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Simultaneous Determination of α-Amanitin and β-Amanitin in Mouse Plasma Using Liquid Chromatography-High Resolution Mass Spectrometry

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Abstract : α-Amanitin and β-amanitin are highly toxic bicyclic octapeptides responsible for the poisoning of poisonous mushrooms such as *Amanita*, *Galerina*, and *Lepiota* by inhibiting RNA polymerase II, DNA transcription, and protein synthesis. A sensitive, simple, and selective liquid chromatography-high resolution mass spectrometric method using parallel reaction monitoring mode was developed and validated for the simultaneous determination of α- and β-amanitin in mouse plasma to evaluate the toxicokinetics of α- and β-amanitin in mice. Protein precipitation of 5 μL mouse plasma sample with methanol as sample clean-up procedure and use of negative electrospray ionization resulted in better sensitivity and less matrix effect. The calibration curves for α- and β-amanitin in mouse plasma were linear over the range of 0.5–500 ng/mL. The intra- and inter-day coefficient of variations and accuracies for α- and β-amanitin at four quality control concentrations were 3.1–14.6% and 92.5–115.0%, respectively. The present method was successfully applied to the toxicokinetic study of α- and β-amanitin after an oral administration of α- and β-amanitin at 1.5 mg/kg dose to male ICR mice.

Keywords: α-amanitin, β-amanitin, LC-HRMS, mouse plasma

Introduction

Amatoxins are highly toxic bicyclic octapeptides found in three genera of poisonous mushrooms, i.e., *Amanita*, *Galerina*, and *Lepiota* including nine different compounds such as α-, β-, γ-, and ε-amanitin, amanullin, amanullinic acid, amaninamide, amanin and proamanullin and cause serious cellular damage by inhibiting RNA polymerase II, DNA transcription, and protein synthesis in eukaryotic and MCF-7 cells,[1-7] α- and β-Amanitin are found to be higher than other amatoxins in poisonous mushrooms and are responsible for poisonings.[8-11]

For the simultaneous determination of α- and β-amanitin in various biological matrices including serum, plasma, urine, and tissues, radioimmuno assay,[12] enzyme-linked immunoassay,[13,14] capillary zone electrophoresis,[15,16] high-performance liquid chromatography (HPLC) with ultraviolet detection or electrochemical detection (ECD),[17-19] LC with mass spectrometry (LC-MS),[20-22] triple quadrupole mass spectrometry (LC-MS/MS),[23-26] or with high-resolution mass spectrometry (LC-HRMS)[27-34] have been reported. There are reports on the analysis of a-amanitin only in plasma, serum, urine, or tissue samples using HPLC-ECD,[35,36] LC-MS/MS,[37,38] or LC-HRMS.[39] Solid-phase extraction (SPE),[18,20-23,25,27,28,30,35,36] SPE combined with liquid-liquid extraction (LLE),[24,32-34] SPE combined with protein precipitation and LLE,[26,37] and on-line SPE[29] have been used as sample clean-up procedures, being expensive, laborious and time-consuming. A few methods used protein precipitation with acetonitrile, methanol, or perchloric acid as sample clean-up procedure.[31,37-39] These methods provided a low limit of quantification (LLOQ) levels, namely 0.05–25 ng/mL for α- and β-amanitin but used large volumes of biological samples (100-5000 μL). There are a few reports on the determination of plasma concentrations of α- and β-amanitin after oral administration of toadstool extract in dogs and rats.[31,37,38,40] Therefore, the toxicokinetic features of α- and β-amanitin after their administration as pure chemical still need to be unveiled. For the toxicokinetic study of α- and β-amanitin, it is necessary to develop a simple and sensitive analytical method using small volume of mouse plasma samples for the simultaneous determination of α- and β-amanitin.

