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Development and Full Validation of a Novel Liquid Chromatography Electrochemical Detection Method for Simultaneous Determination of Nine Catecholamines in Rat Brain

Short Title: Analysis Nine Catecholamines in Rat Brain

Saniye Özcan[a,b], Murat Kozanlı[c], Aysun Geven[a], Nafiz Öncü Can [a,b*]
[a]*Department of Analytical Chemistry, Faculty of Pharmacy, Anadolu University, 26470, Eskisehir, Turkey*
[b]*Central Analysis Laboratory, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey*
[c]*Pharmacy Services, Health Services Vocational School, Bilecik Şeyh Edebali University, 11230, Bilecik, Turkey*

Corresponding Author Information
Nafiz Öncü Can
+90 (222) 335 0580 / 3770
nafizoc@anadolu.edu.tr
https://orcid.org/0000-0003-0280-518X
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ABSTRACT
INTRODUCTION: Chemical neurotransmission, managed by neurotransmitters, has a crucial role in brain processes such as fear, memory, learning, and pain, or neuropathologies such as schizophrenia, epilepsy, anxiety/depression, and Parkinson's disease. The measurement of these compounds is to elucidate the disease mechanisms and evaluate the outcomes of therapeutic interventions. However, this can be quite difficult due to various matrix effects and the problems of chromatographic separation of analytes. In the current study; for the first time, an optimized and fully validated fully according to FDA and EMA Bioanalytical Validation Guidance HPLC-EC method was developed for the simultaneous analysis of nine neurotransmitter compounds which are dopamine (DA), homovanilic acid (HVA), vanilmandelic acid (VA) serotonin (SER), 5-hydroxyindole-3-acetic acid (5-HIAA), 4-hydroxy-3-methoxyphenylglycol (MHPG), norepinephrine (NE), 3,4 dihydroxyphenylacetic acid (DOPAC) and 3-methoxytyramine (3-MT) and simultaneously determined in rat brain samples.
METHODS: The separation was achieved with 150 mm × 4.6 mm, 2.6 µm F5 Kinetex (Phenomenex, USA) column isocratically, and analysis was carried out HPLC equipped with DECADE II electrochemical detector.
RESULTS: The method exhibited good selectivity and correlation coefficient values for each analyte's calibration curves were >0.99. The detection and quantification limits ranged from 0.01 to 0.03 ng/mL and 3.04 to 9.13 ng/mL, respectively. The stability of the analytes and method robustness were also examined in detail in the study, and the obtained results are also presented statistically.
DISCUSSION AND CONCLUSION: The developed fully validated method has been successfully applied to real rat brain samples and important results have been obtained. In the rat brain sample analysis, the least amount of SER and the highest amount of NA were found.
Keywords: Catecholamine, Electrochemical detection, High-performance liquid chromatography, Neurotransmitter, Rat brain

INTRODUCTION

It is an essential group of biogenic amines that contain monoamines, dopamine (DA), noradrenaline (NE), epinephrine (EP), serotonin (SER), and their metabolites in the central nervous system (CNS)[1, 2]. Their main function is neutrotransmission with hormonal or neuronal signals in various physiological events. In addition, the amount of monoamine and dysfunction are closely related to various CNS diseases [3]. These

monoamines are principally obtained from two amino acids, tryptophan and tyrosine [4]. EP and DA are primary catecholamines, which include metanephrine (ME), vanilmandelic acid (VA), and homovanilic acid (HVA) are the emerging metabolites. NE metabolites are two as HVA and VA. It is also known that the last pathways are HVA when transforming into 3-methoxytyramine (3-MT) and 3,4 dihydroxyphenylacetic acid (DOPAC). SER synthesized from tryptophan amino acid turns into 5-hydroxyindole-3-acetic acid (5-HIAA).

The chemical neurotransmission was looked after neurotransmitters and appers in brain processes such as memory, pain, learning, fear or neuropathologies such as epilepsy, schizophrenia, Parkinson's disease, and anxiety/depression. So, changes in decreasing or increasing in their regions are seen as the precursors of many mood disorders, diseases, or irreversible events such as tissue deformation [5, 6]. Electrochemical reactions are often the basis of these deformations. Because these compounds have small and straightforward structure, they contain only non-rigid electroactive functional groups [6]. Also, these properties range from the dominance of electrochemical detectors in their analysis.

In clinical and experimental neuroscience, neurotransmitters are widely studied in different biological samples, such as blood, plasma, urine, dialysates, or supernatants from tissue homogenates. In general, two main methods are followed in tissue analysis of neurotransmitters. The samples are collected in vivo, and their determinations are made by a selected analytical method. Frequently preferred methods in sample preparation are microdialysis or push-pull perfusion techniques. Their determination is mostly achieved with using HPLC. However, most of these compounds do not contain any chromophore groups, so the UV-visible region or PDA cannot be used. Instead, two types of detectors are used, mostly mass spectrometry (MS) and electrochemical. Electrochemical (EC) detector equipment is cheaper and easier to use than MS. Also, it is not required sample derivatization because of the high electro-activity of catecholamines and indoleamine. Moreover, electrochemical detectors are specific and very rare to be affected by the matrix or signals interference of other analytes. Coulometry or amperometry can be used in EC detection. The main advantage of coulometry is the complete oxidation of the compounds entering the detection cell, resulting in a low LOD. Nevertheless, the technology of miniaturization of the cell is currently limited. In contrast, the amperometric cell size can be easily reduced, and when connected to the microhole and capillary columns, amperometry can detect low compound levels despite a weak electrochemical yield (-10%).

