PHARMACOLOGY AND PHARMACY

DEVELOPMENT AND VALIDATION OF A METHOD FOR THE QUANTITATIVE DETERMINATION OF MONOAMINE NEUROTRANSMITTERS AND THEIR METABOLITES IN RAT BRAIN TISSUE USING HPLC-MS/MS

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ABSTRACT

Background. *Determining changes in the content of monoamine neurotransmitters and their metabolites in brain structures is a necessary part of studying the pharmacodynamics of antiparkinsonian drugs. A method for the joint determination of norepinephrine, adrenaline, dopamine, serotonin, 5-hydroxyindole-3-acetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, vanillylmandelic acid in rat brain tissue has not previously been developed.*

The aim of the study. *Development and validation of a method for quantitative determination of noradrenaline, adrenaline, dopamine, serotonin, 5-hydroxyindole-3-acetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and vanillylmindalic acid in rat brain tissue by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).*

Materials and methods. *A method for determining monoamine mediators and their metabolites was developed using the HPLC-MS/MS method. Brain tissue homogenates were prepared using a mechanical hand-operated homogenizer. The effect of various antioxidants on the stability of norepinephrine, adrenaline, dopamine and 3,4-dihydroxyphenylacetic acid in the test samples was studied.*

Results. *Chromatographic separation of sample components was carried out using two Synergi Max RP (20 × 2.0 mm, 2.5 µm) and Synergi Fusion RP 80Å (250 × 4.6 mm, 4 µm) chromatographic columns. Elution was carried out in a gradient mode using a mobile phase based on methanol and a 0.1 % solution of formic acid in water. To prepare homogenate batches, the samples were diluted with a solution of internal standards in methanol. A 5 % aqueous solution of ascorbic acid was chosen as an antioxidant stabilizer.*

Conclusion. *The developed methodology has been fully validated and meets the requirements of Russian and international guidelines. The chosen stabilization method allows samples of brain homogenates to be stored for 30 days after collection.*

Key words: *HPLC-MS/MS, monoamine neurotransmitters, brain tissue, sample stabilization*

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РАЗРАБОТКА И ВАЛИДАЦИЯ МЕТОДИКИ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ МОНОАМИНОВЫХ НЕЙРОМЕДИАТОРОВ И ИХ МЕТАБОЛИТОВ В ТКАНЯХ МОЗГА КРЫС С ПОМОЩЬЮ ВЭЖХ-МС/МС

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РЕЗЮМЕ

Обоснование. *Определение изменения содержания моноаминовых нейромедиаторов и их метаболитов в структурах головного мозга является необходимой частью изучения фармакодинамики противопаркинсонически лекарственных средств. Методика совместного определения норадреналина, адреналина, допамина, серотонина, 5-гидроксииндол-3-уксусной кислоты, 3,4-дигидроксифенилуксусной кислоты, гомованилиновой кислоты, ванилилминдальной кислоты в тканях мозга крыс ранее не была разработана.*

Цель исследования. *Разработка и валидация методики количественного определения норадреналина, адреналина, допамина, серотонина, 5-гидроксииндол-3-уксусной кислоты, 3,4-дигидроксифенилуксусной кислоты, гомованилиновой кислоты, ванилилминдальной кислоты в тканях мозга крыс с помощью высокоэффективной жидкостной хроматографии в сочетании с тандемной масс-спектрометрией (ВЭЖХ-МС/МС).*

Методы. *Методика определения моноаминовых медиаторов и их метаболитов разработана с применением метода ВЭЖХ-МС/МС. Гомогенаты тканей мозга готовились с помощью механического ручного гомогенизатора. Изучено влияние различных антиоксидантов на стабильность норадреналина, адреналина, допамина и 3,4-дигидроксифенилуксусной кислоты в испытуемых образцах.*

Результаты. *Хроматографическое разделение компонентов пробы осуществлялось с помощью двух хроматографических колонок Synergi Max RP (20 × 2,0 мм, 2,5 мкм) и Synergi Fusion RP 80Å (250 × 4,6 мм, 4 мкм). Элюирование проводили в градиентном режиме с применением подвижной фазы на основе метанола и 0,1%-го раствора муравьиной кислоты в воде. Для подготовки проб гомогенатов использовалось разведение образцов раствором внутренних стандартов в метаноле. В качестве стабилизатора-антиоксиданта был выбран 5%-й водный раствор аскорбиновой кислоты.*

Заключение. *Разработанная методика прошла полную валидацию и соответствует требованиям российских и международных руководств. Выбранный способ стабилизации позволяет хранить образцы гомогенатов мозга в течение 30 дней после отбора.*

Ключевые слова: *ВЭЖХ-МС/МС, моноаминовые нейромедиаторы, ткани мозга, стабилизация образцов*

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INTRODUCTION

Parkinson's disease is a neurodegenerative disease in which there is a decrease in the number of dopaminergic neurons in the substantia nigra, which causes a decrease in the concentration of dopamine (Dop) in the striatum. This causes the classic motor symptoms: rigidity, posture disturbances, akinesia, tremor, and bradykinesia. Most of the existing models of this disease involve the use of rats as experimental animals [1]. When studying the pharmacodynamics of new antiparkinsonian medicinal products (MPs), quantification of dopamine and its major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC, 3,4-dihydroxyphenylacetic acid) and homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid; HVA, homovanillic acid) in the striatum is required. One of the promising groups of MPs used in the treatment of Parkinson's disease are inhibitors of the enzyme MAO-B, which selectively catalyses the oxidation of dopamine. In order to study the effect of these MPs on MAO-A activity, it is necessary to measure the concentration of noradrenaline (NA, noradrenaline), serotonin (5HT, 5-hydroxytryptamine) and its metabolite (5-hydroxyindole-3-yl)-acetic acid (5HIAA, 5-hydroxyindoleacetic acid) [1, 2]. To control the correctness of brain and striatum sampling and the absence of contamination by blood and other tissue particles, it is necessary to monitor the content of adrenaline (Adr) and vanillylmindelic acid (2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-acetic acid; VMA, vanillylmandelic acid), which should not normally be detected in these samples [3-7].

