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Determination of mushroom toxins ibotenic acid, muscimol and muscarine by capillary electrophoresis coupled with electrospray tandem mass spectrometry

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ABSTRACT

The CE–ESI-MS/MS method for the identification, separation and determination of mushroom toxins, namely ibotenic acid, muscimol and muscarine, was developed. It proved to be sensitive and thus useful for the real sample analysis with omitting the labor and time consuming pretreatment step. The CE–ESI-MS/MS method was applied on the spiked human urine. The analytical characteristics of the proposed method, such as limits of detection, linearity and repeatability of the peak area and the migration time, were evaluated. The RSD of the migration time and peak area were from 0.93% to 1.60% and from 2.96% to 3.42%, respectively. The obtained LOD values were at the nanomolar concentration level, therefore the developed method is sufficient for the determination and quantification of studied toxins in human urine after mushroom intoxication.

1. Introduction

The isoxazole derivatives ibotenic acid and muscimol are the major low molecular toxins in the hallucinogenic mushrooms *Amanita muscaria* (fly agaric) [1], *Amanita pantherina* (panther cap) [2] and *Amanita gemmata* [2]. The other toxin which occurred in Amanita mushrooms is muscarine. The concentration and distribution of toxins in Amanita mushrooms are variable and depend on several factors, mainly on their origin, the growth state and the storage conditions [3,4]. For example the concentration of ibotenic acid in hallucinogenic mushrooms decreases in time due to its transformation into muscimol (more pharmacologically active toxin) during drying of mushroom [5].

Ibotenic acid and muscimol resemble and act to two main neurotransmitters of the central nervous system, namely to glutamic acid and γ-aminobutyric acid. Ibotenic acid has an excitatory action whereas muscimol produces a depressant effect [1,6]. Muscarine, comparing to the isoxazole derivatives, exhibits minor pharmacological activity [7].

The intoxication by *A. muscaria*, *A. pantherina* and *A. gemmata* could cause intentionally or accidentally. The accidental poisoning is resulted mainly by mistake with edible mushrooms (for example with other *Amanita* mushrooms). On the other hand hallucinogenic mushrooms have been used for recreational purposes, especially by young people experimenting with drugs [6,8]. Unfortunately the abuse of hallucinogenic mushrooms has an increasing trend especially because they are easily accessible and free [1].

The case studies of mushroom intoxication have been described for example by Satora et al. [6,8], Brvar et al. [1] and Stříbný et al. [9]. The human dose for the observation of central nervous system disturbances is about 6 mg for muscimol and from 30 to 60 mg for ibotenic acid [10]. This amount is corresponding to one mushroom of *A. muscaria* and/or *A. pantherina* [11]. The ingested amount of muscimol and muscarine is rapidly excreted into the urine in its unmetabolized form. Ibotenic acid is mostly excreted unchanged into the urine, at the same time part of it is transformed to muscimol. The death caused by the intoxication with those kind of mushrooms is rare [12].

Usually the mushroom poisoning is proved by microscopic examination of spores in the stomach and/or intestinal content [9].

Abbreviations: EIE, extracted ion electropherogram; MRM, multiple reaction monitoring; SIM, single ion monitoring

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That kind of investigation required qualified and experienced staff and is not easy to evaluate. For the mentioned reasons it is necessary to have the reliable and fast analytical method for the screening of intoxication and also the determination of mushroom toxins. Up to now, the several analytical methods dealing with the separation and determination of ibotenic acid, muscimol and muscarine in different matrices including plant and biological materials were published [13–21].

The coupling of liquid chromatography with tandem mass spectrometry (LC–MS/MS) was used for the determination of ibotenic acid and muscimol in Amanita mushrooms [13,14]. Gonmori et al. [13] analyzed ibotenic acid and muscimol in *Amanita* mushrooms by hydrophilic interaction LC–MS/MS, which can provided different selectivity for polar compounds and better suitability for coupling to MS in the comparison with reversed phase LC [22]. Tsujikawa et al. developed gas chromatography with MS (GC–MS) [15] and LC-MS/MS [14] methods for the identification of ibotenic acid and muscimol in the samples which circulated on the drug market, especially in the "smoke shops". Stormer et al. [16] published LC–UV and LC–MS methods for the determination of ibotenic acid in *Amanita muscaria* spores and caps and Gennato et al. [17] used ion-interaction LC–UV for the determination of muscimol and ibotenic acid also in *Amanita muscaria*.

