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Determination of α-Amanitin in Serum and Liver by Multistage Linear Ion Trap Mass Spectrometry

MICHAEL S. FILIGENZI,* ROBERT H. POPPENGA, ASHEESH K. TIWARY, AND BIRGIT PUSCHNER

California Animal Health and Food Safety Laboratory System, Toxicology Laboratory, University of California, Davis, California 95616

This paper describes a rapid LC-MS/MS/MS method for the analysis of α-amanitin in serum and liver. Serum was initially prepared by precipitation of proteins with acetonitrile and subsequent removal of acetonitrile with methylene chloride. Liver was prepared by homogenization with aqueous acetonitrile and subsequent removal of acetonitrile using methylene chloride. For both matrices, the aqueous phase was then extracted using mixed-mode C18/cation exchange SPE cartridges and analyzed on a linear ion trap LC-MS system. Standards were prepared in extracts of control matrix. Seven replicate fortifications of serum at 0.001 μg/g (1 ng/g) of α-amanitin gave a mean recovery of 95% with 8.8% CV (relative standard deviation) and a calculated method detection limit of 0.26 ng/g. Seven replicates of control liver fortified at 1 ng/g gave a mean recovery of 98% with 17% CV and a calculated method detection limit of 0.50 ng/g. This is the first report of a positive mass spectrometric identification and quantitation of α-amanitin in serum and liver from suspect human and animal intoxications.

KEYWORDS: Amanitin; liquid chromatography; mass spectrometry; LC-MS; ion trap

INTRODUCTION

Illnesses and deaths due to the ingestion of toxic mushrooms have been reported since ancient times and remain a problem. The mushrooms most likely to cause fatalities are those that contain amanitins, a family of hepatotoxic cyclopeptides. Three genera, *Amanita*, *Galerina*, and *Lepiota* (*1*), are known to contain hepatotoxic cyclopeptide toxins. The most toxic cyclopeptide-containing mushrooms are *Amanita phalloides*, the ubiquitous Death Cap or Death Angel, and *Galerina sulpices*. *A. phalloides* is found throughout North America, commonly in association with oaks and birch, and is the species most frequently resulting in fatalities in humans (*2*). In Eastern Europe, *G. sulpices* is considered to be the species most commonly associated with human fatalities, followed by *A. phalloides* (*3*). Although there are three groups of cyclopeptides, including the amatoxins, phallotoxins, and virotoxins, only the amanitins have been associated with severe poisonings and lethalities (*4*). Most cases are diagnosed by positive identification of the suspect mushroom along with the occurrence of consistent clinical signs (*5*). In the case of animals, mushroom exposure is even more difficult to prove because animals are often left unattended and a history of ingestion is not available. Detection of amanitins in biological specimens is confirmatory for diagnostic purposes, but such tests are currently limited to the analysis of urine of human origin for α-amanitin (*6*) or are not routinely available to the medical community (*7*). Therefore, the accuracy of the reported frequency of mushroom poisoning is likely to be low because of the lack of methods to confirm exposure to toxic mushrooms. In suspect intoxications a rapid, specific, and accurate method assists in the early recognition of exposure. Early recognition of exposure is critical because survival rates are greatly improved with timely therapeutic intervention (*5*, *10*).

There are nine known amatoxins, with α- and β-amanitin each making up approximately 40% of the total amanitin content in most amanitin-containing mushrooms (*8*). All amatoxins are bicyclic octapeptides differing only in the number of hydroxyl groups and in the presence or absence of an amine group on an aspartic acid residue. The chemical structures of α-amanitin and β-amanitin are represented in **Figure 1**.

There are only limited data on the bioavailability of amanitins in humans and animals. After oral ingestion of *A. phalloides* in humans, α- and β-amanitins were detected in plasma up to 36 h after ingestion and in urine up to 72 h postexposure (*9*). Following intravenous (iv) administration of amanitins in dogs, it was shown that the plasma half-life of amanitins is short, ranging from 25 to 50 min, and that amanitins are not detectable in plasma after 4−6 h (*10*). Therefore, amanitins can be detected in serum or plasma well before any clinical sign of poisoning has occurred, whereas routine laboratory tests such as serum chemistry profiles are unremarkable until liver or kidney damage has occurred. Between 80 and 90% of the iv dose of amanitins is eliminated in urine, and up to 7% is eliminated in the bile (*9*). There is no known metabolism, or plasma protein binding, of the α-amanitin. It has been established that plasma and urine

* Author to whom correspondence should be addressed [telephone (530) 754-5608; fax (530) 752-3361; e-mail msfiligenzi@ucdavis.edu].

