Improved method for the detection of α- and β-amanitin in biosamples by atmospheric pressure ionization electrospray (API-ES) LC-MS

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Abstract

Highly specific detection of amanitins in body fluids is necessary for an early diagnosis of an intoxication entailing a large scale of invasive and expansive therapy. Therefore, we have developed an LC-MS method for the detection of α- and β-amanitin in human urine after a simple solid-phase extraction. In the meantime, this procedure could be improved by developing a gradient elution instead of the isocratic method. This improved method is suitable not only for the analysis of urine but also for mushrooms and plasma.

Keywords: API LC-MS; Electrospray; Amanita phalloides; Amanitin.

1. Introduction

GC-MS is the method of choice for detection and quantification of toxicants volatile under GC conditions [1], whereas non-volatile toxicants require LC-MS. Therefore, we have developed LC-MS procedures using an atmospheric pressure ionization electrospray interface for those compounds. Among the non-volatiles α- and β-amanitin, toxic peptides of amanita mushrooms, are of great relevance in clinical toxicology. After ingestion of toxic *amanita* mushrooms the amatoxins α- and β-amanitin may lead to severe gastrointestinal disorders and irreversible liver damage. Highly specific detection of amanitins in body fluids is necessary for an early diagnosis of an intoxication entailing a large scale of invasive and expansive therapy [2]. In 1992 Dorizzi *et al.* [3] have critically reviewed methods published for the determination of amatoxins in biological matrices. Determination of amanitins in urine by a commercially available radioimmunoassay (RIA) is possible but the tracer is only stable for 1-2 months and the assay is not available throughout the year. Moreover, interferences with urine matrix are possible so that confirmation of the RIA results by alternative techniques is required [3]. Several of the reviewed HPLC methods are suitable for separation of the amanitins. However, electrochemical or UV detection requires a

complex protocol of extraction and purification steps often followed by column switching techniques.

Therefore, we have developed an atmospheric pressure ionization electrospray LC-MS procedure for the detection of α- and β-amanitin in urine [4]. In the following, we describe an improved method via gradient elution of the amanitins.

2. Experimental

2.1. Detection of α- and β-amanitin in biosamples by LC-MS

2.1.1. Chemicals and reagents

α- and β-amanitin were obtained from Sigma-Aldrich (Deisenhofen, Germany). Methanol, water (both HPLC grade), ammonium acetate, acetic acid, 5 M perchloric acid, 5 M aqueous sodium hydroxide (all analytical grade) and the LiChrolut RP-18 (500 mg) cartridges for the solid-phase extraction (SPE) were obtained from E. Merck (Darmstadt, Germany).

2.1.2. Biosamples for LC-MS

Dried *Amanita phalloides* mushrooms were a kind gift of Dr. Johannes A. Schmitt, Institute for Biochemistry, University of Saarland, Saarbruecken, Germany. Blank plasma and urine samples were collected from healthy volunteers. Authentic urine samples were obtained from patients recently intoxicated by amanita mushrooms.

2.1.3. Sample preparation of biosamples for LC-MS

For extraction of mushrooms, we used an extraction with methanol according to Caccialanza et al. [5]. 20 µl of this extract were injected into the HPLC system with complete loop filling mode.

For plasma and urine, a 5 ml volume of sample was first diluted 1:1 with distilled water. 100 µl of 5 M perchloric acid were added to the diluted plasma sample for protein precipitation. After centrifugation at 2500 g for 5 min, 100 µl of aqueous 5 M sodium hydroxide were added to fix a neutral pH.

2.1.4. Liquid chromatography-mass spectrometry

The LC-MS procedure was described in [4]. Gradient separation was achieved on an ODS Hypersil RP-18 narrowbore column (100 x 2,1 mm I.D.) with 3 µm particle size with Hypersil guard column from Hewlett Packard (Waldbronn, Germany). The gradient was methanol-ammonium acetate (0.02 mol/l, adjusted to pH 5) 10:90 to 50:50 in 5 minutes. Before use, the mixture was

degassed for 30 min in an ultrasonic bath. During use, the mobile phase was degassed with helium. The flow-rate for the LC-MS was 75 µl/min. UV detection was performed at 302 nm, before the effluent entered the electrospray interface.

In Fig. 1 the electrospray mass spectra, structures, empirical formulas and molecular masses of α- and β-amanitin are shown.

