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Mass Spectrometry-Based Analytical Methods of Amatoxins in Biological Fluids to Monitor Amatoxin-Induced Mushroom Poisoning

Jin-Sung Choi and Hye Suk Lee*

College of Pharmacy, The Catholic University of Korea, Bucheon 14662, Republic of Korea

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Abstract : Amatoxin-induced mushroom poisoning starts with nonspecific symptoms of toxicity but hepatic damage may follow, resulting in the rapid development of liver insufficiency and, ultimately, coma and death. Accurate detection of amatoxins, such as α-, β-, and γ-amanitin, within the first few hours after presentation is necessary to improve the therapeutic outcomes of patients. Therefore, analytical methods for the identification and quantification of α-, β-, and γ-amanitin in biological samples are necessary for clinical and forensic toxicology. This study presents a literature review of the analytical techniques available for amatoxin detection in biological matrices, and established an inventory of liquid chromatography (LC) techniques with mass spectrometry (MS), ultraviolet (UV) detection, and electrochemical detection (ECD). LC-MS methods using quadrupole tandem mass spectrometry, time-of-flight mass spectrometry, and orbitrap MS are powerful analytical techniques for the identification and determination of amatoxins in plasma, urine, serum, and tissue samples, with high sensitivity, specificity, and reproducibility compared to LC with UV and ECD, enzyme-linked immunoassay, and capillary electrophoresis methods.

Keywords : amatoxin-induced mushroom poisoning, α-amanitin, β-amanitin, γ-amanitin, LC-MS/MS, biological samples

Introduction

Mushrooms are consumed worldwide as an ingredient of many meals, but severe syndromes and even death can be caused by the misidentification of wild poisonous mushrooms as edible.[1-6] Mushrooms such as *Psilocybe* species and *Amantia muscaria* are intentionally abused because of their psychoactive activities.[7] There is some evidence that mushroom poisoning may be increasing, and that exotic species are entering new areas and countries, thereby expanding the range of mushroom poisoning symptoms observed in presenting patients.[8] White et al.[9] classified mushroom toxins based on the clinical types of mushroom poisoning, as follows: primary hepatotoxicity (amatoxins), primary nephrotoxicity (AHDA, orellanine), neurotoxicity (psilocybins, muscarines, ibotenic acid, muscimol), mytotoxicity (saponaceolide B), metabolic/endocrine toxicity (gyromitrins, coprines, trichothecenes, polyporic acid), gastrointestinal irritants, and miscellaneous (entinan, acromelic acid).

Although global data are not available, the absolute number and incidence of mushroom poisoning cases may be increasing based on local studies.[9-12] An emerging mushroom poisoning risk in Europe may be the result of the large migrant influx, a subset of whom forage for food because of poor economic circumstances. This results in the consumption of mushrooms not known to the migrants and an increased incidence of amatoxin-type mushroom poisoning.[3,10] Because most toxic syndromes caused by mushroom toxins start with unspecific symptoms, diagnostic difficulties are most common during the critical first hours after presentation.[3] Therefore, suspected poisonings should be confirmed or excluded to ensure that therapy starts as soon as possible, and to prevent an inappropriate therapy being implemented. The analytical strategies used to identify poisonous mushroom toxins include spore analysis (if mushroom leftovers or gastric content are available) and the identification of various toxins and their metabolites in human biological samples.[3,14,15] It is therefore necessary to develop sensitive, selective, and rapid analytical methods for the identification and quantification of mushroom toxins and their metabolites in the human biological matrix.

The purpose of this study was to review the bioanalytical methods used for the diagnosis of amatoxin-induced mushroom poisoning, which is the main cause of fatal mushroom poisoning.[3,10,13]

*Reprint requests to Hye Suk Lee
https://orcid.org/0000-0003-1055-9628
E-mail: sianalee@catholic.ac.kr