High-performance liquid chromatography with spectrophotometric (HPLC-UV) [8], electrochemical (HPLC-EM) [9-12] and tandem mass spectrometric detector (HPLC-MS) [2, 5-7, 13-20] was used for quantitative determination of the above analytes in biological objects. However, the methodology for co-analysing all eight substances studied together in brain structures has not been previously published. The process of developing this technique is complicated by the fact that noradrenaline, adrenaline, dopamine and DOPAC contain a pyrocatechin fragment in their structure, which contributes to their rapid oxidation in samples as a result of interaction with endogenous substances and air oxygen [4, 21]. The addition of antioxidant solutions to the samples is required to prevent degradation of these compounds. The necessity of using a stabiliser is indicated only in the studies of J. Lu et al. [2], J. Thomas et al. [8], G. Cannazza et al. [12], C. Ji et al. [15], A. Kovac et al. [17]. Consequently, the choice of optimal conditions for stabilisation and storage of selected rat brain tissues is also relevant to ensure the reliability of the results of preclinical studies.

THE AIM OF THE STUDY

Development and validation of a method for the combined quantitative determination of noradrenaline, adrenaline, dopamine, serotonin, 5-hydroxyindole-3-acetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and vanillylmindalic acid in rat brain tissue.

MATERIALS AND METHODS

Study design

The first stage of the study involved the selection of optimal conditions for homogenate sample preparation as well as parameters for chromatographic-mass spectrometric determination. The selection of an antioxidant stabiliser was then performed to prevent degradation of noradrenaline, adrenaline, dopamine and DOPAC. The matrix effects of the methodology were further evaluated. Based on the results obtained, correction of the volumetric ratio of solvent to tissue in the preparation of homogenates was performed. In the next step, a full validation of the bioanalytical methodology was performed. It was then tested by analysing striatum samples from six intact Wistar male line rats weighing 362 ± 25 g (mean ± standard deviation (SD, standard deviation)). The study was approved by the Ethical Committee of the Yaroslavl State Medical University of the Ministry of Health of Russian Federation (Minutes No. 2 dated March 23, 2023).

Equipment

Method development and validation were performed on an HPLC-MS/MS system comprising a QTRAP5500 hybrid tandem mass spectrometer (SCIEX, Canada) and a 1260 Infinity chromatograph (Agilent, USA) (G1312B pump, G1329B autosampler with G1330B thermostat, G1316A column thermostat).

Reagents

Methanol (Cat. No. 1060352500; Merck KGaA, Germany) and formic acid (Cat. No. A117-50; Thermo Fisher Scientific, USA) of «HPLC-MS-Grade» quality were used to prepare the mobile phase. Substances of ascorbic acid (c.p. (chemically pure); cat. No. 160003; JSC «Lenreaktiv», Russia), sodium sulfite (r.g. (reagent grade); cat. No. 130231; JSC «Lenreaktiv», Russia), sodium thiosulfate pentahydrate (cat. No. SO07270500; Scharlau, Spain), sodium pyrosulfite (p. (pure); cat. No. 8.06.00804; JSC «Khimreaktivsnab», USA) were tested as antioxidants. Secondary standard samples produced by Sigma Aldrich (USA) were used as standard samples of the substances to be determined: noradrenaline (Cat. No. A7257-1G), adrenaline hydrochloride (Cat. No. E4642-5G), serotonin (Cat. No. 14927-25MG), dopamine hydrochloride (Cat. No. H8502-5G), (5-hydroxyindole-3-yl)-acetic acid (Cat. No. H8876-1G), 3,4-dihydroxyphenylacetic acid (Cat. No. 850217-1G), homovanilic acid (Cat. No. H1252-1G), vanillylmindalic acid (Cat. No. H0131-1G). The 3,4-dihydroxybenzylamine hydrobromide (3,4-DHBA) substance (Cat. No. 858781-1G) and the pharmacopoeial standard sample of sotalol (USP; Cat. No. 1617408) were used to prepare the internal standards (IS) solution (Fig. 1).

Chromatography-mass spectrometric determination technique

The chromatographic separation was performed under gradient mode using two columns Synergi Max RP (20 × 2.0 mm, 2.5 μm) and Synergi Fusion RP 80Å (250 × 4.6 mm, 4 μm) using 0.1 % aqueous formic acid solution and methanol as mobile phase components (Table 1). These reversed-phase columns had additional

