Express Method for Detection of *Amanita Phalloides* Amanitine Toxins

D. N. Vlaskin, E. T. Gainullina, O. V. Klyuster, I. V. Rybal'chenko, S. B. Ryzhikov, and V. F. Taranchenko

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Spectral studies of the interaction between amanitine and ethidium bromide fluorophore showed the appearance of a new intensive fluorescence band after addition of amanitine to ethidium bromide solution, caused by the formation of a charge-transfer complex. The new fluorescence band is located in a shorter wave region of the spectrum compared to ethidium bromide fluorescence band. Based on the results, a rapid fluorescent method for detection of amanitines was developed.

Key Words: *amanitine; rapid method; fluorescence; ethidium bromide*

Lethal outcome after *Amanita phalloides* poisoning is mainly determined by the toxic effect of amanitines, inhibitors of eukaryotic DNA-dependent RNA polymerase [1].

Amanitine toxicity is 0.1 mg/kg for humans. The concentration of toxins in *Amanita phalloides* is high (0.4 mg/g for amatoxins), and since the lethal dose is only 5-7 mg, even one mushroom can cause death.

Several analytical methods for *Amanita phalloides* toxin detection were proposed [1]. However, the sensitivity of the known methods is inadequate to high toxicity of amanitines, and the technology of analysis by these methods is difficult.

The aim of this study was to develop a rapid method for detection of amanitines, based on the effects of these toxins on the fluorescence spectra of ethidium bromide.

MATERIALS AND METHODS

Sigma preparations 2,7-diamino-10-ethyl-9-phenylphenantridiniumbromide (EB), α- and β-amanitines were used in the study.

Military University of Radiation, Chemical, and Biological Defense, Moscow

Fluorescence spectra were recorded using a Hitachi spectrofluorometer.

α-Amanitine solution (100 μM) in 0.02 M phosphate buffer (pH 8.0), β-amanitine solution (100 μM) in 0.02 M phosphate buffer (pH 8.0), and ethidium bromide solution (50 μM) were used in the study.

Ethidium bromide solution (0.5 ml) was added to 3.5 ml amanitine solution of the needed dilution, the mixture was put into a quartz cuvette (10×10×40 mm), and the fluorescence spectrum was recorded. All measurements were carried out in 0.02 M phosphate buffer (pH 8.0) at 20°C (excitation wavelength λ=470).

The sample was prepared as follows: 5-6 g mushrooms were cut into small fragments, 10 ml phosphate buffer (pH 8.0) was added, thoroughly mixed, and left in a glass cylinder for 10-15 min, after which the upper optically transparent layer of the extract was taken for the analysis. For some experiments the sample was prepared as described previously [1]. The results of the analysis were identical in both cases.

RESULTS

Ethidium bromide well dissolved in aqueous media and characterized by high intensity of fluorescence in the red spectrum (in which the concomitant com-

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pounds do not fluoresce) and characterized by high photostability, thermostability (fluorescence intensity virtually does not depend on medium pH within pH values from 5 to 9) was selected as the analytical fluorophore reagent.

Addition of amanitine to EB solution led to the appearance of a new intensive fluorescence band (Fig. 1). Ethidium bromide at pH 8.0 has a fluorescence band at λ_{max}=610 nm, while in the presence of amanitines new fluorescence bands appear: at 560 nm in the presence of α-amanitine and at 525 nm in the presence of β-amanitine (Fig. 1, *2*, *3*). If both α- and β-amanitines are present simultaneously, each of them can be detected by its fluorescence spectrum (Fig. 1, *4*). Thus, fluorescence bands of α- and β-amanitines are located at 560 and 525 nm, respectively. The analytical effect was observed immediately after addition of the reference amanitine preparation (Sigma) and *Amanita phalloides* extract to EB solution. The analytical effect is stable within at least 30 min.