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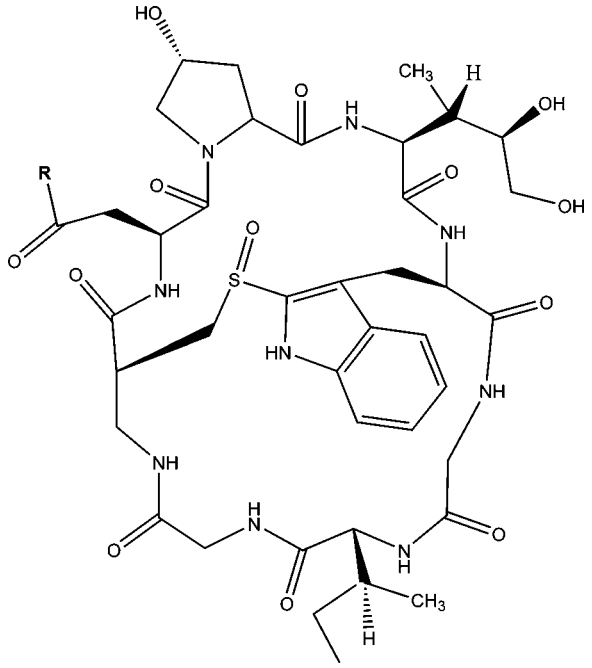

Figure 1. Structure of amanitin: α-amanitin, R = NH_2; β-amanitin, R = OH.

amanitin concentrations do not seem to correlate with the clinical severity or outcome. In post-mortem investigations of humans, α- and β-amanitins have been detected in kidney and liver up to 22 days after the ingestion of *A. phalloides* (*10*).

The goal of this investigation was to develop a rapid, sensitive, and highly specific method for the analysis of amanitins in serum and tissue with detection limits of 1 ng/g or less. Although we were primarily interested in a qualitative diagnostic analysis, we felt that a quantitative method may also be valuable. This procedure is especially suited to medical and veterinary diagnostic or public health laboratory situations for which rapid ante-mortem diagnosis of exposure to amanitin-containing mushrooms is desirable.

MATERIALS AND METHODS

Reagents. Ultrapure water was obtained from a Barnstead Nanopure Infinity system. HPLC grade methanol, acetonitrile, methylene chloride, reagent grade glacial acetic acid, and formic acid were obtained from Fisher Scientific (Pittsburgh, PA). Monobasic and dibasic potassium phosphate (minimum 99% pure) were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Analytical Standards. α-Amanitin and β-amanitin (minimum 90% purity) isolated from *A. phalloides* were obtained from Axxora, LLC (San Diego, CA). Separate stock solutions of 0.5 mg/mL for each amanitin were prepared in methanol. Combined working solutions used for fortifying control samples were prepared by diluting the stock solutions with methanol. Standard solutions for use in LC-MS analysis were prepared by dilution of the stock solutions in water.

Extraction of Amanitin from Serum. The sample extraction and cleanup methods were modified from previously reported procedures (*11*, *12*). Briefly, 1 mL of serum was combined with 4 mL of acetonitrile and vortexed for approximately 30 s. The sample was centrifuged for 10 min at 2000 rpm (approximately 650*g*) using an IEC CU-5000 centrifuge (International Equipment Corp., Needham Heights, MA), and the supernatant was decanted into a 25-mL disposable test tube. Ten milliliters of methylene chloride was added, and the tube was inverted several times and then centrifuged for 5 min at 2000 rpm. One milliliter of water was carefully added to the aqueous (top) layer, and this layer was then transferred to a 16 × 125 mm tube. Xtrackt XRDAH C18/benzenesulfonic acid solid-phase extraction cartridges (United Chemical Technologies, Bristol, PA) were conditioned by sequential rinsing with 3 mL of methanol, 3 mL of water, and 3 mL of 0.1 M, pH 6, phosphate buffer. The aqueous extract was then applied to the cartridge. The cartridge was rinsed sequentially with 5 mL of water, 3 mL of 0.1 M acetic acid, and 5 mL of methylene chloride/methanol (95:5), dried under vacuum for approximately 1 min, and eluted with 4 mL of methanol. The eluent was evaporated to dryness under nitrogen using a TurboVap LV evaporation system (Caliper Life Science, Hopkinton, MA) set at 30 °C. The extract was redissolved in 0.25 mL of water and filtered through a 0.45 μM HPLC syringe filter (Millipore Corp., Milford, MA) into a 2-mL autosampler vial with a 250-μL insert. All control and fortified samples were prepared in the same manner.