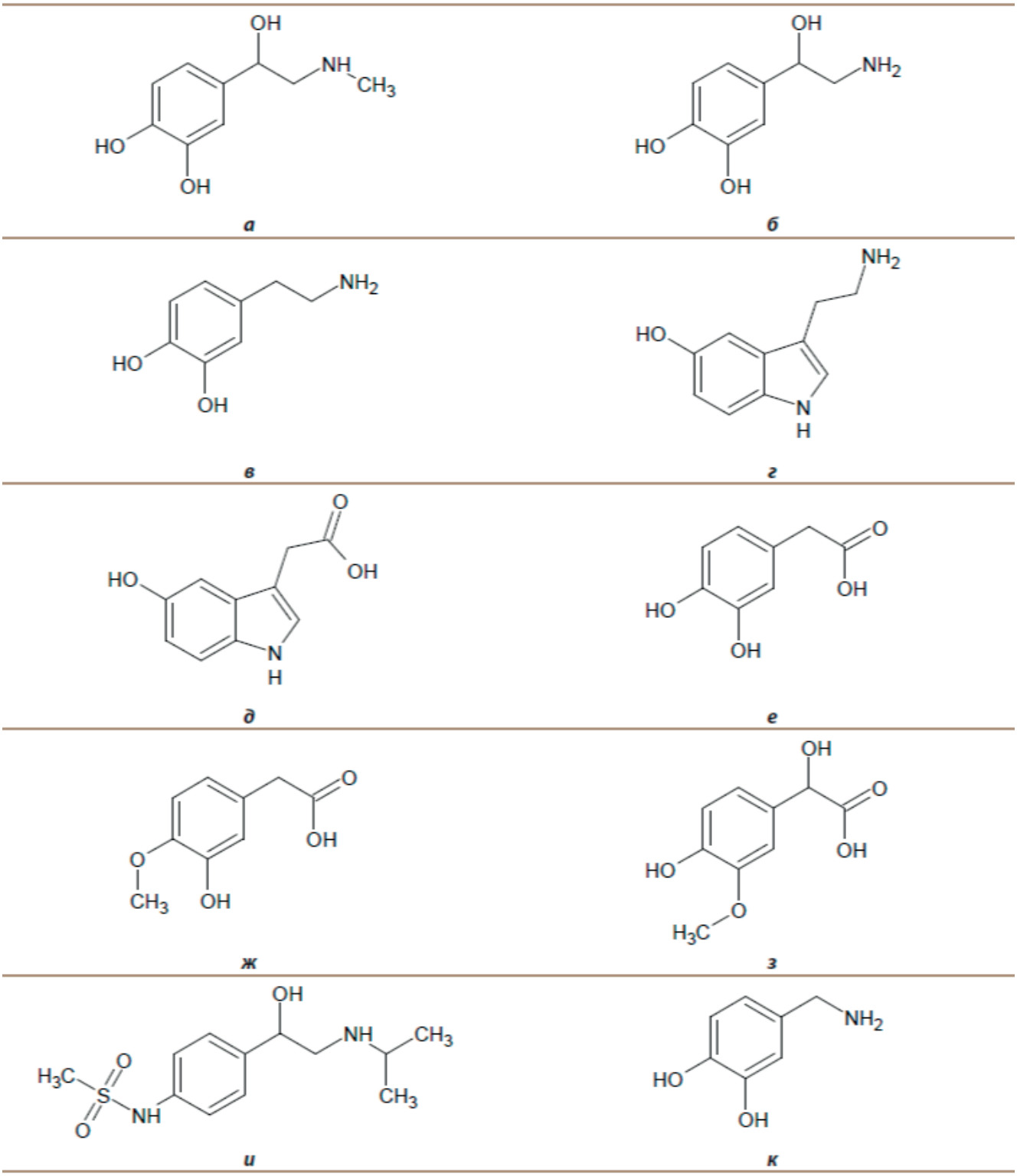

FIG. 1.
Structural formulae of adrenaline (а), noradrenaline (б), dopamine (в), serotonin (г), (5-hydroxyindole-3-yl)-acetic acid (д), 3,4-dihydroxyphenylacetic acid (е), homovanillic acid (ж), vanillylmindalic acid (з) and internal standards of sotalol (и) and 3,4-dihydroxybenzylamine (к)

hydrophilic functional groups required for retention of polar catecholamines. The thermostat temperature of the columns was 40 °C.

Detection was performed in MRM (multiple reaction monitoring) mode (Table 2) using electrospray ionisation (ESI, electrospray ionization). NA, Adr, Dop, 5HT, 5HIAA, and 3,4-DHBA were determined in positive polarity; DOPAC, HVA, and VMA were determined in negative polarity. Sotalol was detected in both polarities: positive for 5HT and 5HIAA concentration calculation; negative for DOPAC, HVA and VMA concentration calculation. This compound was used because of its structural similarity to catecholamines and the closeness of its retention time (10.4 min) to that of 5HT (10.1 min), 5HIAA (12.4 min), DOPAC (11.9 min), HVA (12.9 min) and VMA (10.8 min). 3,4-DHBA was used as an internal standard for the determination of norepinephrine, adrenaline and dopamine. Its choice is based on previously published methods for quantification of these analytes

TABLE 1

GRADIENT ELUTION PARAMETERS

Time, min	Flow rate, µl/min	A, %	B, %
0.0	650	98	2
2.0	650	98	2
9.0	650	20	80
14.1	650	20	80
14.5	1000	20	80
15.0	1000	20	80
15.1	1000	98	2
19.0	1000	98	2
19.2	650	98	2
21.0	650	98	2

Note. Mobile phase: A – 0.1 % aqueous solution of formic acid; B – methanol.

Method validation parameters

Full validation of the method was performed in accordance with the requirements of the guidelines for validation of bioanalytical methods (M10) of the International Council on Harmonisation (ICH) [22], FDA (Food and Drug Administration) guidelines [23], guidelines (Volume 1) of the Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Health of Russia (SCEEMP) [24] and Decision of the Council of the Eurasian Economic Commission (EAC) No. 85 (Annex 5) [25] related to chromatographic methods. Model homogenate mixtures were prepared from whole brain and striatum samples of Wistar rats. These objects were placed in a pre-calibrated tube of a manual homogeniser

TABLE 2

MASS SPECTROMETRIC DETECTION PARAMETERS

No.	Analyte	Polarity	ESI voltage, V	MRM junction		DP	EP	CE	CXP
				Q1	Q3				
1	Adr	+	4500	184.0	77.0	60	10	45	25
2	NA	+	4500	170.0	77.0	60	10	40	25
3	Dop	+	4500	154.0	119.0	60	10	35	25
4	5HT	+	4500	177.0	160.0	60	10	30	25
5	5HTAA	+	4500	192.0	146.0	60	10	20	13
6	DOPAC	–	–4500	167.0	123.0	–60	–10	–10	–30
7	HVA	–	–4500	181.0	122.0	–60	–10	–20	–25
8	VMA	–	–4500	197.0	137.0	–60	–10	–25	–30
9	3.4-DHBA	+	4500	140.0	77.0	60	10	25	25
10	Sot	+	4500	273.0	133.0	60	10	80	13
		–	–4500	271.0	174.0	–60	–10	–40	–16

Note. Sot – sotalol; DP – declustering potential; EP – entrance potential; CE – collision energy; CXP – collision cell exit potential.

