HILIC-MS RAT BRAIN ANALYSIS, A NEW APPROACH FOR THE STUDY OF ISCHEMIC ATTACK

Abstract
Clinicians often rely on selected small molecular compounds from body fluids for the detection, screening or monitoring of numerous life-threatening diseases. Among others, important monoamines – biogenic amines (BAs) – and their metabolites serve as sensitive biomarkers to study the progression or even early detection of on-going brain pathologies or tumors of neuroendocrine origins. Undertaking the task to optimize a reliable method for the simultaneous analysis of the most relevant BAs in biological matrices is of utmost importance for scientists.

Hydrophilic interaction liquid chromatography (HILIC) with mass spectrometry (MS) detection provides a specific and sensitive technique for the separation and assessment of several neurotransmitter concentrations in body fluids (blood, urine, tissues). The present study was focused on the optimization of a straightforward, sensitive and reliable method for the simultaneous analysis of the ten most important BAs and their acidic metabolites from homogenates of rat brain tissues by use of HILIC-MS. Here, we present the optimized experimental workflow in terms of sample preparation, buffer compositions, HILIC and MS settings and data analysis. The presented method is reliable, straightforward and sensitive. Our method permits the unbiased, qualitative and quantitative determination of several BAs and their metabolites simultaneously. The optimized method was applied to the analysis of rat brain tissue samples from healthy hemispheres or those with induced transient ischemic attack (TIA). The undertaken pilot study demonstrated that the proposed approach could be applied to reveal the perturbation in neurotransmitters concentration after TIA in rat brains.

Keywords
• high-performance analytical method • transient ischemic attack • sample preparation • neurotransmitters • biogenic amines

1. Introduction

Biogenic amines (BAs) are known to be associated with numerous life-threatening diseases such as neuroendocrine tumors (NET), neurodegenerative disorders like Alzheimer’s disease or depression, and brain pathologies [1,2]. Due to the wide range of physiological roles possessed by BAs and their unquestionable involvement in the central and peripheral nervous systems, the development of reliable techniques for their extraction and quantification from biological matrices is essential. Much effort has been made to investigate BAs and their important metabolite concentrations in body fluids such as serum, plasma, platelets, cerebral spinal fluid (CSF), saliva, and urine, as well as in the tissues. Nevertheless, the task is highly complicated since BAs occur in biological matrices in extremely low concentrations. Besides this, they possess different physicochemical properties, distinct pKa values and they are hydrophilic and thermo- and photosensitive [3]. The awareness of their structure and properties must be considered at every step of their investigation – from sample collection, across sample transport, storage and preparation, to their final qualitative and quantitative determination with the use of advanced bioanalytical methods.

Nowadays BAs are used as beneficial biomarkers in several pathology diagnoses or to monitor the effectiveness of applied therapies or the progression of the disease. As an example, the L-tyrosine (L-Tyr) derived neurotransmitter – dopamine (DA) is screened together with its main metabolites – 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) for the characterization of the complex neurobiology of schizophrenia. Moreover, increased urinary excretion of DA together with noradrenaline (NA), and epinephrine (A) has been used in the diagnosis of pheochromocytoma – a rare tumor of the adrenal medulla gland. Furthermore, for clinical purposes, BAs derived from exogenous amino acid – L-tryptophan (L-T) are studied. There are several tests for monoamine assays in the diagnosis of gastrointestinal NET (GI-NET) that over-secrete serotonin (5-HT). Also, the elevated concentration in the urine of the main metabolite of 5-HT – 5-hydroxyindole-3-acetic acid (5-HIAA) – could be useful in the estimation of GI-NET tumor prognosis. On the other hand, a high risk of suicide has been extensively correlated with a low concentration of 5-HIAA in CSF, therefore the monitoring of its level can be used as a new potential predictive marker for some cases of suicide. Nevertheless, none of

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of hydrophilic, polar compounds. Still, its more popular for the excellent separation liquid chromatography (HILIC) is becoming (RP-LC). Nevertheless, hydrophilic interaction use of reverse phase-liquid chromatography highlighted.

