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Improved high-performance liquid chromatographic determination with amperometric detection of α-amanitin in human plasma based on its voltammetric study

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ABSTRACT

A sensitive and simple method is described for the selective determination in human plasma of α-amanitin, the most poisonous and prevalent toxin in the lethal fungi of species *Amanita*, using high-performance liquid chromatography with amperometric detection. After an extraction of plasma with disposable C_{18} silica cartridges, the extracts were separated by isocratic reversed-phase chromatography using a macroporous poly(styrene–divinylbenzene) column and a mobile phase of 0.05 *M* phosphate buffer–acetonitrile (91:9) at the apparent pH of 9.5. Amperometric detection was performed by applying an oxidation potential as low as +350 mV (*vs.* Ag/AgCl) to a glassy carbon electrode, in a thin-layer flow-cell. The linear range for α-amanitin was 3–200 ng/ml, and the relative limit of detection in plasma was 2 ng/ml at a signal-to-noise ratio of 2. The intra-assay precision was evaluated at levels of 10 and 200 ng/ml; the coefficients of variation were 4.5 and 2.6% ($n = 5$), respectively. Inter-assay coefficients of variation were 6.5 and 4.2% ($n = 5$) for the same concentrations of toxin. These analytical conditions have been chosen on the basis of a preliminary in batch cyclic voltammetric investigation of α-, β- and γ-amanitins, which has allowed their oxidation process to be clarified and the pH dependence of their oxidation potentials to be determined. All three amanitins are oxidized at the same potential values, and adsorption onto the electrode surface of both reactant and products was found in all cases. This adsorption did not affect the signal recorded for α- and γ-amanitins at the amperometric detector, and for β-amanitin a stronger adsorption for the anodic product was found, which leads to a marked positive shift of the potential required for the oxidation of this isomer in the amperometric detector cell.

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INTRODUCTION

Several hundred deaths every year are caused by intoxication from poisonous mushrooms, in most cases *Amanita phalloides* or *Amanita verna*. Given the latency period and the non-specificity of early symptoms, diagnosis is often uncertain, being grounded merely on anamnestic data. Therefore therapy is often delayed, although it has been reported that early aggressive gastroenteric decontamination can reduce the extent of hepatic damage [1]. Moreover, the lack of specific and objective evidence of intoxication is problematic for the forensic pathologist who is asked to answer in court about a supposed death from poisoning with toadstools. These arguments stress the importance of demonstrating through a reliable laboratory analysis, possibly during the asymptomatic period, whether or not amanitin exposure has occurred.

Toadstools of the genus *Amanita* contain many different toxins, such as phallotoxins, phallolysins, virotoxins and amatoxins, comprising α-, β-, γ- and ε-amanitins (Fig. 1), amanin and amanullin. However, pathological evidence in fatal cases is based almost solely on amatoxins, bicyclic octapeptides and potent inhibitors of RNA polymerase II. Research has been primarily focused on α- and β-amanitins, owing to their high potency (LD_{50} 0.3 and 0.4 mg/kg, respectively, in the mouse) and their high content in *Amanita phalloides* (*ca.* 8 and 5 mg/100 g, respectively, in fresh mushroom).

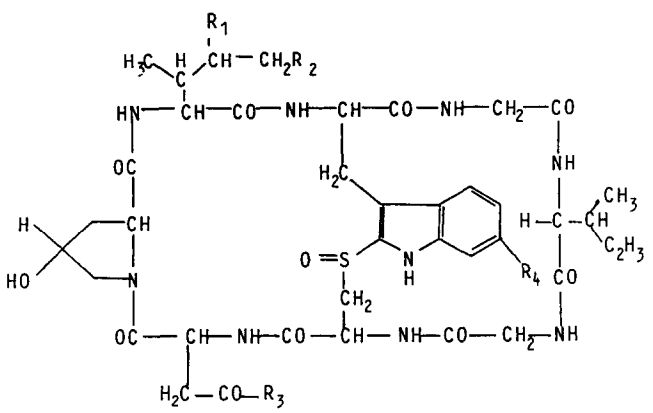

Fig. 1. Structures of amanitins (from ref. 9, with permission).