TABLE 3

CALIBRATION AND QUALITY CONTROL SAMPLE CONCENTRATIONS

Designation	Concentration, ng/g							
	NA	Adr	Dop	5HT	DOPAC	5HTAA	HVA	VMA
K1 (LLOQ)	50	50	1250	75	200.0	150	80	67.50
K2	100	100	2500	150	400.0	300	160	135.00
K3	200	200	5000	300	800.0	600	320	270.00
K4	300	300	7500	450	1200.0	900	480	405.00
K5	400	400	10000	600	1600.0	1200	640	540.00
K6	600	600	15000	900	2400.0	1800	960	810.00
K7	900	900	22500	1350	3600.0	2700	1440	1215.00
K8	1200	1200	30000	1800.0	4800	3600	1920	1620.00
LQC	150	150	3750	225.0	600	450	240	202.50
MQC	500	500	12500	750.0	2000	1500	800	675.00
HQC	975	975	24375	1462.5	3900	2925	1560	1316.25
Dil	1800	1800	45000	2700.0	7200	5400	2880	2430

Note. Dil – concentration to estimate dilution effect; K – calibration concentration.

and homogenised after adding solvent in the required volume. After centrifugation, a standard solution of the analyte mixture was added to the supernatant fluid at a rate of 10 µl of standard solution per 190 µl of supernatant. To study the selectivity of the technique (series 1 in Table 6), as well as the effect of sample dilution, brain samples stored for 2 years at a temperature no higher than -20 °C were used, in which there was no analytical signal of the analytes. Linearity was assessed at 8 concentration levels (K1-K8), accuracy and coefficient of variation at 4 concentration levels (at the lower limit of quantitation (LLOQ, lower limit of quantitation), lower (LQC, lower quality control), middle (MQC, middle quality control) and higher (HQC, higher quality control) quality control levels), and dilution effect at the same concentration level (Dil) (Table 3).

RESULTS

At the initial stage of development, methanol was chosen as a solvent for the preparation of homogenates, since when water was used, the NA peak was absent on the chromatograms (retention time t_R = 4.7 min), and when acetonitrile was used, its signal-to-noise ratio (S/N, signal/noise) was 10 times lower (106/1). Samples were prepared using a manual homogeniser by adding solvent at a rate of 3 µl of solvent per 1 mg of brain tissue[1]. The homogenates were then centrifuged for 5 min at 10000 rpm and 10 µl of methanol standard solution with K4 concentration was added to 190 µl of supernatant (Table 3). A 120 µl methanol solution of a mixture of internal standards of sotalol and 3,4-DHBA was added to 50 µl of the obtained sample. The mixture was stirred on a shaker for 30 s and then centrifuged for 5 min at 10000 rpm.

In the next step, antioxidant (AO) solution selection was performed by studying the short-term stability (STS, short-term stability) of NA, Adr, Dop and DOPAC in brain homogenate samples, as well as the stability of these analytes in prepared samples in autosampler (ASS, autosampler stability). Aqueous solutions of ascorbic acid, sodium sulfite, sodium metabisulfite, and sodium thiosulfate at concentrations of 5 % and 10 % were used as stabiliser [21]. Antioxidant solution was added at a rate of 10 µl of solution per 50 µl of homogenate. The test results are summarised in Table 4.

[1] The tissue sample was weighed in a pre- calibrated homogeniser tube and then methanol was added to it in the desired amount: for example, if the tissue mass was 100 mg, 300 µl of methanol was added to it.

TABLE 4

ANTIOXIDANT STABILISER SELECTION RESULTS

			Antioxidant					
			Without AO (n = 2)	Ascorbic acid		$Na_2S_2O_3$		Na_2SO_3	
				5 % (n = 2)	10 % (n = 2)	5 % (n = 2)	10 % (n = 2)	5 % (n = 2)	10 % (n = 2)
Homogenate in methanol 1:3 (mass/vol.)	NA	ASS (+4 °C, 24 hours)	**56.24**	109.55	100.12	87.24	96.82	**74.82**	**71.43**
		STS (room temperature, 24 h)	**49.02**	91.02	114.71	100.48	103.19	**78.18**	**77.61**
	Adr	ASS (+4 °C, 24 hours)	**62.01**	97.66	101.95	**74.99**	**79.85**	**71.44**	**48.92**
		STS (room temperature, 24 h)	**59.52**	102.19	112.68	**34.19**	**62.90**	**22.20**	**38.45**
	Dop	ASS (+4 °C, 24 hours)	**39.28**	105.03	96.62	92.53	N/A	**83.01**	N/A
		STS (room temperature, 24 h)	**35.21**	101.39	95.96	**54.65**	N/A	**48.12**	N/A
	DOPAC	ASS (+4 °C, 24 hours)	**78.26**	107.21	97.88	93.37	87.44	94.24	90.94
		STS (room temperature, 24 h)	**72.42**	107.52	100.55	**78.27**	87.18	92.37	94.51
Homogenate in methanol 1:7 (mass/vol.)	NA	ASS (+4 °C, 24 hours)	90.00	90.15	–	–	–	–	–
		STS (room temperature, 24 h)	**75.45**	97.35	–	–	–	–	–
	Adr	ASS (+4 °C, 24 hours)	**70.73**	95.98	–	–	–	–	–
		STS (room temperature, 24 h)	**23.17**	100.57	–	–	–	–	–
	Dop	ASS (+4 °C, 24 hours)	88.31	103.93	–	–	–	–	–
		STS (room temperature, 24 h)	**49.93**	98.61	–	–	–	–	–
	DOPAC	ASS (+4 °C, 24 hours)	95.55	97.28	–	–	–	–	–
		STS (room temperature, 24 h)	90.24	95.10	–	–	–	–	–

Note. N/A – no chromatographic peak of the analyte.