One of the works that need to be emphasized is that, in 2011, Parrot et al. developed a method for the analysis of NE, DA, and three metabolites using DOPAC, HVA, 3-MT, SER, and its metabolite 5-HIAA, using UPLC-ECD. For the method, the validation studies were performed by selecting the working range of all compounds 10^{-9}-10^{-6} M, and they applied to rat, cat, and mouse brain tissue [7]. Furthermore, in 2014, benzoyl chloride was used for derivatization to the analysis of cerebrospinal fluids from rat models such as SER, 5-HIAA, HVA, NE, EP, DA, glutamic acid, GABA, DOPAC by LC-MS/MS method [8]. Especially since 2018, LC-MS/MS method has been developed to carry out the potential therapeutic effect of various diseases and many neurotransmitters, including DA, NE, 5-hydroxy-tryptamine, acetylcholine, l-tryptophan, γ-aminobutyric acid, glutamic acid, and aspartic acid [9-13]. Simultaneous determinations in various tissues such as the mouse brain were accelerated. A simple protein precipitation method is generally used for sample pre-treatment. Brain samples were separated by the polar functional group attached column and detected in the LC-MS/MS system equipped with a positive ion in the mass detector and a turbo ion spray source in multiple reaction tracking modes.

The other efficient work is that; in 2018, seven compounds, including monoamines and their metabolites, were analyzed by HPLC-ECD [3]. The method was validated to obtain a working range at ng level and applied to three rat brain regions (posterior-anterior cortex, hippocampus, and striatum). In addition, voltammetry techniques are widely examined when looking at the studies performed until today. However, because of selectivity problems, the analysis was carried out with electrodes that have been modified with various techniques, and very successful results have been obtained [14-16]. For example, in the 2014 study, the covalently bound electrode was designed by the condensation reaction of graphene oxide and 5,15-pentafluorophenyl-10,20-paminophenylporpyrin [17]. The modified electrode was used for DA and SER analyzes at μM levels.

As seen above, much progress has been made, and studies have been published in the analysis of neurotransmitters. The mass detector is particularly promising. However, this detector has an Achilles tendon matrix interference in biological fluid and tissue analysis [18]. The sensor work is still in its infancy. Briefly, more efficient methods should be produced by developing chromatographic techniques. The purpose of current study is to perform more compound analysis of neurotransmitters in brain tissue in a shorter time. For this reason, an efficient and promising HPLC-ECD method was developed to the routine analysis of DA, HVA, VA, SER, 5-HIAA, MHPG, NE, DOPAC, and 3-MT in brain samples, all the method optimization parameters were examined in detail, and full bioanalytical validation parameters were performed according to the international guidelines [13, 19, 20]. Thanks to the current method, a total of

10 eluents, nine analytes, and internal standard (IS) were successfully separated in required chromatographic efficiency and applied to the rat brain samples.

MATERIALS AND METHODS

Chemical

The hydrochloride salts of DA, SER and 3-MT, HVA, VA, 5-HIAA, MHPG, NE, DOPAC, 3,4-Dihydroxybenzylamine hydrobromide (DHBA), triethylamine (TEA), ethylene-diamine-tetra-acetic acid (EDTA) disodium salt, 1-octanesulfonic acid (OSA), potassium dihydrogen phosphate, potassium chloride, citric acid, methanol sodium metabisulfite, and perchloric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water was produced using a Milli-Q system (Millipore, Bedford, MA, USA). The used Whatman® cellulose acetate membranes were purchased from Sigma Aldrich (St. Louis, MO, USA), and PTFE syringe filters were purchased from IsoLab (Wertheim, Germany).

Preparation of stability solution

The stability solution was used to prepare, store standard and brain solutions, and homogenize brain tissue to ensure adequate neurotransmitter substances' adequate stability. The prepared stability solution consists of 0.1 M perchloric acid and 0.1 mM sodium metabisulfite in water [21]. For this purpose, 4.8 mg of sodium metabisulfite and 2.156 µL of perchloric acid were placed in a 250 mL volumetric flask with distilled water, and then the volume was completed with water.

Preparation of standard solutions

Each standard was weighed at approximately 1 mg and placed in a volumetric flask 10 mL, and completed the volumes were with the stability solution. Afterward, they were stored at –4 °C for optimization and validation studies by making necessary dilutions. It was examined whether the solutions made any degradation under the storage conditions and how long they remained in the analysis. As a result, the compounds could be stored safely in the freezer and thawed, and they remained intact for a minimum of 60 hours during the analysis period.

Preparation of mobile phase solution

The mobile phase composition to be used in liquid chromatography was prepared in water with 0.07 M KH_2PO_4, 20 mM citric acid, 5.3 mM OSA, 100 mM EDTA, and 3.1 mM TEA, 8 mM KCl and 11% (*v/v*) methanol. The mobile phase was filtered through a 0.22 µM cellulose acetate filter before used. In these conditions, the mobile phase's measured pH value was optimized as 3.2 ± 0.1 isocratically.

Removal of brain tissues from rats

About 9-10 weeks old male Sprague Dawley rats rats were used for the experiments. Rats were fed on a standard rodent diet and tap water. The animals that were sacrificed without any experimental procedure to obtain tissue were anesthetized with pentobarbital (60 mg kg^{-1}) and decapitated. All animal procedures were performing according to protocols approved by the national ethical requirements for animal research and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Union Directive number 2010/63/EU) [22]. After each animal was sacrificed immediately, all brain tissues were removed and placed in tubes containing prepared stability solutions. The tissues were quickly frozen with liquid nitrogen and stored for electrochemical analysis in a -40 ºC freezer. This study's experimental protocols had been approved by the Local Ethical Committee on Animal Experimentation of XXX University, Eskişehir, Turkey.

