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Short Communication

Direct, simultaneous determination of α-amanitin, β-amanitin and phalloidine by high-performance liquid chromatography

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Introduction

An increase in the number of people who pick mushrooms, despite a lack of basic knowledge of mycology, has led to an increase in the population at risk from poisoning. Indeed, every year during the mushroom picking season, an increasing number of cases of poisoning and death occur following accidental ingestion of mushrooms of the genus *Amanita*, especially *Amanita phalloides*.

At present, the diagnosis of poisoning is based on the symptoms, the incubation time and the account given by the patient. No routine search for the most important mycotoxins in biological specimens from poisoned patients is currently carried out in hospital or poison centres. This is mainly due to the lack of analytical methods that enable these mycotoxins to be promptly and reliably detected.

Although many analytical techniques such as thin layer chromatography (TLC, HPTLC) [1–8], radioimmunoassay (RIA) [9–10] and high-performance liquid chromatography (HPLC) have been proposed for the determination of α-amanitin in blood [11], no simple, accurate and reliable methods for routine clinical practice are yet available.

As a contribution to solving the problem of prompt and reliable diagnosis in mushroom poisoning, an analytical method has been developed to determine simultaneously α-amanitin, β-amanitin and phalloidine in biological fluids and in the fungal matrix.

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Because of the physico-chemical characteristics of the mycotoxins of *Amanita phalloides* and the nature of the matrices, it was thought advisable to develop a reversed-phase HPLC method capable of meeting the following basic requirements: simultaneous determination of α-amanitin, β-amanitin and phalloidine in serum, urine and in the mushroom; sufficient resolution to eliminate any interference due to peaks of other substances present in the matrix; the method should display high sensitivity and be reasonably rapid.

Interest was focussed on α-amanitin and β-amanitin, since these substances are present in relatively large amounts (about 80 and 50 mg/kg of fresh mushroom, respectively) [12]; in addition they can be considered as the most important of the *Amanita* toxins because of their lethal toxicological effects.

Phalloidine was also studied since this toxin is present in the highest amount (100 mg/kg of fresh mushroom) [12] and, although its toxicity is lower, the detection of this toxin in body fluids might be regarded as the most valid indication of poisoning by *Amanita phalloides*.

It was decided not to confine the investigation to biological fluids of human or animal origin, but to study the fungal matrix also, since an early diagnosis of poisoning might be made through an examination of any uneaten food remaining, or of food residues found in the vomit, where taxonomic investigation may be difficult.

Experimental

Chemicals

α-Amanitin and phalloidine were donated by Boehringer Mannheim GmbH; β-amanitin was obtained from Sigma Chemical Co.; methanol, ethanol, chloroform and acetonitrile (Chromasolv) were from Hoechst. Reagent grade acetic acid and ammonium acetate were obtained from Carlo Erba. Distilled water was filtered through a Millipore filter (0.2 μm).

Apparatus

A Varian 5060 HPLC system was used, equipped with a Valco sampling valve, a 10-μl loop, a Varian Varichrom detector and a Varian Vista CDS-401 data system with a printer plotter. The 125 × 4 mm i.d. column was packed with 5-μm Lichrosorb RP-18 (Merck).

HPLC conditions

The operating conditions were: ambient temperature; flow rate, 1 ml/min; detector wavelength, 302 nm; chart speed, 0.3 cm/min. A buffer (pH 5.0) was prepared from acetic acid and ammonium acetate (0.01 M).

Separation of the three mycotoxins was achieved by a linear gradient elution system: phase A, acetonitrile; phase B, 0.01 M acetic acid–ammonium acetate buffer (pH 5.0). The gradient profile adopted was: $t = 0$, %B = 93; 7 min, %B = 93; 30 min, %B = 75.

For each mycotoxin, serial dilutions were prepared in methanol to contain 0.5, 2.5, 5.0, 10.0 and 20 μg/ml; mixtures of all three mycotoxins in equal amounts were also prepared. These solutions were used to spike the serum and urine samples. The samples examined were subjected to a preliminary clean-up procedure.

Sample pretreatment

Biological fluids. To 1.0 ml of serum or urine was added 2 ml of methanol–chloroform (50:50 v/v); the mixture was shaken for 3 min and then centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a Millipore (0.2 μm) filter and concentrated under a stream of nitrogen to 100 μl. A 10 μl aliquot of this solution was injected into the chromatograph.

