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

Pavlína Ginterová [a,*], Barbora Sokolová [b], Peter Ondra [c], Joanna Znaleziona [a], Jan Petr [a], Juraj Ševčík [d], Vítězslav Maier [a]

[a] Regional Centre of Advanced Technologies and Materials, Department of Analytical Chemistry, Faculty of Science, Palacký University in Olomouc, 17. Listopadu 12, Olomouc CZ-771 46, Czech Republic
[b] PR LAB a.s., Agel Laboratory, Revoluční 2214/35, Nový Jičín CZ-741 01, Czech Republic
[c] Department of Forensic Medicine and Medical Law, Faculty Hospital, Hněvotínská 3, Olomouc CZ-771 46, Czech Republic
[d] Department of Analytical Chemistry, Faculty of Science, Palacký University in Olomouc, 17. Listopadu 12, Olomouc CZ-771 46, Czech Republic

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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

* Corresponding author. Tel.: +420 585 634 413; fax: +420 585 634 433.
E-mail address: pavlina.ginterova@upol.cz (P. Ginterová).

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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

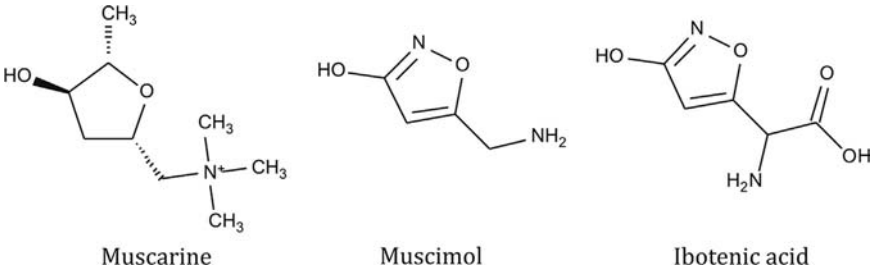

Fig. 1. The chemical structures of analyzed mushroom toxins.

8.43 [25]. The dissociation constant values of ibotenic acid are 3.0, 5.0 and 8.2 [26]. In the strong acidic pH ibotenic acid is partially positively charged and muscimol is fully positively charged therefore electrospray ionization in positive mode could be used for their identification and determination. Muscarine is a quaternary ammonium compound, therefore is permanently positively charged in whole pH range. Thus the CE–ESI-MS/MS analysis was based on the analytes basic properties. For this reason, the acidic background electrolyte (BGE) and electrospray ionization in the positive ion mode were used. The method development included optimization of CE and MS part with respect to obtain best resolution, selectivity and sensitivity for studied analytes.

3.1. Optimization of CE part

Firstly, the optimization of the CE part was provided. It was mainly focused on the type of BGE, its concentration and pH value.

