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New Bühlmann ELISA for determination of Amanitins in urine - Are there false positive results due to interferences with urine matrix, drugs or their metabolites?

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1. Introduction

Amanita phalloides, the so called "death cap", is the most poisonous mushroom, causing the majority of severe mushroom intoxications. It has been estimated that 10-40% of these lead to death (1). Amanita mushrooms contain the amatoxins α-, β-, γ- and ε-amanitin, amanin and amanullin together with phallotoxins and virotoxins. However, the high toxicity is mainly attributable to the amatoxins. In particular α- and β-amanitin are of toxicological interest and serve as target analytes for diagnosis. These mycotoxins occur also in white Amanita species and are found in the small Lepiota and Galerina species (2).

Chemical structure. *Amatoxins are bicyclic octapeptides, the structures of which are shown in Fig. 1.*

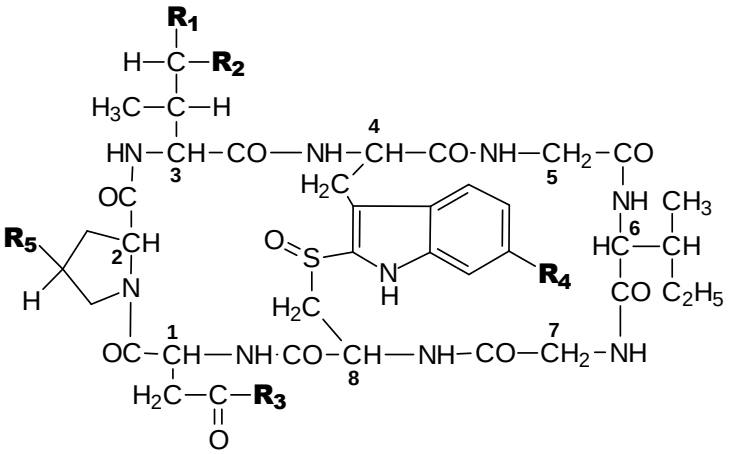

Fig. 1: Structure und Toxicity of Amatoxins (3)

Name	R1	R2	R3	R4	R5	LD50 [mg/kg]
α-Amanitin	CH_2OH	OH	NH_2	OH	OH	0,3-0,6
β-Amanitin	CH_2OH	OH	OH	OH	OH	0,5
γ-Amanitin	CH_3	OH	NH_2	OH	OH	0,2-0,5
ε-Amanitin	CH_3	OH	OH	OH	OH	0,3-0,6
Amanin	CH_2OH	OH	OH	H	OH	0,5
Amaninamid	CH_2OH	OH	NH_2	H	OH	0,5
Amanullin	CH_3	H	NH_2	OH	OH	>20
Amanullinic acid	CH_3	H	OH	OH	OH	>20
Proamanullin	CH_3	H	NH_2	OH	H	>20

Toxicity and mechanism of action. Amatoxins are potent inhibitors of RNA polymerase II, thus interfering with cellular protein synthesis, resulting in necrosis. Due to a fast uptake by a sinusidal bile salt transport system and the high replication rates (4,5) liver cells are mainly affected, resulting in potentially fatal liver damage. However, necrosis is also found in kidney and intestinal cells (6). For further information see ref. (7)

Analytical toxicology. Specific and fast detection of amatoxins in body fluids is necessary for early diagnosis of an intoxication entailing a large scale of invasive and expensive therapy (e.g. liver transplantation). Urine is the most important sample material for the determination of amatoxins. It has to be taken into consideration, that patients usually arrive at the hospital 12 h after ingestion of the mushrooms, since symptoms appear after a corresponding lag time. At that time, the amatoxins have already been eliminated from the plasma (8,9).

Several methods were published for the determination of amatoxins in biological matrices. To our best knowledge, in emergency cases of intoxications, amatoxins have routinely been detected by means of a radioimmunoassay (RIA) (10) or a liquid chromatographic-mass spectrometric assay after immunoaffinity extraction (IAE-LC-MS assay) (8).

Recently, an ELISA kit has been introduced as an alternative method (11). Like with other immunoassays, interferences with urine matrix, drugs or their metabolites may occur, possibly leading to false positive results. For amanitin radioimmunoassays, interferences have been described (12,13). The aim of this study was to determine whether false positive ELISA results occur because of the interferences mentioned above.

2. Experimental

One hundred urine specimens from our daily toxicological routine analysis (emergency toxicology, drugs of abuse testing) were tested according to the manufacturers manual (11) by the new amanitin ELISA kit (EK-AM 1) of Bühlmann Laboratories (Allschwil, Switzerland) distributed by DPC Biermann (Bad Nauheim, Germany).

