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LC-MS/MS-based Quantification of Ten Neurotransmitters in Rat Limbic System and Serum: Application to Chronic Unpredictable Mild Stress-Induced Depression Rats

Mingyan Ma[1]*, Qiangxiang Chen[2], Wen Cao[1], Yubo Zhou[1], Aijuan Yan[1], and Yanru Zhu[3]

[1]Laboratory of Pharmaceutical Analysis, School of Pharmacy, Zhejiang Pharmaceutical University, No. 888, Yinxian Road, Ningbo, Zhejiang Province, 315100, PR China
[2]ShaoXing Institution for Food and Drug Control, No. 286, Century East Street, Mashan Town, Shaoxing, Zhejiang Province, 312000, PR China
[3]Laboratory of Pharmaceutical Analysis, School of Pharmacy, Shenyang Pharmaceutical University, No. 103, Wenhua Road, Shenyang, Liaoning Province, 110016, PR China

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Abstract : As one of the most common mood disorders, numerous studies have shown depression is the main risk factor for non-suicidal self-harm. The pathogenesis of depression is complex, and a comprehensive and rapid measurement of monoamine neurotransmitters and their metabolites will be very helpful in understanding the pathogenesis of depression. Therefore, a rapid and sensitive underivatized liquid chromatography-tandem mass spectrometry method was developed and validated for the simultaneous monitoring of the levels of ten neurotransmitters and their metabolites in rat serum and limbic system and successfully applied to quantify the changes of neurotransmitter levels in chronic unpredictable mild stress-induced rats. The analytes studied were mainly involved in tyrosine metabolism, tryptophan metabolism, and glutamate cycling pathways, which are important in the pathogenesis of depression. It had been verified the method was sensitive and effective, with satisfactory linearity, and met the requirements of biological sample determination. Levels of neurotransmitters in rat serum, hippocampus, amygdala, prefrontal cortex, striatum, and hypothalamus were determined via the method. The results showed serotonin, dopamine, norepinephrine, and their metabolites were decreased, glutamine was increased, and glutamate was disturbed in chronic unpredictable mild stress-induced depression rats. This method provides a new approach to studying the pathogenesis of depression and other neurological disorders.

Keywords : Neurotransmitter, Limbic system, Chronic unpredictable mild stress, Depression, Liquid chromatography - tandem mass spectrometry

Introduction

Depression and anxiety disorders affect roughly a quarter of the general population in a lifetime, which significantly damages the quality of life. According to the World Health Organization, depression will rise to first place in the global burden of disease and become the leading cause of disability globally by 2030.[1] Depression could lead to the interruption of interpersonal relationships, poor academic performance, drug abuse, and even suicide. There is a strong relationship between depression and physical health, including tuberculosis and cardiovascular disease.[2] The mechanisms of depression are quite complex, and a large number of studies have been carried out to clarify the pathogenesis. The current elucidation of the pathogenesis of depression mainly includes the monoamine hypothesis, neuroinflammation hypothesis, receptor hypothesis, etc.[3] Among them, the most important monoamine hypothesis holds that serotonin (5-HT), norepinephrine (NE), dopamine (DA) and other monoamine neurotransmitters at insufficient levels are the biological basis of depression. 5-HT is synthesized from the essential amino acid tryptophan, which plays a biological role mainly by binding to the 5-HT receptor.[4] DA and NE are other monoamine neurotransmitters closely related to depression besides serotonin. DA is

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*Reprint requests to Mingyan Ma
https://orcid.org/0000-0002-2288-4703
E-mail: mamy@zjpu.edu.cn

released by both peripheral and central nervous systems, which has a critical role in the regulation of movement and mood, in addition to the modulation of brain reward and motivation pathways.[5] Glutamate (GLU) is the most important excitatory neurotransmitter in the brain, and previous studies have found that glutamate metabolic circuit disorders are related to the pathological mechanism of depression.[6] Therefore, the use of sensitive targeted analysis methods to study the metabolic transformation of monoamine neurotransmitters and GLU metabolism in the central systems will help to understand the potential mechanisms in depression.

The limbic system, derived from the concept of limbic lobes proposed by Broca,[7] is the general term for the brain tissue and the closely connected neural structures and nuclei which evolved from the ancient cortex in the central nervous system,[8] including hippocampus, amygdala, hypothalamus, etc. The limbic system plays an important role in regulating emotion, perception, and cognitive impairment,[9] which is inseparable from the pathogenesis of depression. The research on the target area of depression has shifted from the hippocampus to other related areas lately, and exploring the method of treating depression with the neural circuit composed of multiple brain regions as the target would become a new trend in the future.

