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Analysis of amatoxins α-amanitin and β-amanitin in toadstool extracts and body fluids by capillary zone electrophoresis with photodiode array detection

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Abstract

Over 90% of the lethal cases of mushroom toxin poisoning in man are caused by a species of amanita. The amatoxins (especially α- and β-amanitin) found in amanita deserve special attention, because of their high pharmacological potency, their high natural concentration and their high chemical and thermal stability. Measures can be taken to improve the survival rates (aggressive gastroenteric decontamination, liver protection therapy) if the poisoning is diagnosed correctly and as early as possible. The standard assay for α-amanitin is a radioimmunoassay (RIA). Among other reagents, this assay uses ^{125}I-labelled α-amanitin, which has a low shelf life. The assay is therefore not available at all hospitals and all year round. In this paper, a first attempt to employ capillary zone electrophoresis (CZE) to quantify amatoxins α- and β-amanitin in urine samples of afflicted patients and in toadstool extracts is described. Diode array detection is used for identification of the resolved substances in the electropherogram. An analysis requires 20 min. The detection limit is 1 μg/ml, i.e., 5 pg absolute. Relative standard deviations are between 1 and 2% for the calibration standards (peak height and area) and ca. 7.5% for the real samples. Advantages of the CZE over the RIA include lower cost, the possibility of quantifying several toxins in one analysis, less consumption of potentially harmful reagents (no radio-labelled substances, no addition of α-amanitin as reagent) and, most importantly, all-year-round availability of the assay. The detection limit is still somewhat high and does not cover the entire clinically relevant range. Attempts to lower the detection limit by the necessary order of magnitude are currently under way in our laboratory. These include application of laser-induced fluorescence detection, liquid chromatography–CZE and CZE–mass spectrometry techniques.

Keywords: Amatoxins; Toxins; Amanitins

1. Introduction

Over 90% of the lethal cases of mushroom toxin poisoning are caused by one of the poisonous species of amanita: *Amanita phalloides*, *Amanita verna*, (*Amanita virosa*) [1–4]. Often the poisoning occurs accidentally, since the various amanita species resemble edible fungi. Poisonous amanita contain different toxins including amatoxins (α-, β-, γ-, ϵ-amanitin, amanin, amanullin) phallotoxins (phalloidin, phalloin, phallisin, phallacidin) and virotoxins (viroidin, viroisin, desoxyviroisin, viroidin, desoxyviroidin) [5].

Efforts towards the detection, recognition, and quantitative analysis of these toxic peptides in body

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fluids have concentrated almost exclusively on the amatoxins. Especially α- and β-amanitin, Fig. 1, are known for their extremely high pharmacological potency (LD_{50} (p.o. human): 0.1 mg/kg body weight) and unusually high chemical and thermal stability. Amanitin is, e.g. not destroyed by cooking and can be found active in food stuffs after prolonged storage below 0°C. In this context *Amanita phalloides* (death cap) deserves special attention because of the high natural concentration of α- and β-amanitin in that species (average 8 and 5 mg/100 g wet mushroom, respectively). In addition, 100 g of fresh *Amanita phalloides* contain on average 0.5 mg γ-amanitin, 10 mg phalloidin, and traces of phalloin [5].

The toxicity of the amatoxins is due to inhibition of eucaryotic RNA polymerase type II, an enzyme involved in the synthesis of mRNA [6]. Lack of RNA polymerase II will thus impede protein synthesis. The poison is preferably taken up by the liver (first-pass-effect) and in addition circulates within the enterohepatic circuit. Liver failure is thus the common cause of death by amanitin poisoning in man [7].

The symptoms of α-amanitin poisoning unfortunately tend to occur only after a latent period of up to 48 h and resemble those of stomach flu. Survival rates (70–80% today [8]) can be considerably improved when the poison is rapidly removed from the body (stomach wash, forced diarrhoea and/or diuresis, haemodialysis, haemoperfusion). Liver protection therapy (Silibinin) is also possible at an early stage [7]. Fast, sensitive, and reliable detection of amanitin in body fluids (urine, gastric juices, (blood/serum)) is highly important in these cases. Analysis of a dish suspected of containing poisonous mushrooms, or verification of a suspected poisoning by a second analytical method are other challenges facing the toxicologist or forensic scientist.