	R_1	R_2	R_3	R_4
α-Amanitin	OH	OH	NH_2	OH
β-Amanitin	OH	OH	OH	OH
γ-Amanitin	OH	H	NH_2	OH
ε-Amanitin	OH	H	OH	OH

Radioimmunoassays (RIAs) have been developed for amanitins by Faulstich *et al.* [2], Fiume *et al.* [3] and, more recently, by Adres *et al.* [4]. Although sensitive, in the range of nanograms per millilitre, RIAs hardly fit the requirements of a non-routine assay, to be performed "on the spot" in emergency cases, because of the need for dedicated areas and personnel, and radiolabelled tracers. Furthermore, owing to the often unforeseeable interferences affecting the antigen–antibody binding, immunoassays need confirmation through alternative techniques, when legal validation is necessary [5].

Thin-layer chromatography (TLC) owing to its limited sensitivity, has been successfully applied only in the study of amanitin-containing fungi [6–8], not in the assay of samples from intoxicated subjects.

A number of high-performance liquid chromatographic (HPLC) methods have been reported [9–13], most of which used reversed-phase (RP) separation and UV detection in the wavelength range 280–303 nm. However, because of the relatively poor sensitivity and selectivity of the detector, absolute limits of detection (LOD) lower than 5–10 ng could not be routinely achieved, and elaborate and time-consuming sample pre-treatment had to be adopted. More recently, a method based on the column-switching technique allowed almost fully automated sample handling, but required costly and dedicated instrumentation [14].

Taking advantage of the presence of an oxidizable 6-hydroxytryptophan residue in the molecules of α-, β- and γ-amanitin (see Fig. 1), we investigated the possibility of using the more sensitive and selective electrochemical detection (ED) [15]. The use of a thin-layer cell with a glassy carbon electrode set at +600 mV *vs.* an Ag/AgCl reference allowed us to lower the absolute LOD to 40–80 pg. Unfortunately, the quite high potential adopted resulted in poor selectivity, and interference from matrix components was observed. Thus the sample preparation could not be simplified at all. Moreover, the electrochemical processes involved in the amanitin detection seemed to deserve further investigation.

This paper describes the results of in-batch and hydrodynamic investigations on the voltammetric behaviour of amanitins, as well as an improved HPLC–ED method, optimized on the basis of the voltammetric study.

EXPERIMENTAL

Standards, chemicals and samples

Extractive standards of α-, β-, and γ-amanitins (from *Amanita phalloides*) were purchased from Fluka (Buchs, Switzerland) and from Serva (Heidelberg, F.R.G.). Stock solutions of 500 μg/ml were prepared in methanol and stored, protected from light, at −20°C. Tritium-labelled α-amanitin, furnished as a component of an RIA kit by H. Faulstich (Max Planck Institute, Heidelberg, F.R.G.), was used in recovery studies, after HPLC purification. 4-Hydroxyindole was furnished by Aldrich (Steinheim, F.R.G.). All the chemicals employed were of analytical-reagent grade or HPLC-grade quality.

In-batch linear sweep voltammograms were recorded in twice-distilled water in which suitable Britton–Robinson or phosphate buffers were dissolved in order to adjust the pH to the desired values. When necessary, sodium perchlorate was added to adjust the ionic strength to 0.1 *M*.

Sample preparation was carried out using RP disposable cartridges (Sep-Pak C_{18}, Millipore, Milford, MA, U.S.A. or Separon SGX C_{18}, Tessek, Prague, Czechoslovakia) and disposable syringe filters (Bio-Rex®) with AG® 1-X8 anion-exchange membrane (pore size < 0.45 μm) (Bio-Rad, Richmond, CA, U.S.A.).

Before use in HPLC, all eluents were filtered through a 0.45 μm Nylon 66 membrane (Alltech, Eke, Belgium) under reduced pressure.