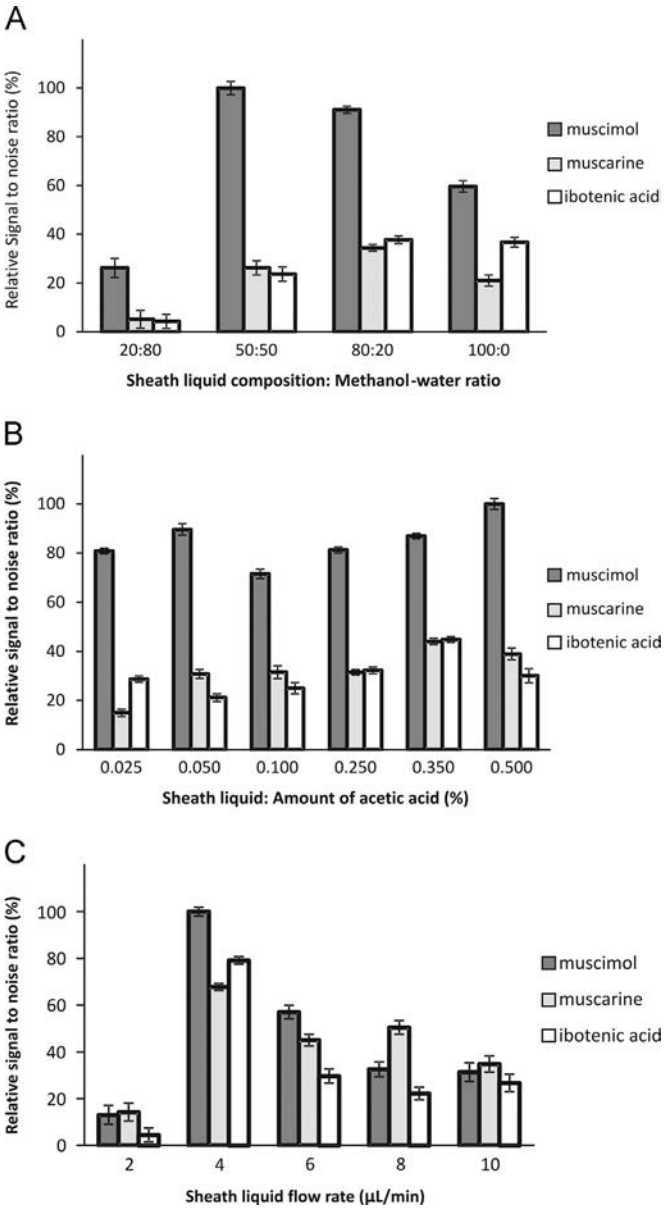

Fig. 2. The influence of sheath liquid composition (graphs A and B) and flow rate (graph C) on the relative signal to noise ratio.

For the necessity to use volatile BGE due to the coupling of CE with MS, formic acid and acetic acid were taken under consideration.

Formic acid concentration was studied in the range from 10 mM to 125 mM, which corresponding with the pH values from 2.3 to 2.9. The obtained results were not satisfied.

Subsequently the BGE containing 100 mM acetic acid was chosen with regarding to the analysis time, especially to the migration time of ibotenic acid. In the case of formic acid as BGE constituent, the migration time of ibotenic acid was around 45 min, whereas the chosen acetic acid as BGE enabled the separation of all analytes within 24 min. Also the resolution of muscarine and muscimol was higher in acetic acid BGE. The noticeable difference in the migration time of ibotenic acid was caused mainly because of dissociation of ibotenic acid (different ratio of positive and negative charge). Additionally the differences of the migration times in both BGEs could be connected with the dissimilarity of ionic strength and also a change in the electroosmotic flow velocity.

In the next step the concentration of acetic acid (in the range from 10 to 500 mM of acetic acid; pH 2.5–3.4) and separation voltage (from 15 to 30 kV) were evaluated based on the peak symmetry, number of theoretical plates and resolution of studied analytes. Firstly the separation voltage was set on 20 kV and the concentration of acetic acid was optimized, thereafter the concentration of acetic acid was constant (200 mM) and the suitable separation voltage was found. The best CE separation was obtained with 200 mM acetic acid as BGE constituent (pH=2.7) and the separation voltage +27 kV.

3.2. Optimization of MS part

To achieve the highest ionization efficiency, which ultimately leads to the lower detection limits of studied analytes, the optimization of electrospray ionization (positive ion mode) in single ion monitoring mode: $[muscarine]^+$ $m/z=174\pm0.5$, $[muscimol+H]^+$ $m/z=115\pm0.5$ and $[ibotenic\ acid+H]^+$ $m/z=159\pm0.5$ was performed.

Primarily the optimization step included the study of the influence of sheath liquid composition and its flow rate to achieve the sufficient analytes ionization. Isopropanol and methanol were tested as a sheath liquid (as a main solvent) at the different ratio of water and acetic acid or formic acid. However isopropanol did not support the ionization of studied analytes. Signal to noise ratio,

Table 1
The values of monitored ions and collision energy used for the MS part and calculated data obtained from CE–ESI-MS/MS analysis of ibotenic acid, muscimol and muscarine.