Current standard assay is a radioimmunoassay (RIA) based on ^{125}I-labelled α-amanitin as competing reagent for the α-amanitin presumably contained in the respective sample [9–11]. This assay, especially the ^{125}I-labelled α-amanitin, has a low shelf life and thus is not available in most hospitals (only 7 centres all over Germany are, e.g. able to perform that particular analysis) and even those who do use it, will not provide it all year round (i.e., usually only

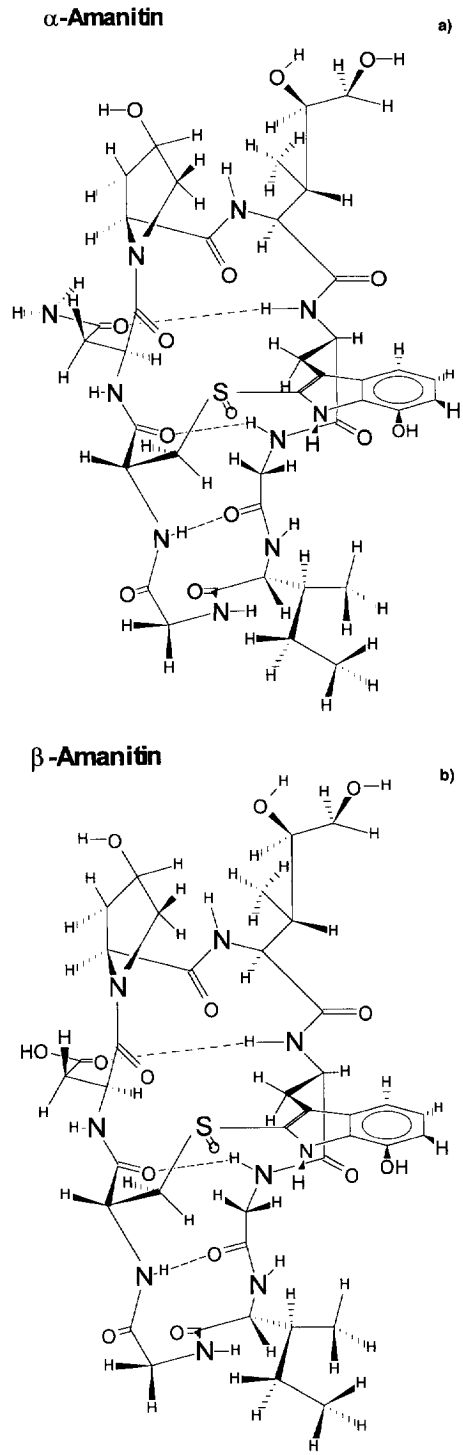

Fig. 1. (a) Structure of α-amanitin, (b) structure of β-amanitin.

within the "mushroom season"). A tendency for cross-reactivities leading to wrong positive or negative results has also been reported for the RIA [6]. Because of the radiolabelled material specialized laboratories and personnel are required.

Alternatively, thin-layer chromatography (TLC) and (high-performance) liquid chromatography (HPLC) have been used in amatoxin analysis. The detection limits of the TLC methods are generally described as too high for analysis of amatoxins in body fluids [6,12–14]. These techniques are used for the quantification, e.g. in mushroom extracts. HPLC techniques on the other hand have been developed to a point where the quantification of α- and β-amanitin together with some other toadstool toxins in body fluids is possible in the clinically relevant range, i.e., down to at least 10 ng/ml [6,15,16]. In the methods described so far, this is achieved by elaborate sample preparation (preconcentration step) and/or the use of sophisticated detectors [17,18]. Also, many of these methods were developed for amatoxin analysis in serum and blood rather than the clinically more relevant matrices urine and gastric juices [19,20]. The major advantage of the chromatographic techniques is that several toxins are quantified simultaneously [21]. Thus an intentional poisoning by pure α-amanitin may by be differentiated from one by ingestion of *Amanita phalloides* by the HPLC techniques but not necessarily by the RIA.

