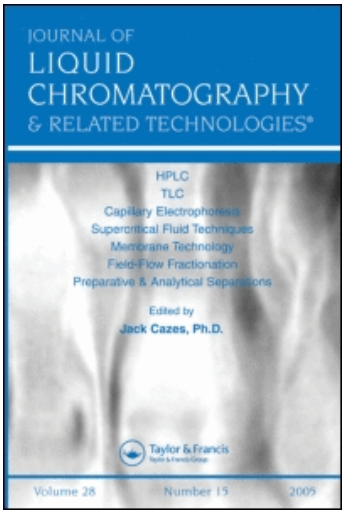

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DETERMINATION OF α-AMANITIN IN SERUM BY HPLC

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ABSTRACT

A reverse-phase high-performance liquid chromatographic method for the quantitative determination of α-amanitin in serum of poisoned patients is described and discussed.The methodology developed is simple and rapid with a minimum of sample preparation steps required.Detection is very sensitive,allowing quantitation of 25 ng of α-amanitin/ml of serum.The technique described is a useful tool to determine the severity of *Amanita phalloides* intoxication during the first 24 h after ingestion of poisonous mushrooms.

INTRODUCTION

Even today,lethal poisoning from mushrooms is still very frequent.Approximately 95% of all fatal mushroom intoxication is almost exclusively attributable to members of the *genus Amanita* which includes a few species of high toxicity (1,2,3).

In Central Europe,in France and in Italy the predominant kind of *Amanita* is the olive-green *Amanita phalloides* Vaill. ex Fr.,but there are also reports of poisoning by the white *Amanita verna* Lamb. ex Fr.

A. phalloides grows from July to the end of October in damp woods,associated with deciduous trees,particularly with beeches and sweet chestnuts.

This mushroom (and its autumnal white form,*A. phalloides* var. *alba* (4)) is very frequently confused with the *Tricholoma equestris* (Linn.) Quélet and *Psalliota arvensis* (Vittad.) Fr.

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Three families of toxic compounds have been isolated from *A. phalloides*, the amatoxins,the phallotoxins and the phallolysins (1,5).All three groups have interesting biological and structural properties.The amatoxins are bicyclic octapeptides containing amino acids (hydroxylated isoleucine and 6-hydroxy tryptophan).The octapeptide ring is bridged through the sulfur atom of the sulfoxide form of cysteine to the 2 position of the indole ring.The phallotoxins are bicyclic heptapeptides containing several unusual hydroxylated amino acids and a molecule of cysteine bridged to tryptophan by a thioether.The phallolysins are high molecular weight proteins.

Of these three groups,the amatoxins are clearly the most toxic,and are considered mainly responsible for fatal human *Amanita* intoxication (6).

Amatoxins cause death by destroying liver cells,via inhibition of DNA-dependent RNA polymerase II,and damaging the secretory cells of the convoluted tubules in the kidney (7,8,9).

In the case of human intoxication by *A. phalloides*the long latency period between eating a dish of mushrooms and the first gastroenteric symptoms is a useful tool for diagnosis,but is enough for the positive identification of *Amanita* poisoning.Mycological identification of *A. phalloides* spores in fresh mushrooms,in cooked mushrooms,in gastric washing or from feces required a specialist usually not present in the hospital.On the other hand identification of *Amanita* poisoning should be made as quickly as possible,to start immediate treatment in order to minimize the exposure time of the parenchymatous organs, expecially the liver, to the toxins.

The development of a rapid,specific and sensitive method for the determination of the concentration of amatoxins in body fluids (blood,urine and in gastro-duodenal aspirate) is therefore important and desirable.

Several chromatographic procedures for quali-quantitative determination of amatoxins providing a varying degree of sensitivity have been published (10,11,12).Radioimmunoassay measurements of amatoxins in the biologic fluids of poisoned patients,with a limit of detection near to 10 ng/ml (urine) were recently reported (13).

The approach taken in this study is to develop a system which exploits the HPLC method to detect,in the nanomole range,α-amanitin in the serum of poisoned patients.

MATERIALS AND METHODS

Blood specimens were obtained from poisoned patients 16-48 h after eating a dish of mushrooms containing *A. phalloides*.

Serum was separated from whole blood by centrifugation.To 2 ml of serum 10 ml of MetOH were added,shaken for 1 minute and then centrifuged for 8 min. (4000 r.p.m.).The upper methanolic layer was carefully removed,and the residue was resuspended in 3 ml of MetOH and again extracted.The combined methanolic phases were evaporated to dryness under reduced pressure in a rotary evaporator (45 °C).The residues were redissolved in 3 ml of MetOH,if necessary centrifuged (5 min. at 2000 r.p.m.),carefully transferred into a 5 ml vial and the organic solvent evaporated under a stream of nitrogen (40 °C).For HPLC analysis the extract was redissolved in 200 µl of MetOH,LiChrosolv(Merck,Darmstadt F.R.G.)