Extraction of Amanitin from Tissue (Kidney and Liver). Kidney tissue was frozen in liquid nitrogen and ground to a powder. Liver tissue was finely chopped. One gram of the ground or chopped tissue was weighed into a 16 × 125 mm test tube and homogenized with 5 mL of 30% 0.1 M phosphate buffer in acetonitrile using an Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, NC). The homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was transferred to a 50-mL test tube and the pellet rinsed twice with 5-mL aliquots of 0.1 M phosphate buffer. The rinses were combined with the initial supernatant, and 30 mL of methylene chloride was added. The tube was shaken by hand and then centrifuged for 5 min at 2000 rpm to remove the acetonitrile. The aqueous layer was transferred to a 25-mL test tube and cleaned up using the solid-phase extraction procedure described for serum samples.

Instrument Calibration Standards. Five-point calibration curves for quantification of serum and tissue samples were prepared by fortification of dried extracts of the appropriate negative control matrix, prepared as per the methods listed above. For each calibrant, an aliquot of the standard solution was added to the dried extract, vortexed, evaporated to dryness on the TurboVap, and then redissolved in 0.25 mL of water. Calibrants were prepared at levels of 1.0, 5.0, 10, 25, and 50 ng/mL.

Quality Control Samples. Each batch of 3−12 samples included a negative control sample and a positive control sample fortified at a level of 1 ng/g of α-amanitin. Fortified samples were prepared by adding an appropriate level of standard solution in methanol to the negative control matrix and allowing it to equilibrate for at least 15 min prior to extraction. Control matrices included human and porcine serum and bovine liver. It should be noted that a single source of each control matrix was used for the preparation of both standards in matrix and fortified control samples. Therefore, this investigation does not take into account potential differences in control matrix composition that could affect measurements of amanitin levels between individuals.

LC-MS/MS/MS Analysis. An Agilent model 1100 binary high-performance liquid chromatograph coupled to a Thermo LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA) was used for all reported analyses. The chromatograph was fitted with a 100 mm × 4.6 mm i.d. Synergi RP-Polar column with a 10-mm RP-Polar guard column (Phenomenex, Torrance, CA). The mobile phases consisted of 0.01 M ammonium acetate in 0.1% v/v aqueous formic acid (channel A) and 0.01 M ammonium acetate in 0.1% v/v methanolic formic acid (channel B). The flow rate was 0.50 mL/min. The initial mobile phase consisted of 60% B held for 5 min and was then ramped immediately to 90% B. It was held at 90% B for 2 min and then returned to the initial conditions and held for 8 min for re-equilibration. α-Amanitin eluted at 4.5 min under these conditions. Chromatographic flow was diverted to waste for the first 2 min and after 6 min. The injection volume was 20 μL.

The LTQ was operated in electrospray mode using positive ionization. Instrument source conditions were optimized by infusion of a 1 μg/mL solution of α-amanitin. α-Amanitin was monitored in MS/MS/MS mode by sequentially fragmenting the sodium adduct of the molecular ion (*m*/*z* 941) to *m*/*z* 746 and then taking the full-scan spectrum of product ions of *m*/*z* 746. Collision energies were 40% for the *m*/*z* 941 → *m*/*z* 746 transition and 25% for fragmentation of *m*/*z* 746. Samples were considered to be positive when a peak matching the retention time for the amanitin standard was detected with a spectrum containing ions at *m*/*z* 395, 488, 659, 718, and 729. No specific ion ratios were required for a positive identification.