As far as we know, capillary electrophoresis (CE) has never been employed in amatoxin analysis. Here capillary zone electrophoresis (CZE) is used to establish a technique for amanitin quantification in urine samples and *Amanita phalloides* extracts. A positive identification of the resolved substances in

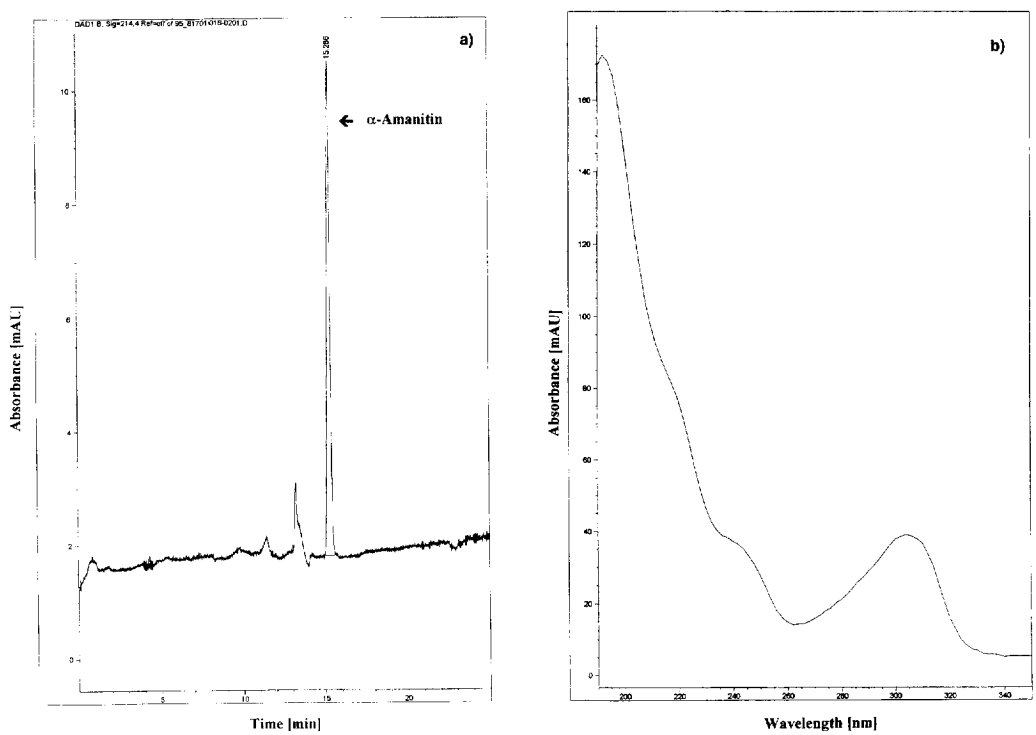

Fig. 2. (a) CZE electropherogram of an α-amanitin standard solution. Sample: Sigma α-amanitin (>95% pure) 100 μg/ml in water. CZE conditions: capillary, 28 cm_{eff} (length inlet to detector, i.e., 36 cm total)×50 μm I.D.; buffer, 100 m*M* phosphate (pH 2.4); temperature, 25°C; voltage, 20 kV (80 μA); injection, 50 mbar (3 s); detection, UV at 214 nm. (b) UV spectrum (190 to 350 nm) obtained from the diode array detector for the major peak (15.286 min) in the electropherogram of the α-amanitin standard, i.e., presumably α-amanitin.

the electropherogram is possible, since a diode array detector is used to record the electrophoretic separations.

2. Experimental

2.1. Chemicals

1 *M* Phosphoric acid was from Sigma, St. Louis, MO, USA (P-0180).

2.2. Standards

α-Amanitin (95%, by HPLC) from *Amanita phalloides* was obtained from Sigma–Aldrich, Deisenhofen, Germany (A-2263).

2.3. Mushrooms

Amanita phalloides were collected in different forests in northern Germany.

2.4. Urine samples

Urine samples of three patients suffering from amanitin poisoning were donated by the Medizinische Hochschule Hannover (Medical School Hannover), Institut für Toxikologie, Hannover, Germany. The samples had been collected in summer/fall 1992 and stored at −20°C since then. Samples were encoded by number to guard the patients right to privacy.

2.5. CE

CE analysis was performed on a Hewlett-Packard 3D-CE instrument. For data collection, data analysis, spectral identification, and system control the HP 3D-CE (Rev.A01.02.) was applied. Detection was by UV absorbance with a photodiode array detector (range normally 190–350 nm). Capillaries were from CS-Chromatographie Service, Langerwehe, Germany.