Human blood plasma (containing sodium citrate anticoagulant) from healthy donors was used as a blank. Spiked plasma samples were left standing for at least 1 h before extraction in order to allow protein-binding equilibration.

Standards of therapeutic drugs and drugs of abuse, supplied desiccated onto glass microfibre discs impregnated with silicic acid (Toxi Disc® Library), were purchased from Analytical Systems (Laguna Hills, CA, U.S.A.).

Instrumentation and procedures

In-batch voltammetric studies. Linear sweep voltammetric experiments were run at room temperature in a small-size three-electrode cell, allowing 1-ml samples to be used. The working electrode was a stationary glassy carbon disc (0.02 cm^2), mirror polished with graded alumina powder prior to each voltammetric scan. A platinum wire was used as the counter electrode, while the reference was an aqueous saturated calomel electrode (SCE), connected to the cell by a salt bridge containing the same medium as in the test solution.

The voltammetric unit was a three-electrode system composed of a Model 552 potentiostat and a Model 568 digital logic-function generator (Amel, Milan, Italy); the recording device was a 7090 A plotting system (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Voltammograms were typically recorded at 0.05 V/s in solutions containing 10^{-4} *M* of each investigated analyte, preliminarily deaerated with nitrogen, previously equilibrated to the vapour pressure of the solution to be tested.

Chromatography and hydrodynamic voltammetric studies. The isocratic HPLC system was composed of a Model 880 PU high-pressure pump (Jasco, Tokyo, Japan), a Model 7125 injector with a 100-μl sample loop (Rheodyne, Cotati, CA, U.S.A.), a Clar 055 column oven (Violet, Rome, Italy) and an LC4B/17A amperometric detector (BioAnalytical Systems, West Lafayette, IN, U.S.A.) with a glassy carbon thin-layer transducer cell and an Ag/AgCl reference electrode. Detector signals were recorded by an Omniscribe (Houston Instruments, Gistel, Belgium) or by a 3390A integrator (Hewlett-Packard).

The column was a PLRP-S 100 Å (150 mm × 4.6 mm I.D.) from Polymer Labs. (Church Stretton, U.K.), packed with 5-μm spherical macroporous poly(styrene–divinylbenzene) particles. An on-line 0.5-μm filter frit (Rheodyne)

placed after the injection valve protected the column from particle contamination.

The mobile phase was 0.05 *M* phosphate buffer–acetonitrile (91:9, v/v) at the apparent pH of 9.5. The flow-rate was 0.5 ml/min, giving, at an oven temperature of 45°C, a pressure of 50–70 kg/cm^2 on the top of the column.

Except during hydrodynamic voltammetric tests, detection was performed using an oxidation potential of +350 mV (*vs.* Ag/AgCl). Under these conditions, sensitivity range down to 0.5–0.2 nA full scale could still be used with acceptable baseline noise and drift.

Because of the lack of a suitable internal standard, external standardization was used. The concentrations of unknown samples were interpolated from a calibration plot of the peak heights of α-amanitin standards in water–acetonitrile (91:9, v/v) *versus* their concentrations, in the range 5–250 ng/ml.

Hydrodynamic voltammograms of α-, β- and γ-amanitins were carried out by repeated injection of 50-ng amounts of each compound under the same chromatographic conditions as above, changing the potential by 50 or 100 mV, and recording the area of the corresponding peaks. Further hydrodynamic voltammograms were carried out with only α-amanitin at different pH values of the mobile phase; the percentage of acetonitrile in the eluent had to be adjusted in order to obtain suitable retention times of the analytes.

Sample preparation. A 2-ml volume of plasma or serum was loaded onto an RP cartridge, previously activated with 5 ml of methanol and rinsed with 10 ml of water. The cartridge was washed with 40 ml of 1% acetic acid in water, and then α-amanitin was eluted with 1 ml of methanol. The eluate was dried under a stream of air with mild warming (hair dryer). The residue was redissolved in 1 ml of water–acetonitrile (91:9, v/v) and passed through an anion-exchange filter (Bio-Rex®/AG® 1-X8), previously rinsed with 5 ml of distilled water. Finally, 100 μl of the filtered extract were injected into the HPLC system.

