Journal of Chromatography B, 879 (2011) 2549–2553

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of muscarine in human urine by electrospray liquid chromatographic–mass spectrometric

Barbora Merová[a,*], Peter Ondra[a], Marie Staňková[a,b], Miroslav Soural[c], Jan Stříbrný[d,e], Lenka Hebká[f], Karel Lemr[f]

[a] Institute of Forensic Medicine and Medical Law, Medical Faculty, Palacky University, Hněvotínská 3, 775 09 Olomouc, Czech Republic
[b] Institute of Forensic Medicine, University Hospital Ostrava, 17. listopadu 1790, 708 52 Ostrava-Poruba, Czech Republic
[c] Department of Organic Chemistry, Faculty of Science, Palacky University, 17. listopadu 12, 771 46 Olomouc, Czech Republic
[d] Military Institute of Forensic Medicine, Central Military Hospital Praque, U vojenské nemocnice 1200, 169 02 Praha 6, Czech Republic
[e] Faculty of Military Health Sciences, Department of Toxicology, Třebešská 1575, 500 01 Hradec Králové, Czech Republic
[f] Regional Centre of Advanced Technologies and Materials, Department of Analytical Chemistry, Faculty of Science, Palacky University, 17. listopadu 12, 771 46 Olomouc, Czech Republic

ARTICLE INFO

Article history:
Received 9 November 2010
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
Muscarine
Determination
Human urine
Liquid chromatography
Mass spectrometry

ABSTRACT

A liquid chromatography–mass spectrometry based method for determination of muscarine in human urine was developed and validated. The method involved a solid phase extraction of muscarine from urine using Strata X-CW column. Separation of muscarine was achieved within 16.0 min on a reversed phase Gemini C18 analytical column (150 mm × 2.0 mm i.d., 5 μm) with a mobile phase consisted of aqueous 8 mmol/L heptafluorobutyric acid and acetonitrile in a gradient mode. Mass spectrometric detection was performed at *m*/*z* 174 and *m*/*z* 216 for muscarine and acetylmuscarine (internal standard), respectively. The linearity was satisfactory with a coefficient of determination (R^2) 0.9993 at concentration range from 0.3 ng/mL to 2.0 μg/mL, LOD and LOQ for muscarine was 0.09 ng/mL and 0.3 ng/mL, respectively. The found out recoveries of muscarine were 96% or 95% for concentration 0.3 ng/mL and 0.2 μg/mL or 2.0 μg/mL, respectively. The precision in the intra-assay-study varied from 0.48% to 1.39% and in the inter-assay-study from 2.39% to 5.49%. The accuracy ranged from −3.3% to −6%. The validation results demonstrated that the method fulfilled satisfactory requirements for precision and accuracy across the calibration curve. The applicability of the method has been demonstrated by analyzing clinical urine samples. The method offers the fast objective identification of intoxication by muscarine and can become a routine screening alternative to more difficult microscopic examination of spores in the gastric content in clinical practice.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Muscarine is the principal toxin in the fungi of genus *Inocybe*, *Clitocybe* and together with isoxazole derivatives is presented in genus *Amanita* (e.g. *Amanita pantherina* and *Amanita muscaria*). It was first isolated from *A. muscaria* in 1868 (structure see Fig. 1). Muscarine causes profound activation of the peripheral parasympathetic nervous system – it mimics the action of the neurotransmitter acetylcholine at muscarinic acetylcholine receptors [1,2]. Its main poisoning symptoms – perspiration, salivation and lacrimation (so-called PSL syndrome) occurred within 15–30 min after ingestion. With large doses, these symptoms may be followed by abdominal pain, nausea, diarrhea and blurred vision. The specific antidote is atropine [3–5]. Although intoxications by mentioned mushrooms are rarely lethal, it is important to determine them soon and to initial a medical treatment [6–8].