Preparation of brain tissues

The brain tissues were removed from the freezer and placed in the sonicator bath, allowing them to dissolve quickly. The sonicator bath's effect on the tissues' dopaminergic substances was tested by applying various parallel sample preparation procedures, and it was found that it did not affect. The defrosted brain samples were gently removed from the stability solution, accurately weighed, and put into the homogenization vessel. The brain tissues were homogenized in an ice-cold stability solution for approximately 25 minutes by adding internal standard (IS) DHBA with a 61.6 ng/mL concentration. The amount of solvent added per mg brain tissue is 7.5 µL [23]. All homogenization processes are carried out with Potter S (B. Braun Biotech International, Berlin, Germany) model homogenizer. The homogenates were then sonicated for 10 min, centrifuged at 11000 rpm at 5 ºC for 40 minutes, and filtered through a 0.22 µm PTFE filter. This process was repeated for the second time, and the filtrate was placed in injection vials and analyzed.

Analysis parameters

The analysis system is equipped with UPLC from Shimadzu (Kyoto, Japan) and DECADE II electrochemical detector (Antec-Leyden, Netherlands) to be used for the chromatographic separation and detection. The system consists of two LC-30AD parallel piston pumps, SIL-30AC refrigerated autosampler, CBM-20A system control unit, and DGU-A5R inline degassing unit. A Decade II electrochemical detector (cell volume 80 nL, Antec, Leyden, Netherlands) equipped with a 2 mm glassy

carbon working electrode, in-situ Ag-AgCl (ISAAC) reference electrode, platinum auxiliary electrode, and a 25 µm spacer at 35 °C.

In the study, separations were made at 35 °C (oven-controlled) using 150 mm × 4.6 mm, 2.6 µm F_5 Kinetex (Phenomenex, USA). The mobile phase is pumped at a flow rate of 0.35 mL/min, and the injection volume is 1 µL. Electrochemical transformations at +800 mV oxidation potential, the signal range is 2 nA, and the filter 0.005 Hz cell temperature was set to 35 ± 0.1 °C, as the column temperature.

Evaluation of results

Peak normalization (PN) technics were used to investigated to results. PN method could utilitze for minimize errors in the analysis. PN values are obtained by dividing the area of the relevant peak by the t_R value. In response to each concentration, the values obtained by dividing the PN values of the analyte and IS, DHBA, were accepted as rational responses. Based on PN values and concentrations of solutions were applied to method validation and optimization applications.

Method validation

The validation studies were investigated according to the bioanalytical method validation guidelines of FDA and EMA [19, 20]. The method validity practices were applied to the method development, and it was checked that the method provided analytically acceptable data such as specificity, precision, accuracy, linearity, LOD, LOQ, and system suitability tests (SST) [24]. Indeed all statistical calculations were made with GraphPad Prism v6.0b (trial version) program.

The SST parameters showing the LC instrument's performance and column used during the analysis is fundamental in the method development step. Parameters such as resolution (Rs), tailing factor (TF) and asymmetry factor (As), theoretical plate number (N), capacity factor (k'), and selectivity factor (α) constitute the system suitability parameters [25]. During method development, each of these parameters was calculated using the Shimadzu LCsolution v1.11 SP1 data analysis program, according to the United States Pharmacopeia (USP) method.

Selectivity

The presence of any matrix component that could interfere with each analyte signal in the detector was investigated. Each analyte's selectivity studies were performed to three concentration levels to rat brain samples homogenates (80%, 100%, 120%).

Linearity

For the linearity studies; all analytes were prepared six concentration levels (20%, 50%, 80%, 100%, 120%, and 150%). Linearity was investigated between intra-day and inter-day slope, correlation coefficient, and intercept at 95% confidence level were calculated with linear regression analysis.

Accuracy

For accuracy studies, low, medium, and high concentrations (80%, 100%, and 120%) neurotransmitter standard substance was added to the brain tissue solution before analyzed. Three sets were prepared and analyzed for each concentration level, and recovery values were calculated.

Precision

Precision studies include intra-day and inter-day measurements. The analyte solutions used in linearity studies (80%, 100%, and 120%) were analyzed three times within the same day and for three consecutive days. In the statistical evaluation of the results, mean, standard error of the mean (SEM), standard deviation (SD), relative standard deviation (RSD) are given at 95% confidence level.

LOD and LOQ

According to ICH recommendations for LOD and LOQ values, the ratio obtained in the calculations using the signal/noise ratio is accepted as 3 and 10, respectively. On the other hand, LOD was calculated using the given in equation 1 for this study. It is recommended in the user manual of the detector [26].

$$C_{LOD} = \frac{3\sigma_{noise}}{signal} C_A \quad (1)$$

σ_{noise} is the length of the 0.2× noise peak, and the C_A is indicated as the analyte concentration injected.

Stability

The stability of standard solutions was examined for one month. For this purpose, the standard solution mixture was analyzed with the developed method for one week, three weeks, and one month in a time period.

RESULTS AND DISCUSSION

Stability of analyte solutions

The extraction of neurotransmitters from tissues and cells was done by homogenization and sonication in stability solutions. The stability solution damaged the tissues, revealed the target compounds, and provided stability of neurotransmitter compounds. In studies, the most active compound in the extraction of neurotransmitters is perchloric acid [27]. While it provides efficient extraction by adsorption of perchlorate ion in perchloric acid structure, it also contributes to stabilizing the compounds thanks to its acidic pH value. Because acidic pH is stable due to neurotransmitters' structure, and as the pH increases, they undergo oxidation effortlessly [28]. Another component of the stability solution is the preservative with

antioxidant activity. Consider the other works, ascorbic acid could be antioxidants components but not sufficient alone, and bisulfite ion or EDTA was added [29]. Sodium metabisulfite was preferred in the current analysis because its preservative feature alone is sufficient. Moreover, the stability solution had to be selected to minimize analyte loss by preventing tissue damage, and also it should not lead to any loss or increase of the detector signal. This mixture was found by using the literature as a result of various stability trials.