In the case of biological material, the urine [9,18,19] and serum [20] samples were analyzed. Stříbrný et al. [9] developed GC–MS method for the separation of ibotenic acid and muscimol in urine. Merová et al. published validated LC–MS methods for the analysis of muscarine in human urine [18,19]. The analysis of ibotenic acid and muscimol in human serum was provided by LC–MS/MS [20]. However, only one work was dealing with the simultaneous separation of all selected mushroom toxins (ibotenic acid, muscimol and muscarine) by LC-MS method and was applied on urine samples [21].

The lowest values of limit of detection were 0.09 ng/mL [19] for muscarine, 1.4 ng/mL [14] for muscimol and 7.8 ng/mL [14] for ibotenic acid. In the case of published works focused on the determination of studied mushroom toxins in urine [9,18,19] the extraction step was provided. The sample extraction could be time consuming and the extraction recovery could be insufficient due to the matrix effect [23]. The advantage of presented work is that the method does not need the complicated sample pretreatment.

The use of capillary electrophoresis (CE) concerning the analysis of mentioned toxins has not been reported yet.

Consequently, this work is focused on the separation and determination of ibotenic acid, muscimol and muscarine by CE coupled with electrospray tandem mass spectrometry (CE–ESI-MS/MS), which could be an alternative method to the commonly used LC and GC methods. The main advantages of CE are extremely small injection volume (typically tens of nanoliters), high separation efficiency and short analysis time. The coupling of CE with MS/MS detection brings other relevant advantages, namely the possibility of analyte identification and low detection limits [24], which are very crucial for analysis of physiologically active compounds in biological fluids. Furthermore works dealing with GC method [9,15] included sample derivatization, which is not necessary in case of CE. Moreover the CE is also characterized as environmentally friendly method due to low solvent consumption in the comparison with LC.

2. Materials and methods

2.1. Instrument

All CE–ESI-MS/MS measurements were carried out using an Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent MSD mass spectrometer 6460 Series (Agilent Technologies) equipped with a triple quadrupole analyzer. For the CE–ESI-MS/MS coupling an Agilent coaxial sheath-liquid sprayer (Agilent Technologies) was used. Sheath-liquid was introduced to the sprayer by 1260 Infinity Isocratic Pump (Agilent Technologies). Electrospray parameters: sheath liquid composition 20:79.65:0.35 (v/v/v) of water, methanol and acetic acid, sheath liquid flow rate 0.4 μL/min, drying gas temperature 250 °C, sprayer voltage +4.5 kV. Both single ion monitoring (SIM) and multiple reaction monitoring (MRM) were used in this study. The obtained data were evaluated by Agilent MassHunter workstation software (Agilent Technologies).

All analyses were performed in uncoated fused-silica capillaries obtained from MicroSolv Technologies (Eatontown, NJ, USA). The positive voltage was applied and separation temperature was set up to 25 °C. Total/effective capillary length (to the MS inlet) was 100 cm (inner diameter 50 μm). Each first conditioning of the capillary included rinses as follows: 20 min with 0.1 M sodium hydroxide, 15 min with deionized water and 15 min with background electrolyte, all with the capillary end outside of the MS inlet. The capillary was rinsed between analyses with background electrolyte for 3 min. The injection of samples was performed hydrodynamically at 100 mbar for 5 s.

2.2. Chemicals

Ibotenic acid monohydrate, muscimol, muscarine hydrochloride, acetic acid, formic acid, sodium hydroxide, isopropanol and methanol (LC–MS grade) were obtained from Sigma (St. Louis, MO, USA). LC–MS water was purchased from Honeywell (Burdick and Jackson, MI, USA) and it was used for the preparation of stock solutions, as well as the background electrolytes and sheath liquid. All chemicals used were of analytical grade. Deionized water was obtained from Simplicity Water Purification System (Millipore, Molsheim, France).

3. Results and discussion

Ibotenic acid, muscimol and muscarine are polar and low molecular substances. Their chemical structures are shown in Fig. 1. Muscimol has values of dissociation constants 4.78 and

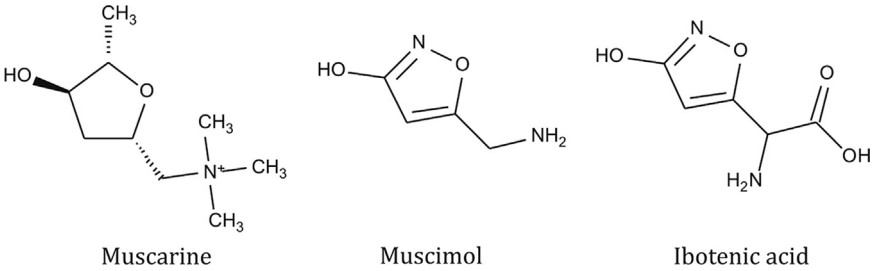

Fig. 1. The chemical structures of analyzed mushroom toxins.