A few articles devoted to muscarine isolation from mushrooms have been published. Eugster et al. [9–11] described isolation of muscarine and its isomers from *A. muscaria* and *Inocybe* species. Stijve [12] described determination of muscarine from *Inocybe* and *Clitocybe* species. Methanolic extract of these mushrooms was analyzed by high performance thin-layer chromatography and modified Dragendorff reagent was used for detection. Muscarine was turned up as an orange spot at Rf 0.55. Unger et al. [13] described the identification of muscarine in *Inocybe napipes* by TLC/MS and LC/SIMS. Iodine vapor was used to detect muscarine. LC/SIMS offered more straightforward muscarine identification (ion at *m*/*z* 174) in comparison to LC/MS equipped with a moving belt interface and chemical ionization. Brown et al. [14] published determination of muscarine in 34 species of *Inocybe* by paper chromatography. Thies and Reuther's reagents were used for detection of muscarine. Chung et al. [15] developed hydrophilic interac-

* Corresponding author. Tel.: +420 585632608; fax: +420 585639572.
E-mail addresses: barbora.merova@upol.cz, baramer@seznam.cz (B. Merová).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.07.009

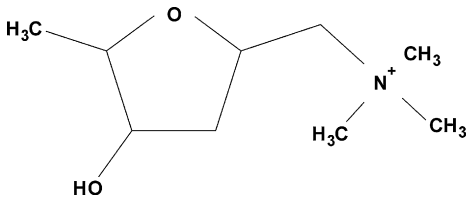

Fig. 1. Structure of muscarine.

tion liquid chromatography (HILIC) separation in combination with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) using a TSK-Gel Amide 80 column and ion-trap. They analyzed muscarine with both amatoxins and phallotoxins in food samples.

In a previous study [16] we tested two procedures for isolation of ibotenic acid, muscimol and muscarine from human urine – liquid–liquid extraction and solid-phase extraction. In this study we reassumed by development of the LC/MS method for rapid and specific quantitation of muscarine. This is the first known method for isolation and determination of muscarine from human urine. Analysis of clinical urine samples collected after suspicion to intoxication of patients by muscarine is also reported.

2. Experimental

2.1. Chemicals and materials

Muscarine hydrochloride, methanol gradient HPLC grade, acetonitrile gradient HPLC grade and chemicals used for the synthesis of acetylmuscarine were purchased from Aldrich (St. Louis, USA). Acetylmuscarine used as an internal standard (IS, hereinafter) was prepared by acetylation of muscarine. HPLC grade water was obtained using a water purification system Aqual (Brno, Czech Republic). Heptafluorobutyric acid (HFBA), formic acid and ammonium acetate were purchased from Fluka (St. Louis, USA). SPE cartridges Strata X-CW and analytical columns, Gemini C18 (50 mm × 2.0 mm i.d., 5 μm), Gemini C18 (150 mm × 2.0 mm i.d., 5 μm) and Synergi Fusion RP 80 (150 mm × 2.0 mm i.d., 4 μm), were purchased from Phenomenex (Torrance, USA).

Urine samples from healthy volunteers were used as blank samples and spiked with different amount of muscarine and IS. Urine from volunteers and urine samples collected during the treatment of real intoxication by muscarine were obtained from the Institute of Forensic Medicine and Medical Law, Medical Faculty, Palacky University, Olomouc.

2.2. Synthesis of IS

Muscarine hydrochloride (10 mg, 0.047 mmol) was dissolved in a mixture of acetic acid (800 μL), acetic anhydride (100 μL) and pyridine (100 μL) and the solution was stirred at room temperature for 20 h. Then the solvents were evaporated with use of rotavapor, the resulting residue was subsequently evaporated three times with toluene and dried *in vacuo* overnight. The crude acetylmuscarine of an excellent purity was obtained as a colorless oil in a quantitative yield, MS $[M+H]^+$ at *m/z* 216.2, ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.20 (d, J = 6.59 Hz, 3H), 1.87–2.15 (m, 2H), 2.04 (s, 3H), 3.12 (s, 9H), 3.37–3.50 (m, 1H), 3.55–3.68 (m, 1H), 3.93–4.07 (m, 1H), 4.41–4.57 (m, 1H), 4.75–4.88 (m, 1H). NMR ^{1}H spectrum of acetylmuscarine was obtained on a Varian Unity*Plus* (299.89 MHz, ^{1}H) instrument. NMR spectrum was recorded at ambient temperature (21 °C) in DMSO-d_6 solution and referenced to the resonance signal of DMSO. Chemical shifts δ are reported in ppm and coupling constants J in Hz.