Importance of studying the concentrations hemisphere [8]. In the presented research, the increased HVA secretion in the CSF of patients metabolism of important BAs manifested by data have demonstrated that deregulated monitor BAs during TIA [7]. Moreover, clinical pathologies and the cases when stroke patients neurotransmitters are disrupted during brain studies on several biomarkers, a mode of poor outcomes (disability or death) after fluids, to be applied as predictive biomarkers (cytokines, peptides) in easily accessible body revealed the potential ability of some molecules (cytokines, peptides) in easily accessible body fluids, to be applied as predictive biomarkers of poor outcomes (disability or death) after stroke [5,6]. Despite the increasing number of ongoing studies on several biomarkers, a method for the determination of BAs and their metabolite concentrations during TIA episodes is still lacking. The strong evidence that neurotransmitters are disrupted during brain pathologies and the cases when stroke patients developed depression have led to the need to monitor BAs during TIA [7]. Moreover, clinical data have demonstrated that deregulated metabolism of important BAs manifested by increased HVA secretion in the CSF of patients with ischemic stroke of the right or left cerebral hemisphere [8]. In the presented research, the importance of studying the concentrations of BAs and their metabolites during TIA is highlighted.

The level of BAs could be determined by use of reverse phase-liquid chromatography (RP-LC). Nevertheless, hydrophilic interaction liquid chromatography (HILIC) is becoming more popular for the excellent separation of hydrophilic, polar compounds. Still, its potential has not been extensively verified for the separation of BAs [3,9,10]. HILIC allows the separation of compounds that are poorly retained on the RP-LC column. Moreover, the use of ion-pairing reagents that are often necessary for PR-LC is omitted, which simplifies the entire analytical platform.

Most commonly, LC is coupled to mass spectrometry (MS) [8], a diode array detector (DAD) [11] and fluorometric or electrochemical detectors [12,13] for BA analysis. With these methods, even nanomolar concentrations of each BA could be detected in biological samples [12,14,15]. BAs possess weak chromophores in their structure and often derivatization is necessary before analysis (for example when using DAD or fluorometric detectors), which could cause the prolongation of the entire process. On the other hand, LC-MS is a great combination of the high separation efficiency of LC and sensitive and specific detection of MS for the determination of a panel of biomarkers. Tandem mass spectrometry (MS/MS) can provide high specificity and sensitivity for the analysis of neurotransmitters. Nevertheless, some of the fragmentation methods, such as electron capture dissociation (ECD), have a limited capacity to shelter changes in the mobile phase composition, therefore this mode of MS/MS is not suitable for the study of a panel of biomarkers. Further studies revealed the potential ability of some molecules (cytokines, peptides) in easily accessible body fluids, to be applied as predictive biomarkers of poor outcomes (disability or death) after stroke [5,6].

2. Materials and Methods

2.1. HILIC-MS materials and reagents

Acetonitrile (ACN), methanol (MeOH), ammonium formate, ammonium acetate (all LC-MS grade) and perchloric acid (HClO₄), as well as analytical standards of dopamine (DA), epinephrine (A), norepinephrine (NA), serotonin (5-HT), tyrosine (L-TY), 3,4-dihydroxyphenylalanine (L-DOPA), tryptophan (L-T), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Sigma (St. Louis, MO, USA). Ultrapure water was obtained from the Milli-Q purification system (Millipore, Bedford, MA, USA). Nitrogen (NM32LANitrogen Generator, Peak Scientific Instruments, Billerica, MA, USA) was used as the drying gas.

2.1.1. Standard solutions

Stock standard mixtures of each BA (concentration 1 mg/mL) were prepared by dissolving 1 mg of each BA in 1 mL MeOH. The stock solutions were stored in dark conditions at -20 °C. Each day the working standard solutions were prepared by the dilution of the stock solutions with acidic water (pH 2.0).