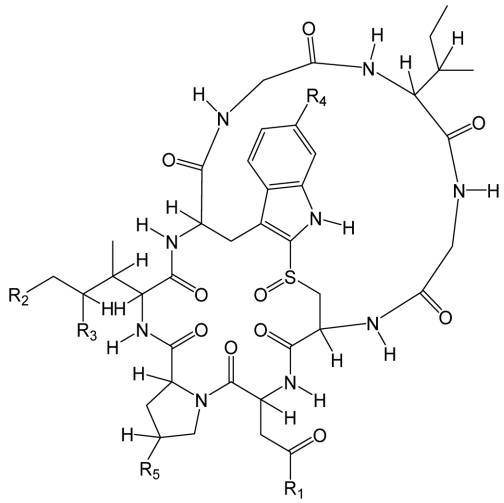
Amatoxins

Amatoxins are highly toxic bicyclic octapeptides (Figure 1). They are the most toxic compounds among mushroom toxins, and are found in the *Amanita*, *Galerina*, and *Lepiota* mushroom species.[4-6] Among these species, *Amanita phalloides* has the most toxin components/weight and is responsible for most cases of fatal poisoning.[15-18] These toxins are classified as neutral substances (α-amanitin, γ-amanitin, amaninamide, amanullin, and proamanullin) or acidic substances (β-amanitin, ε-amanitin, amanine, and amanullic acid), which differ in terms of the number of hydroxyl groups and amide carboxyl exchange (Figure 1).[19,20] Amatoxins are water-soluble, heat-stable, and resistant to enzyme and acid degradation. Therefore, these substances remain unchanged during freezing, drying, and cooking (including frying, grilling, boiling, steaming, and other processing operations, such as digestive processing), and are resistant to gastrointestinal inactivation and metabolic processes.[18,21-23] However, amatoxins can be degraded slowly when stored in an open and aqueous solution, or exposed to sun or neon light for periods of about 7−8 months, which could potentiate the toxicity of amatoxins upon exposure in vivo.[17,20] The content of amatoxins varies among *Amanita* species, but α- and β-amanitin are the most abundant substances. For example, an amatoxin content of 9.3 mg/g was observed in dried mushrooms, while α- and β-amanitin accounted for 56% of the toxins found in dried powder from *A. phalloides*.[24] It has been reported that α- and β-amanitin account for 82% of toxins (2.87 mg of amanitin/3.49 mg peptide toxins/g dried powder) in *A. exitialis*.[25,26] According to Yilmaz et al.,[27] an oral intake of approximately 50 g of fresh *A. phalloides*, equivalent to a dose of 0.32 mg/kg of amatoxins, can be lethal.

The LD_{50} value of α-amanitin was reported to be 0.3–0.6 mg/kg in mice and 4.0 mg/kg in rats (intraperitoneal injection), 0.1 mg/kg in humans (oral administration), and 0.1 mg/kg in dogs (intravenous injection).[17,28,29] The LD_{50} values of β-amanitin, γ-amanitin, ε-amanitin, amanitin, and amaninamide were reported to be 0.5, 0.2−0.5, 0.3−0.6, 0.5, and 0.5 mg/kg, respectively, in mice following intraperitoneal injection.[17] The LD_{50} values for orally administered amanullin, amanullinic acid, and proamanullin in mice were > 20 mg/kg, but these levels are not toxic to humans.[17,28]

Amanita phalloides poisoning can cause acute hepatitis, leading to the rapid development of liver insufficiency and, ultimately, coma and death.[5,15,17] However, nephrotoxicity has been less frequently reported.[30] The main toxicity mechanism of amatoxins is the inhibition of RNA polymerase II, which leads to the inhibition of messenger RNA synthesis and protein synthesis.[31-33] Other toxic mechanisms have been suggested, including oxidative stress-related damage via the increased formation of reactive oxygen species induced by an increase in superoxide dismutase activity and inhibition of catalase activity;[18,34,35] and amatoxin-induced apoptosis caused by the translocation of p53 to the mitochondria, leading to alteration of mitochondrial membrane permeability through the formation of a complex with Bcl-xL and Bcl-2.[17,18,36-39] The cytotoxicity of α-amanitin is 10-fold greater than that of β-amanitin in MCF-7 cells.[40] The main toxicological studies of amatoxins have focused on α- and β-amanitin; therefore, no conclusions have been drawn regarding potential toxicity differences between neutral and acid amatoxins.[15,17]

Toxicokinetic studies of α- and β-amanitin after the intravenous, intraperitoneal, and oral administration of amatoxin in rats and mice have been reported.[14,18,20,21,41,42] Low absolute bioavailability of α-amanitin (3.5–4.8%) and β-amanitin (7.3–9.4%), and substantial transport thereof to



Compound	R_1	R_2	R_3	R_4	R_5
σ-amanitin	NH_2	OH	OH	OH	OH
β-amanitin	OH	OH	OH	OH	OH
γ-amanitin	NH_2	H	OH	OH	OH
ε-amanitin	OH	H	OH	OH	OH
amanullin	NH_2	H	H	OH	OH
amaninamide	NH_2	OH	OH	H	OH
proamanullin	NH_2	H	H	OH	H
amanin	OH	OH	OH	H	OH
amanullinic acid	OH	H	H	OH	OH

Figure 1. Chemical structures of amatoxins.

