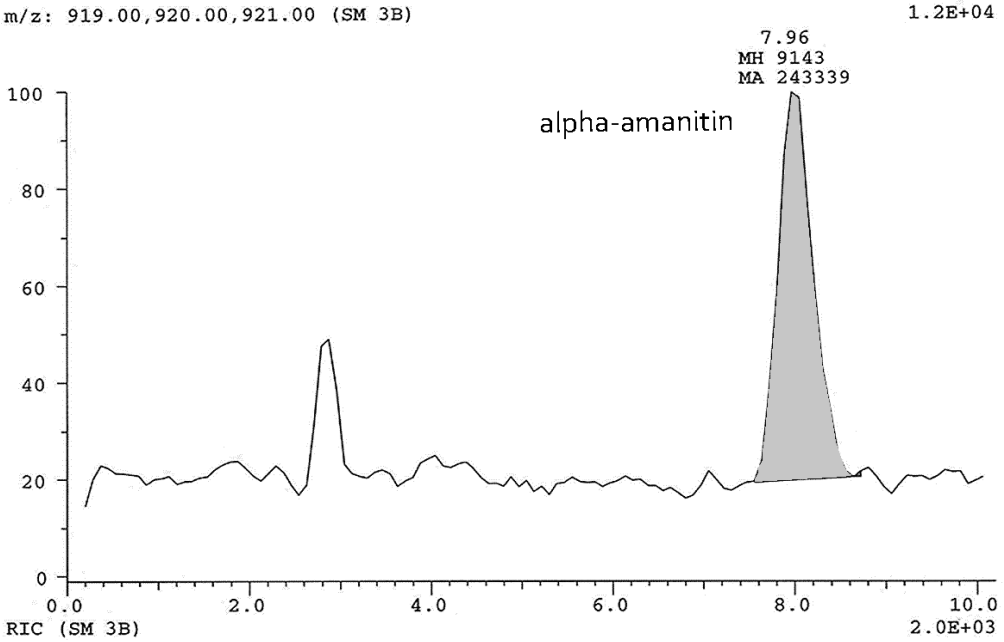

Figure 2. Typical chromatogram of alpha-amanitin standard, 25 ng/mL, Single Ion Monitoring mod (SIM)

A number of methods described in the literature using more advanced SPE have grown considerably (8,12,16) because of their better repeatability, lower solvent consumption and reduced time for sample pre-treatment. SPE is the process with proper purification and concentration effects with satisfactory recovery. In addition, an immunoassay SPE method was reported as a possibility of extraction of alpha-amanitin from serum (21). Described method achieved medium extraction efficiency of 61% and required long-time immunoaffinity column preparation.

In this assay, in order to obtain the optimum parameters of the recovery, purity and run time, all the techniques were examined with different solvents at various pH values. The extraction recoveries were determined by comparing peak areas of serum samples spiked before extraction, with those from standard solutions at the same levels. Two extraction columns were tested for SPE, namely silica-based Sep-Pak Vac C18 and hydrophilic–lipophilic balance (HLB) cartridges. Finally, a neutral solid phase extraction with copolymer adsorbent showed sufficient sample clean-up, no interference with the chromatograms extraction and efficiency more than 90%. Therefore, it was adopted and used in further proceedings of sample preparation. This rapid method of sample preparation allows obtaining results within 20 minutes, which is four times faster than earlier reported data (21).

Validation parameter

Concentration-detector response relationship was evaluated over the concentration range from 5-100ng/ml using five different concentration levels (5-10-25-50-100ng/ml, without zero point). A least square regression model, where x was the concentration of alpha-amanitin, was fitted to each standard curve. The high correlation coefficients ($r>0.99$) for all calibration curves indicated good correlations. Under certain assum-ptions required for valid linear regression, reliable results were obtained and suitable prediction function was defined. First, the linear relationship between the concentrations and peak areas is obvious from the corresponding scatter plots. No significant outliers were noticed. Further, homo-scedasticity of the error terms was established using Hartley's test and the independency of the residuals was proved by using the Durbin-Watson autocorrelation test. Also, the normality of the error terms of the regression line was not violated, as Shapiro Wilk test showed. The lack of fit test was also performed and showed the high coefficients of the correlation ($R>0.99$) between the two variables. The value of $p<0.005$ indicated that the regression model statistically significantly predicts the outcome variable, i.e. is a good fit for the data. The corresponding regression lines were: $y=11054x–35191$, $y=11390x–28268$, $y=9706x+60.03$.