According to previous reports [2,3], the appearance of a new fluorescence band can be explained by the formation of a complex with charge transfer from amanitine (donor) to stimulated EB molecule (acceptor).

The increase in fluorescence intensity of amanitine+EB complex with increasing amanitine concentration is linear for the entire range studied, for both α- and β-amanitines (Fig. 2, *1*, *2*).

For experiments by the method of additions, a sample with a known concentration of β-amanitine was taken (the concentration was established using its calibration curve based on the reference compound; point A in Fig. 2, *1* corresponded to this concentration). Then a preset amount of reference β-amanitine compound, previously used for plotting the calibration curve, was added to the sample. Experimental data (Fig. 2, point B) were compared with estimated concentration of β-amanitine (Fig. 2, point C). Comparison of experimental and estimated data showed good agreement between the calibration curve and amanitine concentrations in the sample. These data indicate a negligible effect of admixture compounds of the sample on the results of β-amanitine analysis by the suggested method.

Hence, the studied fluorescent method is rapid and sensitive, not inferior to liquid chromatography, simple, with a low error of analysis.

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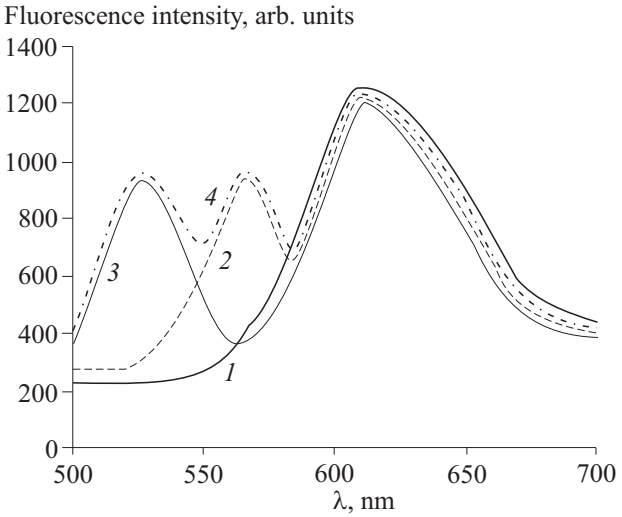

Fig. 1. Ethidium bromide (6 μM) fluorescence spectra in the presence of amanitines and without them in 0.02 M phosphate buffer (pH 8.0) at λ_{stim}=470 nm. *1*) ethidium bromide; *2*) ethidium bromide+α-amanitine (100 μM); *3*) ethidium bromide+β-amanitine (100 μM); *4*) ethidium bromide+α-amanitine (100 μM)+β-amanitine (100 μM).

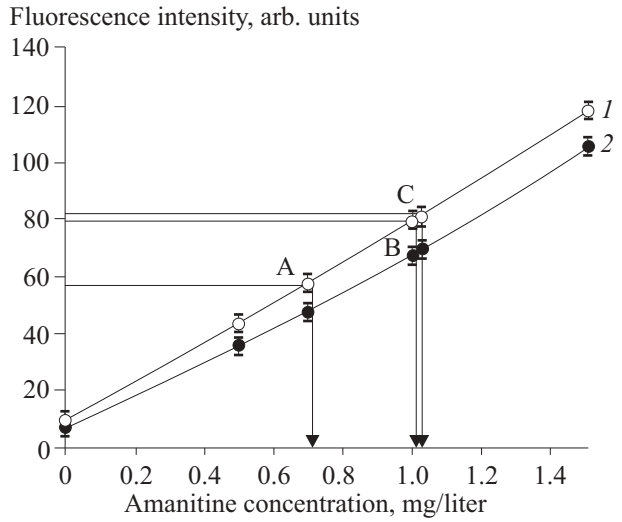

Fig. 2. Relationship between ethidium bromide fluorescence intensity (6 μM) and concentrations of α-amanitine (analytical effect at λ=560 nm; 1) and β-amanitine (analytical effect at λ=525 nm; *2*).

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