The addition of sodium sulfite and sodium thiosulfate solutions failed to prevent oxidation of all analytes (Table 4). For instance, when Na_2SO_3 solutions were used, only the DOPAC concentration fell within the required range of 85–115 % of the initial value. The use of $Na_2S_2O_3$ solution at a concentration of 5 % prevented the oxidation of noradrenaline as well as dopamine and DOPAC in the prepared samples in the autosampler. The chromatographic peak of Dop was not detected in samples with the addition of 10 % solutions of $Na_2S_2O_3$ and Na_2SO_3. When sodium metabisulfite solutions were added to the methanol homogenates, this salt precipitated, so these samples were not analysed. Only when ascorbic acid was used the concentrations of all analytes in the short-term stability and autosampler stability tests were in compliance. For further testing, an aqueous solution of ascorbic acid at the lowest concentration of 5 % was chosen to minimise the risk of contaminating the chromatography column, ion source and ion optics of the mass spectrometer with excessive amounts of this substance.

At the next stage of the study, matrix effects were studied. Freshly sampled rat brain homogenates, freshly sampled striatum homogenates, and brain homogenates stored for 2 years at a temperature not exceeding -20 °C, obtained from 6 different animals, were used

TABLE 5

EVALUATION OF THE MATRIX EFFECT IN THE DETERMINATION OF ANALYTES IN BRAIN HOMOGENATES

	Analytes		Homogenate in a ratio of 1:3	Homogenate in a ratio of 1:7
CV (NMF), %	NA	LQC	13.48	11.39
		HQC	14.09	4.80
	Adr	LQC	13.94	7.46
		HQC	12.76	10.56
	Dop	LQC	8.95	6.07
		HQC	5.15	7.29
	5HT	LQC	13.98	5.93
		HQC	**17.69**	4.37
	5HIAA	LQC	11.14	9.77
		HQC	6.25	5.23
	DOPAC	LQC	**26.82**	7.89
		HQC	**21.38**	7.49
	HVA	LQC	11.10	5.37
		HQC	11.95	8.65
	VMA	LQC	10.90	8.37
		HQC	10.31	5.07
Peak area ratio «analyte/ internal standard» (mean value)	5HT (brain homogenate) (*n* = 4)	LQC	0.183	0.135
		HQC	1.464	0.956
	5HT (striatum homogenate) (*n* = 2)	LQC	0.142	0.132
		HQC	1.021	0.940
	DOPAC (brain homogenate) (*n* = 4)	LQC	4.635	2.223
		HQC	33.255	15.538
	DOPAC (striatum homogenate) (*n* = 2)	LQC	2.701	2.115
		HQC	22.502	15.327

Note. CV (NMF) – coefficient of variation for normal matrix factorization.

for the preparation of model mixtures. According to the requirements of Russian and foreign guidelines for validation of bioanalytical techniques [23-25], each of 6 samples were analysed at the level of LQC and HQC concentrations (Table 3), as well as a sample of each homogenate without the addition of a standard to subtract the signal of endogenous substances (Table 5).

At the initial conditions of sample preparation, the value of the coefficient of variation (CV) of the normalised matrix factorization (NMF) in the determination of 5HT and DOPAC exceeded the permissible limit of 15 % (Table 5). To reduce matrix effects, the ratio of methanol to brain tissue in the preparation of homogenates was adjusted: the sample was prepared at a rate of 7 µl of solvent per 1 mg of tissue. Under these sample preparation conditions, the CV (NMF) result was in compliance for all analytes. Afterwards, the stability of NA, Adr, Dop and DOPAC was re-tested in samples of methanol homogenate prepared in a 1:7 ratio using the previously selected 5 % aqueous ascorbic acid solution (Table 4). The concentrations of all analytes in the ASS and STS tests were within the acceptable range of 85.0–115.0 % of the initial value.

Therefore, in order to quantify the concentration of the studied substances in brain tissue, the homogenate was prepared manually at a ratio of 1:7 (tissue weight/ volume of methanol). The sample was then centrifuged for 5 min at 10000 rpm. And stabilised with an aqueous solution of ascorbic acid at a concentration of 5 % at a rate of 10 µl of solution per 50 µl of supernatant. Thereafter, 120 µl of methanol solution of internal standards was added to 60 µl of the mixture, stirred for 30 s and centrifuged for 5 min at 10000 rpm. The supernatant fluid was transferred to a microtiter plate and analysed by HPLC-MS/MS.

After selecting the final sample preparation conditions and chromatography-mass spectrometric determination, a complete validation of the methodology was performed. The analytical range for Adr and NA was 50–1200 ng/g, for Dop – 1.25–30.00 µg/g, for 5HT – 75–1800 ng/g,

for DOPAC – 200–4800 ng/g, for 5HIAA – 150–3600 ng/g, for HVA – 80–1920 ng/g, and for VMA – 67.5–1620 ng/g. The dependence of the analyte/internal standard peak area ratio on the concentration of each compound was linear. In evaluating the selectivity of the method using brain homogenate samples stored at a temperature not exceeding -20 °C for 2 years, the area of chromatographic peaks in blank matrices for analytes did not exceed 20 % of the peak area in LLOQ samples, for internal standards of sotalol, for internal standards of 3,4-DHBA, the area of chromatographic peaks did not exceed 5 % of the peak area in LLOQ samples (Fig. 2, 3).

The mean values of the calculated determination concentrations of all studied compounds were within 85–115 % of the nominal value for LQC, MQC and HQC concentration levels, within 80–120 % for LLOQ concentration level,