RESULTS AND DISCUSSION

Voltammetric investigations

The voltammetric profile recorded from α-amanitin solutions buffered at pH 3 (Fig. 2) shows that this species undergoes an appreciable reversible anodic process, followed by a rather slow chemical reaction. The presence of the latter chemical step is confirmed by the relative increase in the height of the later peak found when the scan rate was increased progressively.

However, this behaviour was observed only if the electrode surface was cleaned prior to each voltammetric scan. When the surface was not renewed, repetitive cycling led to the sudden disappearance of the later peak and to a progressive, although fairly small, decrease of the earlier peak, which reached an almost constant height after about five cycles (Fig. 2, dashed line). Such a peak-height decrease can be conceivably accounted for by weak adsorption of the

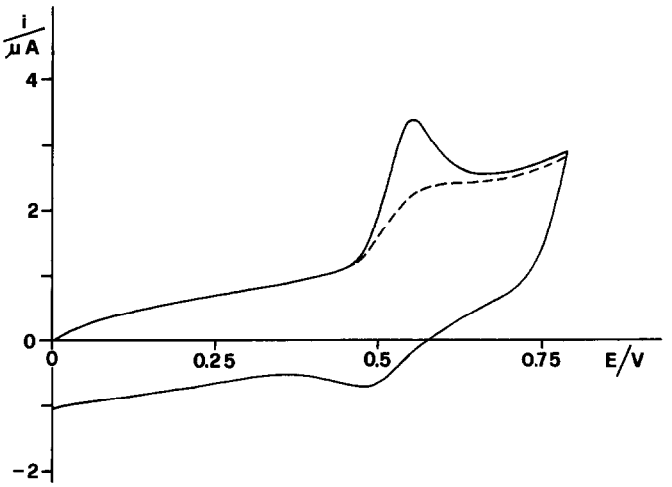

Fig. 2. Cyclic voltammograms recorded at a glassy carbon electrode on an aqueous solution of α-amanitin (10^{-4} *M*) buffered at pH 3. Scan rate, 0.05 V/s. Solid line: first cycle; dashed line: tenth cycle.

oxidation product at the electrode surface [16], which is not unlikely given the peptidic nature of its framework.

The peak current that was recorded on cleaned electrodes, as well as the corresponding constant peak height after repetitive cycling, turned out to increase linearly with the α-amanitin concentration (10^{-4}–10^{-3} *M*). This indicates that the oxidation process was actually diffusion-controlled, in spite of the weak adsorption of the product. This diffusive control for the anodic peak was substantiated by the constant value exhibited by the ratio $I_p/v^{1/2}$ (of the anodic peak current to the square root of the scan rate) for scan rates lower than *ca.* 200 mV/s. Higher scan rates led conversely to increasing value of this ratio, as shown in Fig. 3, which is strongly suggestive of the concomitant occurrence of a weak adsorption of the reagent too [17], attributable once again to the peptidic framework present also in this species.

Similar results were obtained with β- and γ-amanitins, under the same pH conditions. In particular, all the three amanitins turned out to be oxidized at the same potentials, in agreement with the location of the organic groups specific to each analogue, far from the hydroxyindole moiety (the expected electroactive center) and therefore unable to affect its activity. Nevertheless, when the electrode surface was not cleaned prior to each voltammetric scan, peculiar behaviour was observed for the β-isomer, which indicated stronger adsorption of the corresponding oxidation product. Repetitive cycling led in this case to voltammetric responses that indicated that the decrease of the earlier peak and the disappearance of the later peak (mentioned above and shown in Fig. 2) are accompanied by a progressive and more remarkable shift of the oxidation peak towards more positive potential values (*ca.* 150 mV after ten cycles).