the intestines, kidneys, and liver, were observed after they were orally administered to mice at doses of 2, 5, or 10 mg/kg.[41,42] α- and β-amanitin show similarities in terms of the elimination process; they are both eliminated in urine without significant metabolism.[21,41,42] α- and β-amanitin show OATP1B1- and OATP1B3-mediated hepatic uptake, but only β-amanitin shows OAT3-mediated kidney uptake.[42] α- and β-amanitin were detected in serum, plasma, urine, liver, and fecal samples of amatoxin poisoning patients.[15,43–46] Among 43 amatoxin-intoxicated patients, 11 showed plasma concentrations of 8−190 ng/mL for α-amanitin and 23.5−162 ng/mL for β-amanitin.[46] In total, 35 urine, 12 feces, and 4 liver and kidney samples were obtained from 43 amatoxin-intoxicated patients, with ranges of 0.03–3.29 mg for α-amanitin and 0.05–5.21 mg for β-amanitin in 24 of the urine samples; 8.4–152 μg for α-amanitin and 4.2-6270 μg for β-amanitin in 10 fecal samples; and 10–19 ng/g and 122–1719 ng/g for α-amanitin, and 170.8–3298 ng/g and 1017–1391 ng/g for β-amanitin, in liver and kidney samples, respectively, for three of patients.[46] Although cellular uptake (mediated by hepatic or renal transporters) is approximately two-fold higher for β-amanitin than α-amanitin, as is liver and kidney accumulation, the contribution of β-amanitin to in vivo amatoxin toxicity may be lower than that of α-amanitin because of differences in their cytotoxicity.[40]

Benzylpenicillin, silibinin, and *N*-acetylcysteine have been used for the treatment of amatoxin-induced mushroom poisoning.[3,17] The therapeutic effects may be attributed to the hepatoprotective and anti-oxidative activities of silibinin and *N*-acetylcysteine, and the reduced hepatic distribution of α- and β-amanitin resulting from the inhibition of OATP1B3 by benzylpenicillin, silibinin, and cyclosporine.[17,40,47-49]

Analytical methods of amatoxins in biological fluids

Confirmation of the intake of mushrooms containing amatoxins is needed by detecting amatoxins such as α-, β-, and γ-amanitin in biological fluids to avoid expensive and time-consuming treatment for every suspected intoxication case.[15] Sufficient analytical sensitivity is also necessary because hospitalization often occurs late after intake such that only trace amounts of toxins can be found.[15,44,46] Several methods have been reported for the qualification and quantification of amatoxins in biological fluids using liquid chromatography (LC) combined with mass spectrometry (MS),[41-45,50-70] ultraviolet (UV) detection,[71,72,75,77,78] or electrochemical detection (ECD),[71,73,74,76] as well as capillary zone electrophoresis (CZE),[79,80] radioimmunoassay (RIA),[81,82] enzyme-linked immunosorbent assay (ELISA),[83,84] and lateral flow immunoassay (LFA).[85] However, each method has drawbacks. The ELISA and LFA methods have been used for the screening of α-, β-, or γ-amanitin in clinical toxicology, but compared to LC-MS methods they have the disadvantages of low sensitivity (3−10 ng/mL), high workload, false-negative and -positive results, and the requirement for additional confirmation in forensic cases.

The LC–MS methods have the advantages of high specificity, sensitivity, resolution, and rapidity relative to other analytical methods, making them suitable for routine clinical and forensic toxicological analysis of amatoxins. The LC-MS methods that have been developed for the analysis of α-, β-, and γ-amanitin in various biological fluids are summarized in Table 1. High-performance liquid chromatography (HPLC) with UV detection and ECD methods for the quantification of α- and β-amanitin in plasma, urine, liver, and kidney are summarized in Table 2; these methods have drawbacks such as low sensitivity and laborious sample preparation.

Sample preparation

Blood, plasma, serum, urine, bile, and tissue samples have been used for clinical purposes, forensic toxicology, and toxicokinetics of amatoxins.[41-45,51-80] Because the amatoxin concentrations in urine are usually higher than those in serum and plasma,[26,45,46] urine is considered as the biological sample of choice. However, major drawbacks of urine sampling include reduced output in the case of decreased renal function and acute renal failure, which can occur in some amatoxin and other mushroom poisoning cases, and the greater intra- and interindividual variability in the urine as a biomatrix. If therapeutic measures like fluid replacement or forced diuresis are applied, the low amounts of amatoxins in urine could be further diluted. Therefore, blood, plasma, and serum samples are more commonly used in clinics than urine samples for the determination of amatoxins.