	Ibotenic acid	Muscimol	Muscarine
MS part			
Precursor ion	159	115	174
Product ion	113	98	57
Collision energy (eV)	10	10	20
Fragmentor voltage (V)	80	80	100
Calculated parameters			
Limit of detection (ng/mL)[a]	0.15	0.05	0.73
Regression equation	$y=1487.4$ $x-16.643$	$y=4365.6$ $x-25.279$	$y=12565$ $x-43.661$
r^2	0.9993	0.9992	0.9997
RSD_{tm} (%)[b]	1.60	0.93	0.96
RSD_{area} (%)[b]	3.42	2.96	3.16

[a] The limit of detection values were calculated as $S/N=3$.
[b] The values are related to the concentration 50 ng/mL and were calculated from five replicate measurements.

peak areas, peak shapes (symmetry) and number of theoretical plates were compared for the evaluation of ionization efficiency. Surprisingly muscarine provided lower ionization efficiency than muscimol and ibotenic acid. For this reason the higher concentration of muscarine was used for the optimization of MS part.

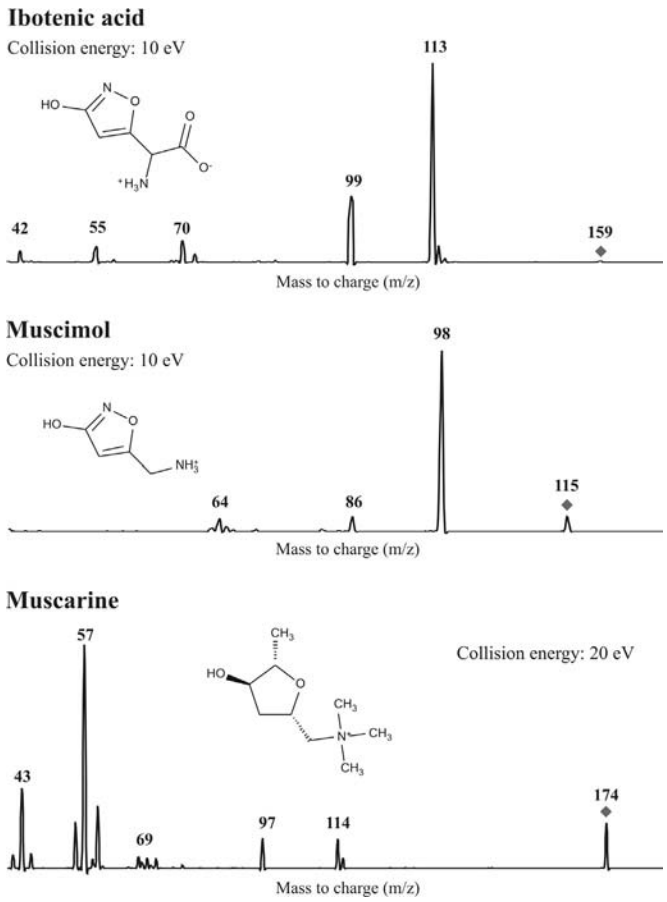

Fig. 3. Fragmentation spectra of studied analytes (product ion scan mode). For more details about fragmentation step see Table 1.

Firstly, the different ratio of methanol and water were studied. The best results were obtained with the sheath liquid consists of methanol and water in a ratio 80:20. In the next step, the effect of acetic acid addition was considered. The addition of acetic acid resulted in the higher ionization efficiency for studied analytes. The amount of 0.35% of acetic acid was used for the following analyses. The proper composition of sheath liquid was as follows: 20:79.65:0.35 (v/v/v) of water, methanol and acetic acid. In the case of sheath liquid flow rate the values from 2 μL/min to 10 μL/min were tested. The highest ionization efficiencies were obtained with the flow rate at 4 μL/min. In Fig. 2 the obtained results corresponding to the influence of sheath liquid on relative signal to noise ratio are demonstrated.