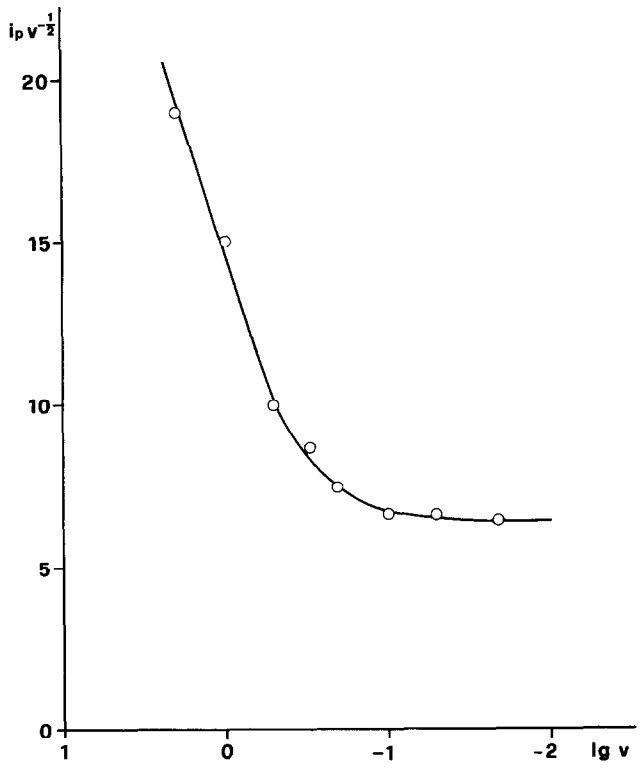

Fig. 3. Plot of the ratio $I_p/v^{1/2}$ *vs.* the logarithm of the scan rate obtained at a glassy carbon electrode on an aqueous solution of α-amanitin (10^{-4} *M*) buffered at pH 3. Peak current and scan rate are in μA and V/s, respectively.

The voltammetric features so far described remained essentially unchanged at increasing pH values, the only difference being a progressive shift of the oxidation peak towards less positive potentials. Only at pH values higher than 11.5 was an appreciable decrease of the peak current, progressive with time, found. Concomitantly, the formation of insoluble gelatinous products was observed, which was apparently due to the decomposition of the electroactive species. In fact, by lowering the pH of these suspensions, clear solutions were obtained, but the voltammetric profiles were not restored.

The trend obtained for all three amanitins by plotting the anodic peak potential *versus* pH is reported in Fig. 4, as well as the unreliable results obtained in strongly basic media. It can be seen that a pH increase causes a linear decrease of E_p with a slope of -59 mV until pH is *ca.* 11.5, value at which a break takes place. This finding indicates that amanitin oxidation occurs through a one-proton one-electron process of the type:

$$AH \leftrightarrow H^+ + e^- + A^\cdot \qquad (1)$$

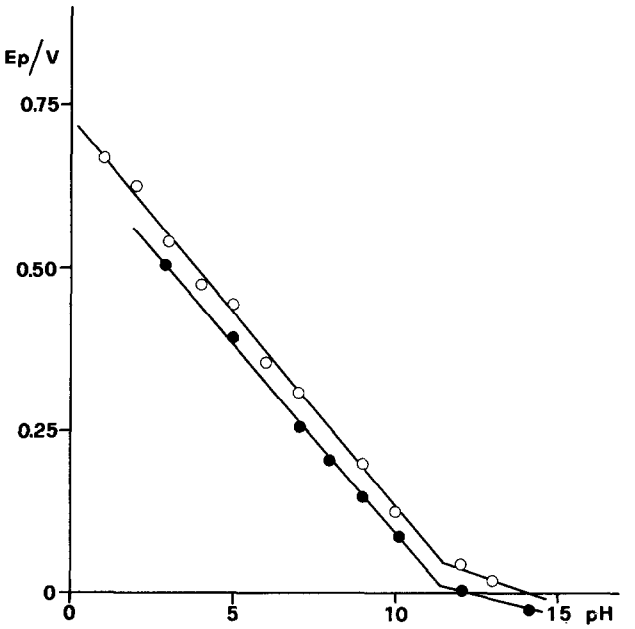

Fig. 4. Plots of peak potential *vs.* pH for (○) α-, β- and γ-amanitins (10^{-4} *M*) (recorded on the first cycle) and (●) 4-hydroxyindole (10^{-4} *M*). Scan rate, 0.05 V/s.