For the determination of α-, β-, and γ-amanitin in human and animal plasma, serum, urine, and tissue samples using HPLC, LC-MS, liquid chromatography-tandem mass spectrometry (LC-MS/MS), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and CZE, several sample preparation techniques have been developed, including protein precipitation with acetonitrile, methanol, or perchloric acid, [41,42,51,52,59,64,71] liquid-liquid extraction (LLE),[78] solid-phase extraction (SPE) with reverse-phase, cation exchange or immunoaffinity cartridges,[43,50,54,56,58,60-62,66-68,70,71,73,74,76] SPE of the aqueous phase obtained after LLE with dichloromethane or chloroform,[55] SPE of the aqueous phase obtained after protein precipitation of the biomatrix with acetonitrile and LLE of the supernatant with chloroform,[44,45,65,69,72,77] and online column switching technique,[63,75] and simple dilution in CZE[79,80] (Tables 1 and 2). These methods use different volumes of biological matrix samples, as follows: serum, 100−5000 μL;[54,60,67,69,72,76-78] plasma, 5−3000 μL;[41,42,44,50-53,57-60,67,68,74,75] and urine, 50−10000 μL[41,42,43,45,50,54-58,60-67,70,73,76-80] (Tables 1 and 2). Two or three sample preparation procedures have

Table 1. The LC-MS and LC-MS/MS methods used for the determination of amatoxins in various biological matrices.

Toxin	Matrix	Sample preparation	Column	Mobile phase	Ionization (mode)	Linearity (LOD)	Transitions	Ref
β-Amanitin	Mouse plasma (5 μL), urine (50 μL), tissues	Protein precipitation with methanol	Atlantis dC18	Gradient elution of 0.1% formic acid and methanol	Negative ESI (PRM)	Plasma: 0.5−200 ng/mL Urine: 2−500 ng/mL; Liver: 50−50000 ng/g; Kidney: 25−5000 ng/g	β-amanitin: *m/z* 918.33185> 900.32123, 4′-hydroxydiclofenac (IS): *m/z* 310.00443>266.01450	42
α-Amanitin, β-Amanitin, γ-Amanitin	Human urine (2000 μL) & plasma (1000 μL)	SPE (immunoaffinity column)	Kinetex Biphenyl	Gradient elution of methanol and 0.005% formic acid	Negative ESI (MRM)	1−200 ng/mL (Plasma: 0.004 ng/mL; Urine: 0.002 ng/mL)	α-amanitin: *m/z* 917.4>899.3, β-amanitin: *m/z* 918.4>900.3, γ-amanitin: *m/z* 901.4>883.3	50
α-Amanitin	Rat plasma (50 μL)	Protein precipitation with methanol and dilution	Acquity BEH C18	Gradient elution of water and acetonitrile	Negative ESI (MRM)	0.9−600 ng/mL	α-amanitin: *m/z* 917.4>205.0, roxithromycin (IS): *m/z* 835.8>484.7	51
α-Amanitin	Mouse plasma (5 μL), urine (50 μL), tissues	Protein precipitation with methanol	Atlantis dC18	Gradient elution of 0.1% formic acid and methanol	Negative ESI (PRM)	0.5−500 ng/mL plasma & urine	α-amanitin: *m/z* 917.34747> 899.33710, verproside (IS): *m/z* 497.12936>153.01897	41
α-Amanitin, β-Amanitin	Mouse plasma (5 μL)	Protein precipitation with methanol	Atlantis dC18	Gradient elution of 0.1% formic acid and methanol	Negative ESI (PRM)	0.5−500 ng/mL	α-amanitin: *m/z* 917.34747> 899.33710, β-amanitin: *m/z* 918.33185> 900.32123, 4′-hydroxydiclofenac (IS): *m/z* 310.00443>266.01450	52
α-Amanitin, β-Amanitin	Human plasma (100 μL)	Protein precipitation with acetonitrile, LLE, online SPE (ODS cartridge)	XBridge BEH C18	Gradient elution of methanol and water	Negative ESI (MRM)	0.05−20 ng/mL (0.02 ng/mL)	α-amanitin: *m/z* 917.4>205.1, β-amanitin: *m/z* 918.4>205.1	45
α-Amanitin, β-Amanitin, γ-Amanitin, Phalloidin, Phallacidin	Human serum (500 μL) & urine (1000 μL), pig liver (5 g)	SPE (β-cyclodextrin collaborated molecularly imprinted polymers)	Acquity BEH C18	Gradient elution of 0.2% formic acid and methanol	Positive ESI (SRM)	Serum: 1.0−30 ng/mL (0.33−0.42 ng/mL) Urine: 0.5−30 ng/mL (0.16−0.33 ng/mL) Liver: 0.1−30 ng/g (0.035−0.056 ng/g)	α-amanitin: *m/z* 460.33>259.19, β-amanitin: *m/z* 461.22>259.19, γ-amanitin: *m/z* 452.29>243.11, phalloidin: *m/z* 847.54>157.1, phallacidin: *m/z* 789.29>330.2	54
α-Amanitin, β-Amanitin	Human plasma (2500 μL)	Protein precipitation with acetonitrile, LLE, and SPE (polymeric SCX cartridge)	Accucore Phenyl-hexyl	Gradient elution of 8 mM ammonium acetate containing 0.05% acetic acid and acetonitrile-methanol	Positive ESI (Orbitrap)	20−2000 pg/mL	α-amanitin: *m/z* 919.3614 β-amanitin: *m/z* 920.3614 identification: *m/z* 259.1275	44
α-Amanitin	Human urine	Protein precipitation with formic acid in acetonitrile-methanol (5:1, v/v), LLE, online SPE (ODS cartridge)	XBridge BEH C18	Gradient elution of methanol and water	Negative ESI (MRM)	0.1−50 μg/L (LOD, 0.03 μg/L)	α-amanitin: *m/z* 917.4>205.1	53