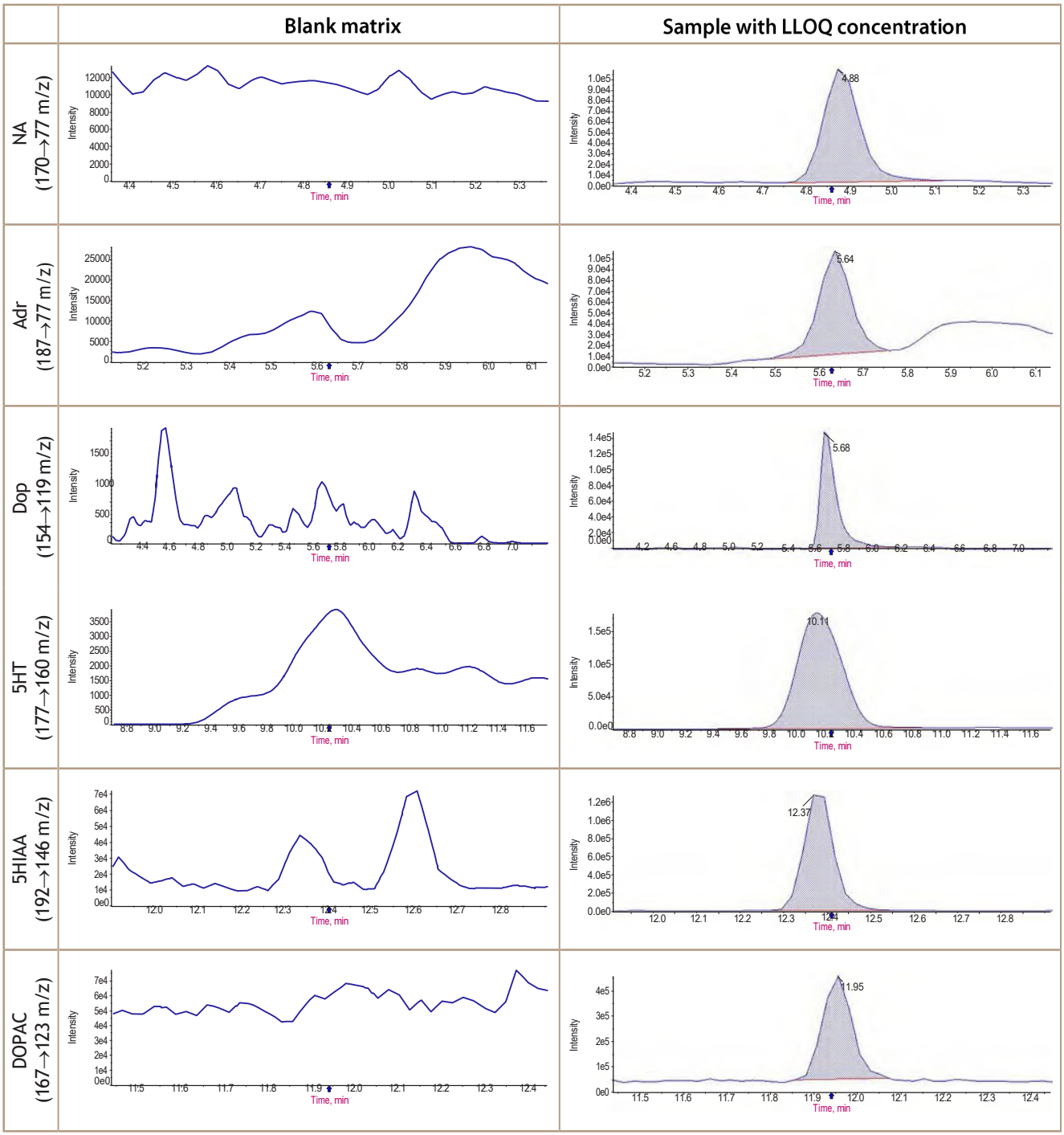

FIG. 2.
Examples of MRM chromatograms of blank matrix and sample with added standard at LLOQ level (analytes - NA, Adr, Dop, 5HT, 5HIAA, DOPAC)