The lowest standard on the calibration curves, 5ng/ml was accepted as the LLOQ with accuracy criteria within ±20% at signal to noise ratio (S/N) 1:10. The accuracy of other spiked QC samples at all concentration levels were within the accepted range ±15%. The limit of detection (LOD) for a method was confirmed at concentration of 2.5ng/ml at signal to noise ratio, S/N, 1:3. According to our knowledge, the limit of quantitation was achieved at the level of the most sensitive methods LC-MS published so far for quantification alpha - amanitin in serum (21).

Evaluation of six replicates at four concentration levels (LLOQ: 5ng/ml; low: 10ng/ml, medium: 25ng/ml and high: 100ng/ml) were assessed within one batch for intra-day and over at 3 days for inter-day measurements, listed in Table 1. Acceptance criteria for RSD (%) were ≤20% for LLOQ and low concentrations, and ≤15% for medium and high concentrations. The mean intra-day RSD (%) was 6.05 (range 3.67-8.9%) and the corresponding value for inter-day was 6.1% (range 3.5-10.1%). The mean intra-day accuracy was 92.6% (range 90.2-95.0%) and corresponding value for inter-day was 92.6% (range 91.1-94.5%). LC-MS analysis of low purity biological samples can cause suppression, elevated background, and other negative matrix effects. Six independent sources of serums were tested (including hemolytic and lipemic samples) to show that there were no significant interferences in the retention times of alpha-amanitin from endogenous and exogenous substances. The retention time of alpha-amanitin was 8.02min, and the total chromatographic run time was 10min.

Table 1. Intra- and inter-day precision and accuracy in serum (n = 6)

Compound	Nominal concentration (ng/ml)	Precision		Accuracy (%)
		Mean ± SD	RSD (%)	
Intraday	5	4.5±0.4	8.9	90.2
	10	9.3±0.7	7.3	92.7
	25	23.2±1.0	4.3	92.6
	100	95.0±3.5	3.7	95.0
Interday (3 days)	5	4.6±0.5	10.1	91.1
	10	9.2±0.6	6.4	92.1
	25	23.2±1.0	4.4	92.9
	100	94.5±3.3	3.5	94.5

SD, standard deviation; RSD, relative standard deviation; n, number of injections

Stock solution stability test was evaluated at one concentration (25ng/ml, n=3). The average area of stored standard solution (5h/room temperature) compared with the freshly prepared standard solution showed no significant static difference (not higher than 5%). Short-term, long-term and on the instrument stability test were evaluated at low (10ng/ml) and high (100ng/ml) concentration levels. Six replicates of QC serum samples at low and high concentrations were assessed at the initial time point and after a period of storage (1 hour) at a room temperature for short-term, at -20°C for long-term, and after 12 hours sample standing on an autosampler stability test. The same concentrations and replicates were applied for analysis freeze/thaw stability after tree cycles. Concentrations of sample stability were back-calculated against freshly prepared calibration curve. The relative decrease was evaluated and did not exceed 15% at high level and 20% at low level. No significant degradation of alpha-amanitin in human serum was observed under all conditions studied.

The carryover effects were studied by analysis of three replicates of the blank sample, which were injected immediately after the sample at high concentration (100ng/ml). The responses of alpha-amanitin did not exceed 25% of the average responses of analytes at LOQ concentration levels.

Extraction recovery of alpha-amanitin was determined by comparing peak areas obtained from extracted serum samples with those found by extracting blank matrices through the extraction procedure and spiking with a known amount of alpha-amanitin. The absolute recoveries of alpha-amanitin for this application were 91.2%

Table 2. Recovery of alpha-amanitin at different spike serum concentrations (5-10-25-50-100 ng/mL, n=6)

Actual concentration, (ng/ml)	RSD (%)	Recovery (%)
5	7.9	91.2
10	4.6	89.9
25	2.7	93.3
50	7.8	92.6
100	3.0	94.3

RSD, relative standard deviation; n, number of injections

(5ng/ml), 89.9% (10ng/ml), 93.3%, (25 ng/ml), 92.6 (50ng/ml) and 94.3% (100 ng/ml) with adequate standard deviations showed in Table 2.

The robustness of the bioanalytical method was studied by analysis of calibrators and QC samples at concentrations low, medium and high with two technicians and on different days. No statistically significant differences were obtained.

Application of the method

This method was successfully used in two cases of suspected mushroom poisoning. Serum samples were sent from the Emergency Toxicology Unit immediately after the patients with abdominal pain, nausea and vomiting were admitted. In both cases, the origin and species of consumed mushrooms were not known, and the previous period of ingestion was longer than 12 hours. After the application of this method, negative results were obtained. The same sample preparations and chromatographic procedures were also applied to urine samples again with negative results. Therefore, the amatoxin poisoning was excluded and hemodialysis as detoxification measure was avoided. After the admission of adequate therapy, both patients survived with successful recovery.