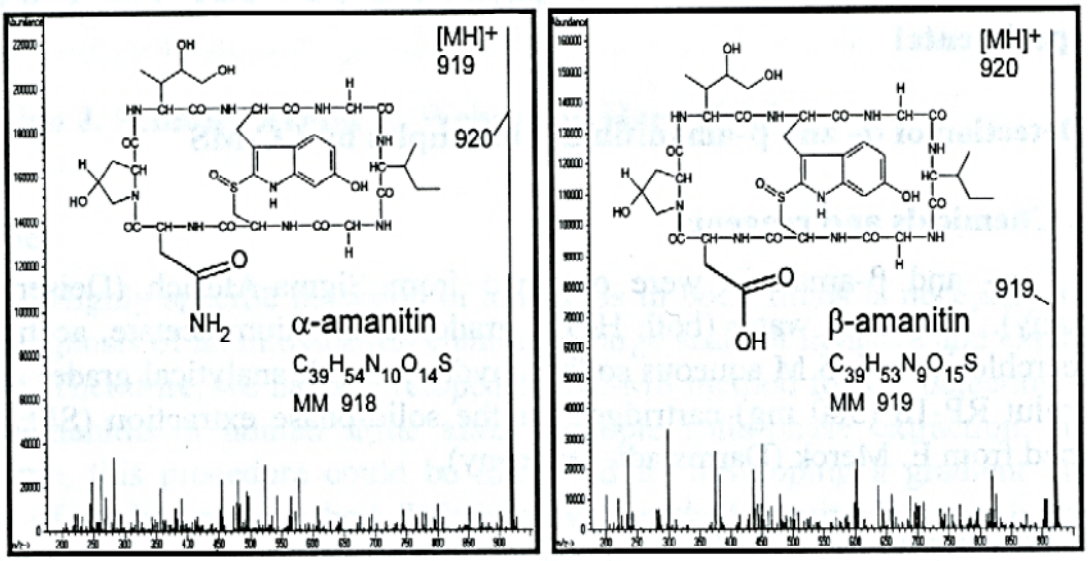

Fig. 1. Electrospray mass spectra, structures, empirical formulas and molecular masses of α- and β-amanitin.

3. Results and discussion

3.1. Sample preparation for LC-MS

The recoveries for the amanitins at a concentration of 100 ng/ml biosample are shown in Table 1. A blank extract of the respective matrix was evaporated to dryness and reconstituted in a standard solution of 5 µg/ml α- and β-amanitin. This solution was used as the external standard.

Table 1. Recoveries [% ± standard deviation] of α- and β-amanitin (100 ng/ ml) from different matrices

Matrix	α-amanitin	β-amanitin
Urine	59±13 %	56±13 %
Plasma	64±12 %	65±11 %

3.2. Comparison between isocratic and gradient system

A comparison between isocratic and gradient system is shown in figure 2. The retention times could be shortened for the amanitins by 30 % at the same flow rate. The peak widths were reduced by 20 % resulting in five times higher peaks. Nevertheless, the improved signal to noise ratio with the gradient system did not result in a better LOD for the detection of amanitins in urine. The methanol part of the eluent at the elution time of the amanitins could be increased (50 % instead of 22 %) resulting in an easier evaporation of the eluent in the electrospray chamber.

