Is LC-High-Resolution-MS/MS a suitable alternative to ELISA in diagnosis of *Amanita phalloides* poisonings? - A two years' experience

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Aims: Poisonings with *Amanita phalloides* toxins require fast diagnosis in order to avoid expensive and unnecessary therapies. Initial clinical assessment in combination with urinary amanitin analysis is necessary for a definite diagnosis. The commonly used ELISA has some disadvantages such as availability of the assay, high costs (for single analysis) and workload. It delivers only preliminary results, which has to be confirmed at least in forensic cases. As alternative, Helfer et al. has described fully validated quantification of α-amanitin in human urine using on-line Turbulent Flow Chromatography (TFC) coupled to LC-HR-MS/MS. The two years' experiences of this approach in routine in comparison to ELISA will be described.
Methods: A total of 44 urine samples after suspected amanitin intake were analyzed by LC-HR-MS/MS and amanitin ELISA and the results were compared. **Results and Discussion:** Fourteen of the 44 urine samples were tested positive by LC-HR-MS/MS and nine by ELISA. In the five urine samples tested negative by ELISA considering the recommended cut-off of 5 ng/mL, amanitin could unequivocally be detected by LC-HR-MS/MS in the concentration range of 1-5 ng/mL. All these urine samples were collected later than 30 h after ingestion. All patients showed already typical symptoms with drastically elevated liver enzymes. The amanitin urine concentrations of proficiency test and authentic patient samples determined by LC-HR-MS/MS were comparable to those given by the ELISA. However, in the authentic samples, the ELISA values were generally higher most probably due to some interferences.
Conclusion: The LC-HR-MS/MS assay allowed reliable diagnostic support in regular cases, in late phase cases, and in forensic cases with the demanded confirmation.

1. Introduction

Ingestion of *Amanita phalloides* mushrooms can lead to fatal poisonings. For efficient treatment or for avoiding expensive therapy, fast diagnosis is essential. Beside the commonly used ELISA assay [1,2], a LC-HR-MS/MS method was published [3] overcoming some disadvantages of the immunoassay and providing a confirmed result as demanded in forensic cases. The aim of this study was the comparison of these two methods for 44 authentic urine samples.

2. Material and Methods

2.1. Chemicals and materials

α- and β-amanitin were purchased from Sigma-Aldrich (Deisenhofen, Germany). γ-Amanitin methyl ether was provided by Prof. Dr. Heinz Faulstich, Max-Planck-Institute for Cell Biology (Ladenburg, Germany). Methanol (HPLC grade) and all other chemicals (analytical grade) were from VWR (Darmstadt, Germany).

Authentic human urine samples were submitted to the authors' laboratory for toxicological diagnostic reasons.

2.2. Sample preparation

For the LC-HR-MS/MS method, sample preparation was according to Helfer et al. [3] Briefly, 100 µL 1 M ammonium acetate buffer pH 5 and 100 µL IS were added to 1 mL urine, vortexed, centrifuged, and the supernatant was transferred into a LC vial. For the ELISA method, sample preparation was according to the Amanitin ELISA description [1]. Briefly, after dilution of the urine with incubation buffer (1:25), 50 µL of urine as well as calibrators and quality controls were added to the prewashed coated wells in duplicate, each. After incubation at room temperature for 30 min, further incubations with 100 µL enzyme label and 100 µL substrate solution were performed in prewashed wells, each for 15 min. Finally, after addition of 100 µL stop solution, the absorbance at 450 nm was read and calibration printed.

2.3. LC and HR-MS/MS apparatus and conditions

Extraction and gradient elution was done via a TurboFlow system consisted of a ThermoFisher Scientific (TF, San Jose, USA) Aria Transcend TLX-I HTLC system, equipped with two TF Accela 1250 pumps, an HTC PAL autosampler, a valve interface module with built-in switching valves, a TF Cyclone (0.5mm×50mm I.D.) and a TF Phenyl TurboFlow extraction column (0.5mm×50mm I.D.) in series, and a TF Accucore PhenylHexyl analytical column (150 mm × 2.1 mm I.D., 2.6 µm particle size) guarded by an UHP filter cart (0.5 µM).

Chromatography was performed at 35°C maintained by an analytical column heater (HotDog 5090, Prolab, Reinach, Switzerland). Software control was done by the TF Aria software version 1.6.3. The mobile phases consisted of eluent A (10 mM ammonium acetate in water with 0.01% formic acid, pH 5), eluent B (acetonitrile with 0.1% formic acid), and eluent C (2-propanol-acetonitrile, 1:1). Gradient elution was performed according to Helfer et al. [3].