We have developed a rapid, simple, and sensitive LC-HRMS method for the simultaneous determination of α- and β-amanitin in mouse plasma samples using the least

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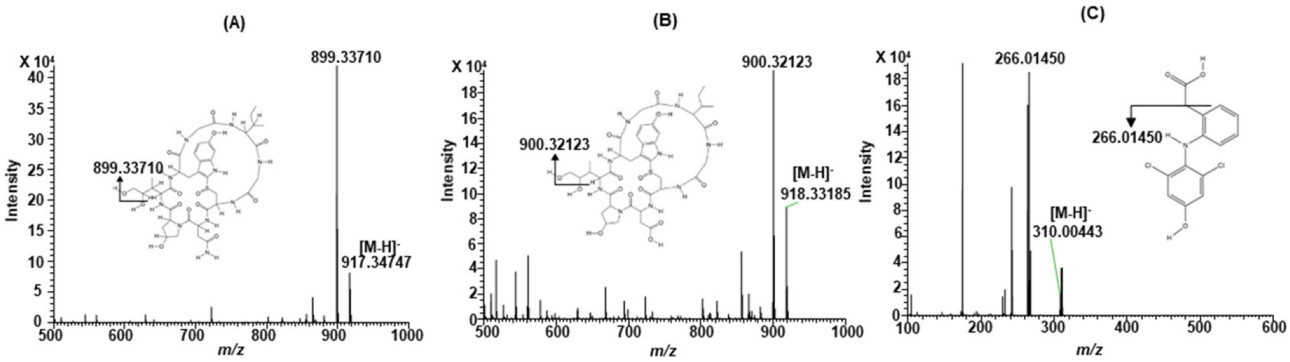

Figure 1. Product ion spectra of (A) α-amanitin, (B) β-amanitin, and (C) 4'-hydroxydiclofenac (internal standard).

mouse plasma volume (5 μL) and successfully applied the method to characterize the toxicokinetics of α- and β-amanitin after concurrent oral administration of α- and β-amanitin at 1.5 mg/kg dose in male ICR mice.

Experimental

Materials

α-Amanitin (purity, 95.0%) and β-amanitin (purity, 95.0%) were obtained from Tocris Bioscience (Bristol, UK) and Cayman Chemical Co. (Ann Arbor, MI, USA), respectively. 4'-Hydroxydiclofenac was obtained from Corning Life Sciences (Woburn, MA, USA). Formic acid was purchased from Honeywell (Charlotte, NC, USA). Methanol and water (LC-MS grade) were supplied from Fisher Scientific (Fair Lawn, NJ, USA).

Sample preparation of α- and β-amanitin in mouse plasma samples

Mouse plasma calibration standard solutions of α- and β-amanitin were prepared at eight different concentrations in the range of 0.5–500 ng/mL. Quality control (QC) plasma samples were prepared by spiking the standard working solutions to blank mouse plasma at 1.5 ng/mL, 75 ng/mL, and 375 ng/mL.

An aliquot (5 μL) of calibration standards, QC samples, and plasma samples were mixed with 15 μL of 4'-hydroxydiclofenac (internal standard, IS, 5 ng/mL) in methanol and then vortexed for 2 min. The mixture was centrifuged at 13,000 rpm at 4°C for 5 min, and 17 μL of the supernatant was then transferred to autosampler vial. An aliquot (5 μL) was injected in the LC-HRMS system for analysis.

LC-HRMS analysis

The LC-HRMS system consisted of a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and UPLC system (Shimadzu, Kyoto, Japan). Analytes were separated on an Atlantis dC18 system (3 μm, 2.1 mm i.d. × 100 mm; Waters Technologies, Milford, MA, USA), using a gradient elution of 0.1% formic acid in 5% methanol (mobile phase A) and 0.1% formic acid in 95% methanol (mobile phase B) at a flow rate of 0.2 mL/min: 5% mobile phase B for 0.5 min, 5%–95% mobile phase B for 3.5 min, 95% mobile phase B for 3 min, 95%–5% mobile phase B for 0.1 min, and 5% mobile phase B for 2 min.

Heated electrospray ionization source settings in negative ion mode were spray voltage, 3.50 kV; sheath gas, 40 (arbitrary units); auxiliary gas, 10 (arbitrary units); capillary gas heater temperature, 250°C; and auxiliary gas heater temperatures, 200°C, respectively. Nitrogen gas (purity 99.999%) was used for higher-energy collision dissociation, and the collision energies for the fragmentation of α-amanitin, β-amanitin, and 4'-hydroxydiclofenac (IS) were 20, 24, and 14 eV, respectively. The parallel reaction monitoring (PRM) transitions of α-amanitin, β-amanitin and 4'-hydroxydiclofenac (IS) were *m/z* 917.34747 → 899.33710, 918.33185 → 900.32123, and 310.00443 → 266.01450, respectively. Xcalibur software (version 3.1.66.10, Thermo Fisher Scientific Inc.) was used for LC-HRMS system control and data processing.