2.3. Instrumentation and experimental conditions

Analysis was carried out using an LC/MS 2010A quadrupole system (Shimadzu, Kyoto, Japan) equipped with electrospray (ESI) and atmospheric pressure chemical ionization (APCI). The parameters of ESI were: spray voltage 2 kV, heated capillary temperature 250 °C, flow rate of nebulizing gas (nitrogen) 1.5 L/min. The chromatographic separation was performed on a Gemini C18 analytical column (150 mm × 2.0 mm i.d., 5 μm) equipped with a guard column using a gradient mode. Mobile phase A: 8 mmol/L heptafluorobutyric acid in water and mobile phase B: acetonitrile were mixed as follows 0–15 min: 0–37% of B, 15–16 min: 37–0% of B. Subsequently, the column was re-equilibrated with mobile phase A for 15 min. The flow rate was 0.2 mL/min. The chromatographic system operated at 30 °C.

2.4. Sample preparation

A Strata X-CW column (60 mg, 3 mL) was prewashed with 2 mL of methanol and 2 mL of 0.1 mol/L hydrochloric acid in water. A 1.0 mL aliquot of urine was loaded, the column was rinsed with 2 mL of acetate buffer (pH 4.5). Muscarine was eluted with 2 mL of 5% formic acid in methanol. The eluate was evaporated to dryness using nitrogen at room temperature. The residue was dissolved in 0.1 mL of water and 5 μL were injected onto an LC/MS system.

2.5. Assay validation for urine analysis

The LC/ESI-MS assay was validated for the quantitation of muscarine in urine samples according to current ICH Harmonised Tripartite Guideline [17].

2.5.1. Standard solutions

A stock standard solution was prepared by dissolving accurately weighed quantity of muscarine hydrochloride in 1 mL of water (1.0 mg/mL). The working standard solutions were prepared by diluting stock standard solution with water. 10 μL of IS (2.0 μg/mL of acetylmuscarine) were added to 1 mL of a solution of muscarine or a sample. Stock and working standard solution were stored at −20 °C and protected from light. The quality control samples (QCs, at three concentration levels 0.0003, 0.2 and 2.0 μg/mL) were prepared by spiking the drug-free human urine samples with appropriate amounts of muscarine and IS. Before the spiking, all drug-free urine samples were tested to ensure there was no endogenous interference at the retention time of muscarine and IS. A six-point calibration standard curve ranging from 0.3 ng/mL to 2.0 μg/mL was constructed using spiked 1 mL of blank urine extracts with appropriate amounts of muscarine and IS.

2.5.2. Selectivity and stability

Thirty blank urine samples from healthy volunteers were analyzed to detect possible peaks interfering with muscarine and IS. Also the muscarine intoxicated patients urine samples were tested for the presence of acetylmuscarine. The stability of muscarine has been investigated under storage conditions (−20 °C) for six months. A sample of acetylmuscarine was treated with 20 mmol/L aqueous solution of heptafluorobutyric acid and stirred at room temperature. No muscarine was detected in the sample after 2 h which indicates the sufficient stability of acetylmuscarine towards acidic hydrolysis.

2.5.3. Linearity of calibration

Linearity was determined using blank urine extracts spiked with 0.0003, 0.001, 0.01, 0.1, 1.0 and 2.0 μg/mL of muscarine. Linear-

ity was calculated using mean peak area ratios of analyte and IS. Linearity has been measured during three following days.

2.5.4. Accuracy and precision

The human urine standards with low (0.0003 μg/mL), medium (0.2 μg/mL) and high (2.0 μg/mL) concentration of muscarine were analyzed in three different days. The determined concentrations were compared with theoretical values. The relative error was employed to estimate accuracy. The relative standard deviation was applied as a criterion of precision.

2.5.5. Limits

The limit of detection (LOD) and the limit of quantification (LOQ) were determined as a signal-to-noise ratio 3:1 and 10:1, respectively. LOD and LOQ have been measured in five repeating.

2.5.6. Recoveries

Recovery of the solid-phase extraction was calculated at three QC concentrations ($n = 5$) by comparing mean absolute peak areas of extracted samples with the mean absolute peak areas of spike-after-extraction that represented 100% recovery.

3. Results and discussion

3.1. Isolation and separation of muscarine

Two sample preconcentration techniques, liquid–liquid extraction and solid-phase extraction, were tested for the isolation of muscarine from urine. Due to a high polarity of muscarine only solid-phase extraction rendered satisfactory results. A Strata X-CW column gave the best results from variety of tested SPE cartridges [16].