Fungal matrix. A 2 g sample of dried mushroom, suspended in 50 ml of a mixture of ethanol–water (50:50 v/v), was stirred with a magnetic stirrer for 15 min at a temperature not higher than 45°C. The suspension was allowed to settle and the supernatant was then separated from the sediment. This step was repeated twice so that 150 ml of final solution was obtained [12].

This solution was then concentrated to dryness and the solid residue resuspended with 5 ml of the water–alcohol mixture; 2 ml of the suspension was diluted to 20 ml with water, filtered through a Millipore (0.2 μm) filter, and 10 μl of the filtrate was then injected into the chromatograph.

Results and Discussion

The mycotoxins exhibited the following retention times: β-amanitin 9.1 min; α-amanitin 14.9 min: phalloidine 27.8 min. The chromatogram for the standard mixture showed clear separation of each mycotoxin (Fig. 1).

The chromatograms of human serum (Fig. 2) and human urine (Fig. 3), each spiked with the standard mixture, clearly show that the elution times of the three toxins allow a wide safety margin in relation to possible interference from other substances that might be in these biological matrices. The chromatogram illustrated in Fig. 4 represents the analysis of the three mycotoxins in a sample of *Amanita phalloides* extract.

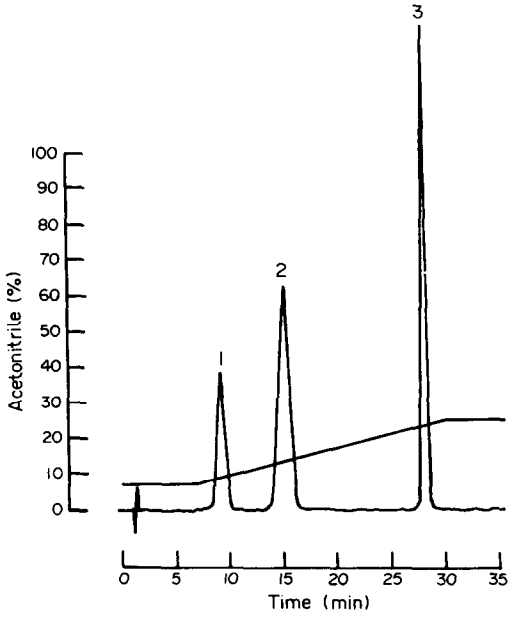

Figure 1
Chromatogram of standard mixture: (1) β-amanitin; (2) α-amanitin; (3) phalloidine. The gradient profile adopted is shown; solvent A was acetonitrile and solvent B was acetic acid–ammonium acetate buffer (0.01 M; pH 5.0). For instrumental conditions, see text.

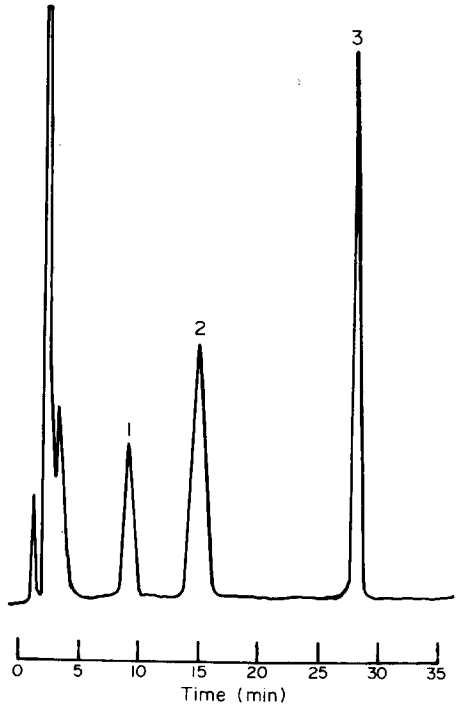

Figure 2
Chromatogram of human serum spiked with standard mixture; key as in Fig. 1.

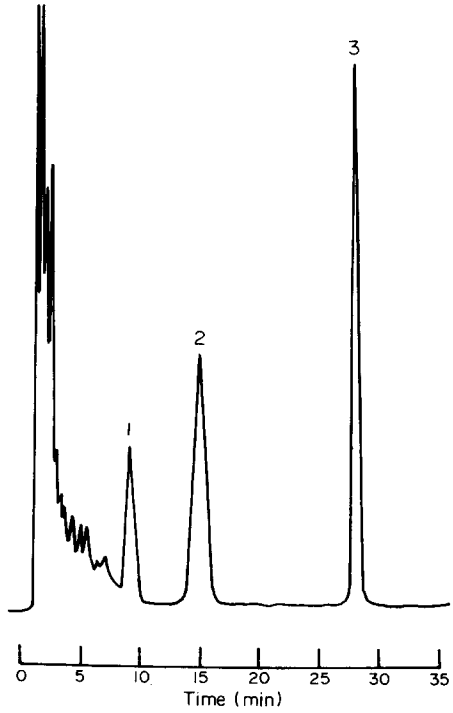

Figure 3
Chromatogram of human urine spiked with standard mixture; key as in Fig. 1.