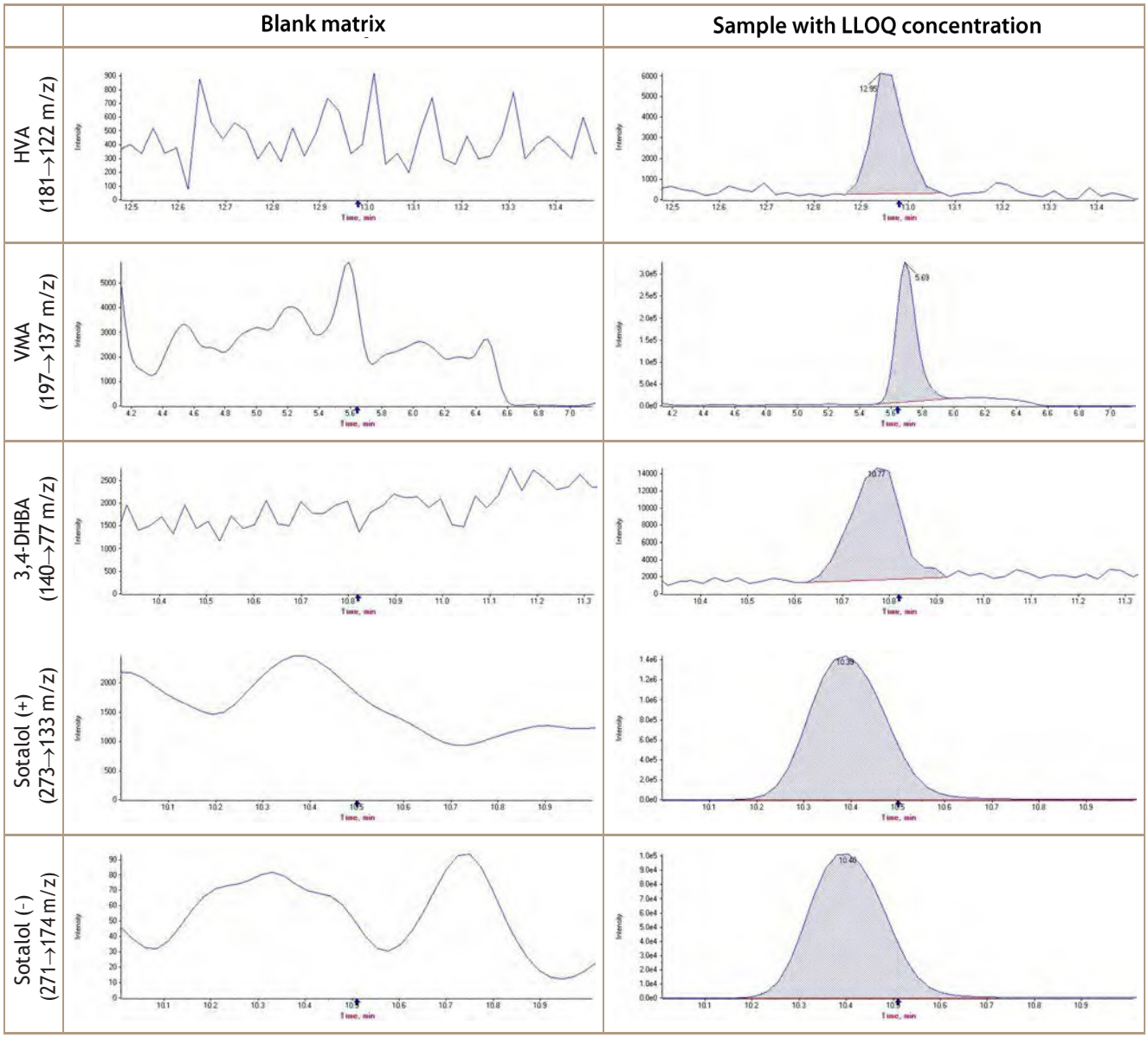

FIG. 3.
Examples of MRM chromatograms of blank matrix and sample with added standard at LLOQ level (analytes - HVA, VMA, 3,4-DHBA (BC), sotalol (BC))

including the assessment of the selectivity of the methodology (Table 6, series 1). The coefficient of variation of the calculated concentrations did not exceed 15 %. In this case, the result of analytical series 2 and 3 (Table 6), performed using samples of freshly collected brain homogenates, was calculated considering the endogenous content of analytes in samples without the addition of the standard. Double dilution of samples with the content of the studied substances exceeding the analytical range (Dil; Table 3) with blank matrix did not affect the metrological characteristics of the method: the value of calculated concentrations of analytes was within the range of 89.76–94.99 % of the nominal value, the CV value – within the range of 2.77–5.88 % (Table 6). There was no carryover of analytes and internal standards from the previous sample.

The selected antioxidant solution provides stability of analytes in homogenate samples during 24 h storage at room temperature, 3 freezing/thawing cycles (FTS, freezing/thawing stability), 30 days storage in a freezer at a temperature not exceeding -20 °C (LTS, long-term stability), as well as stability in prepared samples in an autosampler during 48 h at +4 °C (Table 7).

The developed method was tested by analyzing striatum samples obtained from 6 intact male Wistar line rats. Brain samples were chilled with liquid nitrogen immediately after collection, and the striatum was extracted. Homogenization and addition of stabilizer solution was performed no later than 20 min after sampling. The results of NA, Adr, Dop, 5HT, DOPAC, 5HIAA, VMA, and HVA determinations are presented in Table 8.

TABLE 6

VALIDATION RESULTS OF THE DEVELOPED METHODOLOGY

Indicators		Adr		NA		Dop		5HT		5HIAA		DOPAC		HVA		VMA	
Selectivity		Interference in the retention times of analytes in blank matrices did not exceed 20 % of the LLOQ level, in the retention times of internal samples did not exceed 5 % of the peak area*															
LLOQ		50 ng/g		50 ng/g		1.25 µg/g		75 ng/g		150 ng/g		200 ng/g		80 ng/g		67.5 ng/g	
Calibration range (linear dependence)		50-1200 ng/g		50-1200 ng/g		1.25-30.00 µg/g		75-1800 ng/g		150-3600 ng/g		200-4800 ng/g		80-1920 ng/g		67.5–1620 ng/g	
Accuracy and coefficient of variation		**Acc., %**	**CV, %**	**Acc., %**	**CV, %**	**Acc., %**	**CV, %**	**Acc., %**	**CV, %**	**Acc., %**	**CV, %**	**Acc., %**	**CV, %**	**Acc., %**	**CV, %**	**Acc., %**	**CV, %**
Series 1 ($n = 6$**)	LLOQ	112.73	8.34	109.61	12.06	104.46	13.48	82.64	6.28	103.52	9.49	115.69	7.62	86.68	9.39	100.42	7.97
	LQC	92.25	8.66	96.79	8.94	95.74	7.96	88.31	5.01	98.19	8.97	97.73	9.49	98.98	9.11	102.87	3.34
	MQC	97.49	8.59	90.04	7.47	97.45	6.48	86.98	2.30	111.27	5.75	98.66	6.60	104.73	5.71	109.07	2.72
	HQC	104.51	3.78	91.97	4.04	100.89	5.95	89.17	2.95	114.60	3.25	105.36	3.78	108.76	7.68	110.87	3.96
Series 2 ($n = 6$**)	LLQC	85.44	8.06	94.21	12.11	90.42	6.40	110.94	7.00	108.36	6.91	114.02	5.72	101.01	6.36	111.76	3.39
	LQC	99.82	9.09	92.70	9.06	95.90	10.04	93.18	3.59	106.26	3.19	106.73	5.69	110.72	1.40	102.01	3.73
	MQC	96.97	9.16	91.98	8.04	92.33	8.77	87.29	3.36	113.84	4.22	97.66	5.03	104.49	6.52	96.82	2.55
	HQC	104.38	9.21	101.75	6.78	96.28	6.17	93.33	2.72	111.91	2.13	99.15	3.12	112.17	5.00	102.51	3.01
Series 3 ($n = 6$**)	LLQC	101.48	13.05	98.56	12.01	106.51	5.59	92.11	3.81	116.19	4.06	112.96	4.18	100.72	11.90	117.89	2.41
	LQC	93.43	9.26	93.27	7.95	92.70	4.95	91.44	3.09	110.03	6.76	108.07	3.10	100.96	8.29	109.49	3.73
	MQC	89.70	3.36	96.11	5.02	91.00	2.70	93.15	3.48	95.31	10.62	98.65	3.44	99.25	1.80	105.30	2.93
	HQC	87.67	5.39	99.14	5.51	93.76	7.27	96.03	3.22	86.07	2.35	97.61	3.20	105.11	3.46	106.65	2.61
Accuracy and coefficient of variation between series ($n = 18$**)	LLQC	99.89	15.58	100.79	14.08	100.46	12.13	92.11	3.81	109.36	8.70	114.22	6.29	102.55	12.13	109.99	8.42
	LQC	95.17	9.95	94.26	9.14	94.78	8.37	91.44	3.09	104.82	8.36	104.17	8.01	110.45	8.75	104.80	4.95
	MQC	94.72	8.78	92.71	7.65	93.59	7.37	93.15	3.48	106.81	10.67	98.32	5.36	102.82	5.91	103.73	5.82
	HQC	98.85	10.72	97.62	7.26	96.98	7.35	96.03	3.22	103.31	13.00	100.70	4.90	108.68	6.49	106.68	4.71
Dilution effect ($n = 6$)	Acc., %	93.77		90.73		94.99		94.71		89.76		93.58		91.17		93.36	
	CV, %	4.63		4.05		3.60		5.88		2.77		3.97		4.68		4.24	