Method validation

Method validation was performed according to the methods proposed by the FDA Guidance on Bioanalytical Method Validation (https://www.fda.gov/media/70858/download). The batches of calibration standards and QC samples for α- and β-amanitin were analyzed at 0.5, 1.5, 75, and 375 ng/mL in five replicates and in three different days for the evaluation of intra- and inter-day precisions (coefficient of variation, CV, %) and accuracies (the proximity of the measured mean value to the theoretical value).

The matrix effect was assessed by comparing the peak areas of α- and β-amanitin spiked after extraction into blank plasma extracts originated from six different mice to the mean peak areas for neat solutions of the analytes at 1.5, 75, and 375 ng/mL. The recoveries of α- and β-amanitin were determined by comparing the peak areas of the extract of α- and β-amanitin-spiked plasma with those of α- and β-amanitin spiked postextraction in six different

Table 1. Intra- and inter-day accuracy and coefficients of variation (CV) of α- and β-amanitin in mouse plasma QC samples.

Concentration (ng/mL)	α-amanitin				β-amanitin			
	Intra-day (n = 5)		Inter-day (n = 15)		Intra-day (n = 5)		Inter-day (n = 15)	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.5	115.0	3.1	114.0	4.0	100.0	13.9	100.0	14.6
1.5	94.7	9.1	97.3	12.8	93.3	6.8	93.3	7.9
75	92.5	4.8	97.7	7.6	100.8	7.9	100.1	7.1
375	98.4	5.4	102.5	8.1	98.7	7.7	100.7	7.9

blank plasma extracts at three different concentrations.

The stability of α- and β-amanitin in mouse plasma was evaluated by analyzing 1.5 and 375 ng/mL QC samples in triplicate: postpreparation sample stability in the autosampler at 4°C for 24 h, short-term storage stability following storage of plasma samples on ice for 2 h, long-term storage stability following the storage of plasma samples at -80°C for 2 weeks, and three freeze–thaw cycles.

Toxicokinetic study of α- and β-amanitin in male ICR mice

The study protocol was approved by the Institutional Animal Care and Use Committee of The Catholic University of Korea (approval number, CUK-IACUC-2021-004). Male ICR mice (8 weeks of age) were purchased from Orient Bio Inc. (Seongnam, Korea). Mice were kept in plastic cages with unlimited access to standard mouse diet (Orient Bio) and water before the experiments. For oral administration, the mice (29.4 ± 0.5 g) were fasted for at least 12 h but let free access to water.

α- and β-Amanitin were concurrently dissolved in water and administered to mouse using oral gavage at dose of 1.5 mg/kg, respectively (n = 7). Blood sample (approximately 15 μL) was collected from the retro-orbital plexus under light anesthesia with isoflurane at 0.033, 0.083, 0.166, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, and 8 h after drug administration. Plasma samples (5 μL each) were harvested by centrifugation at 13,000 rpm for 3 min at 4°C and stored at -80°C until the analysis.

The pharmacokinetic parameters such as the area under the plasma concentration–time curve during the period of observation (AUC_{last}), AUC to infinite time (AUC_{inf}), and terminal half-life ($t_{1/2}$) were analyzed by a non-compartment analysis (WinNonlin, Pharsight, Mountain View, CA, USA). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were directly obtained from the experimental data. Data are expressed as mean ± standard deviations (SD).

Results and Discussion

LC-HRMS analysis of α- and β-amanitin in mouse plasma samples

An Atlantis dC18 column showed good peak shape, selectivity, and sensitivity for the analytes using a gradient elution of methanol and 0.1% formic acid compared with a Kinetex PFP (2.6 μm; 2.1 mm i.d. × 50 mm; Phenomenex, Torrance, CA, USA), Accucore C18 (1.6 μm; 2.1 mm i.d. × 50 mm; Thermo Scientific, Waltham, MA, USA), and Halo C18 (2.7 μm; 2.1 mm i.d. × 50 mm; Advanced Materials Technology, Wilmington, DE, USA) columns.