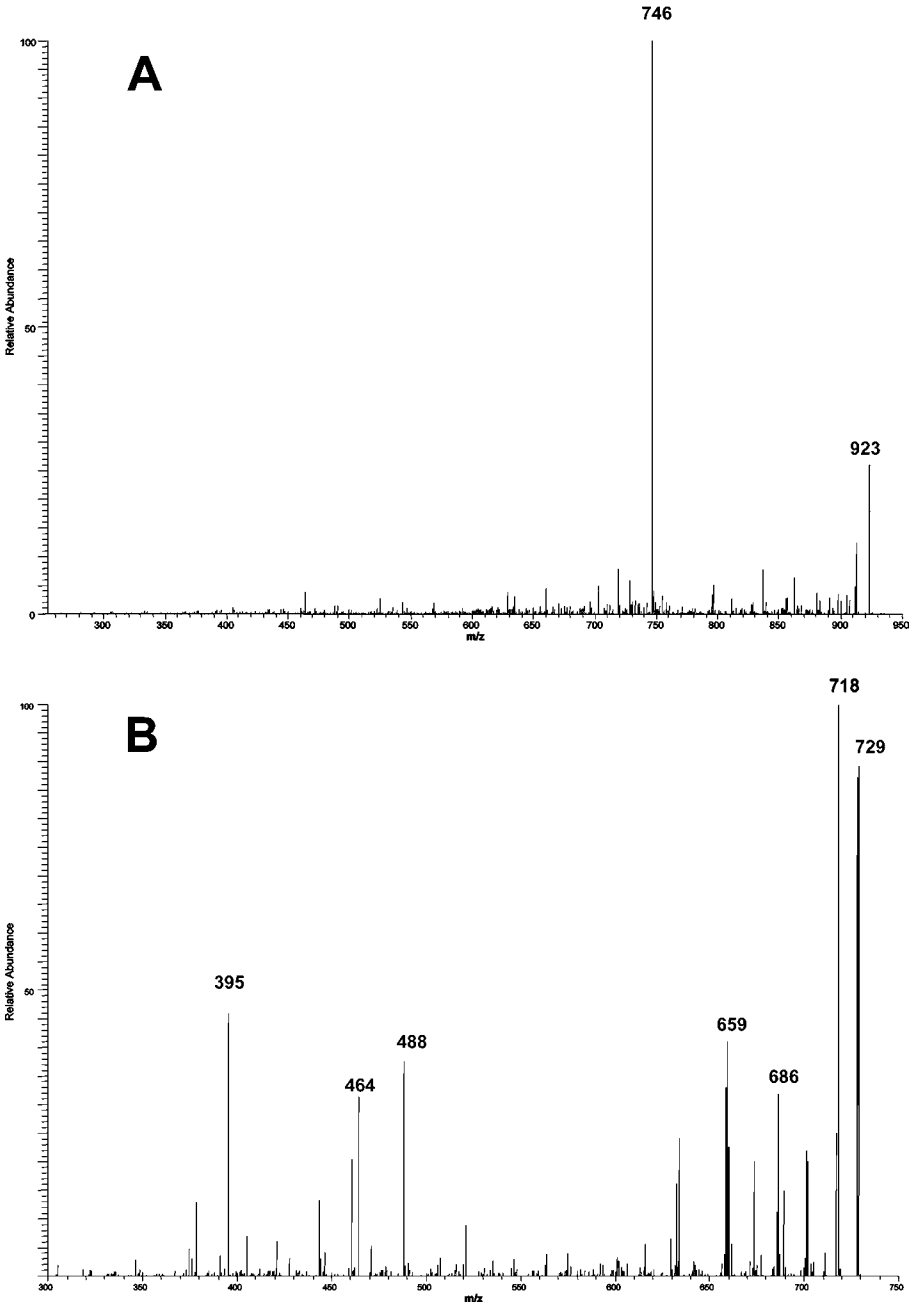

Figure 2. (**A**) MS/MS spectrum of $[M + Na]^+$ at *m*/*z* 941 of α-amanitin. (**B**) MS/MS/MS spectrum of α-amanitin, *m*/*z* 941 → 746 → 300–750.

