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ORIGINAL ARTICLE

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

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ABSTRACT

Objectives: Chemical neurotransmission, managed by neurotransmitters, has a crucial role in brain processes such as fear, memory, learning, and pain, or neuropathology such as schizophrenia, epilepsy, anxiety/depression, and Parkinson's disease. The measurement of these compounds is used to elucidate the disease mechanisms and evaluate the outcomes of therapeutic interventions. However, this can be quite difficult because of various matrix effects and the problems of chromatographic separation of analysts. In the current study; for the first time, an optimized and fully validated high-performance liquid chromatography-electrochemical detection (HPLC-EC) method according to Food and Drug Administration and European Medicines Agency Bioanalytical Validation Guidance was developed for the simultaneous analysis of nine neurotransmitter compounds, namely dopamine, homovanilic acid, vanilmandelic acid, serotonin (SER), 5-hydroxyindole-3-acetic acid, 4-hydroxy-3-methoxyphenylglycol, norepinephrine, 3,4 dihydroxyphenylacetic acid, and 3-methoxytyramine and simultaneously determined in rat brain samples.

Materials and Methods: Separation was achieved with 150 mm x 4.6 mm, 2.6 µm Kinetex F5 (Phenomenex, USA) column isocratically, and analysis was carried out by HPLC equipped with a DECADE II EC detector.

Results: The method exhibited good selectivity, and the 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 analyses and method robustness were also examined in detail in the study, and the obtained results are presented statistically.

Conclusion: The developed and fully validated method has been successfully applied to actual rat brain samples, and important results have been obtained. In the rat brain sample analysis, the lowest number of SER and the highest amount of noradrenaline were found.

Key words: 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 (NA), epinephrine (EP), serotonin (SER), and their metabolites in the central nervous system (CNS).[1,2] Their primary function is neurotransmission with hormonal or neuronal signals during various physiological events. In addition, the amount of monoamines 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) as emerging metabolites. NA metabolites are HVA and VA. The last pathway is HVA, when transforming into 3-methoxytyramine (3-MT) and 3,4-dihydroxyphenylacetic acid

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(DOPAC). SER synthesized from tryptophan amino acid turns into 5-hydroxyindole-3-acetic acid (5-HIAA).

Chemical neurotransmission is considered as neurotransmitters and affects brain processes such as memory, pain, learning, and fear or neuropathology such as epilepsy, schizophrenia, Parkinson's disease, and anxiety/depression. Therefore, changes in decreasing or increasing regions are the precursors of many mood disorders, diseases, or irreversible events such as tissue deformation.[5,6] Electrochemical detection (EC) reactions are often the basis of these deformations. Because these compounds have a small and straightforward structure, they contain only non-rigid electroactive functional groups.[6] In addition, these properties range from the dominance of EC detectors in their analysis.

In clinical and experimental neuroscience, neurotransmitters are widely studied in different biological samples, such as blood, plasma, urine, dialysates, and supernatants from tissue homogenates. In general, two main methods are used in tissue analysis of neurotransmitters. The samples were collected *in vivo*, and their determinations were made by a selected analytical method. Frequently preferred methods in sample preparation are microdialysis or push-pull perfusion techniques. Their determination is mostly achieved using high-performance liquid chromatography (HPLC). However, most of these compounds do not contain any chromophore groups; therefore, the ultraviolet-visible region or photodiode array detector cannot be used. Instead, two types of detectors are used, mostly mass spectrometry (MS) and electrochemical. EC detector equipment is cheaper and easier to use than MS. In addition, it does not require sample derivatization because of the high electroactivity of catecholamines and indolamine.

Moreover, EC detectors are specific and significantly rarely affected by other analysts' matrix or signal interference. Coulometry or amperometry can be used for EC detection. The main advantage of coulometry is the complete oxidation of the compounds entering the detection cell, resulting in a low limit of detection (LOD). Nevertheless, the technology for miniaturization of cells 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 EC yield (~10%).