The following conditions were used unless mentioned otherwise:

Capillary, 28 cm_{eff} (length inlet to detector, i.e., 36 cm in total)×50 μm I.D.; temperature, 25°C; voltage, 20 kV (80 μA); injection, 50 mbar, 3 s (ca. 5 nl); buffer, 100 m*M* phosphate (pH 2.4); detection: for recording of standard electropherograms, 214 nm; diode array detection, 190–350 nm.

Buffer vials were replenished after each run, to prevent changes in the buffer composition and subsequently of the electrophoretic performance of the system.

2.6. Amanita extraction

Following a procedure adapted from Ref. [22], 271 g freshly collected *Amanita phalloides* were broken down into small pieces. A 500 ml volume of MeOH was added and the mixture minced for 30 min at 4°C (Ultra-Turrax) until homogenous. After storage over night at 4°C, the mixture was gently centrifuged (15 min, 3500 rpm) and the amanitin containing supernatant collected. The remainder was re-extracted with 300 ml MeOH. Both MeOH supernatants were combined, reduced by evaporation (rotation evaporator) and redissolved in 80 ml distilled water. $(NH_4)_2SO_4$ was added until saturation and the resulting amanitin containing precipitate recovered by filtration. The supernatant contained no amanitin. The precipitate was dried and suspended in 100 ml MeOH. After 60 min the MeOH phase was recovered by filtration, reduced by evaporation, redissolved in 25 ml water and the aqueous phase extracted thrice with heptane and ethyl acetate.

2.7. Sample preparation for CE measurements

The raw mushroom extract was diluted with water (1:2500) prior to analysis by CZE. Urine samples were thawed and diluted 1:1 with water prior to analysis with CZE. For "spiked" samples a 500 μg/ml α-amanitin solution in water was used for sample dilution.

3. Results and discussion

The α-amanitin used for sample preparation was given >95% pure (HPLC) by the supplier (Sigma). The purity was checked in our laboratory by standard reversed-phase liquid chromatography (RPLC) and

found to be 97%. The major impurity appears to be β-amanitin.

Fig. 2a shows the electropherogram (214 nm) obtained with the CZE method for an α-amanitin standard solution (100 μg/ml). The UV-spectrum shown in Fig. 2b is recorded by the diode array detector for the major peak of the electropherogram. The spectrum was labelled "α-amanitin" and transferred as α-amanitin reference to the library.

A CZE analysis by the established method requires 20 min, which is within the range given for comparative liquid chromatographic (LC) techniques and more than four times less than the RIA (Supplier: Bühlmann Laboratories, Switzerland) currently used as standard by the Institute of Toxicology, Medical School Hannover, which takes 90 min. The CZE should be superior to HPLC in terms of time required for sample preparation, however, since in CZE only a dilution to the analytical range is required.