Each analytical sequence for qualitative screening began and ended with a 1 ng/g standard of amanitins in water. Each sequence for quantitative analysis began and ended with a five-point calibration curve in negative control matrix. Each analytical sequence also included the negative control sample and the fortified control sample prepared with the extraction batch.

Method Validation. Method detection limits (MDLs) were determined by analysis of seven replicates of the appropriate negative control matrix fortified with α-amanitin at 1 ng/g. MDLs for each matrix were calculated by multiplying Student's *t* value by the standard deviation of the α-amanitin values determined in the seven replicate analyses. The method was also tested by analysis of routine diagnostic samples from animals and humans suspected of having been exposed to amanitin mushrooms.

RESULTS AND DISCUSSION

A number of methods have been developed for the diagnostic analysis of α- and β-amanitins in a variety of matrices. Radioimmunoassay was successfully used for the analysis of α- and β-amanitins in human serum, urine, and duodenal fluids and in dog serum (*13*, *14*). This method was sensitive, with a detection limit of 0.5 ng/g in urine, but its use has been limited due to the requirement for several radioactive reagents that are not currently commercially available. Several chromatographic methods have been developed for amanitin analysis in urine, including HPLC with UV detection for α- and β-amanitins (*11*, *15*), HPLC with coulometric detection for α-amanitin (*12*), and HPLC-MS after solid-phase extraction and immunoaffinity extraction for α- and β-amanitins (*16*, *17*). Detection limits for these methods range from 2.5 to 10 ng/g. The latter method was also applied to human serum analysis. Of these methods, the immunoaffinity−LC-MS method offered the highest levels of specificity and sensitivity. However, the immunoaffinity column used for this method required the generation and purification of antibodies, a process that is beyond the capabilities of most diagnostic laboratories. An ELISA kit is commercially available for the analysis of α- and γ-amanitins (Alpco