Table 1. Continued.

Toxin	Matrix	Sample preparation	Column	Mobile phase	Ionization (mode)	Linearity (LOD)	Transitions	Ref
α-Amanitin, β-Amanitin, Ricinine, Psilocin, Bufotenine, Muscarine, Muscimol, Ibotenic acid	Human urine (1500 μL)	Liquid-liquid extraction and SPE (Polymeric SCX cartridge)	Nucleodur HILIC	Gradient elution of methanol, acetonitrile, water, and 120 mM ammonium formate	Positive ESI (Orbitrap)	Limit of identification: α-amanitin, β-amanitin: 1 ng/mL; ricinine, psilocin bufotenine, muscarine: 5 ng/mL muscimol: 2000 ng/mL ibotenic acid: 1500 ng/mL	α-amanitin: *m/z* 919.3614, β-amanitin: *m/z* 920.3455, ricinine: *m/z* 165.0659, muscarine: *m/z* 174.1489, muscimol: *m/z* 115.0502, psilocin/bufotenine: *m/z* 205.1335, ibotenic acid: *m/z* 159.0400, l-tryptophan (IS): *m/z* 210.1285, psilocin-d_{10} (IS): *m/z* 215.1963	55
α-Amanitin, β-Amanitin, γ-Amanitin	Human urine (300 μL)	SPE (Oasis® Hydrophilic-Lipophilic Balance)	Acquity BEH HILIC	Gradient elution of acetonitrile and 20 mM ammonium formate with 0.2% formic acid	Positive ESI (SRM)	α- & γ-amanitin: 1−200 ng/mL, β-amanitin: 2.5−200 ng/mL (LOD: α-: 0.458 ng/mL, β-: 0.930 ng/mL; γ-: 0.169 ng/mL)	α-amanitin: *m/z* 919.3>338.9, β-amanitin: *m/z* 920.3>644.3, γ-amanitin: *m/z* 903.2>855.3, $^{15}N_{10}$-α-amanitin (IS): *m/z* 929.3>911.4, Methionine sulfoxide (IS): *m/z* 889.4>871.4	56
α-Amanitin, β-Amanitin, Phallacin, Phallisacin, Phallacidin	Rat plasma & urine (200 μL), mushroom	Protein precipitation with acetonitrile	Inertsil ODS-3	Gradient elution of 20 mM ammonium acetate containing 0.1% formic acid and acetonitrile	positive ESI (IT-TOF)	Qualitative identification	α-amanitin: *m/z* 919.3600, β-amanitin: *m/z* 920.3400, phallacin: *m/z* 849.3000, phallisacin: *m/z* 863.3200, phallacidin: *m/z* 847.3300	57
α-Amanitin, β-Amanitin, γ-Amanitin, Phallacidin	Dog plasma, urine (1000 μL)	Plasma: dilution and sonication Urine: SPE (Oasis WAX 1cc)	Acquity UPLC HSS T3	Gradient elution of 20 mM ammonium acetate and acetonitrile	positive ESI (MRM)	0.1−100 mg/kg (LOD: 0.01 mg/kg in urine and plasma: LOQ: 0.05 mg/kg)	α-amanitin: *m/z* 919.0>259.1, β-amanitin: *m/z* 920.0>259.1, γ-amanitin: *m/z* 903.0>86.1, phallacidin: *m/z* 848.0>157.1	58
α-Amanitin	Rat plasma (100 μL)	Protein precipitation with 1% formic acid in acetonitrile	Hypersil GOLD C18	Gradient elution of 20 mM ammonium acetate with 0.1% formic acid and acetonitrile	Positive ESI (MRM)	10–1500 ng/mL (LOD, 3.0 ng/mL)	α-amanitin: *m/z* 919.45>259.20, colchicine (IS): *m/z* 400.20>358.20	59
α-Amanitin, β-Amanitin, γ-Amanitin, Phalloidin, Phallacidin	Human plasma, serum, urine (100 μL)	SPE (PRiME HLB μElution 96-well plate)	CORTECS UPLC C18+	Gradient elution of 0.2 formic acid and 0.2% formic acid in methanol	Positive ESI (MRM)	1−100 ng/mL (LOD: α-, β-, γ-amanitin in plasma: 0.5 ng/mL, α-, γ-amanitin in urine: 1 ng/mL, phalloidin, phallacidin: 0.5 ng/mL)	α-amanitin: *m/z* 919.5>86.0, β-amanitin: *m/z* 920.5>86.0, γ-amanitin: *m/z* 903.0>86.0, phalloidin: *m/z* 789.4>157.0, phallacidin: *m/z* 847.0>157.0	60
α-Amanitin, β-Amanitin, Muscarine	Human urine (1000 μL)	SPE (weak cation phase (Strata-X-CW))	Acclaim RS 120 C18	Gradient elution of 2 mM ammonium formate with 0.1% formic acid and acetonitrile	Positive ESI (TOF)	α-, β-amanitin: 1−1000 ng/mL (LOD, 1 ng/mL), muscarine: 0.1-100 ng/mL (LOD, 0.1 ng/mL)	α-amanitin: *m/z* 919.3614, β-amanitin: *m/z* 920.3454, muscarine: *m/z* 174.1489, phallacidin (IS): *m/z* 847.3263	61