Ion sources APCI and ESI in a positive-ion mode were examined. A muscarine standard solution was used for the tuning of ionization. Since muscarine and its acetyl derivative are very polar compounds with a permanent charge, ESI gave expectedly higher response in comparison to APCI. Intensive ions at m/z 174 and m/z 216 were observed for muscarine and for acetylmuscarine, respectively.

Three chromatographic columns were evaluated for muscarine analyses. A Gemini C18 (50 mm × 2.0 mm i.d., 5 μm) column may offer short time of analysis but no retention of muscarine was observed using water: acetonitrile mixtures with ammonium acetate. Neither a longer Gemini C18 column (150 mm × 2.0 mm i.d., 5 μm) nor a Synergi Fusion RP 80 (150 mm × 2.0 mm i.d., 4 μm) column gave satisfactory results with different mobile phases including mixtures containing trifluoroacetic acid as ion pairing agent. Separation of muscarine from compounds originating from a human matrix was insufficient. Finally, mobile phases with heptafluorobutyric acid as ion pairing reagent were tested using a

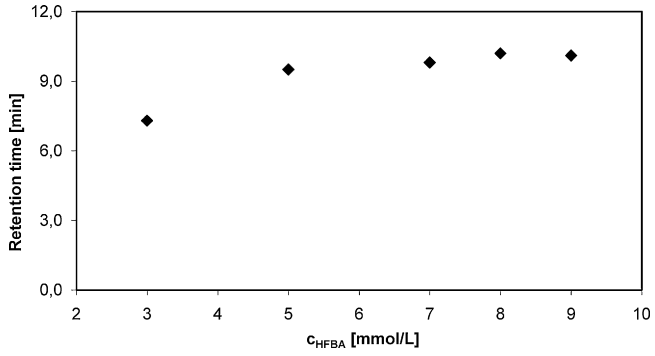

Fig. 2. Concentration effect of heptafluorobutyric acid in mixture with acetonitrile on the retention of muscarine ($c = 1$ μg/mL).

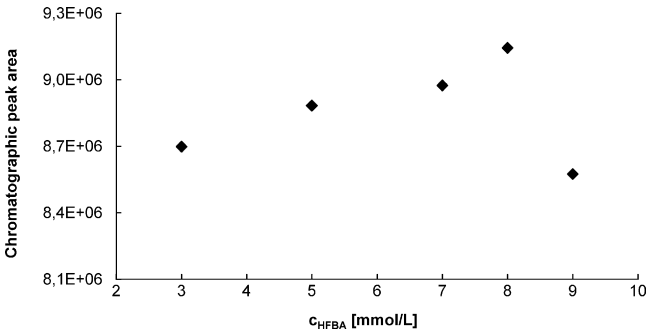

Fig. 3. Concentration effect of heptafluorobutyric acid in mixture with acetonitrile on the response of muscarine ($c = 1$ μg/mL).

Gemini C18 (150 mm × 2.0 mm i.d., 5 μm) column. The retention of muscarine was increased and analyte was successfully separated from matrix compounds. Nevertheless, heptafluorobutyric acid displayed too weak elution strength for its acetyl analog. Finally, gradient elution was applied using acetonitrile as a stronger solvent. Generally, an ion pairing reagent can suppress response of analytes in ESI forming an uncharged ion-pair. Stable retention of muscarine can be reached from 7 mmol/L heptafluorobutyric acid (Fig. 2) and the response of analyte was not importantly different in a tested concentration range of the ion-pairing reagent (see Fig. 3). Concentration 8 mmol/L heptafluorobutyric acid guaranteed robust chromatographic conditions and was selected for further analysis. Comparison of muscarine response for mobile phase without and with the ion-pairing reagent showed the loss of intensity 30% using a quadrupole instrument. Peak area of muscarine extracted from spiked urine was 10% lower in comparison to peak area of muscarine standard solution. It demonstrates negli-