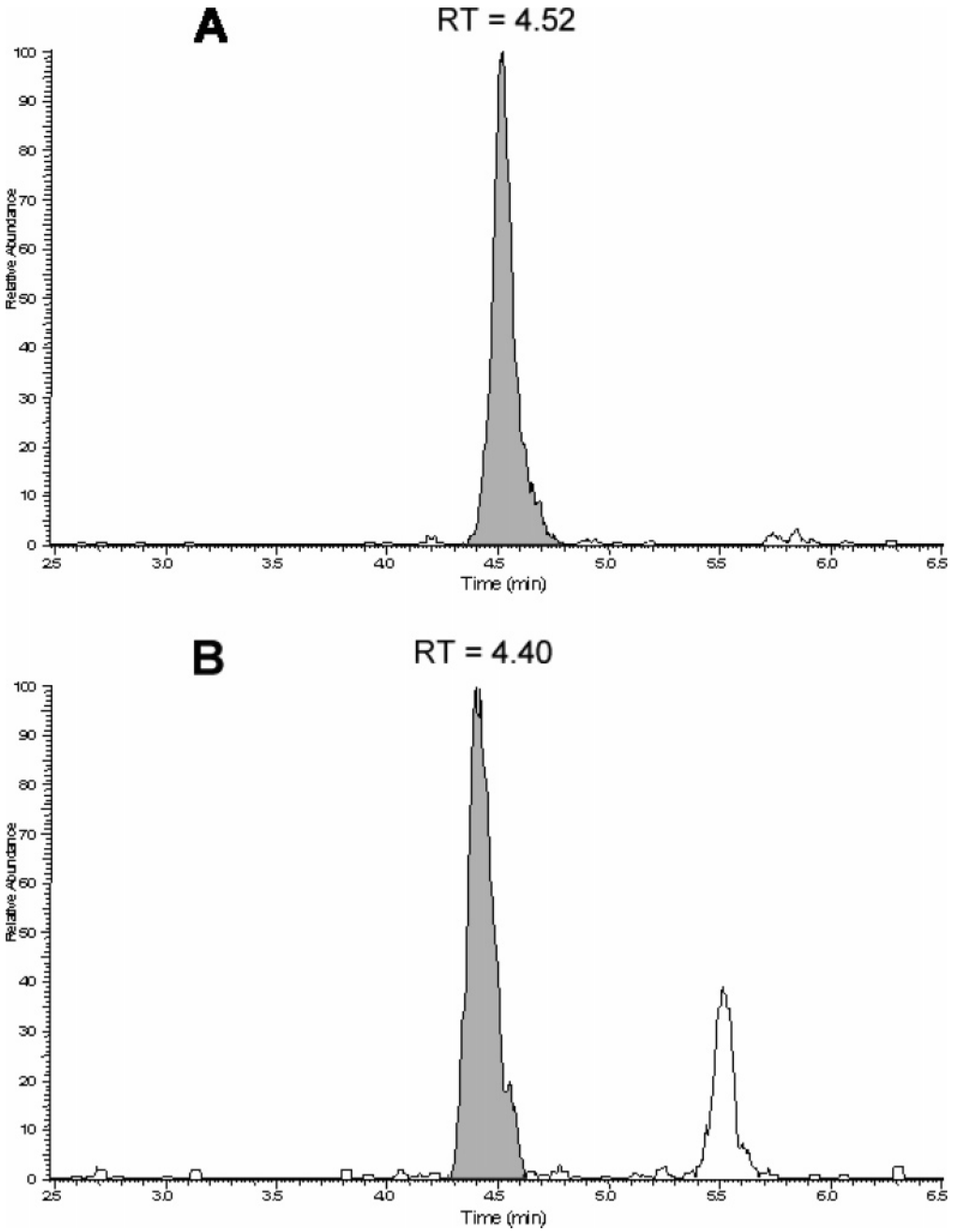

Figure 3. Total ion chromatogram of 1 ng/g α-amanitin fortified into (**A**) control serum extract and (**B**) control liver extract (peak at 4.4 min).

Diagnostics, Windham, NY). Although the manufacturer lists the functional sensitivity of these kits as 1.5 ppb, this level is considered to be a guideline only and a reliable, evidence-based cutoff value for the assay has yet to be determined (*6*, *18*, *19*). Another ELISA test was reported for *β*-amanitin in urine (*7*), although it has not been made commercially available. It should also be noted that ELISA testing is generally not considered to be definitive for the identification of analytes and therefore requires confirmation of positive results by an alternative analytical technique. Analysis of mushrooms and biological fluids using capillary electrophoresis has also been reported (*20*), although detection limits were above those required for clinical use. To our knowledge, there are no published methods for the analysis of amanitins in tissue such as kidney or liver, although levels in liver and kidney have been reported (*9*). Of the methods listed above, none allows for the unambiguous detection of amanitin at low levels in biological matrices with commercially available reagents and equipment. Such a method would facilitate the ante- and post-mortem diagnosis of amanitin exposure and intoxication in humans and animals.

Although α- and *β*-amanitins are structurally closely related, they do not respond in the same manner under electrospray conditions. Upon infusion, α-amanitin gave a protonated molecular ion as the base peak with a sodium adduct at approximately 40−50% abundance. MS/MS dissociation of the $[M + H]^+$ ion resulted in a spectrum with the $[M + H - H_2O]^+$ ion predominating. Fragmentation of this ion using MS/MS/MS gave a spectrum with many low-abundance ions and a level of sensitivity that was not sufficient to detect the low levels expected to be present in serum and tissue. Fragmentation of the $[M + Na]^+$ ion for α-amanitin gave improved results, with a major ion at *m*/*z* 746 (**Figure 2A**). Subsequent fragmentation of the ion at *m*/*z* 746 gave an MS/MS/MS spectrum with numerous diagnostic ions and sensitivity sufficient for low-level detection of α-amanitin in biological fluids and tissues (**Figure 2B**).