One of the works that need to be emphasized is that in 2011, Parrot et al.[7] developed a method for the analysis of NA, DA, and three metabolites using DOPAC, HVA, 3-MT, SER, and its metabolite 5-HIAA, using ultra-high performance liquid chromatography (UPLC)-ECD. For the method, validation studies were performed by selecting the working range of all compounds 10^{-9}–10^{-6} M, and they were applied to rat, cat, and mouse brain tissue.[7] Furthermore, in 2014, benzoyl chloride was used for derivatization for the analysis of cerebrospinal fluids from rat models such as SER, 5-HIAA, HVA, NA, EP, DA, glutamic acid, GABA, and DOPAC by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.[8] Especially since 2018, the LC-MS/MS method has been developed to investigate the potential therapeutic effect of various diseases and many neurotransmitters, including DA, NA, 5-hydroxytryptamine, acetylcholine, *l*-tryptophan, γ-aminobutyric acid, glutamic acid, and aspartic acid.[9-13] Simultaneous determination in various tissues, such as the mouse brain was accelerated. A simple protein precipitation method is generally used for sample pretreatment. 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 the ng level and applied to three rat brain regions (posterior-anterior cortex, hippocampus, and striatum). In addition, voltammetry techniques are widely examined in the studies performed until today. However, because of selectivity problems, the analysis was carried out with electrodes modified using various techniques, and very successful results were obtained.[14-16] For example, in the 2014 study, a 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 analyses at µM levels.

As seen above, much progress has been made, and studies have been published on analyzing neurotransmitters. The mass detector is particularly promising. However, this detector exhibits Achilles tendon matrix interference in biological fluid and tissue analysis.[18] Sensor work is still in its infancy. In brief, more efficient methods should be produced by developing chromatographic techniques. The purpose of the 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 for the routine analysis of DA, HVA, VA, SER, 5-HIAA, MHPG, NA, DOPAC, and 3-MT in brain samples. All method optimization parameters were examined in detail, and full bioanalytical validation parameters were performed according to international guidelines.[13,19,20] Thanks to the current method, 10 eluents, 9 analyses, and an internal standard (IS) were successfully separated in the required chromatographic efficiency and applied to the rat brain samples.

MATERIALS AND METHODS

Chemicals

The hydrochloride salts; DA, SER, and 3-MT, HVA, VA, 5-HIAA, MHPG, NA, 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). 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 the stability solution

The stability solution was used to prepare, store standard and brain solutions, and homogenize brain tissue to ensure adequate neurotransmitter substance stability. The prepared stability solution consisted of 0.1 M perchloric acid and 0.1 mM sodium metabisulfite of 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 the volume was then completed with water.

Preparation of the standard solutions

Each standard was weighed at approximately 1 mg and placed in a volumetric flask (10 mL), and the completed volumes were with the stability solution. Subsequently, they were stored at 4 °C for optimization and validation studies by making necessary dilutions. We 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 safely stored in the freezer and thawed, and they remained intact for a minimum of 60 h during the analysis period.

Preparation of the mobile phase solution

The mobile phase composition for liquid chromatography was prepared in water with 0.07 M KH_2PO_4, 20 mM citric acid, 5.3 mM OSA, 100 mM EDTA, 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 use. Under these conditions, the mobile phase's measured pH value was optimized as 3.2 ± 0.1 isocratically.

Removal of brain tissue from rats

Approximately 9-10-week-old male Sprague-Dawley rats were used for the experiments. Rats were fed a standard rodent diet and tap water. The animals sacrificed without any experimental procedure to obtain tissue were anesthetized with pentobarbital (60 mg/kg) and decapitated. All animal procedures were performed 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 tissue was removed and placed in tubes containing prepared stability solutions. The tissues were quickly frozen with liquid nitrogen and stored in a -40 ºC freezer for EC analysis. The experimental protocols of this study were approved by the Local Ethical Committee on Animal Experimentation of Anadolu University, Eskişehir, Türkiye (Decision no: 2022-12, date: 06.04.2022).