2.3. LC-MS equipment

LC-MS experiments were performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, autosampler and UV wavelength detector coupled to an Agilent 6120 mass detector (MS) with quadrupole analyzer (SingleQuad, Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray (ESI) ion source operated in positive or negative ion mode. The separation of analytes was achieved on a polar XBridgeAmide™ BEH column (Waters, Millford, Massachusetts, USA) (3.0 x100 mm, 3.5 mm particle size) thermostated at 25°C. ChemStation software (Agilent Technologies, Santa Clara, CA, USA) was used to collect HPLC data.

2.4. Biological samples – origins

A total of 3 adult Wistar rats (250-350 g) of both sexes were used in the study. All animals underwent a surgical procedure of cerebral TIA induction of the left hemisphere. The right hemisphere consisted of a control group. The survival period for operated animals was 1 week. The protocol was approved by the local Ethical Committee of the Medical University of Gdansk. Animal care and treatment guidelines outlined by the European Community Council Directive 2010/63/EU for animal experiments were followed.
2.4.1. TIA induction
The induction of anesthesia was achieved with 5 vol. % sevoflurane (Sevorane, Abbott, UK) administered by a Sigma Elite Vaporizer (Penlon, UK) and maintained at a concentration of 2 vol. %, and the basic physiological parameters were monitored during the surgical procedure and maintained within the normal physiological ranges throughout the whole procedure. Transient focal cerebral ischemia was induced by occlusion of the left middle cerebral artery (MCAO) for one hour with the intraluminal filament technique, according to the modified method of Koizumi [17]. Briefly, a 4-0 monofilament nylon suture (Ethilon, UK) was inserted through the left external carotid artery to obscure the origin of the middle cerebral artery. Following surgery, the rats were allowed to recover spontaneous breathing and were kept in standard conditions, with free access to food and water. Sham-operated animals were anesthetized in an identical manner and underwent a midline cervical incision without occlusion of the middle cerebral artery. The operated animals demonstrating apparent neurological deficits in the form of contralateral paresis, assessed according to the Bederson scale [18], were included in the study. Seven days after reperfusion the animals were deeply anesthetized with sodium pentobarbital (thiopental sodium, Biochemie GmbH, Germany; 80 mg/kg of body weight, i.p.) and decapitated. The brains were removed from the skulls and the tissue coming from the region of the cerebral cortex and the striatum was dissected from the ischemic hemispheres in the operated rats and from the same regions in the control, not operated animals.

2.4.2. Brain sample preparation
1 g of brain tissue (healthy as well as diseased) was homogenized using 1 mL of 0.2 M HClO₄. Next, 4 mL of 0.2 M HClO₄ was added and the homogenization was repeated. Then both solutions were combined and centrifuged at 14,000 g/15 min/4 °C. After centrifugation, 100 µL of the resultant supernatant was mixed with 50 µL of 200 mM ammonium formate, 50 µL of water and 800 µL of ACN, transferred to LC-MS vial and HILIC-ESI-MS analysis was carried out.

3. Results

3.1. Sample preparation for the HILIC-MS analysis
The LC-MS technique is very sensitive to all impurities, salts and detergents that are present in biological matrices. Signals from the background could cause a “matrix effect” and this tends to be the main analytical problem when using the LC-MS technique. This affects the detection capability and accuracy, and suppresses the ions, which are essential for conclusive small, polar molecule detection [19]. Therefore, the purification and extraction procedure were optimized to provide high recovery of the studied compounds and allow their simultaneous analysis by HILIC-ESI-MS. In the present study, the extraction of brain BAs was accomplished using liquid-liquid extraction (LLE) [20]. There was no need to evaporate the samples prior to analysis because, as demonstrated in further experiments, the evaporation did not improve the extraction yields of the neurotransmitters from the brain samples.