Subsequently, the sprayer voltage was changed from +2.5 kV to +4.5 kV, the value +4.5 kV provided the best signal intensity for all studied compounds. Other studied parameter was drying gas temperature, evaluated in the range 100–300 °C (in 50 °C increments). Best results were obtained with drying gas temperature 250 °C.

Quantification of studied mushroom toxins in real biological samples such as urine and serum is difficult task due to the trace levels of toxins concentration in case of intoxication and it is necessary to achieve the high sensitivity and specificity. The sensitivities (LOD values) obtained in SIM mode for studied toxins were not sufficient (LODs varied at μmol/L levels). From this point of view MRM mode was chosen for sensitive and more specific quantification of studied analytes.

Firstly, the product ion scan mode of selected precursor ions was performed. The *m*/*z* values of precursor ions for each analyte are presented in Table 1. The fragmentation parameters, namely collision energy (10–50 eV) and fragmentor voltage (60–180 V) for each analyte were studied. The chosen fragmentation conditions are described in Table 1. The obtained fragmentation spectra of all separated analytes are shown in Fig. 3.

Afterwards the MRM mode based on the selection of product ions in third quadrupole (after fragmentation of the selected

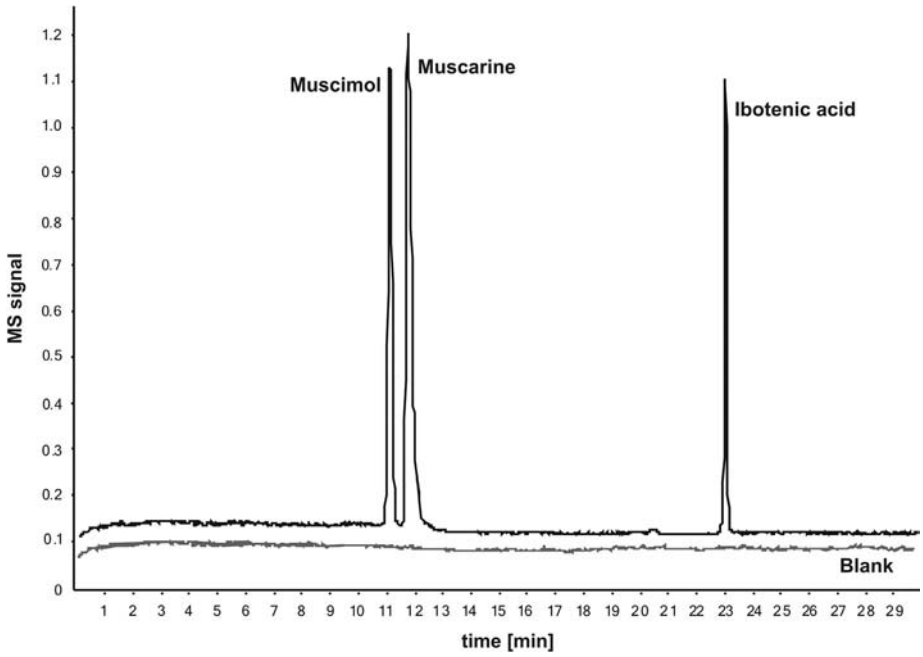

Fig. 4. Total ion electropherogram (MRM mode) of studied analytes (analyte concentration: 50 ng/mL for ibotenic acid and muscimol, 500 ng/mL for muscarine; blank: deionized water). Separation conditions: CE part: 200 mM acetic acid (pH=2.7), separation voltage +27 kV. MS part: sheath liquid composition 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. For the fragmentation parameters see Table 1.

precursor ions) was carried out. The product ions of ibotenic acid, muscimol and muscarine had the m/z values 113 ± 0.5, 98 ± 0.5 and 57 ± 0.5, respectively. For the identification, two MRM transitions were monitored for each analyte and were as follows: 159→113 and 159→99 for ibotenic acid, 115→98 and 115→86 for muscimol, 174→57 and 174→43 for muscarine.

The CE–ESI-MS/MS separation (total ion electropherogram of MRM mode) of standards of selected mushroom toxins at final conditions is presented in Fig. 4.