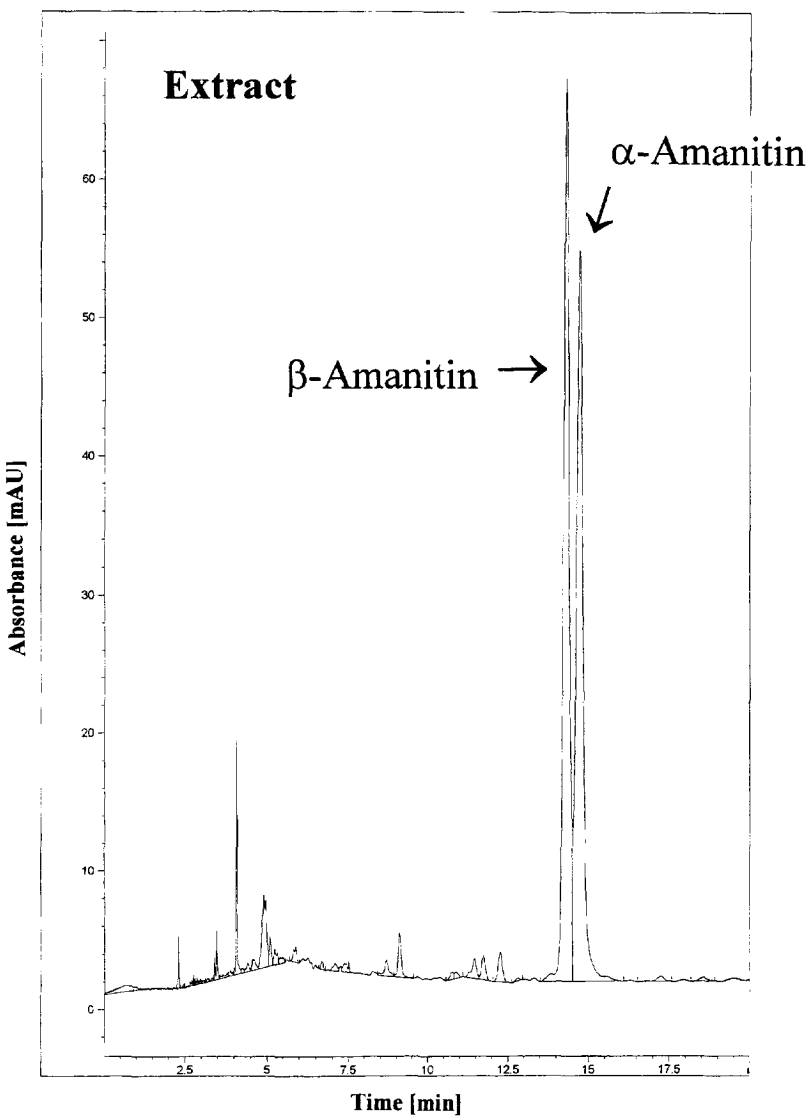

Fig. 3. Electropherogram of diluted mushroom extract.

On the basis of a day-to-day comparison ($n=50$), the relative standard deviation (R.S.D.) of the migration time of the α-amanitin was 3.5%, even when the buffer vials of the CZE were replenished each time to prevent changes in the electrophoretic behaviour of the systems. Identification of the substances found in the separated peaks via their UV spectra was therefore judged necessary in the case of real samples.

Peak heights and peak areas could be reproduced with an R.S.D. of 1.79 and 2.46%, respectively ($n=50$). This compares well with the R.S.D. of the RIA used in Hannover, which averages around 8% (intra assay) and also that reported for various HPLC techniques (e.g., 6.6 and 4.3% [18], 1.9% [19], <10% [20]). A major difficulty in reproducing CE separation is currently the introduction of the sample into the capillary by pressure. We often find that the build-up phase of the pressure shows some variations between runs and thus different volumes are indeed injected, even if nominally the same pressure is applied for the same amount of time, i.e., here 50 mbar for 3 s. A higher reproducibility of the migration time should also help to improve the reproducibility of the peak height and areas.

Table 1
Reproducibility data for α- and β-amanitin quantification in diluted toadstool extracts

	R.S.D. peak area	R.S.D. peak height
α-Amanitin	12.8%	7.3%
β-Amanitin	11.1%	7.7%

The calibration curve for both the peak height and the peak area (constructed from triplicate injection of 10 α-amanitin standard solution with different concentrations) is linear ($y=0.094x+0.052$, $R=0.99945$ (height), $y=1.23x+0.272$, $R=0.99942$ (area)) over 4 orders of magnitude (1–1000 μg/ml).

The absolute detection limit was established to be 5 pg (i.e., 5 nl of a 1 μg/ml solution). The absolute detection limit is thereby in the range of the RIA