α-amanitin (Calbochem-Behring,San Diego,CA,U.S.A.) of known purity (greater then 95%) was used without furter purification.The stock solution of 0.1 mg/ml was prepared by dissolving 1 mg of α-amanitin,exactly weighed,in MetOH and diluting to volume in a 10 ml volumetric flask.A 1 ml volume of stock solutions was quantitatively diluted to 10 ml with MetOH (solution 2),and a convenient working standard was prepared by diluting 0.5 ml of solution 2 to 10 ml with MetOH.Use of 10 µl of this solution led to acceptable peak height when the U.V. detector attenuation was 0.02 AUFS.

A standard curve was obtained by using four methanolic dilutions of solution 2.Although standard solutions are stable at 4 °C,fresh working standard must be made weekly.

HPLC analyses were run on a Perkin-Elmer Series 3B (Norwalk,Connecticut, U.S.A.) liquid chromatograph; the eluted components were monitored with a LC-75 variable wavelength detector (160-600 nm) equipped with a LC-75 Autocontrol.The columns used were a Hibar-LiChrosorb RP 18 10 µm (25 x 0.4 cm I.D.;Merck,Darmstadt,F.R.G.) and a Polygosil 60-10 C 18 (25 x 0.4 cm I.D.;

Macherey-Nagel,Düren,F.R.G.).The separations reported were achieved under the following conditions: mobile phase 40% MetOH (LiChrosolv,Merck) in water purified by a Water I (Gelman Sciences,Michigan,U.S.A.);flow rate,1 ml/min.; temperature,25 °C;wavelength,303 nm;chart speed,0.5 cm/min. Graphs were generally obtained with an attenuation setting corresponding to 0.08 AUFS on a 10 mV Perkin-Elmer mod. 561 recorder.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram and the U.V. spectrum obtained for a standard solution of α-amanitin.The overall precision of the retention time was

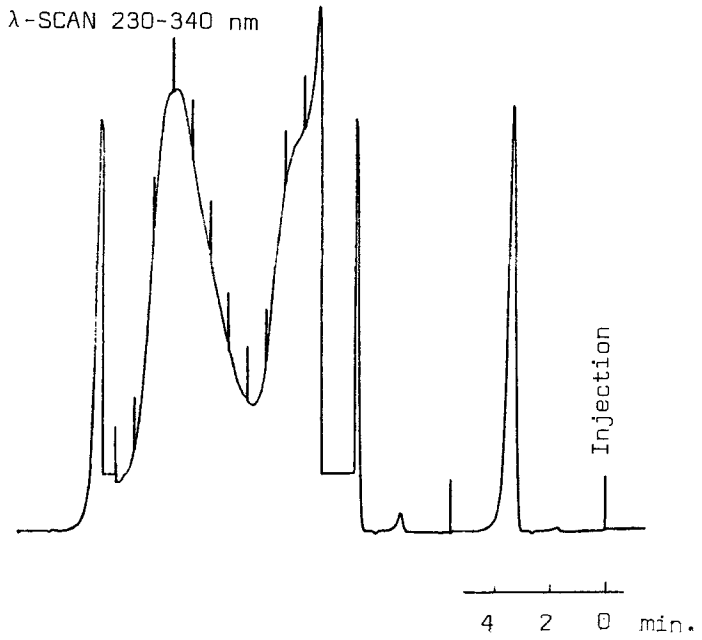

FIGURE 1

HPLC chromatogram of a solution of α-amanitin standard and its U.V. spectra. Column:Hibar-Lichrosorb RP 18 10 µm (25 x 0.4 cm I.D.);mobile phase,MetOH:H_2O (40/60);flow rate 1 ml/min.:wavelength,303 nm;temperature,25 C;chart speed,0.5 cm/min. The U.V. spectrum was recorded in λ-SCAN MODE from 230 to 340 nm.

