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Note

Determination of α-amanitin by high-performance liquid chromatography

L. PASTORELLO and D. TOLENTINO

Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan (Italy)

M. D'ALTERIO

Biolabo Instruments, Milan (Italy)

R. PALADINO and A. FRIGERIO*

Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan (Italy)

and

N. BERGAMO and A. VALLI

Trent Hospital, Trent (Italy)

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Amanita phalloides is one of the mushrooms most frequently responsible for fatal mushroom poisoning [1]. Various amatoxins (α-, β-, γ- and ε-amanitin, amanin, amanullin) (Fig. 1) have been identified, and of these α-amanitin is one of the most toxic and abundant [2—6].

Most published analyses of α-amanitin have involved separation by thin-layer chromatography [7—13] and radioimmunoassay [14, 15].

This paper reports a method using reversed-phase high-performance liquid chromatography (HPLC) with UV detection for the determination of α-amanitin.

MATERIALS AND METHODS

Chemicals and reagents

Standards were obtained from commercial sources; acetonitrile and methanol (HPLC grade) were obtained from Merck, Darmstadt, G.F.R. α-Amanitin was obtained from Boehringer, Mannheim, G.F.R. Water was demineralized, distilled in glass and filtered before use. Human blank serum was obtained

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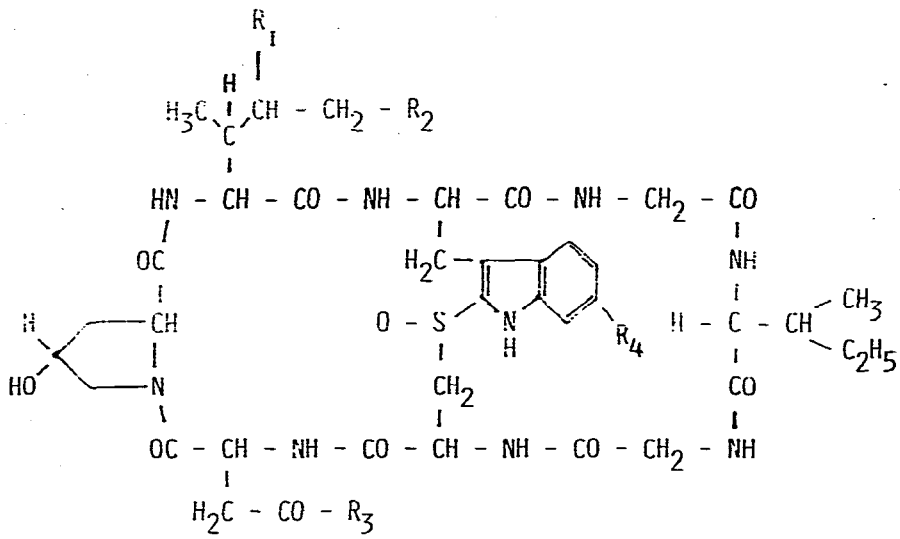

	R_1	R_2	R_3	R_4
α-Amanitin	-OH	-OH	$-NH_2$	-OH
β-Amanitin	-OH	-OH	-OH	-OH
γ-Amanitin	-OH	-H	$-NH_2$	-OH
ε-Amanitin	-OH	-H	-OH	-OH
Amanin	-OH	-OH	-OH	-H
Amanullin	-H	-H	$-NH_2$	-OH

Fig. 1. Chemical structures of amatoxins.

from A.V.I.S., Milan, Italy. The serum of poisoned patients was supplied by Trent Hospital, Trent, Italy.

Instrumentation

A Gilson (Villiers-Le Bel, France) high-performance liquid chromatograph was used equipped with a Holochrome (Gilson) dual-beam UV detector set at 302 nm, the wavelength of maximum absorption of α-amanitin. The column system consisted of a Spherisorb 5-μm ODS precolumn (Phase Separations, Queensferry, Great Britain) and a Spherisorb 5-μm (Phase Separations) analytical column (25 cm × 4.6 mm I.D.). It was eluted with acetonitrile—water (15 : 85) at a flow-rate of 1 ml/min. Injections were made with a Rheodyne 6-port valve fitted with a 20-μl sample loop. The output of the detector was displayed on a recorder (Model N 2, Gilson) having a full-scale range of 100 mV. Chart speed was 0.5 cm/min.

(a)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(min)
(b)
0 1 2 3 4 5 6 7 8 9
(min)