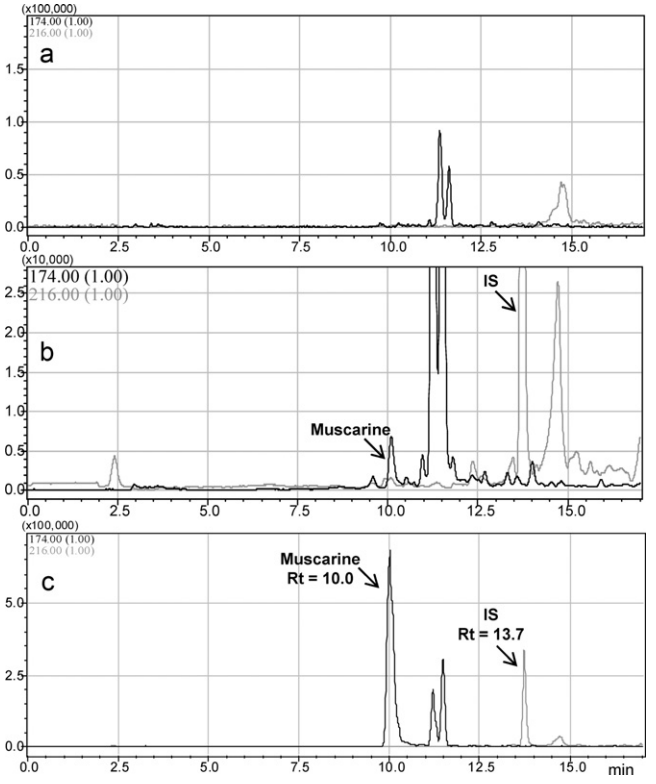

Fig. 4. LC/MS analysis of (a) negative urine, (b) urine sample spiked with standard mixture of muscarine ($c = 0.3$ ng/mL) and acetylmuscarine ($c = 0.02$ μg/mL) and (c) urine sample spiked with standard mixture of muscarine ($c = 0.2$ μg/mL) and acetylmuscarine ($c = 0.02$ μg/mL).

Table 1
The intra-day and inter-day accuracy and precision for the determination of muscarine (n = 3 days, 5 replicates per day).

Spiked concentration (μg/mL)	Intra-day			Inter-day		
	Mean calculated concentration (μg/mL)	Precision (RSD, %)	Accuracy (rel. error, %)	Mean calculated concentration (μg/mL)	Precision (RSD, %)	Accuracy (rel. error, %)
0.0003	0.00029 ± 3.11E−06	0.48	−3.3	0.00029 ± 1.12E−05	4.02	−3.33
0.2	0.19 ± 0.012	0.66	−5.0	0.194 ± 0.011	5.49	−3.0
2.0	1.91 ± 0.009	1.39	−4.5	1.88 ± 0.045	2.39	−6.0

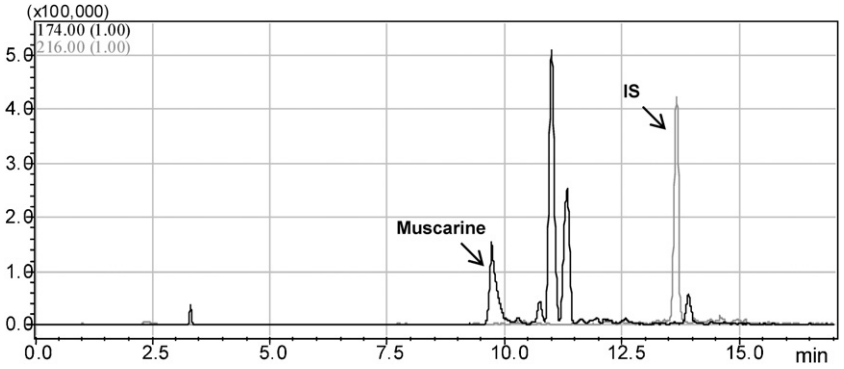

Fig. 5. LC/MS analysis of patient's urine.

gible ion suppression due to matrix components as the mentioned loss of signal occurs partly due to an isolation step. Finally, mobile phase consisted of mixture of 8 mmol/L heptafluorobutyric acid and acetonitrile was ensured mutual separation of analyte and internal standard as well as their separation from matrix components (Fig. 4).

3.2. Partial validation of the method

Thirty human blank urine samples were extracted using Strata X-CW columns and analyzed. No endogenous compounds interfered with the muscarine and the internal standard. It was also verified that muscarine solution was stable for six months when it was stored at −20 °C. The calibration curve (area = $(8.09 \times 10^6 \pm 8.9 \times 10^3)$ concentration + (6448 ± 55)) was linear within the range from 0.0003 to 2.0 μg/mL. The coefficient of determination 0.9993 indicates the sufficient linearity of the method for its application in the clinical diagnosis. The limit of detection and the limit of quantification was 0.09 ng/mL and 0.3 ng/mL, respectively, when 1.0 mL human urine sample was extracted and 5 μL injection volume was used. It offers to improve the limits extracting larger sample volume of urine.