The effect of stability solution on brain tissue also was studied in detail. For this purpose, the fresh brain tissue taken from the rat immediately after decapitation was divided into symmetrically two parts. One of the brain tissue was immediately homogenized and analyzed. The other brain tissue, which was then subjected to the same experimental treatment, was immediately put into the stability solution and frozen for analysis later with liquid nitrogen. Then, this tissue was prepared and analyzed according to the procedure mentioned above, and it was calculated whether there was a significant difference between the analysis of fresh brain tissue. As a result of the analyzes, it was seen that there was no significant difference.

Chromatographic separation

The separations were firstly made in the reverse phase of the Ascentis (St Louis, MO, USA) brand 10 cm × 4.6 mm with C_{18} functional group produced with core-shell silica technology with a 2.7 µm particle diameter operating at high efficiency. However, due to the increasing number of analytes after a while, the column was poor separation, and method optimization was continued with Kinetex (Phenomenex, USA) brand 15 cm × 4.6mm, 2.6 µm column filled with core-shell silica particles with F_5 functional group. Both column's features are compared, particularly in Table 1. The chromatographic separation studies were obtained that column functional group change did not have much effect on the retention time of the compounds, while the separation efficiency significantly increased. In this effect, of course, the contribution of using 15 cm Kinetex® core-shell column instead of the 10 cm long column of Ascentis® cannot be ignored. Although it had no dominant effect on the peak morphology, it showed excellent performance in the separation of structures close to each other analyte. However, as the SER and 3-MT eluents, which were already quite late in the previous short column, had slightly higher retention time in the long column, an increase in tailing was observed in their peak morphology.

It is observed that the compounds with free carboxylic acid in their structure were eluted firstly and had short retention times. The retention times had started to increase as the free amine group exists. In particular, SER, which does not contain any methyl or carboxylic acid in the structure, was the last to leave the column, while 3-MT with a free methyl group remained less in the column for about 9 minutes. The mixture standard solution's chromatogram obtained is given in Figure 1. It was also checked whether the stability solution gave any peaks by injecting, as shown in Figure 2. Also, SST is applied to show the performance of the LC device and method. In these tests, parameters such as N, R_s, TF, A_s, α, and k' were calculated and given in Table 2.

Method development

This study's purpose was a development method for the separation and determination of DA, HVA, VA, SER, 5-HIAA, MHPG, NE, DOPAC, 3-MT, and DHBA for the presence of many interfering compounds in the rat brain. Nine neurotransmitter compounds were analyzed by electrochemical detection reverse-phase ion-pair chromatography, DHBA was used as an IS, and so the unpredictable factors internal or external were eliminated. In experiments with matrix effects, such as biological analyzes, the use of the internal standard is highly recommended. Today, with the development of analytical instruments, lower detection and quantitation limits can be obtained, and the effect of various attempts from interference components more critical. DHBA is the most preferred internal standard in the analysis of neurotransmitters. Structural similarity and acting like catecholamines in chromatographic separation are the main reasons for preference. While calculating analytical data, the error was minimized using the PN method, which considers both the retention time of the analyte and IS and their peak areas.

The effect of each of the mobile phase components on separation is examined in detail with various mobile phases using in the literature [7]. According to the results obtained, EDTA reduces the noise by holding free metal ions and provides a lower level of LOD and LOQ values with achieving a smoother baseline. However, no effect of increasing after a specific EDTA concentration was observed. The EDTA concentration was chosen at this breaking point. The KCl was used in the analyzes as the reference electrode. This system, called in-situ Ag-AgCl (ISAAC), was designed as electrochemical cell T, and KCl concentration was determined according to the potential to be analyzed. Such reference electrodes are very stable but have the disadvantage that the electrochemical reaction media volume is relatively small, and although the diffusion increases, the mobile phase prepared must be renewed in 48 hours at the latest [30]. KH_2PO_4 and citric acid were used to control of pH mobile phase. It was observed that the optimum pH value for buffering was around 3.2. This pH value is around the pKa value of analytes to be distinguished. Lower acidity values can provide more stable better interaction but, at the same time, reduce the life of

the stationary phase. It should be kept in mind that especially the optimum working values of silica-based columns are in the acidity range of 3-7. Also, citric acid was acted as an ion-pairing reagent with OSA, which was observed as the lead ion-pair reagent in optimization studies. It was regulated the peak morphology and increase the eluent column interactions. As OSA concentration increased, it provided more sharp peaks, and because of its micelle feature, it also increases the viscosity of the mobile phase. Another anti-tailing additive component is TEA. In the mobile phase, two different types of anti-tailing components were used because the dominant functional group of some neurotransmitters was carboxyl acid, and some were amine. The analytes with amine groups interact with TEA, and those with the carboxyl group interact with the OSA. Another critical point was the effect of the anti-tailing mobile phase additive on the mobile phase's pH. TEA is a strong-basic substance, its pK_a 10.2, and while the concentrations of TEA were increased, the mobile phase pH was tried to stable at 3.2. For the organic phase of the mobile phase, methanol and acetonitrile were examined optimum separation. Acetonitrile fastly eluted all compounds from the column, and its separation efficiency was inferior. On the other hand, methanol provided a slower elution rate, increased the stationary phase's interaction with analytes, and optimum separation efficiency was obtained.

An electrochemical detector detected signals with flow cell VT-03. The cell's characteristics, which have a three-electrode configuration, are as follows: glassy carbon working electrode (3.00 mm in diameter and 0.18 µL internal volume with 25 µm spacer seal), platinum auxiliary electrode, and in-situ Ag-AgCl (ISAAC) comparison electrode.

Method validation

Linearity/calibration curve

One of the most valuable experiences we gained from our studies with the electrochemical detector is that it is not difficult to achieve excellent linearity when optimum conditions are provided. This study evaluated the calibration curve with the linear regression value at ng/mL level. The correlation coefficient (R^2) of linearity greater than 0.99 was consistently good for all determinations on the respective calibration ranges (Table 3).