The other parameters (e.g. method linearity, limit of detection and relative standard deviation of migration times and peak areas) of the developed CE–ESI-MS/MS method were calculated.

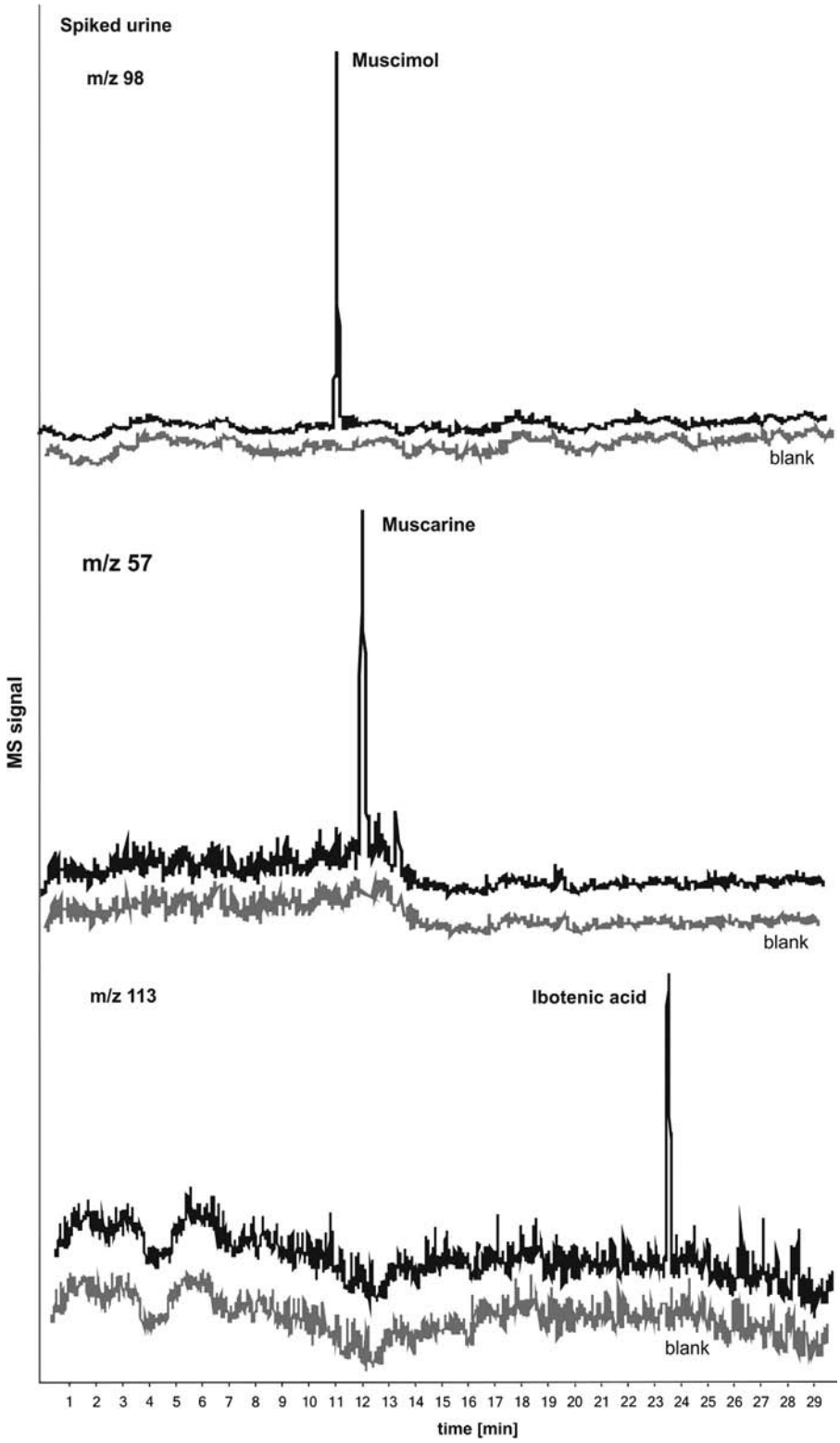

Fig. 5. Extracted ion electropherogram (MRM mode) of spiked real sample (human urine). Analyte concentration: 2 ng/mL for ibotenic acid and muscimol, 5 ng/mL for muscarine; blank: healthy volunteer urine. Separation conditions: CE part: 200 mM acetic acid (pH=2.7), separation voltage +27 kV. MS part: 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. For the fragmentation parameters see Table 1.