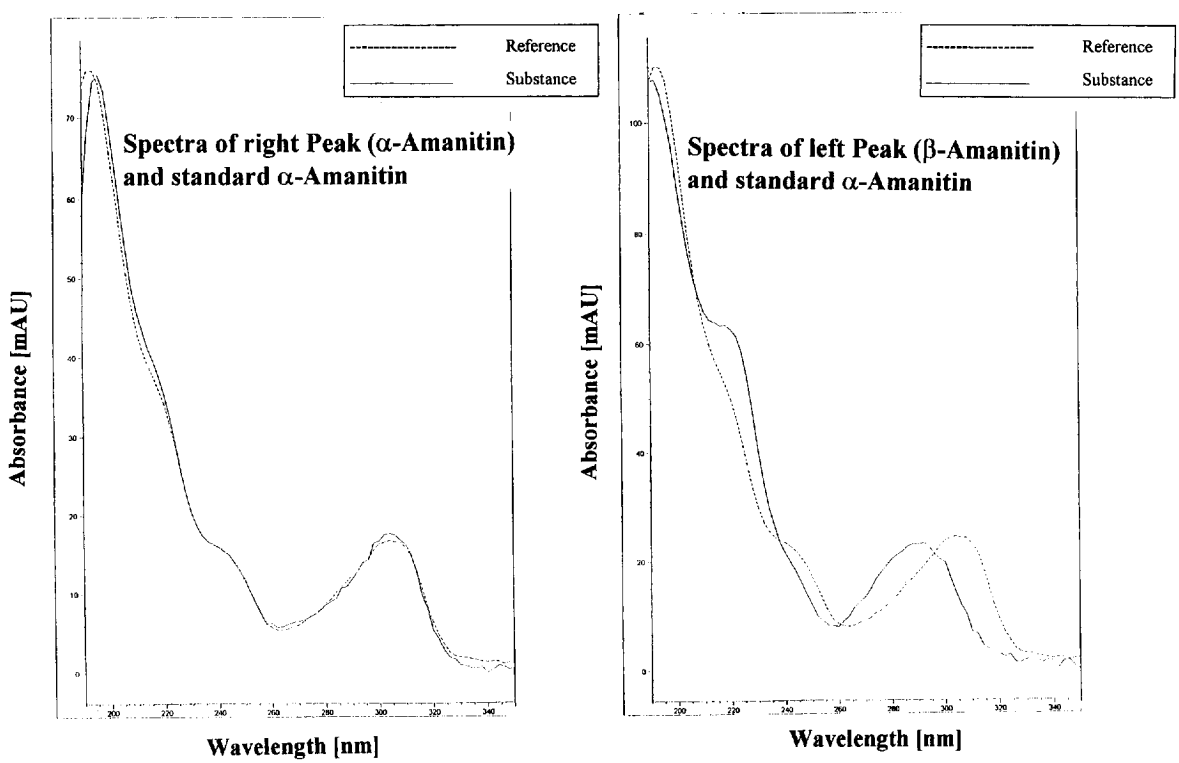

Fig. 4. UV-spectra recorded for the two major peaks found in the electropherogram depicted in Fig. 3 in comparison to the α-amanitin spectrum from the library. Left, "α-amanitin"; right, "β-amanitin".