Preparation of the brain tissues

The brain tissue was removed from the freezer and placed in a sonicator bath, allowing it to dissolve quickly. The effect of the sonicator bath on the tissues' dopaminergic substances was tested by applying various parallel sample preparation procedures, and it was found that it did not affect them. The defrosted brain samples were gently removed from the stability solution, accurately weighed, and placed in the homogenization vessel. The brain tissue samples were homogenized in an ice-cold stability solution for approximately 25 min by adding the IS DHBA at a concentration of 61.6 ng/mL concentration. The amount of solvent added *per* milligram of brain tissue is 7.5 µL.[23] All homogenization processes were carried out with Potter S (B. Braun Biotech International, Berlin, Germany) model homogenizer. The homogenates were then sonicated for 10 min, centrifuged at 11,000 rpm at 5 °C for 40 min, and filtered through a 0.22 µm PTFE filter. This process was repeated again, and the filtrate was placed in injection vials and analyzed.

Analysis parameters

The analysis system is equipped with UPLC from Shimadzu (Kyoto, Japan) and a DECADE II EC detector (Antec-Leyden, Netherlands) for chromatographic separation and detection. The system comprises two LC-30AD parallel piston pumps, a SIL-30AC refrigerated autosampler, a CBM-20A system control unit, and a DGU-A5R inline degassing unit. A Decade II EC detector (cell volume 80 nL, Antec, Leyden, Netherlands) equipped with a 2 mm glassy carbon working electrode, an *in situ* Ag-AgCl (ISAAC) reference electrode, a platinum auxiliary electrode, and a 25 µm spacer at 35 °C.

Separation was performed at 35 °C (oven-controlled) using 150 mm x 4.6 mm, 2.6 µm Kinetex F_5 (Phenomenex, USA). The mobile phase is pumped at a flow rate of 0.35 mL/min, and the injection volume is 1 µL. EC 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.

The evaluation of the results

Peak normalization (PN) techniques were used to investigate the results. The PN method could be used to 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 analyst and IS, DHBA, were accepted as reasonable responses. Based on the PN values, the concentrations of solutions were applied to method validation and optimization applications.

Method validation

The validation studies were conducted according to the bioanalytical method validation guidelines of the Food and Drug Administration and the European Medicines Agency.[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, limit of quantitation (LOQ), and system suitability tests (SST).[24] All statistical calculations were performed with the GraphPad Prism v6.0b (trial version) program.

The SST parameters showing the LC instrument's performance and the column used during the analysis are fundamental in the method development step. Parameters such as resolution (Rs), tailing factor (T_f) 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 method of the United States Pharmacopeia.

Selectivity

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

Linearity

For the linearity studies; all analyses were prepared at six concentration levels (20%, 50%, 80%, 100%, 120%, and 150%). Linearity was investigated between intra-day and inter-day slopes, and correlation coefficients and intercepts at 95% confidence levels were calculated using linear regression analysis.

Accuracy

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

Precision

Precision studies include intra-day and inter-day measurements. The analyst solutions used in the 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, the mean, standard error of the mean, standard deviation (SD), and relative SD are given at 95% confidence level.

LOD and LOQ

According to the 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, the LOD was calculated using the given in equation 1 for this study. This is recommended in the user manual of detector.[26]

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

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

Stability

The stability of the 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.

RESULTS

Stability of the analyte solutions

The extraction of neurotransmitters from tissues and cells was performed by homogenization and sonication in stability solutions. The stability solution damaged the tissues, revealed the target compounds, and stabilized the neurotransmitter compounds. In previous studies, perchloric acid is the most active compound in the extraction of neurotransmitters.[27] While it provides efficient extraction by adsorption of perchlorate ions in the perchloric acid structure, it also contributes to stabilizing the compounds because of 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 a preservative with antioxidant activity. In other studies, ascorbic acid could be an antioxidant component 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 should not lead to any loss or increase of the detector signal. This mixture was found by using the literature because of various stability trials.

The effect of the stability solution on brain tissue was also studied in detail. For this purpose, fresh brain tissue taken from the rat immediately after decapitation was divided into two symmetrical parts. Brain tissue was immediately homogenized and analyzed. The other brain tissue, subjected to the same experimental treatment, was immediately placed in 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. Because of the analyses, it was seen that there was no significant difference.