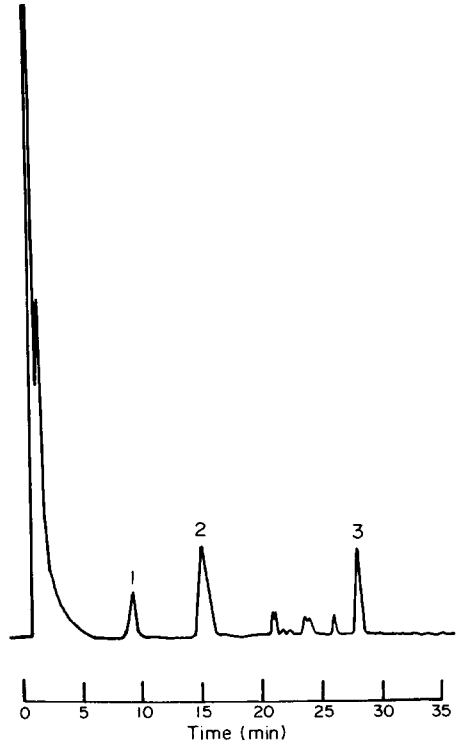

Figure 4
Chromatogram of *Amanita phalloides* extract; key as in Fig. 1.

In order to check the linearity of response, calibration graphs of peak height against concentration were constructed, using serial dilutions of standard solutions of each mycotoxin. The corresponding regression equations (Table 1) demonstrate good linearity for amounts in the range 0.5–20 μg/ml.

In biological fluids, the lowest detectable amount at 0.02 a.u.f.s. was approximately 10 ng for α- and β-amanitin, and 5 ng for phalloidine, for a signal-to-noise ratio of 2:1. Recoveries of β-amanitin, α-amanitin and phalloidine from serum and urine are detailed in Table 2.

The total time of analysis, including the clean-up procedure and chromatography, was less than 1 h in every test. This is certainly consistent with the need for a speedy diagnosis in cases of suspected poisoning from *Amanita phalloides*. Other methods that have been

Table 1
Regression data*

Mycotoxin	Regression equation†	r‡
α-Amanitin	$y = 184.6x - 0.112$;	0.9999
β-Amanitin	$y = 202.0x + 17.7$;	0.9999
Phalloidine	$y = 134.7x + 0.87$;	0.9999

* $n = 5$, each point representing triplicate injections in the range 0.5–20 μg/ml.

† x = concentration in μg/ml; y = peak height; 0.02 a.u.f.s.

‡ r = correlation coefficient.

Table 2
Accuracy and precision of analysis of mycotoxins in serum and urine

	Spiked concentration (μg/ml)									
	0.5		2.5		5		10		20	
	Recovery*	RSD†	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
Serum										
α-Amanitin	81.1	0.91	86.2	1.05	90.1	0.70	93.2	0.71	98.1	0.76
β-Amanitin	80.6	1.42	85.5	0.69	88.7	0.58	92.6	1.19	96.0	0.81
Phalloidine	89.6	0.55	90.7	0.97	90.6	0.84	97.0	0.84	98.5	0.47
Urine										
α-Amanitin	81.1	1.08	86.6	0.69	89.4	1.04	93.2	0.74	98.0	0.49
β-Amanitin	81.0	1.21	85.3	1.08	89.2	0.78	92.5	0.93	97.3	1.00
Phalloidine	88.9	0.90	88.7	0.84	90.8	0.50	97.6	0.81	98.6	0.55

* Recovery is expressed as a percentage of the spiked concentration.
† RSD = relative standard deviation (n = 5).

suggested (TLC, RIA) either require a longer time of analysis (about 24 h) or enable only one mycotoxin to be identified [1–8].

The short analysis time and the results of the present work suggest that the method is suitable for an initial routine screen in clinical practice for cases of suspected mushroom poisoning.

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