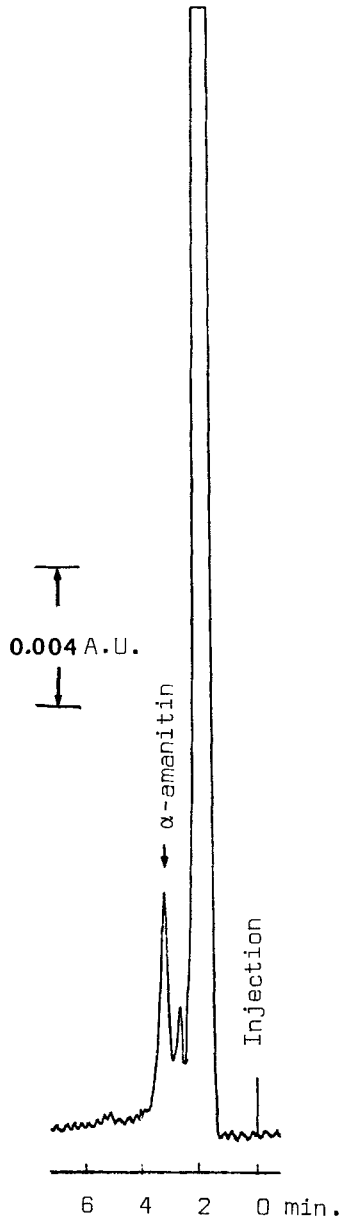

FIGURE 2

HPLC chromatogram illustrating the separation of α-amanitin in a sample of human serum fortified with α-amanitin standard (50 µg of α-amanitin/ml of serum). Injection volume: 15 µl (75 ng of α-amanitin). Chromatographic conditions as in Fig.1

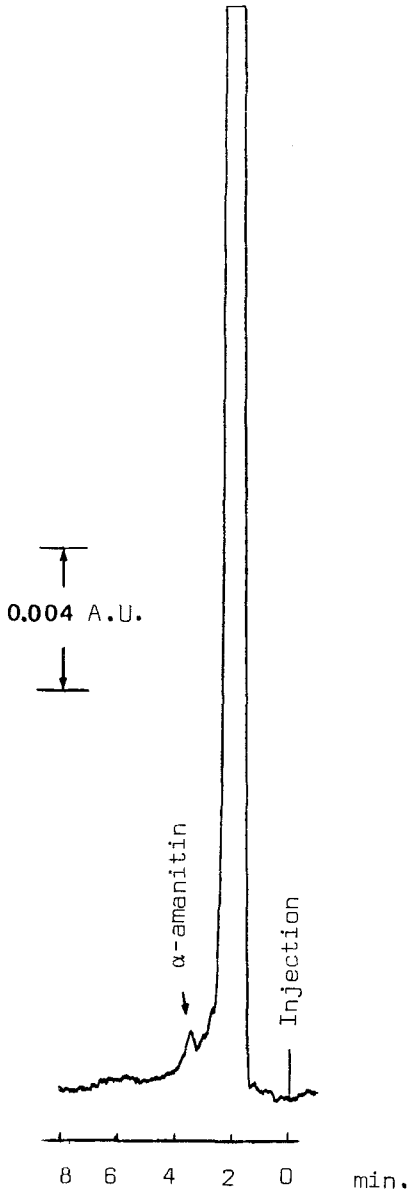

FIGURE 3

HPLC chromatogram obtained from the serum of a patient intoxicated by *A. phalloides*. Chromatographic conditions as in Fig.1.

studied with respect to run-to-run precision,day-to-day precision and overall column-to-column reproducibility.The run-to-run precision for ten runs within a single day averaged 1.15% (relative standard deviation,RSD),while the day-to-day precision over 3-week period using a single column (Hibar-LiChro_ sorb) averaged slightly higher,at about 1.9% (RSD).The results of the column-to-column reproducibility studies show that the absolute retention time for the α-amanitin averaged about 5% (RSD).The two columns used for this comparison were at different stages of their useful lifetime,ranging from factory fresh to used for about 100 runs of serum samples (Polygosil).

The calibration curve of peak area (or peak height) against quantity of α-amanitin injected was constructed for triplicate injections of four standard solutions of α-amanitin,and it was found to be rectilinear over a 0.05-5.0 μg range (y=0.9569x + 0.0032).For routine quantitative determination,peak heights were measured to the baseline extrapolated from the leading edge of the peak. Figure 2 shows a chromatogram of a serum from a normal donor fortified with 50 μg/ml of serum of α-amanitin.A typical chromatogram obtained from the serum of a patient intoxicated by *A. phalloides* (18 h after eating the mushrooms) is illustrated in Figure 3.

Human serum pool containing α-amanitin showed a recovery in the range of 87-92% with an average recovery of 90.5%.The minimum detectability,with instrument sensitivity of 0.02 AUFS,is 20-25 ng;below this level baseline detector noise exceeded peak height.

CONCLUSION

The HPLC methodology here is simple and precise with a minimum of sample preparation steps required.The method described allows the quantitation of α-amanitin in patient's blood with a suitable precision only if the blood specimens are taken during the first 24 h after the ingestion of mushroom. The excretion of the circulation toxins by the kidneys is a very rapid process (amatoxins which are not bound to serum albumin,and have a molecular weight of about 900 dalton,are easily dialysable),and after 30 h the serum concentration of α-amanitin falls to below the limit od detection.

The application of the HPLC method to the quantitation of α-amanitin in the urine of poisoned patient is currently in progress in our laboratories.

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