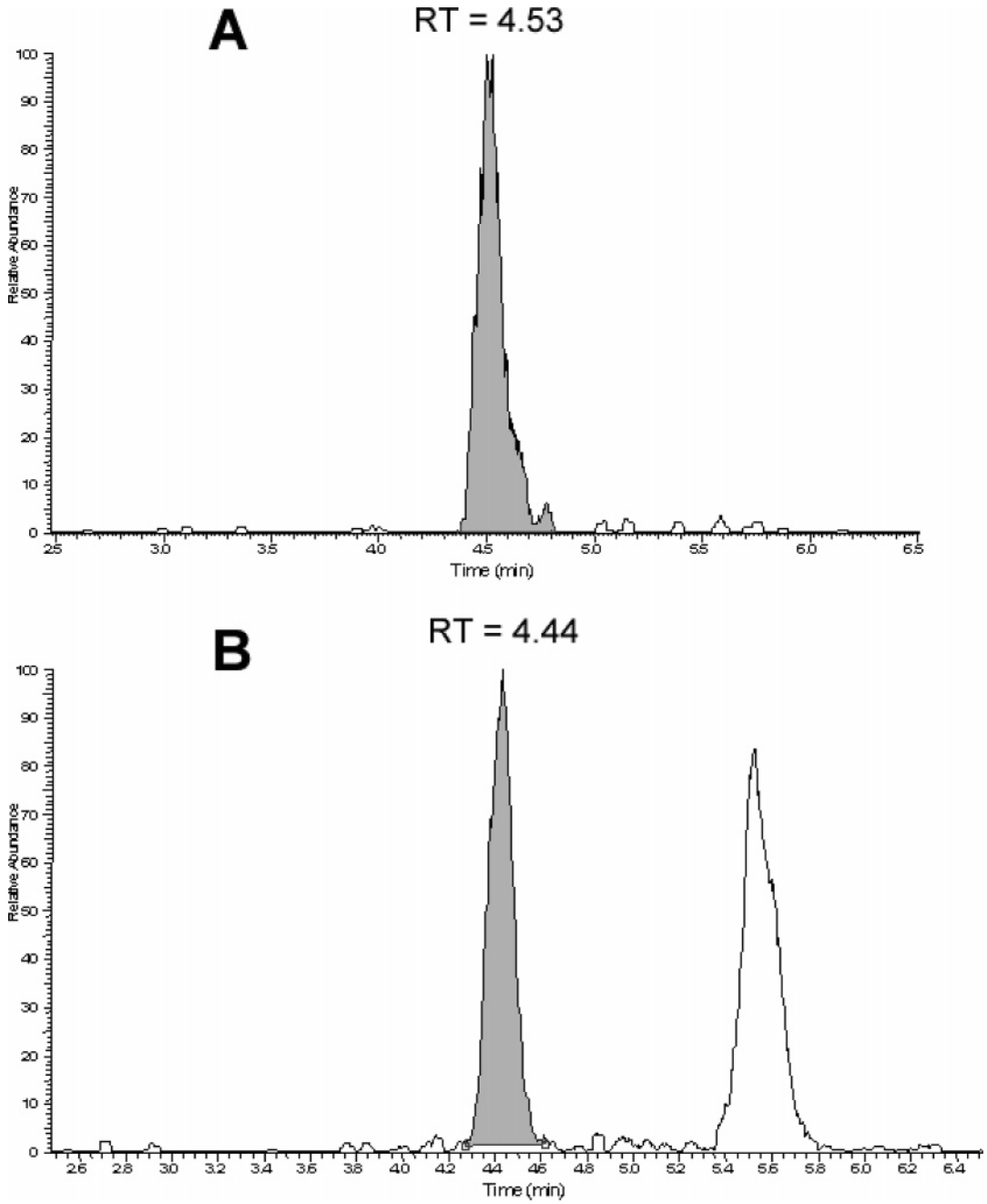

Figure 4. Chromatogram of α-amanitin in (**A**) serum taken from an individual after ingestion of *Amanita ocreata* mushrooms (peak at 4.53 min) and (**B**) liver from a dog exposed to amanita mushrooms (peak at 4.44 min).

Infusion of β-amanitin showed a base peak at *m*/*z* 920, corresponding to the $[M + H]^+$ ion. MS/MS of this ion produced a spectrum primarily consisting of the $[M + H - H_2O]^+$ ion, with poor sensitivity using MS/MS/MS fragmentation. The signal for the $[M + Na]^+$ ion for β-amanitin was only approximately 10% of that for the $[M + H]^+$ ion, and fragmentation of the sodium adduct gave significantly poorer sensitivity as compared to α-amanitin. Therefore, this investigation focused on analysis for α-amanitin only.

The relatively poor sensitivity for β-amanitin represents a potential challenge to this method. In one study, several instances were reported in which β-amanitin was detected in urine or serum and α-amanitin was not (*9*). Although the method used in that study was less sensitive for α-amanitin than the current method, the possibility that a sample from an amanitin-exposed individual would not contain α-amanitin at a detectible level cannot be ignored. Further investigation involving cases of exposure will be required to determine the necessity of analyzing for β-amanitin and the level of sensitivity required for diagnostic relevance.