Quantitative and detection limits (Sensitivity)

LOD and LOQ were calculated for all analytes according to the method section. The obtained results were given in Table 4, respectively. The lowest quantitation limit belongs to NE, and SER is the highest.

Recovery

The precision and accuracy results of the three concentrations level calculated are given in Table 5. Both precision and accuracy results were within acceptable limits according to validation parameters. For the three concentrations levels, SD was lower than 1.5 for each compound. The accuracy of all concentration levels for all analytes tested was 96-100%. The average absolute recovery for each analyte measured in triplicate for all three concentrations level was consistently achieved above 95%.

Stability

Stability results of standard solution and three rat brain tissue samples are shown in Table 6. When all monoamines and their metabolites were injected at 24 h and 48 h, they were stored at autosampler 5 °C. As seen in the results, there is no significant change in any analyte signal for the standard solution and brain samples.

Sample applications

The optimized and validated method was applied to three different rat brain tissues. Brain samples from three different rats were taken, prepared, and analyzed according to the procedure mentioned above. The IS was used to calculate each analyte's concentration in brain tissue samples [31].The chromatogram of brain tissue is given in Figure 3 below. According to available data, in a rat brain tissue, VA 28.20 ng MHPG 5.59 ng, NA 147.15 ng, DOPAC 11.07 ng, DOPA 81.95 ng, 5-HIAA 48.89 ng, HVA 10.68 ng, 3-MT 1.71 ng, and SER 0.30 ng per µg tissue was found. The results obtained are also compatible with the literature data [32, 33].

CONCLUSION

For the first time, in this study, an optimized and fully validated method was developed for the simultaneous analysis of nine neurotransmitter compounds, which are DA, HVA, VA, SER, 5-HIAA, MHPG, NE, DOPAC, and 3-MT in the rat brain for best knowledge. Kinetex®F_5 column separated successfully and DECADE II electrochemical detector determined signal of target compounds. DHBA was used as an IS. The most important advantages of this study are; fast and efficient, does not require sophisticated sample pre-treatment procedures, can provide the stability of analytes, and is analytically evaluated. In the study, all parameters affecting the separation of neurotransmitters were examined in detail. Besides, the applicability of the method was demonstrated by the analysis of three rat brains. The study's weaknesses could be that the separation efficiency of stationary phases with different structures cannot be examined, and more brain sample applications cannot be performed. Although it is

possible to reduce the analysis time by using stationary phases with a shorter length and smaller particles called ultra-high-performance liquid chromatography columns, it will be useful to consider that such columns in ECD systems are short-lived, and the routine analysis cost increases significantly.

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References

1. Drayer, B.; Burger, P.; Darwin, R.; Riederer, S.; Herfkens, R.; Johnson, G., MRI of brain iron. American Journal of Roentgenology 1986, 147 (1), 103-110.
2. Koolman, J.; Röhm, K.-H.; Wirth, J.; Robertson, M., Color atlas of biochemistry. Thieme Stuttgart: 2005; Vol. 2.
3. Viljoen, F.; Du Preez, J.; Wessels, J.; Aucamp, M., HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples. Die Pharmazie-An International Journal of Pharmaceutical Sciences 2018, 73 (10), 563-569.
4. Allen, G. F.; Land, J. M.; Heales, S. J., A new perspective on the treatment of aromatic L-amino acid decarboxylase deficiency. Molecular genetics and metabolism 2009, 97 (1), 6-14.
5. Burtis, C. A.; Ashwood, E. R.; Bruns, D. E., Tietz textbook of clinical chemistry and molecular diagnostics-e-book. Elsevier Health Sciences: 2012.
6. Wise, R. A., Catecholamine theories of reward: a critical review. Brain research 1978, 152 (2), 215-247.
7. Parrot, S.; Neuzeret, P.-C.; Denoroy, L., A rapid and sensitive method for the analysis of brain monoamine neurotransmitters using ultra-fast liquid chromatography coupled to electrochemical detection. Journal of Chromatography B 2011, 879 (32), 3871-3878.
8. Kovac, A.; Somikova, Z.; Zilka, N.; Novak, M., Liquid chromatography–tandem mass spectrometry method for determination of panel of neurotransmitters in cerebrospinal fluid from the rat model for tauopathy. Talanta 2014, 119, 284-290.
9. Kursinszki, L.; Szoke, E., HPLC-ESI-MS/MS of brain neurotransmitter modulator lobeline and related piperidine alkaloids in Lobelia inflata L. J Mass Spectrom 2015, 50 (5), 727-733.
10. Liu, Y. Y.; Wei, M. Y.; Yue, K. X.; Hu, M. X.; Li, S. Z.; Men, L. H.; Pi, Z. F.; Liu, Z. G.; Liu, Z. Y., Study on Urine Metabolic Profile of A beta 25-35-Induced Alzheimer's Disease Using UHPLC-Q-TOF-MS. Neuroscience 2018, 394, 30-43.
11. Lovden, M.; Karalija, N.; Andersson, M.; Wahlin, A.; Axelsson, J.; Kohncke, Y.; Jonasson, L. S.; Rieckman, A.; Papenberg, G.; Garrett, D. D.; Guitart-Masip, M.; Salami, A.; Riklund, K.; Backman, L.; Nyberg, L.; Lindenberger, U., Latent-Profile Analysis Reveals Behavioral and Brain Correlates of Dopamine-Cognition Associations. Cereb Cortex 2018, 28 (11), 3894-3907.
12. Smith, M. K.; Bose, U.; Mita, M.; Hall, M. R.; Elizur, A.; Motti, C. A.; Cummins, S. F., Differences in Small Molecule Neurotransmitter Profiles From the Crown-of-Thorns Seastar Radial Nerve Revealed Between Sexes and Following Food-Deprivation. Front Endocrinol 2018, 9.
13. Sun, Z.; Li, Q.; Bi, K. S., Rapid HPLC-ESI-MS/MS Analysis of Neurotransmitters in the Brain Tissue of Alzheimer's Disease Rats before and after Oral Administration of Xanthoceras sorbifolia Bunge. Molecules 2018, 23 (12).
14. Ly, S. Y., Detection of dopamine in the pharmacy with a carbon nanotube paste electrode using voltammetry. Bioelectrochemistry 2006, 68 (2), 227-231.
15. Pınar, P. T.; Yardım, Y.; Şentürk, Z., Electrochemical oxidation of ranitidine at poly (dopamine) modified carbon paste electrode: Its voltammetric determination in pharmaceutical and biological samples based on the enhancement effect of anionic surfactant. Sensors and Actuators B: Chemical 2018, 273, 1463-1473.
16. Sultan, S. C.; Sezer, E.; Tepeli, Y.; Anik, U., Centri-voltammetric dopamine detection. Rsc Adv 2014, 4 (59), 31489-31492.
17. Han, H. S.; Lee, H. K.; You, J.-M.; Jeong, H.; Jeon, S., Electrochemical biosensor for simultaneous determination of dopamine and serotonin based on electrochemically reduced GO-porphyrin. Sensors and Actuators B: Chemical 2014, 190, 886-895.
18. Taylor, P. J., Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. Clinical biochemistry 2005, 38 (4), 328-334.
19. Food, F.-U., Guidance for Industry-Bioanalytical Method Validation. http://www. fda. gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107. pdf 2001.