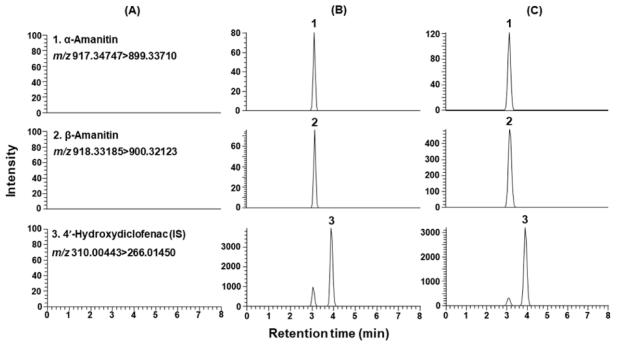

Figure 2. Representative parallel reaction monitoring chromatograms of α-amanitin, β-amanitin, and 4'-hydroxydiclofenac (internal standard). (A) mouse blank plasma, (B) mouse plasma spiked with α- and β-amanitin at LLOQ level (0.5 ng/mL) and 4'-hydroxydiclofenac at 5 ng/mL, and (C) mouse plasma obtained 2 min after an oral administration of α- and β-amanitin at 1.5 mg/kg dose in a male ICR mouse. 1, α-amanitin; 2, β-amanitin; 3, 4'-hydroxydiclofenac.

PRM transitions of the precursor ion ($[M-H]^-$) to the intense product ion were selected on the basis of MS/MS spectra for data acquisition due to the high selectivity and sensitivity (Figure 1). The negative ion mode yielded better sensitivity (LLOQ, 0.5 vs 2.5 ng/mL) and less matrix effect than the positive ion mode for α- and β-amanitin.

The analysis of blank plasma samples from mice revealed no significant interference peaks in the retention times of the analytes, indicating good method selectivity of the present method (Figure 2A). Figure 2B shows a typical PRM chromatogram of a mouse plasma sample spiked with α- and β-amanitin at 0.5 ng/mL.

Method validation

The calibration curves for α- and β-amanitin in mouse plasma were linear over the concentration ranges of 0.5–

Table 2. Matrix effects and recoveries of α- and β-amanitin in mouse plasma samples (n = 6).

Concentration (ng/mL)	α-amanitin			β-amanitin		
	Matrix effect (%)		Recovery (%)	Matrix effect (%)		Recovery (%)
	Mean	CV		Mean	CV	
1.5	94.5	7.6	89.2 ± 6.8	98.3	13.9	82.8 ± 6.4
75	95.1	5.8	90.3 ± 6.4	93.0	5.1	86.9 ± 2.0
375	92.6	5.0	87.3 ± 5.6	98.6	4.5	88.9 ± 8.0

Table 3. Stability of α-and β-amanitin in mouse plasma QC samples (n = 3).

Stability conditions	α-Amanitin concentration (ng/mL)		β-Amanitin concentration (ng/mL)	
	1.5	375	1.5	375
Storage on ice for 2 h				
Accuracy (%)	94.7	97.9	97.6	96.1
CV (%)	3.6	6.2	6.7	7.4
Three freeze–thaw cycles of –80°C to room temperature				
Accuracy (%)	97.2	94.8	95.5	88.7
CV (%)	10.5	7.5	14.3	3.6
Long-term storage for 2 weeks at -80°C				
Accuracy (%)	99.4	93.2	105.0	90.4
CV (%)	6.4	5.9	9.1	3.7
Post-preparative (24 h at 4°C)				
Accuracy (%)	91.2	92.4	90.7	94.1
CV (%)	6.9	7.5	7.1	6.8

500 ng/mL with coefficients of determination (r^2) ≥ 0.9952 using linear regression analysis with a weighting of 1/ concentration, and CV of the slopes were ≤ 5.2%, indicating the repeatability of the method.