3.2. HILIC-MS optimization

3.2.1. Chromatography column selection and optimization of the mobile phases’ compositions
Here, HILIC optimization was carried out using mixtures composed of commercially available standard solutions of the studied analytes. The ten BAs and their metabolites chosen for the study were too hydrophilic to be well retained in RP-LC. Moreover, the use of normal phase LC (NP-LC) for BAs was not optimal as the analytes are not completely soluble in the non-polar solvents used as a mobile phase for NPLC. Therefore, HILIC was applied for the separation and analysis of chosen compounds [3]. HILIC employs traditional polar stationary phases such as silica or modified silica and solvents similar to those in the RP mode of liquid column chromatography [21–23].

The selection of the most appropriate HILIC column was made by checking data already published in the literature about the most commonly used HILIC columns for the analysis of small molecular weight compounds. Most of the HILIC columns were those which are amide-bonded [3]. Experimental data demonstrated that this kind of column allows great separation efficiency for all the analytes. To obtain the best working conditions, repeatability of the results and to keep the retention times (RT) of compounds constant, the on-column temperature was kept constant at the level of 25°C.

Additionally, the composition of the mobile phase (MP) was carefully studied. The obtained experimental data revealed that the optimal separation of a standard BA mixture was achieved when the MP was composed of water (H₂O, mobile phase A – MPA) and acetonitrile (ACN, Mobile Phase B – MPB). The optimal percentage of each of the solvents in the mobile phases was tested. The most sufficient elution gradient was evaluated. As shown in Table 1, the best separation, together with the highest intensity of the signals, was achieved when the initial MP was composed of 70% MPA. The percentage of MPB increased to 95% in 10.10 min. The total cycle time was 20 min. Prolonging the increased content of MPA to 20.10 min, resulted in lowering the intensities of the signals.

Further optimization of the MP composition aimed to carefully balance the ionic strength of MPA and MPB with the solubility of the analytes. The presence of salt in the MP is important for ionic analyte elution and good peak shape. Here, ammonium acetate and ammonium nitrate were evaluated as mobile phases A and B.

### Table 1. Optimal HILIC elution gradient for simultaneous separation of 10 BAs and their metabolites.

| Analysis time (minutes) | MPA (water) | MPB (ACN) |
|------------------------|-------------|-----------|
| 10.00                  | 17.5%       | 82.5%     |
| 10.10                  | 5.0%        | 95.0%     |
| 20.00                  | 5.0%        | 95.0%     |

HILIC, hydrophilic interaction liquid chromatography; MPA, mobile phase A; MPB, mobile phase B; ACN, acetonitrile.
formate were studied since they are soluble in a high amount of organic solvent and could be used when coupling HILIC with the MS detector. Moreover, the pH value was kept at a constant level of 3 during the entire analysis, since it has been previously demonstrated that acidic pH prevents the degradation of BAs [1, 16]. In the presented study, to achieve an acceptable peak shape and resolution, different concentrations of ammonium formate (from 5 – 30 mM) as an additive to MPA and MPB, were tested. The use of MPs with a lesser ionic strength (5 mM ammonium formate) resulted in a significant improvement of separation of all the studied compounds. The increase of ionic strength to 10 mM ammonium formate in both MPs, resulted in shortened retention times and higher intensities of the signals of analytes. On the other hand, an undesirable peak tailing and poor resolution of some of the analytes was observed while increasing the ammonium formate concentration to 10 mM or higher (30 mM). Thus, the optimized mobile phases contained 5 mM ammonium formate.

3.2.2. MS detector settings

HILIC could be conveniently coupled to MS. Therefore, MS with ESI ionization was chosen for the presented research. The positive ionization mode was applied, since it is the most suitable for almost all the studied compounds. The negative ion mode was only considered for acidic metabolites of BAs (HVA, DOPAC and 5-HIAA). Nevertheless, after the optimization of the fragmentary voltage, even acidic compounds were detected with good sensitivity in the positive ion mode.