followed by the chemical decay of radical A$^{\bullet}$ through dimerization or an alternative route, in agreement with reaction pathways reported for the oxidation of the phenol moiety present in other organic species [18]. This notwithstanding, the pK_a value (*ca.* 11.5), which can be inferred from the E_p *versus* pH plot, seems to be too high for a phenol acid dissociation reaction, even though this value is surely affected by the poor stability exhibited by amanitins at high pH values.

This rather ambiguous result prompted us to verify whether the oxidation site in the amanitin molecule is just the hydroxyindole moiety, as postulated above. With this aim, the voltammetric tests were repeated on solutions of 4-hydroxyindole, *i.e.* a commercially available prototype species that is as similar as possible to the predicted electroactive moiety present in all three amanitins. At any pH value an oxidation process was observed at potentials only slightly less positive (*ca.* 45 mV) than those found for amanitins. The slope of the E_p *versus* pH plot is again equal to -59 mV (see Fig. 4), until a break appears at a pH of *ca.* 11. This indicates that such a pK_a value does indeed characterize the hydroxyindole acid dissociation reaction. In this case, however, no evidence for decomposition was observed at high pH values, which suggests that the gelatinous precipitate formed with amanitins was due to their peptidic sequence. Moreover, unlike amanitins, for 4-hydroxyindole no cathodic peak associated with the oxidation peak was detected in the reverse scan, in agreement with a faster decay of the radical

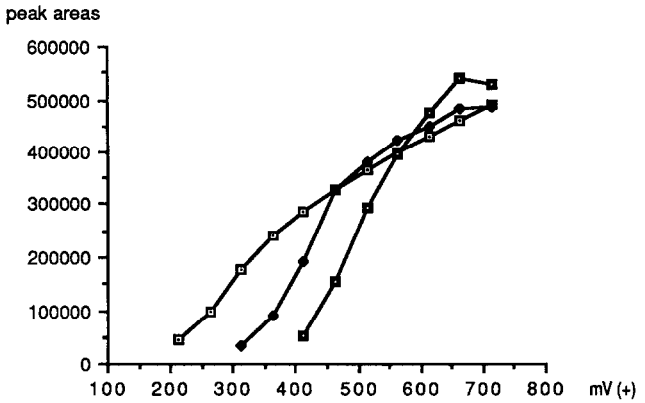

Fig. 5. α-Amanitin hydrodynamic voltammograms at different pH values: (⊡) pH 9.5; (●) pH 6.8; (■) pH 5.0. Injection, 50 ng. Peak areas (ordinate) are plotted *versus* electrode potentials (abscissa); the mobile phase composition is described in the text.

produced in the electrode reaction (eqn. 1) which is expected when it is not confined in a peptidic cage. In this connection, it should be said that such a faster occurrence of the chemical reaction following the electrode transfer would also account for the less positive potentials required to oxidize free hydroxyindole with respect to the same moiety bound into a peptidic framework.

All these voltammetric results confirm that an electrochemical approach can be adopted successfully to detect amanitins after their HPLC separation, provided that the pH of the mobile phase is lower than *ca.* 11.