Table 1. Continued.

Toxin	Matrix	Sample preparation	Column	Mobile phase	Ionization (mode)	Linearity (LOD)	Transitions	Ref
α-Amanitin, β-Amanitin, Phalloidin	Human urine (500 μL)	SPE (Bond Elut Agilent C18)	C18 Accucore	Gradient elution of 10 mM ammonium acetate with 0.1% formic acid and 0.1% formic acid in acetonitrile	Positive ESI (Orbitrap)	1−100 ng/mL (LOD: α-amanitin, phalloidin: 0.25 ng/mL, β-amanitin: 0.5 ng/mL)	α-amanitin: *m/z* 919.3614, β-amanitin: *m/z* 920.3455, phalloidin: *m/z* 789.3257, flurazepam (IS): *m/z* 388.1586	62
α-Amanitin, β-Amanitin	Human urine (100 μL)	On-line turbulent flow chromatography	Accucore Phenyl-hexyl	Gradient elution of 10 mM ammonium acetate with 0.01% formic acid and acetonitrile with 0.1% formic acid	Negative ESI (Orbitrap)	1−100 ng/mL	α-amanitin: *m/z* 917.3458; β-amanitin: *m/z* 918.3298; γ-amanitin methyl ether (IS): *m/z* 915.3665	63
α-Amanitin, β-Amanitin	Human urine (200 μL)	Protein precipitation with acetonitrile and dilution with water	Schzero SM-C18	Gradient elution of 5 mM ammonium formate and methanol	Positive ESI (Q-TOF) MS/MS	α-: 0.01−5 μg/mL β-: 0.005−5 μg/mL	α-amanitin: *m/z* 919.361>259.1289, β-amanitin: *m/z* 920.345>259.1287	64
α-Amanitin, β-Amanitin	Human urine (1000 μL), liver (1 g)	Protein precipitation with acetonitrile, LLE, and SPE (Oasis HLB 6 cc cartridge)	Acquity UPLC HSS T3	Gradient elution of 20 mM ammonium acetate (pH 5) and acetonitrile	Positive ESI (MRM)	10−200 ng/mL or ng/g (LOD: α-: 0.22 ng/mL urine, 10.9 ng/g liver; β-: 0.2 ng/mL urine, 9.7 ng/g liver	α-amanitin: *m/z* 919.48>901.53, β-amanitin: *m/z* 920.48>902.44, tilmicosin (IS): *m/z* 869.60>696.50	65
α-Amanitin, β-Amanitin, Phalloidin	Human urine (400 μL)	SPE (Oasis HLB 1 cc cartridge)	-	-	MALDI (TOF)	10-500 ng/mL (LOD: 5 ng/mL)	α-amanitin: *m/z* 941, β-amanitin: *m/z* 942, phalloidin: *m/z* 811, microcystin (IS): *m/z* 1038	66
α-Amanitin, β-Amanitin, Phalloidin	Human serum, plasma, urine, rat urine (500 μL)	SPE (Oasis HLB 3 cc cartridge)	Acquity UPLC BEH Shield RP18	Gradient elution of 0.1% formic acid in water and methanol	Positive ESI (MRM)	2−420 ng/mL (LOD: 1 ng/mL in human plasma, 1.5 ng/mL in human serum, 0.5 ng/mL human urine, 1.5 ng/mL rat urine)	α-amanitin: *m/z* 919.6>919.6, β-amanitin: *m/z* 920.6>920.6, phalloidin: *m/z* 788.9>616, virginiamycin B (IS): *m/z* 868>663	67
α-Amanitin, β-Amanitin	Human plasma (1000 μL)	SPE (Discovery DSC-18, 500 mg)	Capcell Pak C18 UG120	Gradient elution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile	Positive ESI (SIM)	10−500 ng/mL (LOD: 0.5 ng/mL)	α-amanitin: *m/z* 919-921, β-amanitin: *m/z* 920-922	68
α-Amanitin	Human serum (1000 μL), dog liver	Protein precipitation (acetonitrile), LLE, and SPE (Xtrackt XRDAH C18/ benzenesulfonic acid)	Synergi RP-Polar	Gradient elution of 10 mM ammonium acetate with 0.1% formic acid and acetonitrile	Positive ESI (MS/MS/MS mode)	LOD: 0.26 ng/g serum, 0.5 ng/g liver	α-amanitin: *m/z* 941>746>300	69