Intra- and inter-day accuracy and CV values for α- and β-amanitin in LLOQ, low-, medium-, and high-QC samples ranged from 92.5% to 115.0% and from 3.1% to 14.6%, respectively (Table 1), indicating an acceptable method accuracy and precision. The LLOQ for α- and β-amanitin was 0.5 ng/mL using 5 μL mouse plasma, which showed signal-to-noise ratio > 10, CV < ± 20%, and accuracy, 80%–120%. Matrix effects of α-amanitin, β-amanitin, and 4'-hydroxydiclofenac were 92.6%–95.1%, 93.0%–98.6%, and 92.5%, respectively, indicating little matrix effect (Table 2). The average recoveries of α-amanitin, β-amanitin, and 4'-hydroxydiclofenac (IS) in mouse plasma were 87.3%–90.3%, 82.8%–88.9%, and 80.1±7.8%, respectively (Table 2), indicating that the protein precipitation using methanol was suitable for sample preparation. Three freeze–thaw cycles, short-term storage for 2 h on ice, long-term storage for 2 weeks at -80°C, and post-preparation stability for 24 h in 4°C autosampler showed negligible effect on the stability of α- and β-amanitin (Table 3).

Table 4. Pharmacokinetic parameters of α- and β-amanitin after an oral administration of α- and β-amanitin at 1.5 mg/kg dose to male ICR mice (mean ± SD, n = 7).

Pharmacokinetic parameters	α-amanitin	β-amanitin
C_{max} (ng/mL)	24.6 ± 4.2	30.1 ± 6.7
T_{max} [a] (min)	45 (45-60)	45 (45-90)
AUC_{last} (ng·min/mL)	3285.0 ± 951.4	4236.9 ± 1585.6
AUC_{inf} (ng·min/mL)	3398.3 ± 1026.7	4497.0 ± 1764.7
$t_{1/2}$ (min)	68.1 ± 10.2	82.0 ± 12.4

[a] T_{max} presented median values with the range in parentheses.

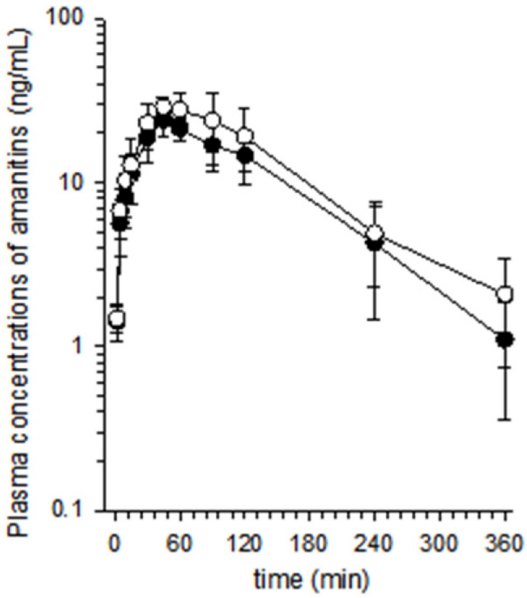

Figure 3. Mean plasma concentration-time curves of α- and β-amanitin after an oral administration of α- and β-amanitin at 1.5 mg/kg dose in male ICR mice. ● : α-amanitin; ○ : β-amanitin. Each point represents mean ± SD (n = 7).

Toxicokinetics of α- and β-amanitin in mice

Mean plasma concentration-time curves and the pharmacokinetic parameters of α- and β-amanitin following an oral administration of α- and β-amanitin at 1.5 mg/kg dose to male ICR mice are shown in Figure 3 and Table 4, respectively. A representative PRM chromatograms of a plasma sample at 2

min after oral administration of α- and β-amanitin at 1.5 mg/kg dose to a mouse is shown in Figure 2C.

α-Amanitin and β-amanitin showed no statistically significant difference between AUC_{last} (3285.0 ± 951.4 vs 4236.9 ± 1585.6 ng·min/mL) and $t_{1/2}$ (68.1 ± 10.2 vs 82.0 ± 12.4 min) values, and they were detected at the first blood sampling time point (2 min) with T_{max} value of 45 min (Table 4), indicating that the toxicokinetic profiles of α- and β-amanitin in mice may be similar.

Conclusions

A sensitive and rapid LC-HRMS method using protein precipitation as a sample clean-up procedure was developed and validated for the simultaneous determination of α- and β-amanitin with LLOQ level of 0.5 ng/mL in 5 μL of mouse plasma. This method was successfully applied to the toxicokinetic study of α- and β-amanitin after an oral administration of α- and β-amanitin at 1.5 mg/kg dose to male ICR mice.

Acknowledgments

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