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CHROMATOGRAPHIC DETECTION OF MUSHROOMS TOXINS OTHER THAN *AMANITA PHALLOIDES*

Abstract - D. MICHELOT, J. P. BROUARD & R. LABIA - Chromatographic detection of mushrooms toxins other than *Amanita phalloides*.

Mushrooms poisonings occur every year, in several cases they lead to deaths or failure of organ such as kidneys. Chromatographic methods for identification of higher fungi toxins have been developed in order, on the one hand, to detect the toxic agents, since the victim often does not associate his condition with a mushroom meal consumed several hours or days earlier, and, on the other hand, to monitor the appropriate treatment oriented towards the elimination of the toxin.

In the numerous reviews dealing with mushrooms poisonings, informations are scarcely available on statistics about the different responsible species since most of the cases are not properly reported (for informations see: BORNET et Alii 1983; BORNET et Alii, 1984; TRESTAIL, 1991). *Amanita phalloides* (Fr.) Link., *Amanita verna* (Bull: Fr.) Lamk. and *Amanita virosa* (Fr.) Bertillon are the species most frequently involved; *A. phalloides* causes the majority of the intoxications; 10-40% (several hundreds every year) of these intoxications lead to death (FLOERSHEIM et Alii, 1982; OLSON et Alii, 1982; BRESINSKY and BESL, 1990; GARNIER et Alii, 1990).

Besides *A. phalloides*, many other mushrooms species are poisonous, and detection of the toxins or their metabolites in biological fluids could help the management of the poisonings. The patient is usually unable to associate his

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condition with a prior consumption of mushrooms and to describe the responsible fungi. The identification of the toxic mushrooms species (especially by the means of the spores) in the remains of the meals, in the stomach or in the feces turns out to be a really tedious task for the mycologist. Only a few examples of detection of non-*Amanita phalloides* toxins in the body fluids are mentioned. In some cases, levels of toxins (or metabolites) detected in biological fluids are only diagnostic, but in some others, they could allow a decision to be taken concerning the therapeutic (for instance the appropriate treatment: hemodialysis, peritoneal dialysis, etc.).

In this paper we will try to review the several different methods employed in order to detect mushrooms toxins (other than *A. phalloides*; for this topic see the article of Doctor Franco Tagliaro in the present book; Dorizzi et Alii, 1992), or we will mention the analytical techniques readily compatible. In other respects, we will not describe the different methods available for finding hallucinogenic substances in mushrooms or in body fluids; these techniques are the concern of forensic science rather than hospital intensive care (in a few cases determination kits are sold by fine chemical products companies).

We must remind that biological samples, as opposed to synthetic organic chemistry mixtures, are constituted of various kinds of substances with quite different chromatographic behavior, therefore, sample pretreatment before chromatography is of the highest importance. The cleaner is the sample, the more sensitive and accurate is the analysis.

Poisonings by *C. orellanus* (Fr.) and *C. speciosissimus* Kühn. & Romagn., both members of the genus *Cortinarius* (Moser) section *Orellani* occur yearly and cause acute and chronic renal failure appearing after an unusually long latency period (2-3 weeks) (Michelot & Tebbett, 1990). This kind of poisoning because of the persistence of the toxins in the systemic circulation and because of the irreversible fixation of the metabolites or conjugates in the kidneys aroused the interest among pharmacologists and toxicologists. Orellanine 1 and its breakdown products orellinine 2 and orelline 3 are the main toxins (presented in Figure 1). The amount of orellanine, a 3,3',4,4'-tetrahydroxy-2,2'-bipyridine-*N,N'*-dioxide, the principal toxins, has been estimated in extracts by TLC methods and more recently by HPLC methods using UV and EC detectors.

Because of the low stability (UV light, pH and oxidation-reduction reactions in some particular solvents), poor solubility and high polarity of the substance, TLC yielded unsatisfactory results. The best results were obtained using a cellulose layer and butano1/acetic acid/water elution mixture (3/1/1 v/v). A procedure for the assay of orellanine in biological fluids has been reported. Plasma samples were loaded on an Amberlite XAD-4 resin (Rohm & Haas) adjusted to pH 2 and elution was carried out with water brought to pH 2 with hydrochloric acid, methanol/water and methanol. Two-dimension TLC on cellulose allowed

separation of orellanine which was quantified by fluorescence (at 366 nm) of its first breakdown product orellinine. This method surprisingly demonstrated very large quantities of this compound (6 mg/ml) still present in the systemic circulation even 10 days after consumption of the mushrooms (Rapior et Alii, 1989).

HPLC has also been developed. The mobile phase used with the Rosil column CN 5 μm (150 cm x 4.6 mm, Alltech) was phosphoric acid at pH 1 (Cantin et Alii, 1989). Alternatively, ion pairing chromatography was used: with Nucleosil C18 5 μm (20 cm x 4,6 mm, Waters), the mobile phase was 0.05 M phosphate citrate buffer pH 4.5 containing 15.4% methanol and PIC B6 1-hexanesulphonic acid (Holmdahl et Alii, 1987); and, with μ Bondapak C18 5 μm (15 cm x 3,9 mm, Waters) phosphoric acid PH 1-acetonitrile (94/6 v/v) and 1-octanesulphonic acid 2.5 mM (Cantin et Alii, 1989). Detection was accomplished with a UV detector (at 260 and 290 nm) or with a EC detector (working potential 900 mv vs Ag/AgCl ref).