Note. * – selectivity evaluation was performed within series 1; ** – number of samples at each concentration level; Acc. (accuracy) – deviation of the mean value of calculated concentrations from the nominal value.

TABLE 7

RESULTS OF STABILITY ASSESSMENT OF ANALYTES IN BRAIN HOMOGENATES

Indicators			Adr	NA	Dop	5HT	5HIAA	DOPAC	HVA	VMA
% of initial concentration	STS (24 hours at room temperature) (*n* = 6*)	LQC	100.88	96.58	99.85	91.80	96.93	97.80	99.01	101.79
		HQC	100.47	94.03	97.51	90.64	105.85	102.21	103.88	107.47
	FTS (*n* = 6*) (3 cycles)	LQC	100.60	101.04	101.40	95.56	97.26	100.69	102.62	105.00
		HQC	103.22	96.78	103.04	97.63	101.48	101.13	102.01	107.44
	ASS (48 h at +4 °C) (*n* = 6*)	LQC	102.81	98.42	98.76	96.21	99.61	100.65	101.47	100.41
		HQC	102.48	95.96	96.22	93.82	100.83	97.95	100.79	104.58
	LTS (30 days at a temperature not exceeding -20 °C) (*n* = 6*)	LQC	103.11	100.12	104.84	95.50	98.41	102.41	102.43	96.99
		HQC	102.25	99.08	100.74	98.70	98.75	97.84	98.65	98.85

Note. * – number of samples at each concentration level; FTS – freezing/thawing stability after 3 cycles; LTS – long-term stability.

TABLE 8

RESULTS OF QUANTIFICATION OF NORADRENALINE, ADRENALINE, DOPAMINE, SEROTONIN, DOPAC, 5HIAA, VMA, AND HVA IN RAT STRIATUM SAMPLES

	Concentration, ng/g							
	Adr	NA	Dop	5HT	5HIAA	DOPAC	HVA	VMA
Mean values (*n* = 6)	less than LLOQ	354.85	9181.30	595.86	1180.49	1851.35	896.26	less than LLOQ
SD	–	33.43	1497.84	137.25	244.61	283.25	266.38	–
CV, %	–	9.42	16.31	23.03	20.72	15.30	29.72	–

DISCUSSION

Following the selection of optimal analysis conditions for the preparation of brain tissue samples after their mechanical homogenisation, sample dilution with methanol solution of internal standards was chosen. This significantly improves sample preparation throughput compared to work that has used liquid-liquid extraction [18], dialysis [13, 17, 19], and analyte derivatisation [13, 14]. The use of HPLC-MS/MS provides an advantage in selectivity and sensitivity of the method over HPLC-UV [8] and HPLC-EM [9-12] in performing pharmacodynamic studies of drugs. For instance, when using these methods, the molecules of MPs under study may be extracted and coeluted together with the analytes, thus giving overestimated quantification results [3]. The chromatographic analysis time is 21 min, which is longer than in the methods of E. Grouzmann et al. [5], N. Hwang et al. [6], L. Fang et al. [7], S. Greco et al. [14], C. Ji et al. [15]. These studies, however, analyse a smaller number of monoamine neurotransmitters and their metabolites.

A stabiliser selection approach was adopted by pre-assessing the short-term stability of analytes in homogenates and the stability of samples in the autosampler both without and with added antioxidants. A similar approach was used for blood plasma samples in [21], but it did not involve ASS testing. Reasonable results with the choice of antioxidant were achieved only when aqueous solutions of ascorbic acid in a volume ratio of 1:5 were added to the homogenate supernatant (Tables 5, 7). This is due to the fact that in methanol samples, salt reducing agents (Na_2SO_3, $Na_2S_2O_3$, $Na_2S_2O_5$) dissolve much worse than the selected stabilizer. In the study of long-term stability, a temperature of -20 °C or less is considered to be sufficient for sample storage (Table 7).

The concentrations of Dop, NA, 5HT, 5HIAA, DOPAC, and HVA in rat striatum samples obtained in the course of method validation coincide with the data published in J. Lu et al. [2] and N.N. Khlebnikova et al. [10]. No Adr and VMA chromatographic peaks were revealed in the chromatograms of the tested samples, indicating that the samples were collected correctly.

CONCLUSION

The developed method for quantitative determination of norepinephrine, adrenaline, dopamine, serotonin, DOPAC, 5HIAA, VMA, HVA in rat brain samples conforms to the requirements of NCESMP, EAC, ICH, FDA guidelines in terms of selectivity, calibration dependence, accuracy and coefficient of variation within and between cycles, dilution effect, carry-over effect from previous sample, matrix effect, stability. The implementation of the chosen stabilisation method prevents oxidation of analytes during sample preparation and analysis, as well as their storage for at least 30 days. It substantially reduces the risks of unreliable results in preclinical pharmacodynamic testing and exclusion of pharmacologically effective compounds from the experiment as a consequence of falsely underestimated concentrations of neurotransmitters and their metabolites in the samples.

Study limitations

This method has been validated and shown to be convenient for the determination of analytes in rat brain tissue samples. The use of the method for quantification of the substances under study in another animal species would require partial validation by assessing the matrix effect, calibration dependence and selectivity.

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Conflict of interest

The authors declare no conflict of interest.

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