

MANTAR ZEHİRLENMELERİNDE KANDA HPLC İLE α -AMANİTİN MİKTAR TAYİNİ

DETERMINATION OF α -AMANİTİN IN SERUM BY HPLC IN CASES OF MUSHROOM POISONING*

Afife MAT** - Türkan YURDUN*** - Zülal TÜMKOR** - Ayşen AYANOĞLU*** -
Vecdet ÖZ***

SUMMARY

In Turkey, mushroom poisoning is especially seen in spring and autumn may even result in death. The main reasons for poisoning with mushrooms are their widespread usage as food and the inexperience of the gatherers in distinguishing the edibles from the poisonous. The diagnosis should be made as quickly as possible to start immediate treatment. *Amanita phalloides* and *A. verna* are the most dangerous species which give rise to fatal poisoning. α -Amanitin is the main compound responsible for poisoning in Phalloides syndrome. Its determination in serum is highly helpful to the diagnosis. We have therefore investigated various HPLC methods for the determination of α -amanitin in serum and developed a rapid and sensitive method which can be applied to routine analysis. According to this method, 2 μ g/ml of α -amanitin in serum can be detected.

Key words: mushroom poisoning, Phalloides syndrome, *Amanita phalloides*, α -amanitin, HPLC

ÖZET

Türkiye'de özellikle ilkbahar ve sonbahar aylarında ölümlü sonuçlanan mantar zehirlenmeleri görülmektedir. Halk arasında ormanlardan mantar toplayıp yeme alışkanlığının yaygın olması ve toplayıcıların yenen ve zehirli mantarları iyi tanımaması zehirlenmelerin başlıca nedenleridir. *Amanita phalloides* ve *A. verna* öldürücü zehirlenmelere neden olan en tehlikeli türlerdir. Phalloides sendromundan sorumlu başlıca bileşik ise α -amanitindir. Mantar zehirlenmelerinde tedaviye hemen başlayabilmek için doğru teşhisin kısa sürede yapılması gerekir. Serumda α -amanitin tayini yapılması

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** Department of Pharmacognosy, Faculty of Pharmacy, University of Istanbul.

*** Toxicology Department, Istanbul Public Health Institute

**** Director of Istanbul Public Health Institute

teşhise büyük ölçüde yardımcı olmaktadır. Bu amaçla, rutin analizlerde uygulanabilecek, hızlı ve hassas bir HPLC metodu geliştirilmiştir. Bu metoda göre serumda 2µg/ml α-amanitini tayin etmek mümkündür.

Anahtar kelimeler: Mantar zehirlenmesi, Phalloides sendromu, *Amanita phalloides*, alfa-amanitin, HPLC

INTRODUCTION

Every year, especially in spring and autumn many fatal cases resulting from mushroom poisoning are reported in Turkey. Gathering mushrooms from forests and using them as food is a widespread habit among people living in rural areas or people who move to the cities from these areas. The edible and the poisonous mushrooms grow together and are very alike. The inexperience of the gatherers in distinguishing them leads to fatal cases.

Poisoning by fungi are observed in two groups according to the length of the latent period (1,2). The first group includes cases of poisoning with a short latent period which are caused by different species. Nausea, vomiting, diarrhoea and other symptoms appear within 2 hours after ingestion of fungi. Treatment is limited in the first instance to eliminating the poison from the gastrointestinal tract by inducing vomiting and purging. The rest of the treatment is symptomatic. The second group with a late latent period is almost always serious and require immediate hospitalisation. Among this group the Phalloides syndrome caused by *Amanita phalloides* and *A. verna* are of primary importance because they account for about 90 per cent of all fatal cases of poisoning by fungi (3). The latent period may vary from 6 to 24 hours. The gastrointestinal phase, in which there is sudden onset of nausea with abdominal pain and colic, together with vomiting and cholera-like watery, and then bloody diarrhoea, lasts for 12 to 24 hours, and there follows, after appropriate treatment, a deceptive period of improvement. In the course of second latent period, which lasts for 1 to 2 days, there are changes in blood coagulation and transaminase (SGOT, SGPT) values, and the first signs of liver damage become evident. In the hepatorenal phase the following symptoms are observed: an enlarged and pressure-sensitive liver, jaundice, stomach and intestinal bleeding, oliguria to anuria, and disturbances of consciousness. If it is not treated, 4 to 7 days after the fateful meal of fungi death occurs during hepatic coma. The amatoxins, cyclopeptide compounds which are liver toxins, mainly α-amanitin, are responsible for poisoning in Phalloides syndrome (3,4). In addition to symptomatic treatment, the elimination of toxins from blood should be realised by haemoperfusion in 48 hours after the ingestion of mushroom, as proposed by different authors (5,6,7). The diagnosis

should be made as quickly as possible to start immediate treatment in order to minimise the exposure time of the liver to the toxins. For this purpose the determination of α -amanitin in body fluids (blood, urine and in gastro-duodenal aspirate) is highly helpful to the diagnosis.