To our knowledge, there is only one published study in which tissue levels of amanitins in exposed individuals were determined (*10*). In that study, amanitins were detected in kidney and liver of four exposed humans, with levels in kidney generally being higher than those in liver. Although these data would indicate that kidney is the preferred matrix for amanitin detection, the number of tissue samples analyzed remains small, and further investigation of tissue levels is warranted, particularly in cases involving animals.

The extraction procedures we report gave acceptable recoveries for α-amanitin in serum and liver. Calibration curves for biological fluid and tissue analyses prepared in control matrix extracts gave linear calibration curves with $r^2 > 0.990$ in the range of 1−50 ng/g in matrix. Analysis of seven control serum

samples fortified at 1 ng/g gave a mean recovery of 95%, a within-day percent relative standard deviation (%RSD) of 8.8, and a calculated MDL of 0.26 ng/g. Analysis of seven control liver samples fortified at 1 ng/g gave a mean recovery of 98%, a %RSD of 17, and a calculated MDL of 0.50 ng/g. **Figure 3A** shows a typical chromatogram for an extract of control serum fortified with 1 ng/g of α-amanitin. **Figure 3B** shows a chromatogram for an extract of control liver fortified with 1 ng/g of α-amanitin. The extraction and cleanup procedures used for biological fluids and tissues combined with LC-MS/MS/MS provided chromatograms with minimal background interference. α-Amanitin was not detected in any negative control sample.

This method has several advantages over those previously described for the analysis of α-amanitin in serum and tissues and therefore will be more useful diagnostically. The use of LC-MS/MS/MS provides abundant qualitative information and unambiguous identification of amanitin, particularly when compared to ELISA methods; one potential application of this method would be to confirm presumptive positive identifications by ELISA. Sample preparation is straightforward and involves only commercially available reagents. An analyst can prepare a set of eight samples with the associated quality control samples within approximately 4 h, and analytical results can be available within 1 day of sample receipt by the laboratory. Furthermore, this method gives detection limits as low as or lower than those of previously described methods. This is extremely important in a diagnostic setting due to the low levels found in serum even within 24 h of exposure. This method also should be useful for quantitative applications.

This method has been used in the analysis of a variety of samples submitted for investigations of suspect amanitin intoxications in humans and animals. Sera from individuals suspected to have ingested amanitin-containing mushrooms were analyzed qualitatively for α-amanitin. Two of the samples were taken within 24 h of mushroom ingestion, and three were taken between 24 and 48 h after ingestion. The two samples taken within 24 h of ingestion of the mushrooms were both positive for α-amanitin, whereas the other three were negative. **Figure 4A** shows a chromatogram of one of the positive serum samples. Serum amanitin concentrations vary depending on the time interval between exposure and sample collection and are most likely also influenced by the species affected by the intoxication. However, on the basis of the preliminary data, a detection limit of 0.20 ng/g for α-amanitin provides an adequate diagnostic tool for case investigations of suspect exposures to amanitin-containing mushrooms.

A liver sample from a dog suspected of having died of amanitin poisoning was analyzed qualitatively. This animal died approximately 24 h after the suspected exposure and was necropsied 3 days later. The liver was stored frozen for approximately 4 months prior to analysis. **Figure 4B** shows a chromatogram from the analysis of that liver sample. Kidney tissue from the same animal was later analyzed and also found to be positive for α-amanitin.

To our knowledge, this is the first reported method for LC-MS/MS/MS analysis of amanitins in serum and tissue. This method offers a significant improvement in diagnosis of amanitin intoxications in that it provides a rapid and unequivocal determination of amanitins in serum and liver. Although there is no specific antidote for amanitins, prompt and aggressive measures are required to improve prognosis. Thus, rapid diagnosis is essential to reduce the mortality rate from amanita poisoning.

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Supporting Information Available: Chromatograms of negative control samples and mass spectra of α-amanitin from the analyses shown in **Figures 3A−4B**. This material is available free of charge via the Internet at http://pubs.acs.org.

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