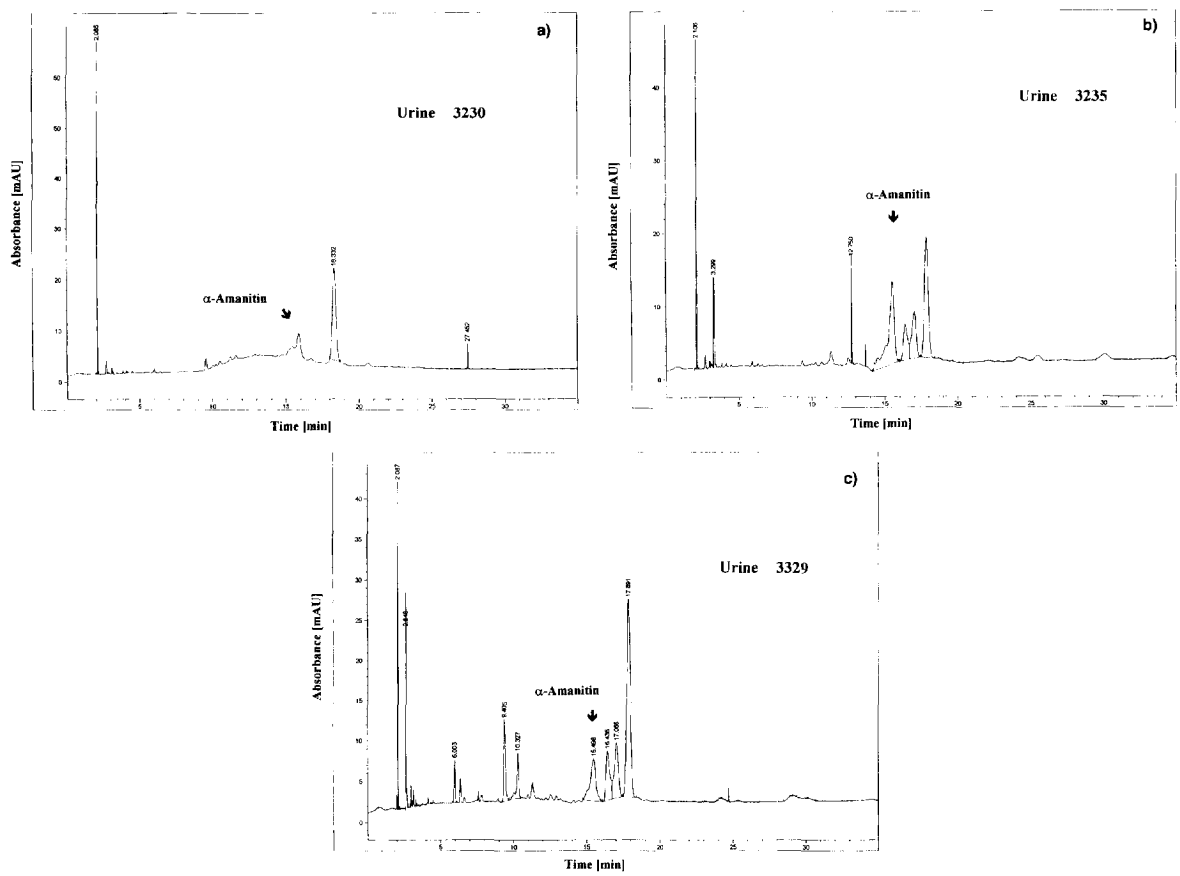

Fig. 5. (a–c) Analysis of three different urine samples obtained from patients suffering from amatoxin intoxication. α-amanitin peaks are indicated by arrow. Samples are identified by their code number.

(detection limit 3 ng/ml) and superior to that given for the HPLC techniques. Due to the small sample volume, however, the detectability of the CZE is not yet in the range currently requested for toxicological or forensic analysis. Three courses are currently under investigation in our laboratory to improve this aspect of the CZE. One is to couple a pre-concentration LC step using membrane adsorbers to the CZE. This has in the past shown to improve the detection limit of proteins in CZE by at least one order of magnitude while increasing the analysis duration only by a few minutes and hardly influencing the reproducibility of the analysis at all [23]. A similar approach has also successfully been used in HPLC detection of amatoxins [6]. The second approach would be to use a more sensitive detector, such as MS, to reach the clinically relevant range. The third approach forsakes the comparative simplicity of the reagent-free approach described above and uses immunoanalytical CZE (FACE, fluorescence affinity/immuno capillary electrophoresis [24,25]). The latter approach requires the development of a competitive CE–immunoassay using e.g. FITC-labelled α-amanitin. It would have the advantage over the RIA of using a more stable reagent.

3.1. Analysis of toadstool (Amanita phalloides) extracts

Mushrooms were collected and the extract prepared as described above. A direct analysis of the diluted extract (1:2500 with water) yielded the electropherogram depicted in Fig. 3. A double peak was recorded between 14 and 15 min. The UV