Table 1. Continued.

Toxin	Matrix	Sample preparation	Column	Mobile phase	Ionization (mode)	Linearity (LOD)	Transitions	Ref
α-Amanitin, β-Amanitin	Human urine (5000 μL)	SPE (immunoaffinity column)	Hypersil RP-18	Gradient elution of 10 mM ammonium acetate (pH 5) and methanol	Positive ESI (SIM)	5−75 ng/mL (LOD: 2.5 ng/mL)	α-amanitin: *m/z* 919, 920, 921, β-amanitin: *m/z* 920, 921, 922, γ-amanitin methyl ether (IS): *m/z* 917, 918, 919	43
α-Amanitin, β-Amanitin	Human urine (5000 μL)	SPE (LiChrolut RP-18)	Kromasil RP-18	Methanol : 20 mM ammonium acetate (pH 5) (22:78, v/v)	Positive ESI (SIM)	50−500 ng/mL (LOD, 10 ng/mL)	α-amanitin: *m/z* 919, β-amanitin: *m/z* 920	70

LLE: liquid-liquid extraction; SPE: solid-phase extraction; SCX: strong cation exchanger; LOD: limit of detection; ESI: electrospray ionization; PRM: parallel reaction monitoring; SIM: selected ion monitoring; SRM: selected reaction monitoring; MRM: multiple reaction monitoring; TOF: time of flight; IS: internal standard.

Table 2. The HPLC and capillary zone electrophoresis methods for the determination of amatoxins in various biological matrices.

Toxins	Matrix	Sample preparation	Column	Mobile phase	detection	Linearity (LOD)	Ref
HPLC							
α-Amanitin	Rat liver, kidney (1 g)	Protein precipitation with perchloric acid	Spherisorb RP-18	20% methanol in 50 mM citric acid, 0.46 mM octanesulfonic acid (pH 5.5 adjusted with 10 mM NaOH)	DAD 305 nm; Electrochemical detection	UV: 0.33−10 μg/g liver; 0.5−10 μg/g kidney (LOD: 0.05 μg/g liver; 0.125 μg/g kidney) ECD: 0.21−10 μg/g liver; 0.11−10 μg/g kidney (LOD: 0.015 μg/g liver; 0.05 μg/g kidney)	71
α-Amanitin	Human serum (500 μL)	Protein precipitation with 1% acetic acid-acetonitrile, aqueous LLE, and SPE (SCX or C18/polymeric SCX)	Diamonsil C18	30% methanol in 10 mM ammonium acetate with 0.1% formic acid	UV 302 nm	SPE with polymeric SCX: 20−500 ng/mL (LOD 6.0 ng/mL) SPE with C18/polymeric SCX: 10−500 ng/mL (LOD 3.0 ng/mL)	72
α-Amanitin	Human urine (10000 μL)	SPE (Bond Elut Certify containing C8 and SCX)	Supelcosil LC18	10% acetonitrile in 5 mM bisodic phosphate (pH 7.2)	Coulometric detection	10−200 ng/mL (LOD, 10 ng/mL)	73
α-Amanitin	Human plasma (2000 μL)	SPE (SepPak C18)	Polystyrene-divinyl benzene	9% acetonitrile in 50 mM phosphate buffer	Ampherometric detection	3−200 ng/mL (LOD, 2 ng/mL)	74
α-Amanitin, Phalloidin	Human plasma (3000 μL)	Column-switching (precolumn: MPLC cartridge RP-8 Spheri-5)	MPLC cartridge RP-8 Spheri-5	acetonitrile-water (16.67 : 83.33, v/v)	UV 303 nm	10−100 ng/mL (LOD: 10 ng/mL)	75
α-Amanitin, β-Amanitin, γ-Amanitin	Human serum (2000 μL) & urine (1000 μL)	SPE (serum: SepPak C18 and silica; urine: immunoaffinity sorbent)	Hypersil WP300 butyl	8% acetonitirile in 20 mM ammonium acetate (pH 5) containing 0.5 mM EDTA	Ampherometric detection	20−200 ng/mL (LOD 2.5 ng/mL urine)	76
α-Amanitin, β-Amanitin	Human serum (5000 μL) & urine (100 μL), stomach washings (250 μL)	Protein precipitation with acetonitrile, LLE, and SPE (SepPak C18)	Ultrasphere ODS	12% acetonitirile in 20 mM ammonium acetate (pH 5)	UV 280 nm	20−500 ng/mL serum	77
α-Amanitin, β-Amanitin, Phalloidin	Human serum & urine (1000 μL), mushroom (2 g)	Aqueous phase after LLE with methanol-chloroform	Lichrosorb RP18	Gradient elution of 10 mM ammonium acetate (pH 5) and acetonitrile	UV 302 nm	0.5−20 μg/mL (LOD: 10 ng for α-, β- amanitin; 5 ng for phalloidin)	78
capillary zone electrophoresis							
α-Amanitin, β-Amanitin	Human urine (10 μL)	Dilution with BGE (1:20)	Fused-silica capillary	5 mM borate buffer (pH 10)	UV 214 nm	5−100 ng/mL (LOD: 2.5 ng/mL)	79
α-Amanitin, β-Amanitin	Human urine, mushroom	Dilution with water (urine, 1:1; mushroom extract, 1:2500)	Capillary	100 mM phosphate (pH 2.4)	UV 214 nm	1−1000 μg/mL mushroom; urine: qualification	80