Chromatographic separation

The separations were first made in the reverse phase of the Ascentis Express (St Louis, MO, USA) brand 10 cm x 4.6 mm with a 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 analyses after a while, the column had poor separation, and method optimization was continued with Kinetex F5 (Phenomenex, USA) brand 15 cm x 4.6 mm, 2.6 µm column filled with core-shell silica particles with F_5 functional group. Both columns' features are compared, particularly in Table 1. The chromatographic separation studies presented that column functional group change did not have much effect on the retention time of the compounds, whereas the separation efficiency significantly increased. In this effect, of course, the contribution of using a 15 cm Kinetex F5 core-shell column instead of the 10 cm long column of Ascentis Express cannot be ignored. Although it had no dominant effect on the peak morphology, it showed excellent performance separating of structures close to each analyte. However, as the SER and 3-MT eluents, already quite late in the previous short column, had slightly higher retention times 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 first and had short retention times.

The retention times began to increase as the free amine group existed. 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 in the column for approximately 9 min. The mixture standard solution chromatogram obtained is given in Figure 1. We also checked whether the stability solution gave any peaks by injecting, as shown in Figure 2. In addition, SST is applied to show the performance of the LC device and method. In these tests, parameters such as N, R_s, T_f, A_s, α, and k' were calculated and are given in Table 2.

Method development

The purpose of this study was to develop a method for the separation and determination of DA, HVA, VA, SER, 5-HIAA, MHPG, NA, DOPAC, 3-MT, and DHBA for the presence of many interfering compounds in the rat brain. Nine neurotransmitter compounds were analyzed by EC detection reverse-phase ion-pair chromatography, DHBA was used as an IS, and thus, the unpredictable factors internal or external were eliminated. In experiments with matrix effects, such as biological analyses, the use of an IS 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 becomes more critical. DHBA is the most preferred IS in the analysis of neurotransmitters. Structural similarity and acting similar to catecholamines in chromatographic separation are the main reasons for preference. While calculating the analytical data, the error was minimized using the PN method, which considers both the retention time of the analyst and IS and their peak areas.

Table 1. Features of Ascentis Express C_{18} and Kinetex F5 columns

Properties	Ascentis® Express	Kinetex F5
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)	450	200
Carbon load (%)	25	9
Total permeability (m^2) (k_0)	-	5.81×10^{-11}
Pore volume/diameter (Å)	90	100
Range of pH	2-9	1.5-8.5
Surface coating (µmol/m^{-2})	3.7	2.8

The effect of each of the mobile phase components on separation is examined in detail using various mobile phases 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, while 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. KCl was used in the analyses as the reference electrode. This system, called *in situ* Ag-AgCl (ISAAC), was designed as EC cell T-, and the KCl concentration was determined according to the potential to be analyzed. Such reference electrodes are very stable but have the disadvantage that the EC reaction media volume is relatively small. Although the diffusion increases, the mobile phase prepared must be renewed in 48 h at the latest.[30] KH_2PO_4 and citric acid were used to control the pH of the mobile phase. It was observed that the optimum pH value for buffering was approximately 3.2. This pH value is around the pK_a value of the analytes to be distinguished. Lower acidity values can provide more stable and better interaction but, at the same time, reduce the life of