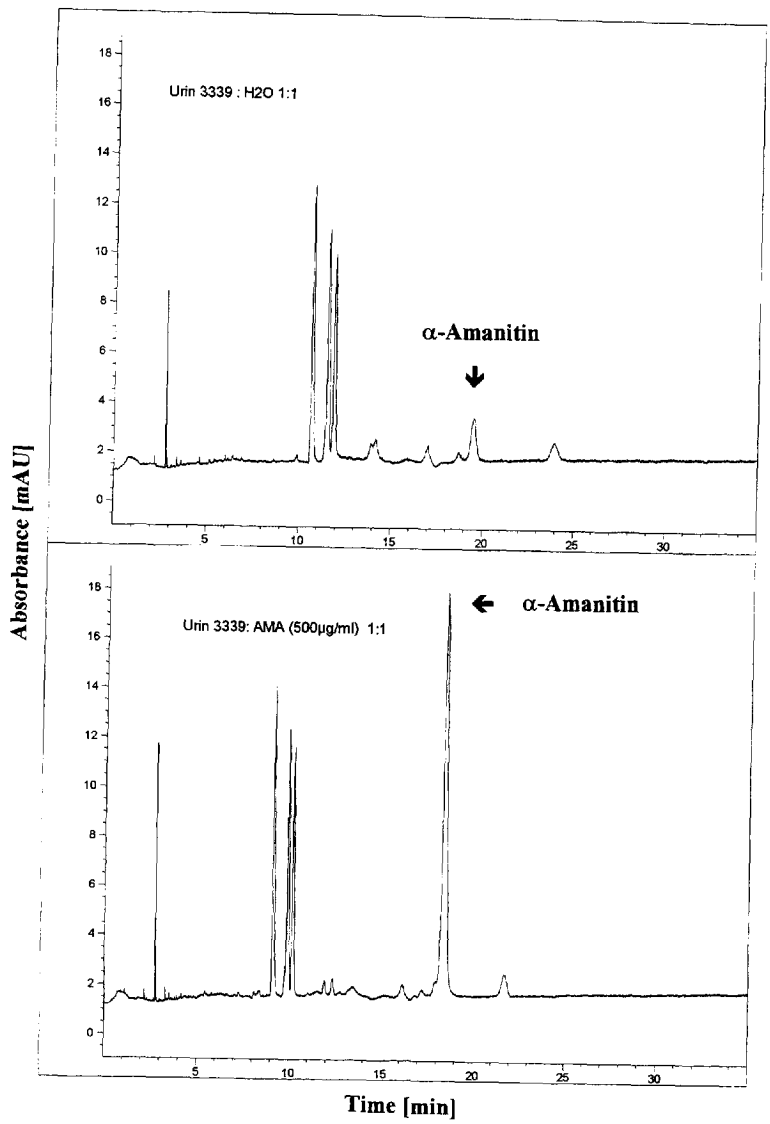

Fig. 6. Analysis of urine sample 3339 with (bottom) and without (top) spiking with α-amanitin standard (500 μg/ml).

spectrum recorded for the substance peak after 14.75 min correlates well with that of the standard α-amanitin (Fig. 4, left). The UV spectrum recorded for the peak after 14.25 min (Fig. 4, right), differs somewhat from that of α-amanitin and was ascribed to β-amanitin on grounds of the closeness in migration time. α- and β-amanitin were subsequently quantified according to peak height and area. From the peak height, a concentration of 484.71 mg/ml α-amanitin was calculated, the respective value from the peak's area was 470.13 mg/ml. The value from the peak height corresponds to an α-amanitin concentration of 11.16 mg/100 g fresh mushroom (or 10.84 mg/100 g according to peak area). Both values are well within the normal biological range of the α-amanitin content of *Amanita phalloides*. Repeated (n=20) analysis of the extract yielded the reproducibility data compiled in Table 1.

3.2. Analysis of urine samples of patients suffering from Amanita intoxication

Electropherograms recorded for three urine samples collected from different patients suffering from *Amanita* intoxication (Fig. 5a–c) all show a more or less pronounced double-peak between 19 and 20 min. Urine from healthy individuals usually shows no comparative signal in that particular area of the electropherograms. The absolute amount of substance in the peaks, however, does not allow direct identification by the UV spectrum. Instead, the presence of α-amanitin in the urine samples could be verified indirectly by spiking with pure α-amanitin (Fig. 6). The second peak of the double peak is significantly enhanced by the spiking. A spectral analysis of the spiked peak shows a correlation, which is indistinguishable from that shown in Fig. 4 (left panel). This argues for a pure substance zone rather than a mixture of the α-amanitin from the standard and an unknown substance, which by pure chance has the same migration time as α-amanitin proper. As pointed out before, the β-amanitin, which presumably gives rise to the first peak of the double peak in the electropherograms, was too lowly concentrated, for identification by UV spectroscopy concomitantly, no standard was available for spiking.

4. Conclusions

The analysis of amanitin by capillary electrophoresis instead of RIA has a number of advantages, especially when a specific method for detection, i.e., a diode array detector is used. The analysis is faster, less costly, requires less consumption of putatively harmful reagents (no radio-labelling, no toxins) and smaller samples. Most importantly, the assays could be performed in any standard analytical laboratory and even "on-the-spot" in emergency cases. Since no labile reagents are involved, an amanitin analysis would be available all-year-round.

Compared to the RIA, the detection limit of the CE is still unsatisfactory. 1 μg/ml barely touches the clinically relevant range, which is at least one order of magnitude lower. To improve this, we are currently investigating three possibilities: the use of LC–CE, CE–MS, and the adaption of the FACE method developed in our laboratory to amanitin quantification. In the latter case, FITC is used as label for α-amanitin or its corresponding antibody.

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