3. Results and discussion

According to the manufacturers manual, the detection of amanitins in urine in concentrations higher than 10 ng/ml by means of their ELISA kit should indicate an amanitin intoxication. All of the one hundred urine specimens showed a negative ELISA result. Moreover, all results were below the so called functional assay sensitivity of 1.5 ng/ml (Fig.2). These data indicate that the new ELISA kit shows no interferences with the tested urine matrix.

It has to be noted that the concentration values determined by the ELISA or RIA results of positive amanitin cases cannot be compared due to the fact that the two kits use different antibodies. The quantitatively most important target analytes of amanita phalloides are α-amanitin and β-amanitin (2). The RIA detects α- and β-amanitin, however with different cross reactivities, whereas the ELISA assay is more selective for α-amanitin (Table 1). A selective determination of both amanitins was possible by IAE-LC-MS (8)

Table 1: Specificity of the different assays for determination of amanitins

Assay	α-amanitin	β-amanitin	γ-amanitin	ε-amanitin	Phalloidin	Phallacidin
RIA	100%	44%	100%		>0,01%	
ELISA	100%	0,1%	90%	0,1%		
LC-MS	100%	100%				

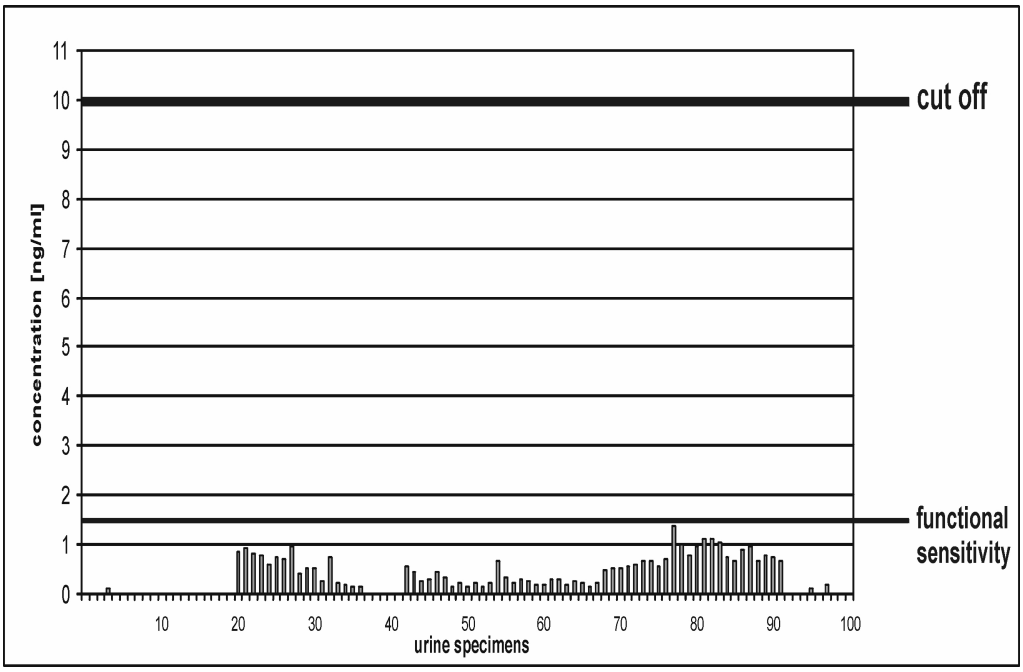

Fig. 2: Amanitin ELISA results of the tested urine specimens

Based on these differences several question arise: According to the manufacturers manual, both assays have the same cut off value of 10 ng/ml despite the different cross reactivities. However, assuming a positive amanita phalloides intoxication would ave been analysed by the RIA assay giving a result of 10 ng/ml, this result would have to be interpreted as positive according to the manufacturers manual (10). Due to the different cross reactivities (Table 1) and assuming that the ingested mushroom contained α- and β-amanitin, the same case would give a result lower than 10 ng/ml by ELISA. Such a result must be interpreted as negative according to the manufacturers manual (11).

It has to be discussed whether the ELISA cut off value has to be lower than 10 ng/ml. Another question is whether a mushroom species containing only β-amanitin exists, with the consequence that an intoxication would not be detected by the ELISA assay. However, to our best knowledge there is no such mushroom, but we cannot exclude its existence.

4. Conclusions

Although some questions remain to be discussed, the amanitin ELISA has several advantages over the RIA. No safety precautions concerning radioactivity have to be taken and there is no radioactive waste. Needed time as well as handling are comparable. The most convincing argument is that the ELISA kit is available throughout the whole year. On account of the limited stability of the ^{125}I tracer, the RIA kit was only available during the mushroom season and therefore, ingestion of e.g. deep frozen amanita mushrooms could not be monitored in the off season. This was the most criticized point of the RIA and set our study group to develop a liquid chromatographic-mass spectrometric assay after immunoaffinity extraction (IAE-LC-MS assay) (8).

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