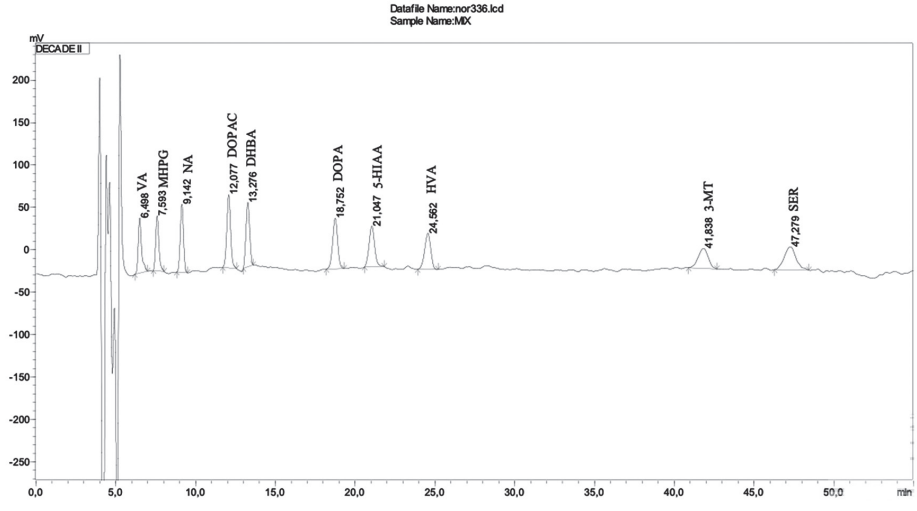

Figure 1. The chromatogram of the standard mixture (VA 89.60 ng/mL, MHPG 14.43 ng/mL, NA 148.0 ng/mL, DOPAC 35.30 ng/mL, DOPA 34.80 ng/mL, 5-HIAA 27.40 ng/mL, HVA 42.30 ng/mL, 3-MT 5.50 ng/mL and SER 0.186 ng/mL) in optimized conditions

VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, NA: Noradrenaline, DOPAC: 3,4 Dihydroxyphenylacetic acid, DOPA: 3,4-Dihydroxyphenylalanine, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin

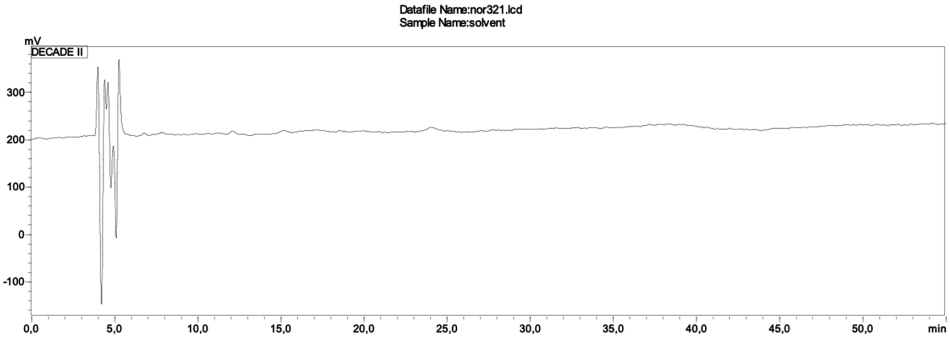

Figure 2. The chromatogram of the stability solution recorded under the optimized conditions.

Table 2. SST results obtained in the analysis of monoamines in the Kinetex F5 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	35.504	40.818	48.736	61.713	73.442	109.916	100.321	98.880	106.521	91.375	> 2.000
HETP	28.166	24.499	20.519	16.204	13.616	9.098	9.968	10.113	9.388	10.944	-
T_f	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
α	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	-

SST: System suitability test, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, T_R: Retention time, SD: Standard deviation, N: Number of theoretical plate, T_f: Tailing factor, HETP: Height equivalent of theoretical plate, As: Asymmetry factor, k': Capacity factor, α: Selectivity factor, R_s: Resolution, USP: United States Pharmacopeia, NA: Noradrenaline

the stationary phase. It should be kept in mind that the optimum working values of silica-based columns are in the acidity range of 3-7. In addition, citric acid acted as an ion-pairing reagent with OSA, which was observed as the lead ion-pair reagent in optimization studies. It regulated the peak morphology and increased the eluent column interactions. As the OSA concentration increased, it provided more sharp peaks, and because of its micelle feature, it also increased the viscosity of the mobile phase.

Another antitailing 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 carboxylic acid and some were amines. Analytes with amine groups interact with TEA, and those with carboxyl groups interact with 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 stabilize at 3.2. For the organic phase of the mobile phase, methanol and acetonitrile were examined for optimum separation. Acetonitrile rapidly eluted all compounds from the column, and its separation efficiency was inferior. On the other hand, methanol provided a slower elution rate, increased the interaction of the stationary phase with analysts, and optimum separation efficiency was obtained.