For the optimization of appropriate fragmentary voltage, a flow injection analysis (FIA) was applied for each analyte. FIA serves as a quick and easy way to study ion formation directly by injecting the sample into MS. The chromatographic column is circumvented in this procedure but all the chromatographic parameters are kept constant as in the whole HILIC-MS analysis. FIA enables detailed information to be obtained about the optimal fragmentor voltage for each of the analytes, which will enable its effective and most favorable fragmentation.

To conduct FIA, at first the most intense ion for each analyte from the scan ion chromatogram was chosen. Subsequently, optimization of the fragmentor voltage was carried out for each of the chosen ions (see Table 2). Fragmentor voltages from 75 up to 375 (every 50 kV) were tested for each of the analytes and examples of the spectra obtained for 5-HT and HVA are depicted in Figure 1. Our data revealed that the most intense formation of the ions for 5-HT (Fig. 1a) and for HVA (Fig. 1b) was observed when 75 kV was applied. Owing to the optimization of the composition of the HILIC MPs, as well as the MS fragmentation voltage, the simultaneous separation and detection in the positive ion mode was achieved.

Table 2. Demonstration of the most intense ions determined for each of the studied analytes in negative ion mode. Second part demonstrated the most appropriate fragmentor voltage chosen from the FIA analysis.

| Compound | Ion M+H | Fragmentor voltage (kV) |
|----------|---------|-------------------------|
| 5-HIAA   | 192     | 125                     |
| 5-HT     | 177     | 75                      |
| HVA      | 183     | 75                      |
| DA       | 154     | 175                     |
| A        | 184     | 75                      |
| L-T      | 205     | 175                     |
| NA       | 152     | 125                     |
| L-TY     | 182     | 75                      |
| L-Dopa   | 198     | 375                     |
| DOPAC    | 169     | 75                      |

FIA, flow injection analysis, M+H - quasi-molecular ion, 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HT, serotonin; HVA, homovanillic acid; DA, dopamine; A, epinephrine; L-T, L-tryptophan; NA, norepinephrine; L-TY, L-tyrosine; L-Dopa, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxynphenylacetic acid

![Fig. 1. FIA analysis revealed the great influence of fragmentor voltage on the ion formation for all of studied BAs, for example for (a) serotonin (5-HT), and (b) homovanillic acid (HVA). In both cases 75kV was the most appropriate.](image-url)
ion mode of several BAs together with their acidic metabolites was achieved (Fig. 2). The optimized method was applied to the analysis of real samples.

4. Discussion

The goal of the presented research was to optimize the HILIC-MS method for the simultaneous analysis of ten chosen small molecular compounds in biological samples obtained from rats with induced, one hemisphere TIA. Particularly, the most error-producing stages of the entire workflow were under careful optimization. The elaborated method was applied to the analysis of real samples (healthy and ill rat brain tissues). The undertaken pilot study allowed the perturbation in neurotransmitter concentration after TIA in rat brains to be revealed. The presented research aimed to confirm that the developed HILIC-MS method could be applied to the simultaneous analysis of endogenous compounds in biological samples with high reliability and accuracy. Moreover, the highlighted analytes could serve as a potential new panel of biomarkers for the diagnosis and monitoring of TIA in the future.

The optimized LLE-HILIC-MS method for the simultaneous extraction and separation of neurotransmitters was applied to the analysis of real samples. The concentrations of BAs in rat brain tissues with or without TIA were compared. The preliminary data demonstrated that some of the neurotransmitters and their metabolites (HVA, DA, A, L-T, L-TY) were differentially secreted while the animal experienced the TIA incidence (Table 3). The table below demonstrates the concentrations of chosen analytes in healthy and diseased stages of the brain of each of the studied animals.

This pilot study finds clear evidence that the secretion of neurotransmitters in the healthy male animal is higher when compared to the female population. Moreover, a visible decrease in the concentration of neurotransmitters was demonstrated when the TIA appeared in the rats’ brains.