Many cases of Phalloides syndrome which occurred in Istanbul since 1990 have been successfully treated by heamoperfusion (8,9). But in these cases it was not possible to determine the α -amanitin level in blood of the poisoned patients. To our knowledge, this analysis had not yet been realised in Turkish hospitals and laboratories. For that reason, the quantitation of α -amanitin in serum by HPLC was studied by us and a routine analysis method which can be realized in a laboratory equipped with a simple HPLC apparatus was developed. This investigation was carried out as an aid for diagnosis and therapeutic control of suspected cases of *Amanita* poisoning.

MATERIALS AND METHODS

Standard and solvents: α -Amanitin standard was purchased from Fluka (Art.No.06422). All solvents (Merck) were HPLC grade. Water was obtained daily from Milli-Q 50 (Millipore) system.

Apparatus and Chromatographic Conditions: The HPLC apparatus composed of the following units : Model 501 solvent delivery pump, U6K Universal Injector, Model 486 Tunable Absorbance Detector at 303 nm, Model 746 Data Module (all Waters, Milford, Massachusetts, U.S.A.). The chart speed was 0.5 cm/min. Separations were performed at ambient temperature on a reversed phase μ -Bondapak C₁₈ column (300x3.9 mm, 10 μ m, Waters). The mobile phase was a mixture of methanol-water (40:60 v/v) run at a flow rate of 1 ml/min. The mobile phase was filtered through an HV-0.45 m Millipore filter and degassed in an ultrasonic bath (Bandelin Sonorex TK52). The samples were filtered through a Millex HV-0.45 m filter. The blood samples were centrifuged at 3000 rpm/min. in a Hettich Universal centrifugator.

Preparation of α -amanitin standard solution: The stock solution of 0.1 mg/ml was prepared by dissolving 1 mg of α -amanitin in methanol and diluting to volume in a 10 ml volumetric flask (solution 1). A 1 ml of the stock solution was quantitatively diluted to 10 ml with methanol (solution 2). Use of 20 μ l of this solution led to acceptable peak height when the UV detector attenuation was 32. A standard curve was obtained by using nine methanolic dilutions of solution 2. The standard solutions were stored at 4⁰ C.

Preparation of *Amanita phalloides* extract: Different methods of extraction were assayed (10-19) and this of Stijve (17) was adopted. 1 g dried mushroom was extracted with 100 ml of methanol under reflux for 1 hour and allowed to cool. The extract was filtered through a plug of glass wool into a 250 ml flask and the residue was re-extracted with 50 ml of methanol for 30 minutes. The second extract after filtration was added to the first and evaporated to dryness under vacuum. The residue was dissolved in 2 ml of methanol and transferred into a 100 ml volumetric flask and then made up to 100 ml with methanol. 10 μ l of this solution containing the toxins were injected to the apparatus.

Preparation of serum samples: Blood specimens were obtained from healthy individuals. Serum was separated from whole blood by centrifugation. Before extracting with methanol the serum samples were spiked consecutively with 200 μ l, 160 μ l, 120 μ l, 80 μ l, 40 μ l of α -amanitin standard solution 1 and 100 μ l of an extract of *A. phalloides* containing 0.51 mg/g dry weight of α -amanitin. For the extraction of the toxin from serum samples, the method of Belliaro (21) was modified. To 1 ml of serum spiked with toxin, 1 ml of methanol was added, shaken for 1 minute and then centrifuged for 8 minutes (1000 rpm). The upper methanolic layer was carefully removed and the residue was resuspended in 1 ml of methanol and again extracted. The combined methanolic phases were evaporated to dryness under a stream of nitrogen. The residue was redissolved in 1 ml of methanol and centrifuged for 5 minutes (1000 rpm), the clear supernatant phase was carefully transferred into a 1 ml vial and adjusted to volume with methanol when necessary. 20 μ l of this extract were used for injection. The same extraction method was employed for the blank serum.