Some of these HPLC experiments have been performed at the limits of the use specifications of the equipment (very low pH, short retention times, high concentration of the samples, wide peaks, ...) and the immediate application for the detection in body fluids seems arduous. The procedure can be improved by the use of polymeric acid resistant stationary phases: styrene divinyl benzene Biogel PRP 170-5 (150 x 4.6 mm, Biorad) with aqueous trifluoroacetic acid and methanol (Michelot & Labia, 1991). An alternative technique would be the derivatization of orellanine or orellinine (with silylating or alkylating reagents); such a chemical modification would lead to the use of GC-MS which could unambiguously demonstrate the persistence of the toxin in the systemic circulation (Michelot & Brouard, in preparation).

Reports have been published on TLC and HPLC methos for analysis of Cortinarins A, B and C, toxins from *C. speciosissimus*, separation was carried out using a ODS 5 μm (25cm x 4.5 mm, Jones Chromatography) column with

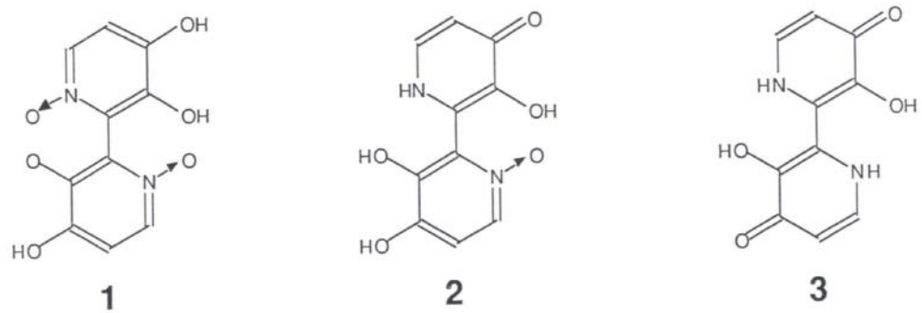

Fig. 1 - Chemical formulas of orellanine, orellinine and orelline.

a mobile phase of acetonitrile-water (25/75 v/v) (Tebbett & Caddy, 1983). Very recently, these latter results have been disputed, the authors claimed that they were not able to reproduce isolation and subsequently chromatographic separation of the fluorescent peptides supposed to be cortinarins (Matthies & Llaatsch, 1991).

Gyromitra esculenta (Pers.: Fr.) Fr. mushrooms have caused severe poisonings and even deaths in man (Michelot & Toth, 1991). Clinical data are primarily characterized by vomiting and diarrhoea followed by jaundice convulsions and coma. The sale and in some way the consumption of these mushrooms were lately forbidden in different European countries, and we hope that poisonings by «false morels» will be scarce in the future. Quantitative analysis of the toxin - gyromitrin (acetaldehyde methylformylhydrazone) 4 - and of its hydrolysis byproducts, *N*-methylformylhydrazine 5 and *N*-methylhydrazine 6 (presented in Figure 2) in human body fluids has never been published, but they have been detected in the peritoneal fluid of mice (Wright et Alii, 1978) and in urine of rabbits after *per os* administration (Mäkinen et Alii, 1977, Savolainen et Alii, 1977). They were detected by gas chromatography as the benzaldehyde derivatives of the hydrazines produced during *in vivo* hydrolysis. Gyromitrin was also detected by infrared or ultraviolet spectroscopy and TLC in the viscera during a post-mortem examination of a poisoned patient (Giusti & Carnevale, 1974).

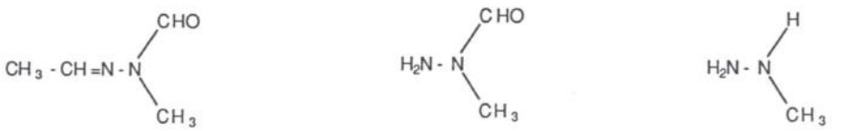

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Fig. 2 - Chemical formulas of gyromitrin, *N*-methylformylhydrazine and *N*-methylhydrazine.

Poisonings by *Amanita pantherina* (DC.: Fr.) Krombh. are characterized by dizziness, incoordination, ataxia, muscular jerking, hyperkinetic activity, stupor and hallucinations. The responsible toxins is ibotenic acid 7 which readily decarboxylates *in vivo* yielding muscimol 8 (presented in Figure 3) (Catalfomo & Eugster, 1970; Waser, 1979). This latter type of poisoning, although «disturbing» the poisoned victims is not responsible for fatalities, and methods of detection have not been efficiently developed. The contents of ibotenic acid and muscimol have been roughly estimated in a crude mushroom extract. The chromatographic system employed for ibotenic acid was LiChrosorb-NH_2 10 μm (5 cm x 4,6 mm, Merck) followed by a second column Nucleosil 5 μm (15

cm x 4.6 mm, Macherey-Nagel) with 0.05 M sodium acetate buffer pH 4 as the mobile phase, and for muscimol LiChrosorb-NH_2 10 μm (20 cm x 4.6 mm) with 0.05 M sodium acetate buffer p'H 5-methanol (1/9 v/v) (Lund, 1979). Unfortunately, these preliminary results do not seem convenient for detection in body fluids. Muscimol has been detected by gas chromatography-mass spectrometry as silylated derivatives and attempts to detect muscimol in urine as trifluoroacetylated derivative are in progress (Repke et Alii, 1978; Michelot & Brouard, in preparation).

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Fig. 3 - Chemical formulas of ibotenic acid and muscimol.

We point out that detection and quantitation of non-*Amanita phalloides* mushrooms toxins in body fluids in the case of poisonings is not usually essential as compared to the phalloidian ones; nevertheless, in some peculiar situations such as *Cortinarius* poisonings, improvements in identification, detection and quantitation of the toxins and metabolites may provide useful informations on the poisoning process and hence indicate the appropriate treatment oriented towards elimination of the toxin from the blood circulation and the target organ, and moreover suggest a therapy directed toward the rehabilitation of the damaged organ.

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