3.3. Partial method validation

After optimization of CE–ESI-MS/MS method, the values of limit of detection (LOD) were calculated for each analyte. For the calculation of LODs the signal to noise ratio ($S/N=3$) method was used. The obtained LODs of ibotenic acid, muscimol and muscarine were at the nanomolar concentration level (see Table 1). The evaluated LODs are sufficient for monitoring of studied toxins in human urine after the mushroom intoxication.

In the next step some additional parameters, namely linearity and relative standard deviation (RSD) of migration time and peak area, were evaluated. Good linearity (in the studied concentration range) of the obtained calibration curves is indicated by the correlation coefficient values (r^2), which were higher than 0.999. The calibration curves (5 points) were constructed in the concentration range from 0.01 to 1.00 μg/mL for ibotenic acid and muscimol and from 0.10 μg/mL to 10.00 μg/mL for muscarine. The calculated values of LODs together with the calculated analytical parameters are presented in Table 1.

3.4. Analysis of real sample

Finally, the real sample, namely spiked urine sample, was analyzed by using developed CE–ESI-MS/MS method. For the sample preparation, the minimum of pretreatment steps were used. The urine sample was spiked with ibotenic acid, muscimol and muscarine in the concentration 10 ng/mL, 10 ng/mL and 25 ng/mL, respectively. Subsequently the spiked urine sample was five times diluted with LC–MS water and filtered through a 0.22 μm membrane micro-filter (Millipore). The obtained recovery values were from 92.6% to 95.4%. The extracted ion electropherogram (EIE) of MRM mode showing the separation of mushroom toxins in spiked urine is illustrated on the Fig. 5. The presented electropherogram demonstrates also that there is any interfering peak at migration times corresponding to studied analytes.

For the CE–ESI-MS/MS analyses five urine samples of healthy volunteers (included urine pool sample) were used. All analyzed urine samples provided results without interfering peaks under final conditions.

The matrix effect was determined by the comparison of calibration curve slopes of the matrix standards (urine of healthy volunteer spiked with studied toxins) and the aqueous standards (water spiked with studied toxins). The obtained calibration curve slopes were not statistically different ($\alpha=0.05$), thus the matrix effect was not significant.

The repeatability of method is expressed via RSD values. In the case of spiked urine samples the RSD values of peak areas were from 2.98% to 3.57%.

4. Concluding remarks

The developed CE–ESI-MS/MS enables the fast separation and high efficiency in the CE step as well as the identification and low detection limits owing to the triple quadrupole analyzer. All of three mushroom toxins were separated within 24 min at nanomolar concentration levels. In the comparison with previous reported methods, used for the determination of selected toxins, presented method provides comparable or lower total analysis time and limits of detection and thus could be used as alternative method. Moreover, comparing to LC the separation of such polar compounds in the reversed mode of LC results in weak retention and thus poor resolution of compounds what required more laborious optimization step than CE.

The presented CE–ESI-MS/MS was successfully applied on the human urine spiked with studied mushroom toxins. The sample pretreatment step avoided the complicated and time consuming sample preparation. The proposed CE–ESI-MS/MS method can be used for simultaneous and routine analysis of ibotenic acid, muscimol and muscarine in human urine after intoxication by *A. muscaria*, *A. pantherina* and *A. gemmata*. For the reason, that mentioned toxins are rapidly excreted in urine in unmetabolized form, the obtained LODs are sufficient for their identification and quantification.

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