5. Conclusions

The HILIC-ESI-MS method for the simultaneous separation and detection of several BAs and their acidic metabolites from biological matrices was proposed. The method allowed the analysis of nanomolar concentrations of BAs in rat brain

Table 3. Concentration (ng/mL) of chosen BAs and their metabolites and precursors registered for rat brain tissue samples.

| Tissue                          | HVA   | DA     | A      | L-T  | L-TY |
|---------------------------------|-------|--------|--------|------|------|
| Brain 1, female, left hemisphere| 203.863| 19.450 | 19.139 | 1.640| 400  |
| Brain 1, female, right hemisphere| 292.493| 33.646 | 26.830 | 2.419| 800  |
| Brain 2, male, left hemisphere | 300.549| 34.372 | 27.694 | 2.273| 782  |
| Brain 2, male, right hemisphere| 314.337| 38.319 | 28.920 | 1.580| 946  |
| Brain 3, female, left hemisphere| 243.935| 35.390 | 22.720 | 1.355| 788  |
| Brain 3, female, right hemisphere| 231.786| 30.083 | 21.168 | 1.119| 1030 |

HVA, homovanilic acid; DA, dopamine; A, epinephrine; L-T, L-tryptophan; L-TY, L-tyrosine.

Fig. 2. TIC obtained while the optimized HILIC-MS settings were applied for the analysis of mixture composed of 10 analytes (BAs and their metabolites) each one at concentration of 1 µg/mL. Y axis stands for ion intensity, while the X axis stands for time of the analysis (in minutes). Legend: HVA, homovanillic acid; 5-HIAA, 5-hydroxyindole-3-acetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, serotonin; DA, dopamine; A, epinephrine; L-T, L-tryptophan; NA, norepinephrine; L-TY, L-tyrosine; L-Dopa, 3,4-dihydroxyphenylalanine;
tissues. The optimized method was successfully applied to the analysis of rat brain tissue samples. The analytes were extracted from brain samples by use of a simple LLE method. Differences in the content of BAs between the sexes, as well as pathological and physiological stages of the brain were demonstrated. Further studies are being carried out to reveal if the presented method is suitable for the study of BAs from different biological samples - easily accessible body fluids, such as serum or urine - derived from animals and from patients suffering from TIA and if the proposed panel of biomarkers could be a new tool for the early detection and/or monitoring of the disease.

**Author contributions**

Designed the study: N.M., L.K.; Performed the experiments: N.M., PK.; Analyzed the data: J.M., T.B., N.M.; Wrote the paper: N.M., PK.; Wrote the manuscript: N.M.

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**References**

[1] Miękus N., Kowalski P., Oleżycka I., Plenis A., Bień E., Miękus A., et al., Cyclodextrin-modified MEKC method for quantification of selected acidic metabolites of catecholamines in the presence of various biogenic amines. Application to diagnosis of neuroblastoma, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 1003, 2015, 27–34.

[2] Salkovic-Petrisic M., Knezovic A., Hoyer S., Riederer P., What have we learned from the streptozotocin-induced animal model of sporadic Alzheimer's disease, about the therapeutic strategies in Alzheimer's research, J. Neural Transm. 120, 2013, 233–252.

[3] Chirita R.I., West C., Finaru A.L., Elfakir C., Approach to hydrophilic interaction chromatography column selection: Application to neurotransmitters analysis, J. Chromatogr. A. 1217, 2010, 3091–3104.

[4] Montaner J., Perea-Gainza M., Delgado P., Ribó M., Chacón P., Rosell M., et al., Etiologic Diagnosis of Ischemic Stroke Subtypes With Plasma Biomarkers, Stroke. 39, 2008, 2280-2287.

[5] Whiteley W., Wardlaw J., Dennis M., Lowe G., Rumley A., Sattar N., et al., The Use of Blood Biomarkers to Predict Poor Outcome After Acute Transient Ischemic Attack or Ischemic Stroke, Stroke. 43, 2011, 86-91.

[6] Laskowitz D.T., Kasner S.E., Saver J., Remmel K.S., Clinical usefulness of a biomarker-based diagnostic test for acute stroke: the Biomarker Rapid Assessment in Ischemic Injury (BRAIN) study., Stroke. 1217, 2009, 3091–3104.