Recovery of muscarine by the described method was well reproducible and ranged from 96% to 95% over the broad interval of analyte concentration. Recovery of internal standard was 61%.

The intra- and inter-day precision and accuracy obtained using the quality control samples were fully satisfactory for the method application in routine clinical laboratories (Table 1).

3.3. Analysis of a real sample

The method was used to analyze samples obtained from four patients intoxicated by muscarine. Here, the case of 55-year old man who consumed an unknown amount of *A. muscaria* is presented. The urine sample was taken at autopsy. His urine analysis was compared with the analyses of the blank urine and the urine spiked with muscarine (0.2 μg/mL). The patient's urine contained 0.045 μg/mL muscarine (Fig. 5). Urine of the other patients contained the traces amounts (under the LOQ) of muscarine due to late urine taking.

Positive detection and quantitation of muscarine proved intoxication by muscarine containing mushrooms and demonstrated applicability of the method in clinical practise.

4. Conclusion

There were 450 suspicions of mushrooms poisoning in the region of North and Central Moravia in the last five years. 98 cases out of that were positive on *A. pantherina* or *A. muscaria* intoxication. The problem of today's forensic toxicology is the absence of an objective analytical method namely for identification or determination of the *A. pantherina* and *A. muscaria* toxins from biological materials. Therefore we developed the first validated LC/MS method for specific identification and quick screening determination of muscarine in human urine. The method is simple, fast and is applicable for the routine determination of muscarine in human urine in a concentration within the range from 0.3 ng/mL to 2 μg/mL. The sensitivity of the method is sufficient for a diagnostic examination of intoxication by the muscarine containing mushrooms. Its results can contribute to appropriate treatment of patients or conduce in crime investigation. The method represents the first objective analytical tool for the isolation, identification and determination of muscarine in urine for emergency investigation and it has potential to replace more difficult microscopic examination of spores in the gastric content.

Acknowledgements

This work was supported by the Ministry of Health of the Czech Republic (IGA NS10269-3), the Ministry of Education, Youth and Sports of the Czech Republic (MSM6198959216) and by Operational Program Research and Development for Innovations – European Social Fund (project CZ.1.05/2.1.00/03.0058 of the Ministry of Education, Youth and Sports of the Czech Republic).

References

[1] P.J. Fraser, Br. J. Pharmacol. 12 (1957) 47.
[2] A.M. Watanabe, B.B. Katzung, in: B.G. Katzung (Ed.), Základní a klinická farmakologie, H & H, Brno, 1992, p. 83.

[3] S. Schonwald, in: R.C. Dart (Ed.), Medical Toxicology, Lippincott Williams and Wilkins, Philadelphia, 2004, p. 1719.
[4] L. Jahodář, in: L. Houdek (Ed.), Přírodní toxiny a jedy, Galén, Praha, 2004, p. 109.
[5] D. Michelot, L.M. Melendez-Howell, Mycol. Res. 107 (2003) 131.
[6] M. Brvar, M. Možina, M. Bunc, Wien. Klin. Wochenschr. 118 (2006) 294.
[7] L. Satora, D. Pach, K. Ciszowski, L. Winnik, Toxicon 47 (2006) 605.
[8] L. Satora, D. Pach, B. Butryn, P. Hydzik, B. Balicka-Slusarczyk, Toxicon 45 (2005) 941.
[9] C.H. Eugster, Fortschr. Chem. Org. Naturst. 27 (1969) 261.
[10] C.H. Eugster, E. Schleusener, Helv. Chim. Acta 52 (1969) 708.
[11] C.H. Eugster, P.G. Waser, Experientia 10 (1954) 298.
[12] T. Stijve, Mitt. Geb. Lebensmittelunters. Hyg. 72 (1981) 44.
[13] S.E. Unger, A. Vincze, R.G. Cooks, Anal. Chem. 53 (1981) 976.
[14] J.K. Brown, M.H. Malone, D.E. Stuntz, V.E. Tyler Jr., J. Pharmacol. Sci. 51 (1962) 853.
[15] W.C. Chung, S.C. Tso, S.T. Sze, J. Chromatogr. Sci. 45 (2007) 104.
[16] B. Merová, P. Ondra, M. Staňková, I. Válka, Neuro Endocrinol. Lett. 29 (2008) 744.
[17] ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures Q2(R1), Step 4 version, November, 2005.