Until now, several chromatographic analysis methods have been reported for neurotransmitter determination, such as ion exchange chromatography,[10] high- performance liquid chromatography[11] and liquid chromatography-mass spectrometry (LC-MS),[12,13] etc. Among them, the LC-MS methods had been widely reported because of high sensitivity, selectivity and reproducibility. Previous studies have been developed for neurotransmitters provided valuable experience for our analysis,[14,15] but they have all undergone more complicated pre-treatments such as chemical derivatization or micro-extraction, which may lead to time-consuming operations, unstable derivatization, and inaccurate determinations. Therefore, it is imperative to develop an analysis method with easy pre-processing operation. At present, the background subtraction method[16,17] is often used to simplify the pre-processing method and obtain accurate results. However, this method is only suitable for determination when the level of endogenous substances is consistent, which is difficult to determine the variable endogenous level such as depression. Based on this, the alternative matrix method is used to avoid this problem, which requires similar matrices effect and extraction recoveries in both alternative and original matrix.[18,19] Lisha et al.[20] developed a liquid chromatography - tandem mass spectrometry (LC-MS/MS) method with an alternative matrix for the quantification of tryptophan metabolites and neurotransmitters in the serum and brain of mice, and María et al.[21] developed an LC-MS/MS method for the determination of neurotransmitters and amino acids in mouse cerebrospinal fluid. Referring to previous research, we established and validated an accurate, sensitive and underivatized LC-MS/MS method to quantitative the monoamine neurotransmitters, excitatory neurotransmitter and their precursors and metabolites (Figure 1) in peripheral serum and different brain regions of the central limbic system including the amygdala (AM), striatum (ST), prefrontal cortex (PFC), hypothalamus (PO), and hippocampus (HIP) simultaneously. Compared with Lisha et al, this method was faster and simultaneously measured the levels of DA and NE and their metabolites DOPAC, HVA and MHPG in the tyrosine. The LC-MS/MS method was successfully applied to explore the variable metabolites in chronic unpredictable mild stress (CUMS)-induced depression in rats.

Materials and methods

Reagent and chemicals

Serotonin (5-HT, 98.0% purity by HPLC) was obtained from Sigma (St. Louis, MO, USA). NE, GLU, L-glutamine, 5-hydroxytryptophan, 3,4-dihydroxyphenylacetic acid, and 5-hydroxyindole acetic acid (GLN, 5-HTP, DOPAC, 5-HIAA, 98.0% purity by HPLC) were obtained from Shanghai Xiyuan Biotechnology Co., Ltd. (Shanghai, China). 3-methoxy-4-hydroxy-phenylglycol (MHPG, 98.0% purity by HPLC) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). DA (98.0% purity by HPLC) was obtained from Shanghai Maclin Biochemical Technology Co., Ltd. (Shanghai, China). Homovanillic acid (HVA, 98.0% purity by HPLC) was obtained from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). [^{2}H5]-L-glutamic acid (Internal standard, IS, 99.0% purity by HPLC) was obtained from Shanghai Zhenzhun Biological Technology Co., Ltd (Shanghai, China). Acetonitrile and methanol (HPLC grade) were bought from Sigma (St. Louis, MO, USA). Formic acid (HPLC grade) was purchased from Concord Technology Co., Ltd (Tianjin, China). Purified water was supplied by a Milli-Q Integral 10 system (MA, USA).

Animals

Four-week-old specific pathogen-free healthy male Sprague Dawley rats were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Studies were carried out under the principles for Animal Experimentation of Shenyang Pharmaceutical University. The protocol was approved by the Animal Ethics Committee of the institution (No. SYPU-IACUC-C2021-4-6-204).

Instrumentation and conditions

AB SCIEX Triple Quad^{TM} 5500 with ACQUITY UPLCI-Class UPLC system (Waters, Milford, MA, USA), equipped with an electrospray ionization source (SCIEX, USA) were used to analyze the biological samples. A Phenomenex Synergi Fusion-RP 80Å LC column (50 × 2 mm, 4 μm, Phenomenex, USA) was used to complete the sepa-