[7] Feng C., Fang M., Liu X.Y., The neurobiological pathogenesis of poststroke depression, Scientific World Journal, 2014, 1-8.

[8] Ferrarese C., Bassi S., Frattola L., Locatelli P., Trabucchi M., Different patterns of CSF neurotransmitter metabolism in patients with left or right hemispheric stroke, Acta Neurol. Scand. 73, 1986, 581–585.

[9] Gianotti V., Chiuminatto U., Mazzucco E., Gosetti F., Bottaro M., Frascarolo P., et al. A new hydrophilic interaction liquid chromatography tandem mass spectrometry method for the simultaneous determination of seven biogenic amines in cheese, J. Chromatogr. A. 1185, 2008, 296–300.

[10] Danaceau J.P., Chambers E.E., Fountain K.J., Hydrophilic interaction chromatography (HILIC) for LC–MS/MS analysis of monoamine neurotransmitters, Bioanalysis. 4, 2012, 783–794.

[11] Gosetti F., Mazzucco E., Gennaro M.C., Marengo E., Simultaneous determination of sixteen underivatized biogenic amines in human urine by HPLC-MS/MS, Anal. Bioanal. Chem. 405, 2013, 907–916.

[12] Hasanazadeh M., Shadjou N., Omidinia E., A novel electroanalytical method for simultaneous detection of two neurotransmitter dopamine and serotonin in human serum, J. Neurosci. Methods. 219, 2013, 52–60.

[13] Kema I.P., Meijer W.G., Meiborg G., Ooms B., Willemsen PH., de Vries E.G., Profiling of tryptophan-related plasma indoles in patients with carcinoid tumors by automated, on-line, solid-phase extraction and HPLC with fluorescence detection., Clin. Chem. 47, 2001, 1811–1820.

[14] Vlčková M., Schwarz M.A., Determination of cationic neurotransmitters and metabolites in brain homogenates by microchip electrophoresis and carbon nanotube-modified amperometry, J. Chromatogr. A. 1142, 2007, 214–221.

[15] Román D.A., Carretero A.S., Blanco C.C., Gutiérrez A.F., Subminute and sensitive determination of the neurotransmitter serotonin in urine by capillary electrophoresis with laser-induced fluorescence detection, Biomed. Chromatogr. 18, 2004, 422–426.

[16] de Jong W.H.A., de Vries E.G.E., Kema I.P., Current status and future developments of LC-MS/MS in clinical chemistry for quantification of biogenic amines, Clin. Biochem. 44, 2011, 95–103.

[17] Fluri F., Schuhmann M.K., Kleinschmitz C., Animal models of ischemic stroke and their application in clinical research., Drug Des. Devel. Theral. 9, 2015, 3445–3454.

[18] Bederson JB., Pitts LH., Tsuji M., Nishiumi M.C., Davis RL., Bartkowski H., Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination, Stroke. 17, 1986, 472–476.

[19] Mallet C.R., Lu Z., Mazzeo J.R., A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts, Rapid Commun. Mass Spectrom. 18, 2004, 49–58.

[20] Guzman D.C., Garcia E.H., Mejia G.B., Olguin H.J., Gonzalez J.A.S., Labra Ruiz N.A., Effect of morphine and lacosamide on levels of dopamine and 5-HIAA in brain regions of rats with induced hypoglycemia, Pakistan J. Biol. Sci. 17, 2014, 292–296.

[21] Alpert A.J., Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds., J. Chromatogr. 499, 1990, 177–196.

[22] Hemström P., Irgum K., Hydrophilic interaction chromatography, J. Sep. Sci. 29, 2006, 1784–1821.

[23] Buszewski B., Noga S., Hydrophilic interaction liquid chromatography (HILIC) - a powerful separation technique, Anal. Bioanal. Chem. 402, 2012, 231–247.