The HR-MS/MS system consisted of a TF Q-Exactive system equipped with a heated electrospray ionization (HESI)-II source. Software control was done by TF Xcalibur Qual and Quan Browser version 2.2 SP1.48 in combination with a processing method for automated data evaluation. Within the processing method, mass accuracy was set to 5 ppm. Detailed HESI-II source conditions were according to Helfer et al. [3]

3. Results and Discussion

For 14 of the 44 tested urine samples, confirmed positive results could be achieved by LC-HR-MS/MS. The corresponding patients showed already typical symptoms with drastically elevated liver enzymes. The ELISA achieved nine positive results considering the recommended cut-off of 5 ng/mL. In the five urine samples tested negative by ELISA, the concentrations ranged from 1-5 ng/mL in both methods and the collection time was later than 30 h after ingestion. These results are in accordance with the manufactures recommendations and with Butera et al. [4] who described that the sample collection must be within 36 h after ingestion. Amanitin concentrations, time after assumed ingestion, and analytical results are given in Tab. 1. In authentic patient samples and a proficiency test sample, LC-HR-MS/MS and ELISA provided comparable results. However, in the authentic samples, the ELISA values were generally up to twofold higher most probably due to interferences, e.g. other amanitins with reported cross-reactivities of up to 90 % (γ-amanitin) [1]. Figure 1 shows the correlation of the given assays for the fourteen positive results.

Tab. 1. Amanitin concentrations determined by LC-HR-MS/MS and ELISA, time after assumed ingestion, and analytical assessment of the 44 urine samples.

Urine sample, No.	Time after assumed ingestion, h	Creatinine, mg/dL	Amanitin concentration, ng/mL		Analytical assessment	
			LC-HR-MS/MS	ELISA	LC-HR-MS/MS	ELISA
1	8	20			-	-
2	8	20			-	-
3	10	100	58.8	116	+	+
4	13	100	70.4	53.1	+	+
5	13	100	42.9	22.4	+	+
6	13	100	29.9	17.3	+	+
7	14	100	37	66.6	+	+
8	15	100	49.3	91.1	+	+
9	17	20	24.2	30.6	+	+
10	19	10		1.1	-	-
11	27	10			-	-
12	27	10			-	-
13	29	100	14.1	30.6	+	+
14	29	20	0.9	1.6	+	-
15	30	20	5.4	4.9	+	-
16	40	100	3.3	18.3	+	+
17	41	100	3.1	1.7	+	-
18	41	100	1.9	1.4	+	-
19	41	100		1.1	-	-
20	58	100		1.1	-	-
21	72	100	0.7	2.1	+	-
22	72	100			-	-
23	72	100			-	-
24	72	20			-	-
25	82	100			-	-
26	82	50			-	-
27	82	20			-	-
28	89	20			-	-
29	90	100			-	-
30	90	50			-	-
31	94	100			-	-
32	94	10			-	-
33	100	100		2.1	-	-
34	100	20		1.5	-	-
35	103	10			-	-
36	119	20			-	-
37	119	20			-	-
38	119	20			-	-
39	129	100			-	-
40	129	20			-	-
41	144	20		1.9	-	-
42	> 144	100			-	-
43	> 144	20			-	-
44	> 144	20		1	-	-

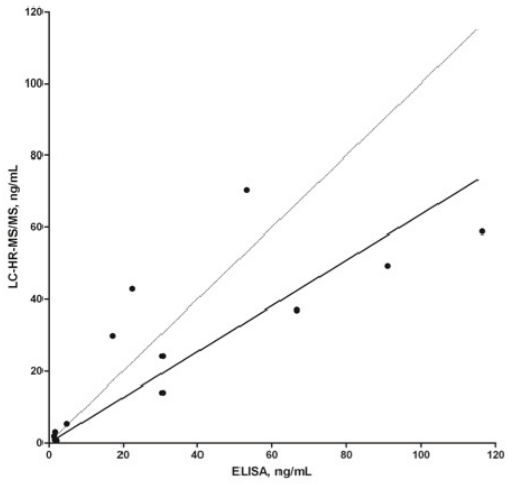

Fig. 1. Correlation of the given assays. The continuous line represents the linear fit, the dotted line the angle bisector.

4. Conclusions

All ELISA positive samples were confirmed by LC-HR-MS/MS. Furthermore, MS/MS-confirmed positive results below the ELISA cut-off could be achieved in later phases of excretion, what could be a demand especially in forensic cases. Moreover, the LC-HR-MS/MS method allowed reliable diagnostic support and has proven to be an alternative to the ELISA assay.

5. References

[1] Bühlmann Laboratories. Amanitin-ELISA, instruction manual, Allschwil (Switzerland): Bühlmann Laboratories, 2000:

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