LLE: liquid-liquid extraction; SPE: solid-phase extraction; SCX: strong cation exchanger; LOD: limit of detection

been combined in an attempt to avoid the matrix effect, but this has disadvantages such as high labor requirements and a long turnaround time (~24 h).

LC-MS methods

Reverse-phase chromatography using C_{18}, C_8, C_4, or phenyhexyl columns is the most common technique for chromatographic analysis of α-, β-, and γ-amanitin in biological fluids. Gradient elution of mobile phase A (ammonium acetate or formic acid) and mobile phase B (acetonitrile or methanol) has been used as the mobile phase for LC-MS methods (Table 1), whereas isocratic elution is used in HPLC methods (Table 2). Hydrophilic interaction chromatography has been used for the simultaneous determination of α-, β-, and γ-amanitin, and six mushroom toxins in human urine, to increase the retention and ionization efficiency.[55,56]

Positive and negative electrospray ionization (ESI) modes have been used for the ionization of amatoxins when applying LC-MS methods (Table 1). Negative ESI mode has higher sensitivity and smaller matrix effects compared to positive ESI mode.[41,42,45,50-53,64] MALDI-TOF MS has been used for qualitative analysis of α-amanitin, β-amanitin, and phalloidin in human urine.[66]

For the quantification of α-, β-, and γ-amanitin, selective ion monitoring mode with quadrupole MS,[43,68,70] multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) mode with triple quadrupole tandem MS (MS/MS),[45,50,51,53,54,56,58-60,65,67] and parallel reaction monitoring (PRM) mode using orbitrap MS[41,42,44,55,62,63] have been used (Table 1). LC-MS/MS methods using quadrupole MS/MS and orbitrap MS are powerful techniques with high sensitivity (lower limit of quantification [LLOQ] = 0.02–50 ng/mL plasma for α-, β-, and γ-amanitin), reproducibility, and specificity. Recently, LC-MS/MS methods performed in PRM mode and protein precipitation of plasma samples (5 mL) for sample clean-up showed good sensitivity (LLOQ 0.5 ng/mL for α- and β-amanitin), selectivity, and speed.[41,42,52]

Conclusions

Because the incidence of amatoxin-induced mushroom poisoning has increased globally, early detection of amatoxins in cases of suspected mushroom poisoning is necessary to improve patient outcomes through aggressive and immediate supportive care among other potential therapies. Early diagnosis of amatoxins has been achieved using LC-MS/MS methods. These methods may be suitable for routine clinical and forensic toxicological analysis of amatoxins in plasma, serum, urine, and tissue samples due to the high specificity, sensitivity, and reproducibility relative to other analytical methods. However, protein precipitation, LLE, and SPE have been combined for sample preparation, to minimize matrix effects and achieve high sensitivity, but with high labor requirements and a long turnaround time. There is a need to improve sample preparation procedures for rapid clinical and forensic toxicological analyses.

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