20. Smith, G., European Medicines Agency guideline on bioanalytical method validation: what more is there to say? Bioanalysis 2012, 4 (8), 865-868.
21. Bhattacharjee, N.; Borah, A., Oxidative stress and mitochondrial dysfunction are the underlying events of dopaminergic neurodegeneration in homocysteine rat model of Parkinson's disease. Neurochemistry international 2016, 101, 48-55.
22. Directive, C., Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 and of the council of 22 September 2010 on the protection of animals used for scientific purposes. OJEU 2010, 276, 33-78.
23. Glowinski, J.; Iversen, L. L., Regional studies of catecholamines in the rat brain-I: the disposition of [3H] norepinephrine,[3H] dopamine and [3H] dopa in various regions of the brain. Journal of neurochemistry 1966, 13 (8), 655-669.
24. Guideline, I. H. T. In Validation of analytical procedures: text and methodology Q2 (R1), International conference on harmonization, Geneva, Switzerland, 2005.
25. USP, C., The United States Pharmacopeia. National Formulary 2008, 14.
26. Virag, L.; Whittington, R. A., Highly sensitive chromatographic assay for dopamine determination during in vivo cerebral microdialysis in the rat. Journal of Chromatography B 2002, 772 (2), 267-272.
27. Vaarmann, A.; Kask, A.; Mäeorg, U., Novel and sensitive high-performance liquid chromatographic method based on electrochemical coulometric array detection for simultaneous determination of catecholamines, kynurenine and indole derivatives of tryptophan. Journal of Chromatography B 2002, 769 (1), 145-153.
28. Miki, K.; Sudo, A., Effect of urine pH, storage time, and temperature on stability of catecholamines, cortisol, and creatinine. Clinical Chemistry 1998, 44 (8), 1759-1762.
29. Sutor, B.; Ten Bruggencate, G., Ascorbic acid: a useful reductant to avoid oxidation of catecholamines in electrophysiological experiments in vitro? Neuroscience letters 1990, 116 (3), 287-292.
30. Ferry, B.; Gifu, E.-P.; Sandu, I.; Denoroy, L.; Parrot, S., Analysis of microdialysate monoamines, including noradrenaline, dopamine and serotonin, using capillary ultra-high performance liquid chromatography and electrochemical detection. Journal of chromatography B 2014, 951, 52-57.
31. Causon, R.; Carruthers, M.; Rodnight, R., Assay of plasma catecholamines by liquid chromatography with electrochemical detection. Analytical biochemistry 1981, 116 (1), 223-226.
32. Coyle, J.; Henry, D., Catecholamines in fetal and newborn rat brain. Journal of neurochemistry 1973, 21 (1), 61-67.
33. Felice, L. J.; Felice, J. D.; Kissinger, P. T., Determination of catecholamines in rat brain parts by reverse-phase ion-pair liquid chromatography. Journal of neurochemistry 1978, 31 (6), 1461-1465.