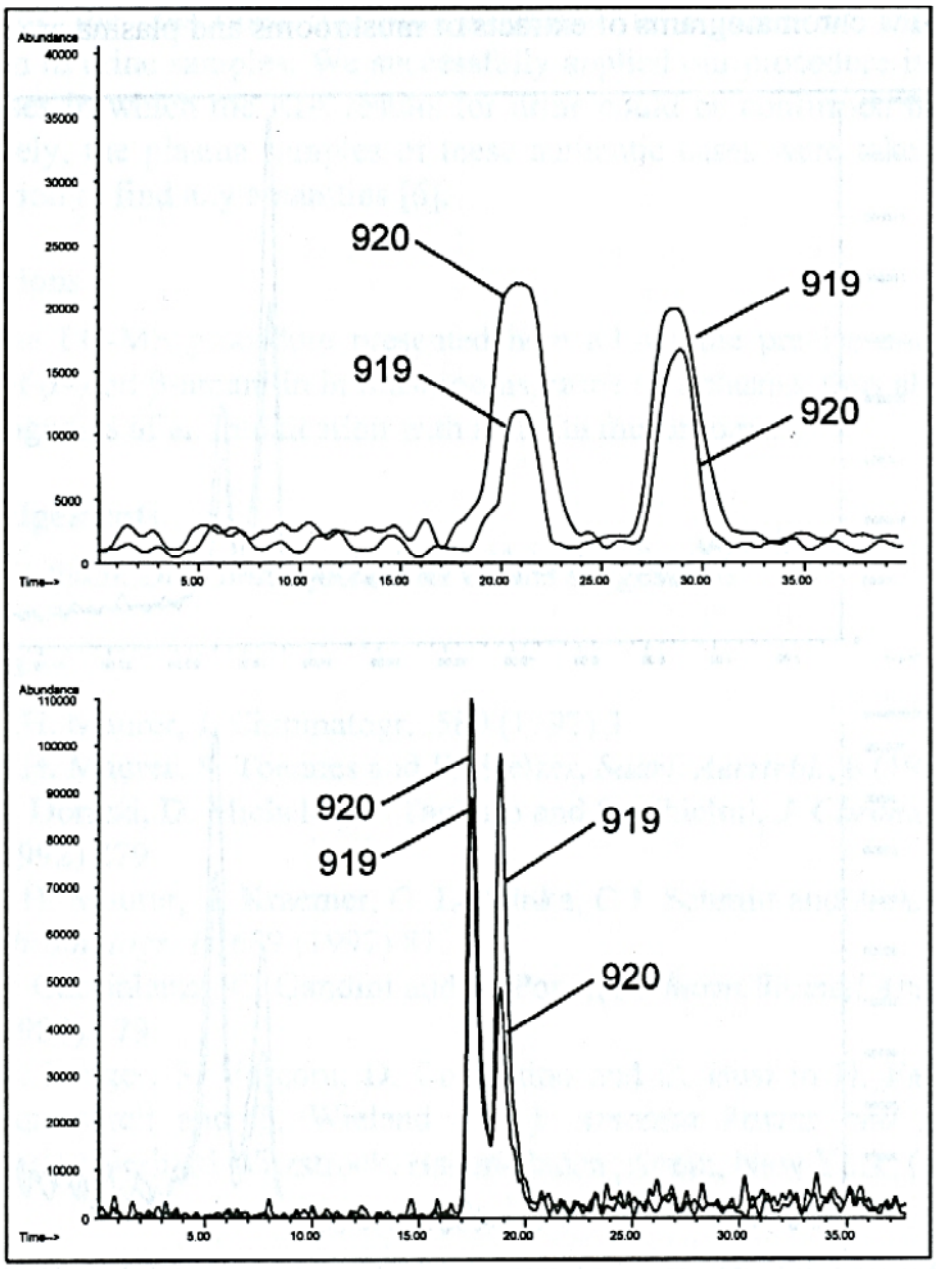

Fig. 2. Smoothed SIM chromatograms with the ions *m/z* 919 and 920 of extracts of a blank urine sample spiked with 100 ng/ml of α- and β-amanitin. Comparison of isocratic (top) and gradient system (bottom).

3.3. Detection by LC-MS

In our system, the optimal flow-rate was 75 µl/min. Using the described method we were able to separate α- and β-amanitin in extracts of biosamples. The applied electrospray LC-MS technique allowed the ionization and detection of both amatoxins in the positive mode. Since both spectra contain the ions *m/z* 919 and 920 in different abundances both ions were selected as diagnostic ions for detection of the amatoxins in the SIM mode. Blank samples of the different matrices were analysed to show that there were no interferences. The limits of detection for both toxins in the different biosamples are shown in Table 2. In figure 3, SIM chromatograms of extracts of mushrooms and plasma are shown.