Conclusions

The liquid chromatography-mass spectrometry with collision-induced ionization analytical method for determination of alpha-amanitin, described and validated in this report, was found to perform satisfactorily with all validation parameters. Three different sample extraction methods indicated that the neutral solid phase extraction procedure was optimal for sample preparation and able to achieve the limit of quantification of 5ng/ml. The LC-ESI-MS method proved to be fast, specific and highly sensitive for the determination of alpha-amanitin in serum in short analysis time of 20min, including sample preparation. This makes the method applicable and useful in clinical toxicological analysis, especially in early stage of poisoning with A. phalloides when symptoms lag (within 6h). The method is also applicable in forensic toxicology in determination of the cause of death under unexplained and suspicious circumstances.

References

1. Karlson-Stiber C, Persson H. Cytotoxic fungi - an overview. Toxicon 2003; 42: 339–49. [CrossRef]
2. Li C, Oberlies NH. The most widely recognized mush room: Chemistry of the genus Amanita. Life Sci 2005; 78: 532-8. [PubMed] [CrossRef]
3. Luo H, Hallen-Adams HE, Scott-Craig JS, Walton JD. Ribosomal biosynthesis of α–amanitin in Galerina marginata. Fungal Genet Biol 2012; 49: 123- 9. [PubMed] [CrossRef]
4. Bushnell DA, Cramer P, Kornberg RD. Structural basis of transcription: alpha-amanitin-RNA polymerase II cocrystal at 2.8 A resolution. Proc Natl Acad Sci 2002; 99: 1218–22. [PubMed] [CrossRef]
5. Zheleva A, Tolekova A, Zhelev M, Dobreva Z, Halacheva K, Popova S. In vivo antioxidant and pro oxidant properties of amanita phalloides mushroom toxins. Trak J Sci 2005; 3: 34-8.
6. Fiume L. Mechanism of action of amanitins. Curr Prob Clin Biochem 1977; 7: 23-8. [PubMed]
7. Bühlmann Laboratories. Amanitin-ELISA, instruction manual, Bühlmann Laboratories, 2000, Allschwil, Switzerland.
8. Filigenzi MS, Poppenga RH, Tiwary AK, Puschner B. Determination of alpha-amanitin in serum and liver by multistage linear ion trap mass spectrometry. J Agric Food Chem 2007; 55: 2784-90. [PubMed] [CrossRef]
9. Enjalbert F, Rapior S, Nouguier-Soulé J, Guillon S, Amouroux N, Cabot C. Treatment of amatoxin poiso ning: 20-year retrospective analysis. J. Toxicol. Clin. 2002; 40: 715-57. [CrossRef]
10. Dorizzi R, Michelot D, Tagliaro F, Ghielmi S. Methods for chromatographic determination of amanitins and related toxins in biological samples. J Chromatog 1992; 580: 279-91. [CrossRef]
11. Andres Y R, Frei WK. Amatoxin and anti-amatoxin for radioimmunoassay prepared by a novel ap proach: Chemical and structural considerations. Toxicon 1987; 25 (9): 915-22. [CrossRef]
12. Jehl F, Gallion C, Birckel P, Jaeger A, Flesch F, Minck R. Determination of α-Amanitin and β-Amanitin in Human Biological Fluids by High-Performance Liquid Chromatography. Anal Biochem 1985; 149: 35-42. [CrossRef]
13. Nishizawa C, Yamaura Y. Determination of amani tatoxins by HPLC. Chudoku Kenkyu 2003; 16(4): 441-5. [PubMed]
14. Defendenti C, Bonacina E, Mauroni M, Gelosa L. Vali dation of a high performance liquid chroma tographic method for alpha amanitin determination in urine. Forensic Sci Int 1998; 92: 59-68. [CrossRef]
15. Kilibarda V, Glišović L, Jovanović Đ. Ciklopeptidni toksini gljiva *Amanita species* i mogućnost njihovog određivanja. Vojnosanit Pregl 1993; 50: 511-6. [PubMed]
16. Tagliario F, Schiavon G, Bontempelli G, Carli G, Marigo M. Improved high-performance liquid chroma tographic determination with amperometric detection of alpha-amanitin in human plasma based on its voltammetric study. J Chromatogr 1991; 563: 299-311. [CrossRef]
17. Helfer AG, Meyer MR, Michely JA, Maurer HH. Direct analysis of the mushroom poisons α- and β-amanitin in human urine using a novel on-line turbulent flow chromatography mode coupled to liquid chromato graphy-high resolution-mass spectrometry/mass spectrometry. J Chromatogr A 2014; 1325: 92-8. [PubMed] [CrossRef]
18. Maurer HH, Kraemer T, Ledvinka O, Schmitt CJ, Weber A.A. Gas chromatograph (GC – MS) and liquid chromatography-mass spectrometry (LC – MS in) toxicological analysis: Studies on the detection of clobenzorex and its metabolites within a syste matic toxicological analysis procedure by GC – MS and by immunoassay and studies on the detection of α – and β – amanitin in urine by atmospheric pressure ionization electrospray LC – MS. J Chromatogr B 1997; 689: 81-9. [CrossRef]
19. Caccialanza G, Gandini C, Ponci R. Direct, simulta neous determination of α-amanitin, β-amanitin and phalloidine by high-performance liquid chromato graphy. J Pharmcol Biomed Anal 1985; 3: 179-85. [CrossRef]
20. Belliardo F, Massano G. Determination of α-Amanitin in Serum by HPLC. J. Liq Chromatogr 1983; 3: 551-8. [CrossRef]
21. Maurer HH, Schmitt CJ, Weber AA, Kraemer T. Validated electrospray liquid chromatographic-mass spectrometric assay for the determination of the mushroom toxins α- and β-amanitin in urine after immunoaffinity extraction. J. Chromatogr. B. 748: 125-35. [CrossRef]
22. Rogatsky E, Stain D. Evaluation of Matrix Effect and Chromatography Efficiency: New Parameters for Validation of Method Development. J. Am. Soc. Mass Spectrom 2005; 16: 1757-9. [PubMed] [CrossRef]
23. ICH Q2A/Q2B. 1996. Q2B Validation of Analytical Procedures: Methodology, Guidance for Industry, International Conference on Harmonization of Technical Requirements for Registration of Phar maceuticals for Human Use (ICH), U.S. Depart ment of Health and Human Services. http://www.ich.org/ truncated]