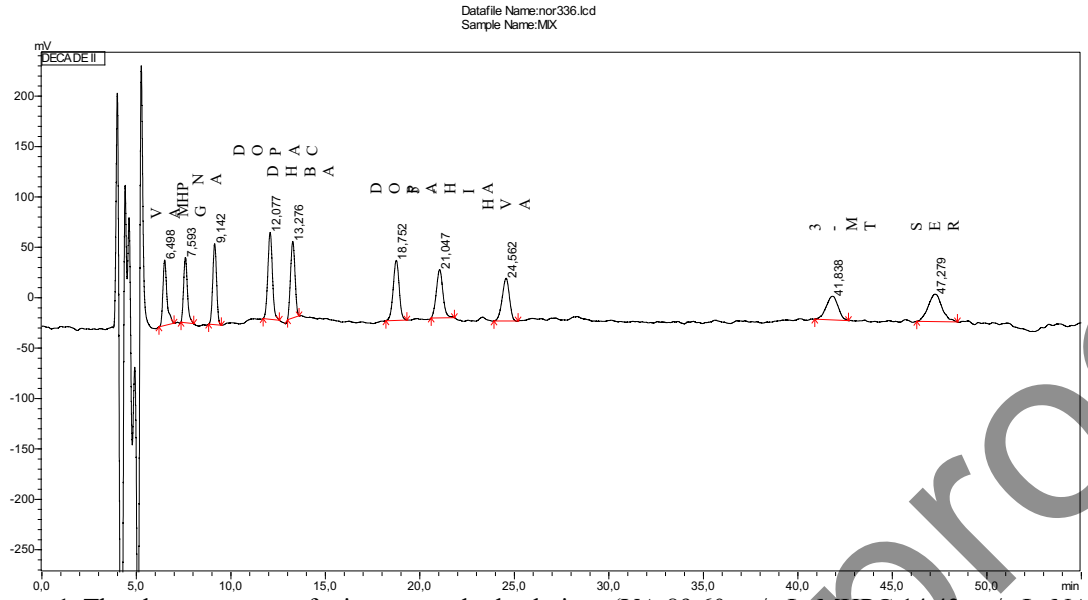

Figure 1. The chromatogram of mixture standard solutions (VA 89.60 ng/mL, MHPG 14.43 ng/mL, NA 148.0 ng/mL, DOPAC 35.30 ng/mL, DOPA 34.80 ng/mL, 5-HIAA 27.40 ng/mL, HVA 42.30 ng/mL, 3-MT 5.50 ng/mL and SER 0.186 ng/mL) in optimized conditions.

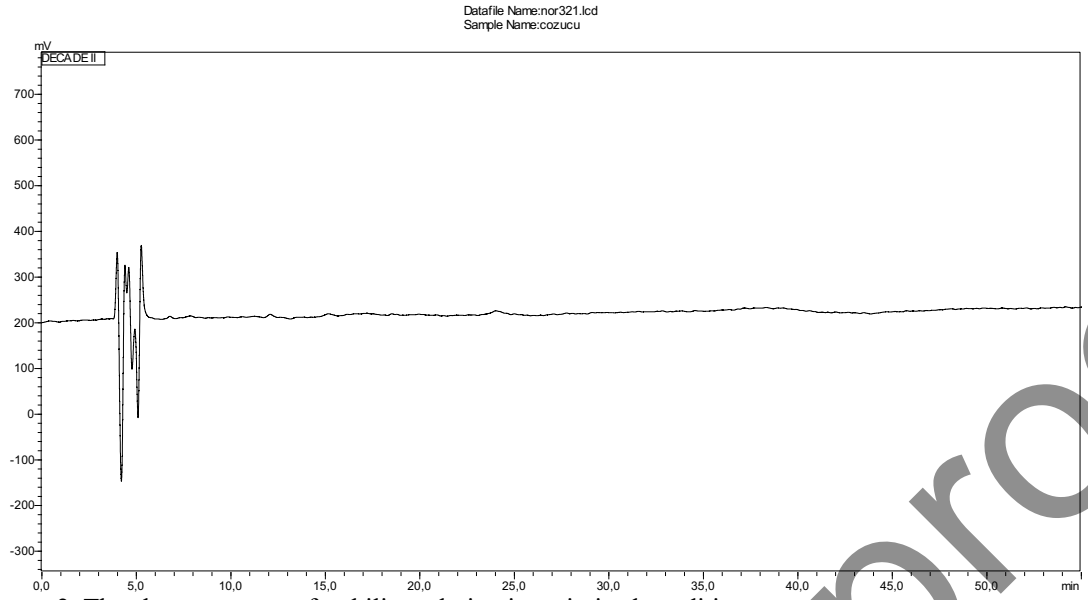

Figure 2. The chromatogram of stability solution is optimized conditions.

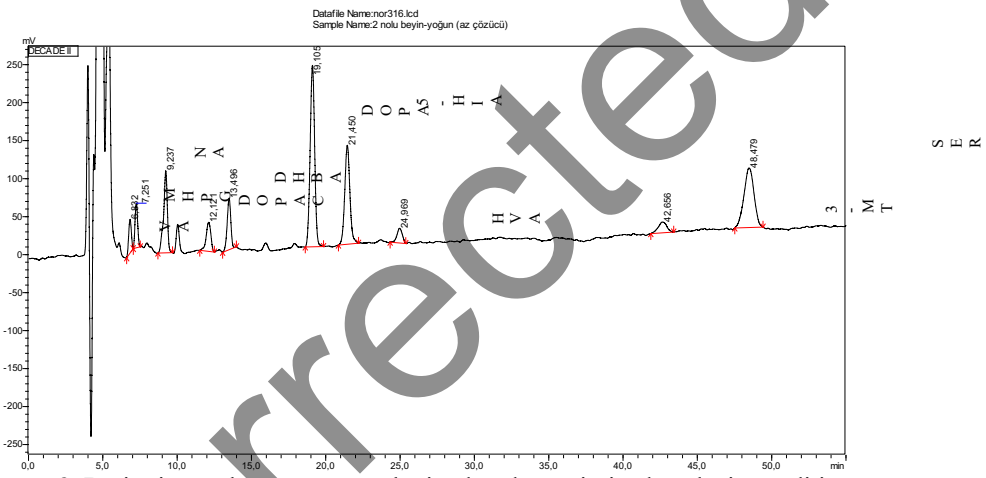

Figure 3. Brain tissue chromatogram obtained under optimized analysis conditions.

Table 1. Features of Ascentis® C_{18} and Phenomenex® F_5 columns.