The hydrodynamic voltammograms carried out with α-amanitin at apparent pH values of 5.0, 6.8 and 9.5 are substantially in agreement with the findings of

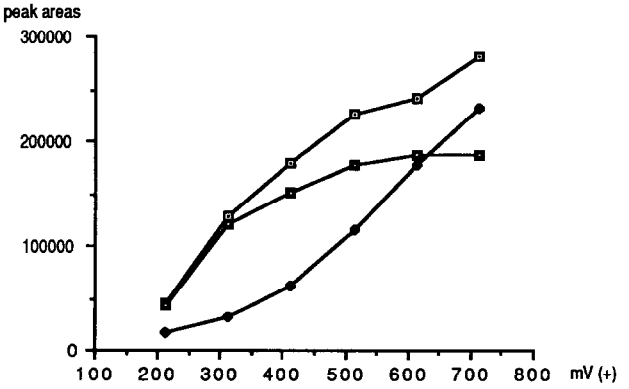

Fig. 6. Hydrodynamic voltammograms at pH 9.5: (⊡) α-, (●) β- and (■) γ-amanitin. Injection, 50 ng. Peak areas (ordinate) are plotted *versus* electrode potentials (abscissa); the mobile phase composition is described in the text.

in-batch studies, since they show a cathodic shift of the curves at increasing pH values (Fig. 5).

A comparison of the three amanitins, carried out at pH 9.5, shows a remarkable difference in the voltammetric curve of β-amanitin, which is oxidized at more anodic potentials (Fig. 6). This finding is in agreement with in-batch studies, and is consistent with our early hydrodynamic voltammograms performed at pH 5 [15]. Such a difference can be accounted for by considering the observed stronger adsorption of the oxidation product of β-amanitin, which, of course, markedly affects the hydrodynamic voltammograms, which are reconstructed from current measurements recorded under potentiostatic conditions.

On the basis of these findings, it was decided to adopt for the HPLC–ED assay a basic eluent and a relatively low electrode potential of +350 mV (*vs.* Ag/AgCl) in order to improve the selectivity and to reduce the background current. Of course, given the instability of silica at basic pH values, the use of a pH-stable polymeric column packing, such as macroporous poly(styrene–divinylbenzene) copolymer, was essential.

HPLC assay

The RP chromatographic separation allowed complete resolution of α-, β- and γ-amanitin, but simultaneous determination was not possible, owing to the large difference in their polarities. α-Amanitin ($k' = 10.7$) was the analyte of choice because of its highest toxicity and constant presence in *Amanita* species. Its elution time was *ca.* 20 min.

Under the adopted conditions, k' values for β- and γ-amanitin were 4.8 and >30, respectively. The latter value is clearly too high to allow the determination of γ-amanitin; in addition, the use of gradient elution was precluded by the well known problems associated with ED. On the other hand, the relatively low electrode potential of +350 mV sacrificed the detectability of β-amanitin in favour of a more selective α-amanitin detection. Under these conditions the background current was 2–3 nA, allowing a fairly stable baseline and rapid equilibration even at full scale deflection ranges as low as 0.2–0.5 nA. Electrode durability was excellent, allowing several weeks of work without resurfacing. The absolute LOD for α-amanitin was 0.2 ng (signal-to-noise ratio of 2).

Both Sep-Pak C_{18} and Separon SGX C_{18} cartridges proved to be suitable for plasma extraction, but the latter gave cleaner extracts and so were used in the final method. The adoption of a filtration step through an anion-exchange membrane considerably reduced the interference due to injected coextractives. The reasons are still unclear, but seem to be related to anionic species, present in the extract from the C_{18} cartridge, that are oxidized at the electrode or, any otherwise disturb the background current. The extraction efficiency was evaluated by using both tritium-labelled α-amanitin and cold toxin, the precision by using only the labelled compound. The recovery of α-amanitin from the whole sample pretreatment from 2-ml plasma samples was 80.0 ± 2.93 and 82.5 ± 1.92% ($n = 10$ in

both cases) at 10 and 200 ng/ml, respectively. The recovery of β-amanitin was *ca.* 50%, and that of γ-amanitin was not evaluated; however, as discussed above, these analytes were of minor interest in the present study.

The linearity of the assay was tested on seven-point dilution curves (in triplicate) in the range 3–200 ng/ml, using the least-squares reduction method. It was described by the equation $y = -0.277 + 2.027x$ ($r = 0.999\,91$), where x was the dose (ng/ml) and y was the peak height (mm). The intra-assay precision of the whole procedure was evaluated at 10 and 200 ng/ml in plasma, resulting in coefficients of variation (C.V.) of 4.5 and 2.6% ($n = 5$), respectively. Inter-assay C.V. were 6.5 and 4.2% ($n = 5$), respectively for the same concentrations of toxin.