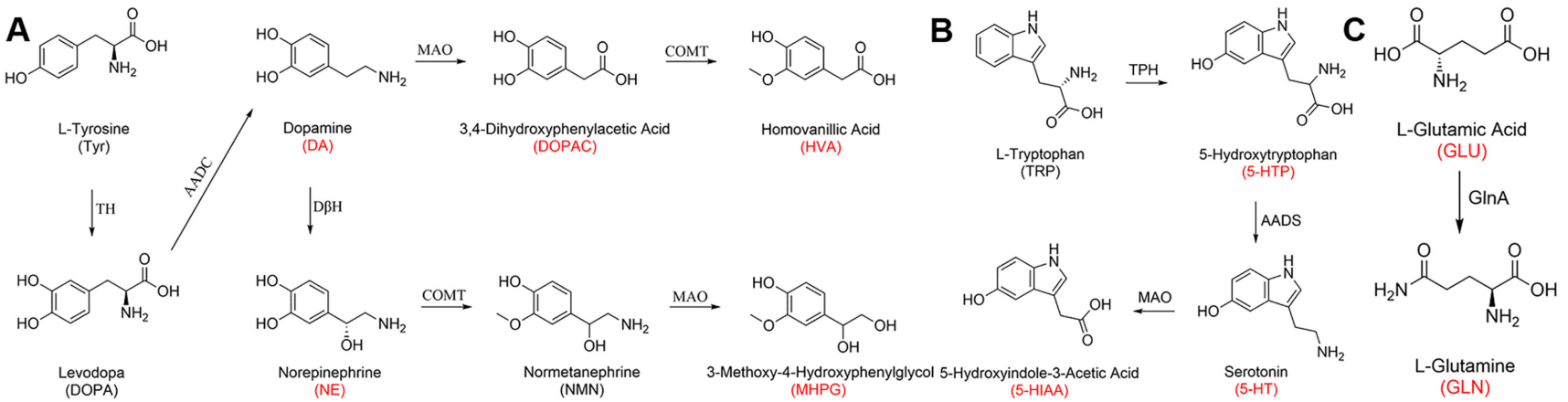

Figure 1. Structures and metabolic transformation of the compounds used in the study. (A) Tyrosine metabolic pathway, (B) Tryptophan metabolic pathway, (C) Glutamate metabolic pathway. AADC: Aromatic l-amino acid decarboxylase, COMT: Catechol-O-methyltransferase, DβH: Dopamine beta hydroxylase, GluA: glutamine synthetase, MAO: Monoamine oxidase, TH: Tyrosine hydroxylase, TPH: Tryptophan hydroxylase. Abbreviations for the components are listed below in parentheses and the targeted analytes are red.

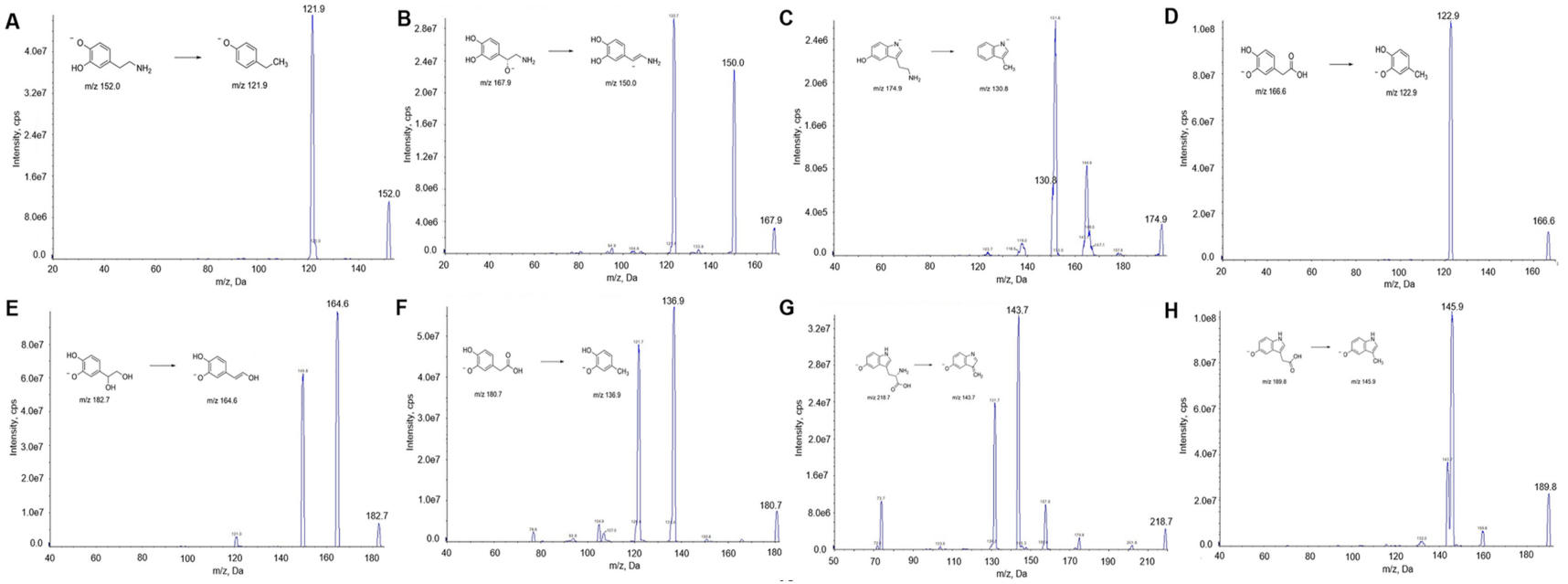

Figure 2. Full-scan product ion spectra of $[M-H]^-$ ions for neurotransmitters and $[^2H5]$-L Glutamic Acid. (A) DA, (B) NE, (C) 5-HT, (D) DOPAC, (E) MHPG, (F) HVA, (G) 5-HTP, (H) 5-HIAA.