ODREĐIVANJE ALFA-AMANITINA U SERUMU PRIMENOM METODE TEČNE HROMATOGRAFIJE SA MASENIM DETEKTOROM NAKON TEČNO-ČVRSTE EKSTRAKCIJE

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Alfa-Amanitin je ciklični polipeptid koji pripada velikoj grupi otrova gljiva poznatih kao amatoksini. Kako su ovi toksini odgovorni za većinu trovanja gljivama sa smrtnim ishodom, zahtevaju brzu detekciju i izlučivanje iz organizma. U skladu sa ovim potrebama, razvijena je jednostavna i precizna metoda za efikasnu identifikaciju i kvantifikaciju alfa-amanitina u serumu pomoću elektrosprej-tečne hromatografije sa masenom spektrometrijom (LC-ESI-MS) nakon disocijacije indukovane kolizijom (CID). Metoda je usklađena sa utvrđenim protokolima Međunarodne konferencije o harmonizaciji i validaciji analitičkih metoda (ICH) Q2A/Q2B 1996. Svi validacioni parametri: linearnost, preciznost, ekstrakcioni prinos, efekat matriksa i testovi stabilnosti standarda i opterećenih seruma sa alfa-amanitinom pod različitim uslovima čuvanja, ispunili su kriterijume protokola za prihvatanje. Dobijene su kalibracione krive, linearne u opsegu koncentracija 5-100ng/ml, sa donjom granicom kvantifikacije (LLOQ) od 5ng/ml i granicom detekcije (LOD) od 2,5ng/ml. Određene su srednja intra- i inter-dnevna preciznost i tačnost merenja od 6,05%, odnosno ±15% od nominalnih vrednosti. Neutralna tečno-čvrsta ekstrakcija sa kopolimernim hidrofilno-lipofilnio izbalansiranim kertridžima usvojena je kao optimalni postupak za pripremu uzoraka u daljem radu. Srednji prinos ekstrakcije iznosio je 91, 94%. Postavljena metoda pokazala je visoku osetljivost i selektivnost za određivanje alfa-amanitina u serumu, što je čini izuzetno pogodnom za klinička i forenzička toksikološka merenja pri niskim koncentracijama. *Acta Medica Medianae 2015;54(1):12-19.*

Ključne reči: *alfa-amanitin, tečna hromatografija-masena spektrometrija, tečno-čvrsta ekstrakcija, serum*