RESULTS AND DISCUSSION

Various methods of HPLC for the determination of α -amanitin in serum have been reported by many authors (20-24). All these methods have been assayed and a rapide, sensitive and simple method which can be applied to routine analysis in our laboratory conditions has been developed. According to this method, 2 μ g/ml of α -amanitine in serum can be detected.

The results of the chromatographic conditions described above are shown in figure 1.

The calibration curve of peak area gave good linearity over a 40 - 200 ng range (figure 2).

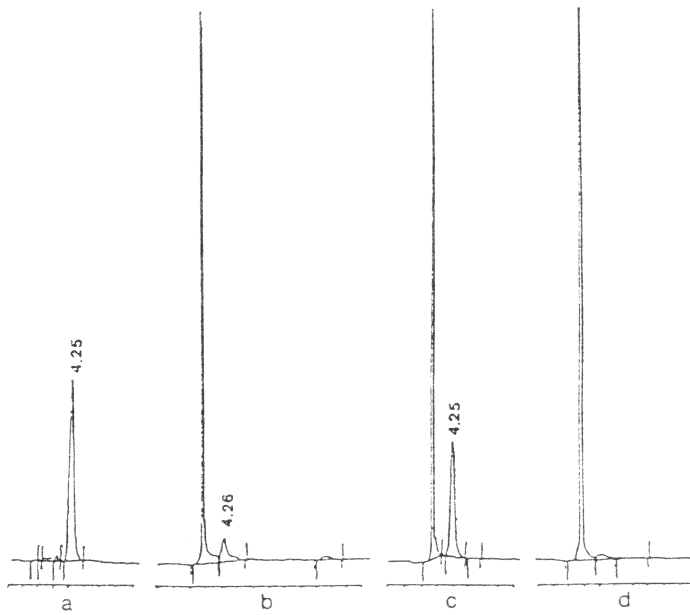


Fig. 1 : HPLC chromatograms, a) α -amanitin standard solution; b) Human serum spiked with *A.phalloides* extract; c) Human serum spiked with α -amanitin standard; d) Blank serum.

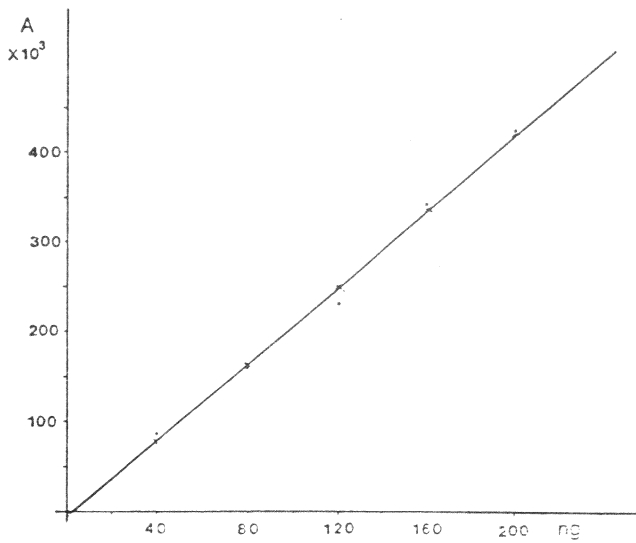


Fig. 2 : Calibration curve of α -amanitin. $y = 2155.076x - 9695$; $r = 0.9967$.

Human serum pool containing α -amanitin showed recovery in the range of 76.14-106.16 % with an average recovery of 90.5 % (Table 1).

Table I: Recovery of α - amanitin in serum samples

α -amanitin added ($\mu\text{g/ml}$)	Recovery (\pm confidence limit) (n=4)	RSD %
2	106.16 (\pm 3.59)	2.11
4	92.06 (\pm 5.19)	3.55
6	91.37 (\pm 1.59)	1.09
8	76.14 (\pm 0.50)	0.42
10	76.64 (\pm 4.07)	2.56

In 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 serum with a suitable precision. The blood specimens should be taken during the first 24 hours after the ingestion of mushroom. The excretion of the toxins by the kidneys is a very rapid process, and after 30 hours the serum concentration of α -amanitin falls below the limit of detection (21).

Many cases of poisoning resulting from ingestion of cultured mushrooms have been reported in Istanbul recently. The long latent period and the similarity of symptoms to Phalloides syndrome in these cases necessitated the determination of α -amanitin in blood. The analytical results obtained using the above described method showed that no α -amanitin was present in blood and poisoning was caused by mushrooms preserved under unsuitable conditions.

Thus, it will be possible to help and to confirm the diagnosis in cases of suspected mushroom poisoning , at least occurring in Istanbul district.

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