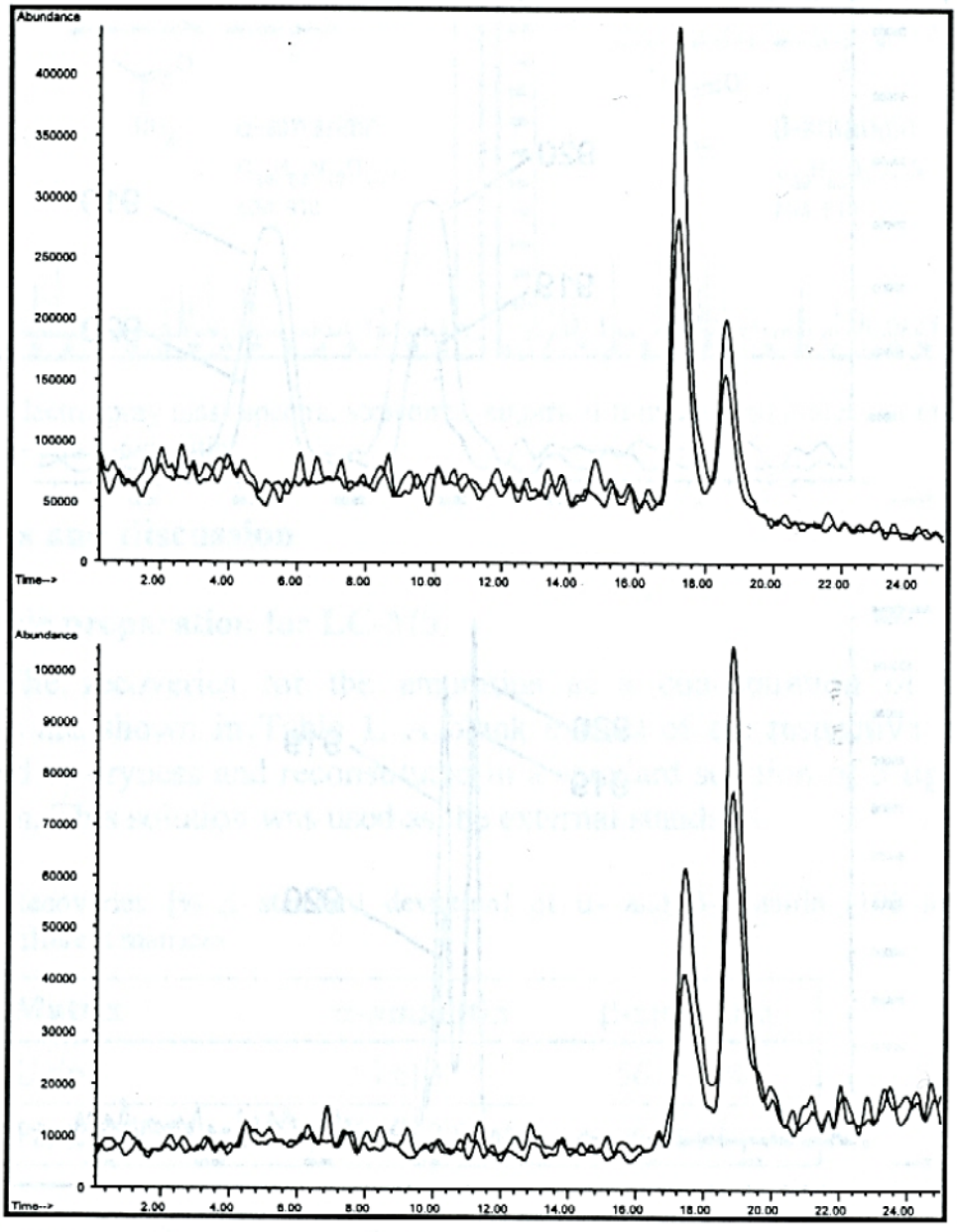

Fig. 3. Smoothed SIM chromatograms with the ions *m/z* 919 and 920 of a mushroom extract (upper part) and an extract of a plasma spiked with α- and β-amanitin (lower part)

Table 2. Limits of detection [ng/ml] for α- and β-amanitin in different matrices

Matrix	α-amanitin	β-amanitin
Urine	10	10
Plasma	10	10

Symptoms of an intoxication with amanita mushrooms do not appear before a lag time of about 8 to 24 h. In our experience, urine concentrations of α- and β-amanitin at this time range between 50 to 500 ng/ml. Therefore, the detection of the described LC-MS procedure is suitable to diagnose an intoxication in urine samples. We successfully applied our procedure in authentic clinical cases in which the RIA results for urine could be confirmed by LC-MS. Unfortunately, the plasma samples of these authentic cases were taken too long after ingestion to find any amanitins [6].

4. Conclusions

The LC-MS procedure presented here allows the precise and sensitive detection of α- and β-amanitin in mushrooms, urine and plasma, thus allowing the specific diagnosis of an intoxication with amanita mushrooms.

Acknowledgements

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