An EC detector detected signals with the 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 EC 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 the ng/mL level. The correlation coefficient (R^2) of linearity greater than 0.99 was consistently good for all determinations in the respective calibration ranges (Table 3).

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

SD: Standard deviation, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, LOD: Limit of detection, LOQ: Limit of quantitation, NA: Noradrenaline

Table 4. 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

SD: Standard deviation, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, NA: Noradrenaline

Quantitative and detection limits

LOD and LOQ were calculated for all analyses according to the method section. The obtained results are given in Table 3. The lowest quantitation limit belongs to NA, and SER is the highest.

Recovery

The precision and accuracy results of the three calculated concentrations are given in Table 4. Both precision and accuracy results were within acceptable limits according to the validation parameters. For the three concentrations, SD was lower than 1.5 for each compound. The accuracy of all concentration levels

for all analyses tested was 96-100%. The average absolute recovery for each analyst measured in triplicate for all three concentration was consistently above 95%.

Stability

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

Table 5. 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

SD: Standard deviation, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, NA: Noradrenaline

Sample applications

The optimized and validated method was applied to three rat brain tissues. Brain samples from three rats were collected, prepared, and analyzed according to the above-mentioned procedure. IS was used to calculate each analyte's concentration in brain tissue samples.[31] The chromatogram of brain tissue is shown in Figure 3. According to available data, in rat brain tissue, VA was 28.20 ng, MHPG was 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, DA, HVA, VA, SER, 5-HIAA, MHPG, NA, DOPAC, and 3-MT, in the rat brain The Kinetex F5 column separated successfully, and the DECADE II EC detector determined the signal of the 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 analysts, and is analytically evaluated. In the study, all parameters affecting the separation of neurotransmitters were examined in detail. In addition, 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 UPLC columns, it will be useful to consider that such columns in ECD systems are short-lived, and the routine analysis cost increases significantly.

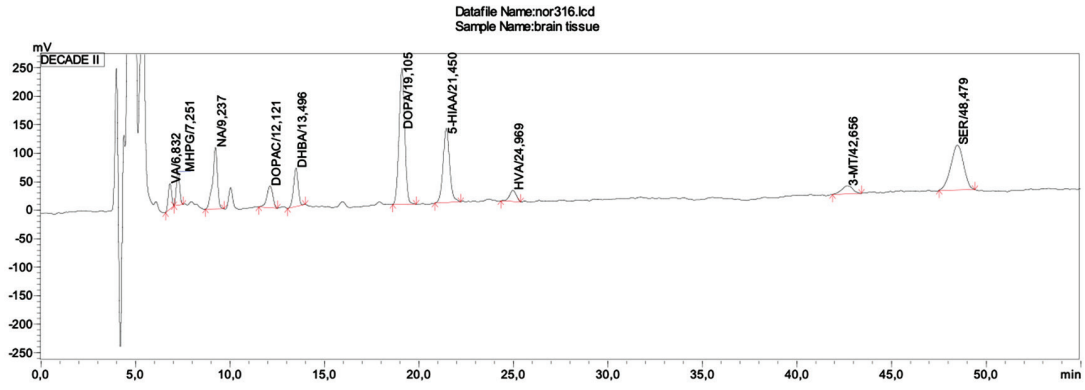

Figure 3. Brain tissue chromatogram obtained under optimized analysis conditions

Ethics

Ethics Committee Approval: The experimental protocols of this study were approved by the Local Ethical Committee on Animal Experimentation of Anadolu University, Eskişehir, Türkiye (decision no: 2022-12, date: 06.04.2022).

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.Ö., Concept: S.Ö., N.Ö.C., Design: S.Ö., N.Ö.C., Data Collection or Processing: S.Ö., A.G., M.K., Analysis or Interpretation: S.Ö., A.G., Literature Search: S.Ö., M.K., Writing: S.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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