Properties	Ascentis® Express	Phenomenex® F_5
Particle size (µm)	2.7	2.6
Functional group	C_{18}	F_5
Macropor/mesopor size (µm)	10	0.35
Core size (µm)	2.7	1.9
Surface area ($m^2.g^{-1}$)	450	200
Carbon load (%)	25	9
Total permeability [m^2] (k_0)	-	5.81×10^{-11}
Por Volume/diameter (Å)	90	100
Range of pH	2 - 9	1.5 – 8.5
Surface coating (µmol.m^{-2})	3.7	2.8

Table 2. SST results obtained in the analysis of monoamines in Kinetex® column

Parameter	VA	MHPG	NA	DOPAC	DHBA	DOPA	5-HIAA	HVA	3-MT	SER	Recommended
T_R	6.49	7.59	9.14	12.07	13.27	18.73	21.03	24.55	41.75	47.22	
SD of T_R	0.07	0.01	0.01	0.01	0.21	0.03	0.03	0.02	0.13	0.08	
N	35504	40818	48736	61713	73442	109916	100321	98880	106521	91375	>2000
HTP	28166	24499	20519	16204	13616	9098	9968	10113	9388	10944	-
T	1.410	1.255	1.046	1.184.	1.206	0.921	1.023	1.007	1.891	1.063	<2
A_s	1.137	1.105	1.18	1.161	1.050	1.13	1.052	0.991	1.200	1.030	$0.95<A_s<1.2$
k'	0.235	0.443	0.737	1.295	1.521	2.560	2.998	3.667	6.927	7.973	2<k<10
a	1.887	1.663	1.757	1.175	1.683	1.171	1.223	1.889	1.151	1.530	>1
R_s	3.683	2.946	3.791	6.313	2.364	10.04	3.637	4.712	16.136	3.753	>2
USP Width	0.356	0.388	0.427	0.501	0.505	0.583	0.685	0.806	1.318	1.611	-

Table 3. Linearity studies (20% - 150%).

	VA	MHPG	NA	DOPAC	DOPA	5-HIAA	HVA	3-MT	SER
Linearity range (ng/mL)	17.9 – 134.4	2.9 – 21.65	29.6 – 222	7.1 – 52.9	6.9 – 134.4	5.5 – 41.1	8.5 – 63.5	1.1 – 8.25	0.037 – 0.279
Slope ±SD (intraday,*n*=6, Ratio)	0.0143±0.0004	0.0954±0.0043	0.00821±0.0001	0.02943±0.0008	0.01889±0.0004	0.0192±0.0006	0.01137±0.0004	0.04591±0.003	1.4474±0.067
Intercept±SD (intraday, *n*=6, mA)	0.1508±0.034	0.10182±0.061	0.01756±0.016	0.15497±0.029	-0.00157±0.014	-0.01537±0.016	-0.01474±0.017	0.00158±0.0157	0.00716±0.012
Regression coefficient (intraday,*n*=6)	0.9970	0.9917	0.9992	0.9968	0.9982	0.9959	0.9947	0.9937	0.9915
LOD (ng/mL)	2.15	0.02	3.04	0.71	1.02	1.01	1.64	0.36	0.01
LOQ (ng/mL)	6.45	0.06	9.13	2.12	3.06	3.03	4.93	1.08	0.03

Table 5. Recovery results (n=3).

Compound	Concentration (ng/mL)	% Recovery (Mean ± SD)	%Difference
VA	71.70	98.15 ± 1.43	-1.85
	89.60	99.03 ± 0.15	-0.97
	107.5	98.83 ± 0.78	-1.17
MHPG	11.50	99.25 ± 1.01	-0.75
	14.43	99.13 ± 0.84	-0.87
	17.30	98.46 ± 0.56	-1.54
NA	118.4	99.75 ± 0.77	-0.25
	148.0	98.89 ± 1.07	-1.11
	177.6	97.99 ± 0.06	-2.01
DOPAC	28.20	99.01 ± 0.28	-0.99
	35.30	98.53 ±0.72	-1.47
	42.40	98.78 ± 1.14	-1.22
DOPA	27.80	101.1 ± 0.52	+1.10
	34.80	99.56 ± 0.45	-0.44
	41.80	100.8 ±0.34	+0.80
5-HIAA	21.90	98.82 ± 0.67	-1.18
	27.40	99.14 ± 0.43	-0.86
	32.90	100.3 ± 1.00	+0.30
HVA	33.80	98.56 ± 1.09	-1.44
	42.30	99.20 ± 0.89	-0.80
	50.80	101.2 ± 0.63	+1.20
3-MT	4.40	99.76 ± 0.47	-0.24
	5.50	98.44 ± 1.07	-1.56
	6.60	97.14 ± 0.35	-2.86
SER	0.149	96.58 ± 1.20	-3.42
	0.186	97.15 ± 0.64	-2.85
	0.223	99.26 ± 1.32	-0.74

Table 6. Stability studies of catecholamines (n=3).

		% Stability of Compounds (mean ± SD)	
		24 h	48 h
Standard solution	VA	99.89 ± 1.46	99.85 ± 1.86
	MHPG	99.81 ± 0.57	99.73 ± 1.35
	NA	99.09 ± 1.66	98.68 ± 1.90
	DOPAC	99.03 ± 1.89	98.85 ± 2.18
	DOPA	99.32 ± 1.16	99.18 ± 1.65
	5-HIAA	99.34 ± 1.28	99.26 ± 1.76
	HVA	99.05 ± 1.05	98.86 ± 0.63
	3-MT	100.52 ± 0.76	99.72 ± 0.83
	SER	99.13 ± 0.56	99.72 ± 0.84
Rat brain tissue	VA	99.78 ± 0.08	97.48 ± 1.14
	MHPG	99.11 ± 0.77	99.11 ± 0.34
	NA	98.21 ± 0.29	98.23 ± 2.39
	DOPAC	97.45 ± 0.58	99.89 ± 1.02
	DOPA	99.75 ± 1.81	98.01 ± 0.27
	5-HIAA	99.59 ± 0.26	98.98 ± 0.45
	HVA	96.23 ± 2.39	93.46 ± 3.57
	3-MT	97.62 ± 0.36	99.20 ± 0.16
	SER	98.23 ± 0.07	97.86 ± 1.28