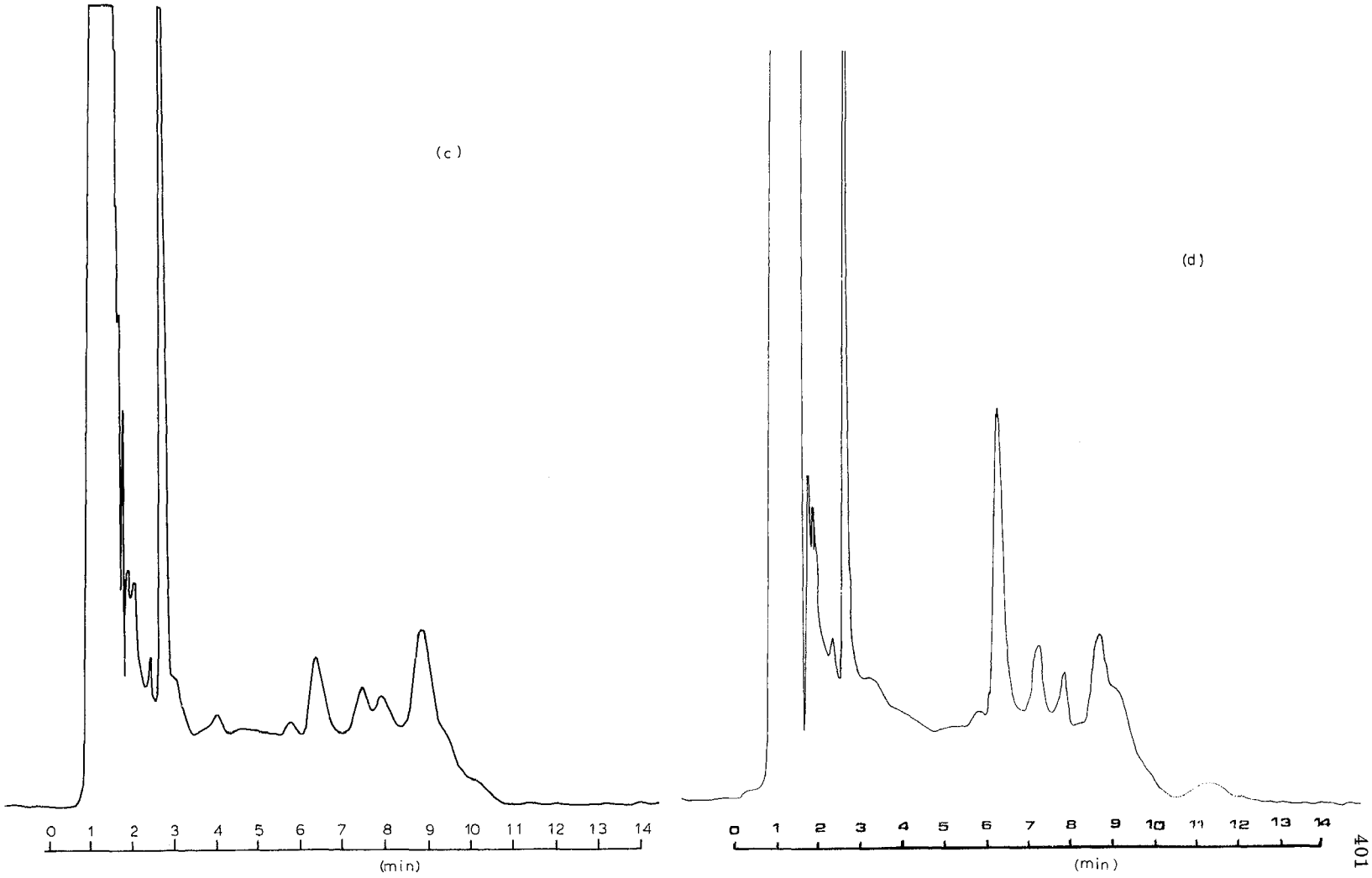

Fig. 2. HPLC profiles of: (a) an extract of blank serum; (b) a standard solution of α-amanitin; (c) an extract of a serum sample of a patient poisoned with *Amanita phalloides*; (d) an extract of a serum sample from a poisoned patient, spiked with α-amanitin.

Analytical procedure

At the beginning a calibration curve was obtained. Methanolic standard solutions corresponding to 20, 10, 5, 2.5 and 0.5 μg/ml α-amanitin were prepared. Each point is the mean of three determinations. Every injection was carried out filling the sample loop. A least-squares regression relationship between concentration and the peak height was calculated.

Serum of some patients poisoned by *Amanita phalloides* was extracted with methanol (1:2, v/v) modified from ref. 10). The sample was centrifuged twice at 2500 *g* for 15 min and the supernatant obtained was evaporated to a volume of 100 μl; 20 μl filling the sample loop were injected into the instrument. The same extraction method was carried out for the blank serum.

RESULTS AND DISCUSSION

The calibration curve of the standard solution gave good linearity. Least-squares linear regression analysis was used to determine the slope, *y*-intercept, and correlation coefficient. Using peak height: $y = 0.4710X - 0.0149$ ($r = 0.9999$).

The minimum detectable amount of α-amanitin by this method was about 10 ng (20 μl of a 0.5 μg/ml solution).

The chromatograms of samples from some patients poisoned by ingestion of *Amanita phalloides* show a peak at the same retention time of the standard, not present in the blank serum and becoming higher if the serum of the poisoned patient is spiked with α-amanitin.

A chromatogram obtained from blank plasma is shown in Fig. 2a. Fig. 2b shows a chromatogram of a standard solution of α-amanitin in methanol. Fig. 2c is a chromatogram of a sample from a patient who died from ingestion of *Amanita phalloides*. Fig. 2d shows a chromatogram of serum of a poisoned patient spiked with α-amanitin standard.

Samples from patients poisoned with *Amanita phalloides*, compared with authentic α-amanitin, show values approximately between 70 and 90 ng/ml of serum.

In conclusion the HPLC method described has the following advantages: (a) α-amanitin is separated from other serum components; (b) the chromatographic analysis can be completed within 15 min; (c) microquantities (ca. 50 ng/ml α-amanitin in human serum) can be determined.

The values we have found for α-amanitin in the serum of the poisoned patients relate to a limited number of cases. For this reason it will be necessary to obtain a larger number of samples to be able to effect a statistical evaluation. These studies should also take into consideration other biological fluids such as urine.

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