The relative LOD in plasma was 2 ng/ml, with a signal-to-noise ratio of 2. At this level the intra-assay C.V. was 6.5% ($n = 5$); the inter-assay C.V. was not evaluated.

Typical chromatograms of control and spiked human plasma are shown in Fig. 7.

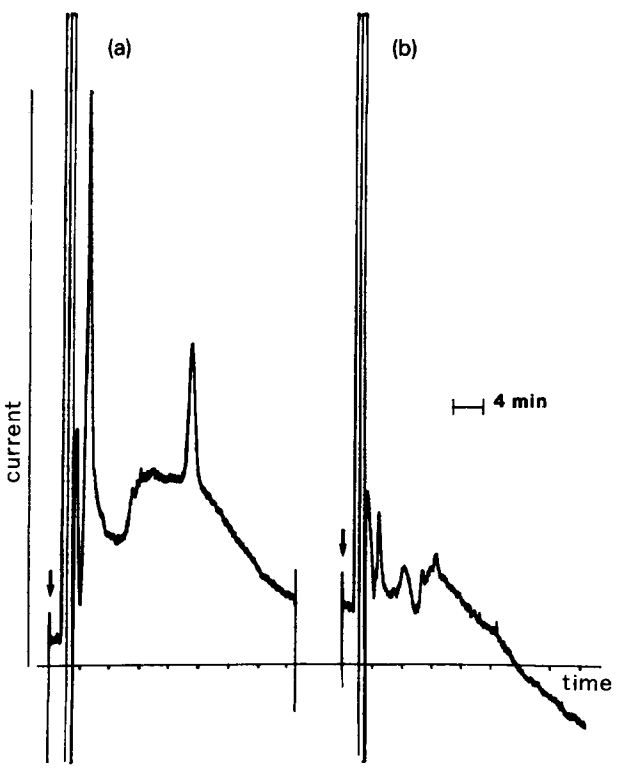

Fig. 7. (a) Chromatogram of plasma spiked with 12.5 ng/ml α-amanitin (range 0.5 nA full scale). (b) Chromatogram of an extract of blank plasma (range 0.5 nA full scale). The elution time of α-amanitin is 20 min.

Possible interferences from more than 80 therapeutical and abusive drugs (see ref. 15 for a list) up to 20 μg/ml were excluded.

CONCLUSIONS

The results reported here show that, after a preliminary voltammetric study, HPLC–ED determination of α-amanitin is selective and sensitive enough for use in cases of clinical interest. As a consequence of the cathodic shift of the α-amanitin oxidation curve at basic pH, a quite low (and therefore very selective) potential can be used, which allows a rapid, though rough, sample pre-treatment to be adopted.

However, two drawbacks had to be accepted: the sacrifice of determining β-amanitin (which could be measureed by an *ad hoc* increase in the electrode potential, although with the consequence of a large loss of selectivity and baseline stability), and the need to use a pH-stable RP polymeric column, which is inherently less efficient than its counterparts based on derivatized silicas. Nonetheless, the present HPLC–ED method is by far the most sensitive chromatographic assay of α-amanitin in real matrices yet reported, and requires only simple sample pretreatment.

During the development of the present method we were unable to obtain samples form real clinical cases. Yet, the ruggedness of the described analytical approach (HPLC using basic eluents, polymeric columns and electrochemical detection) even in the assay of very complex and 'dirty' biological samples (*e.g.* cadaveric serum and tissue extracts from bodies in a state of decomposition) has been widely confirmed in our laboratory for other purposes [15].

In conclusion, the described method, based on a detailed preliminary voltammetric study of amanitins, allows the measurement of circulating α-amanitin levels in intoxicated subjects [19], while being simple and rapid enough to be performed in an emergency room environment.

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