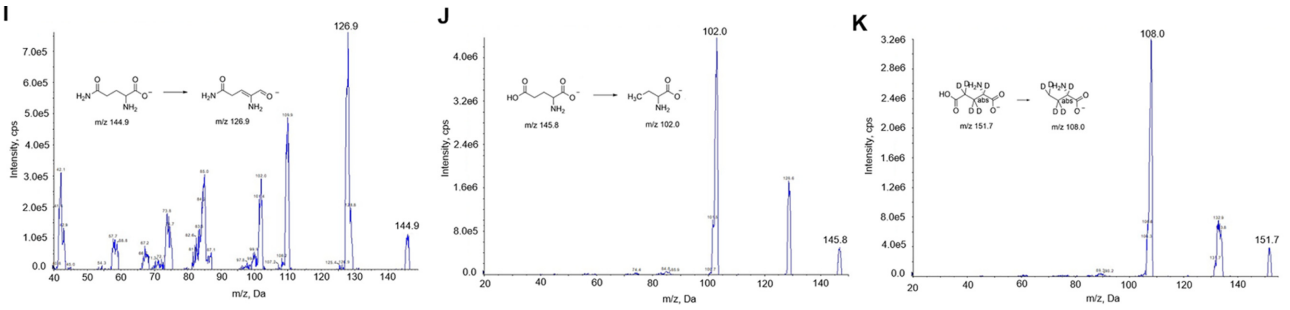

Figure 2. (Continued) (I) GLN, (J) GLU, (K) IS.

ration of multiple neurotransmitters. Elution was accomplished using the mobile phase of 0.1% formic acid-H_2O (A) and acetonitrile (B). The analytes were eluted using an isocratic method with 50% A at a flow rate of 0.3 mL·min^{-1}.

The mass spectrometer was operated in the negative mode using multiple reaction monitoring (MRM). Full-scan product ion spectra of [M-H]$^-$ ions for neurotransmitters were shown in Figure 2. The other parameters were as follows: ion spray voltage of -4500 V, source temperature of 450°C, the ion source gas I at 45 psi, gas II at 50 psi, curtain gas at 20 psi, collision energy (CE) and declustering potential (DP) were shown in Table S1. The chromatograms and data were integrated by the Analyst 1.6 software.

Preparation of calibration standards and QCs

Stock solutions

Each neurotransmitter standard was accurately weighed. Except 5-HT and NE were dissolved in methanol, and GLU and GLN were dissolved in formic acid, the other standards were dissolved in mixture of formic acid-H?O-methanol (10:45:45, *v/v/v*) to prepare stock solutions. The concentration of DA, NE, 5-HT, DOPAC, MHPG, HVA, 5-HTP, 5-HIAA, GLN and GLU stock solutions were 1.0 mg·mL^{-1}, 1.0 mg·mL^{-1}, 5.0 mg·mL^{-1}, 1.0 mg·mL^{-1}, 1.0 mg·mL^{-1}, 5.0 mg·mL^{-1}, 0.5 mg·mL^{-1}, 5.0 mg·mL^{-1}, 10.0 mg·mL^{-1}, and 10.0 mg·mL^{-1}, respectively. Before use, the stock solutions were mixed and serially diluted with 0.2% formic acid-H_2O and methanol (1:1, *v/v*) to give a mixed working solution series. 0.15 μg·mL^{-1} of IS were prepared for further analysis. All solutions were stored in a refrigerator at -80°C for further analysis.

Calibration standards and QC solutions

The maximum concentration mixed standard solution of DA, NE, 5-HT, DOPAC, MHPG, HVA, 5- HTP, 5-HIAA, GLN, GLU (10, 10, 50, 10, 10, 50, 5, 50, 100, 100 μg·mL^{-1}) were prepared by diluting the stock solution with solvent (0.2% formic acid-H_2O: methanol, 1:1, *v/v*). A series of solutions at concentrations of DA (10, 5, 2.5, 1, 0.5, 0.2, 0.1 μg·mL^{-1}), NE (10, 5, 2.5, 1, 0.5, 0.2, 0.1 μg·mL^{-1}), 5-HT (50, 25, 12.5, 5, 2.5, 1, 0.5 μg·mL^{-1}), DOPAC (10, 5, 2.5, 1, 0.5, 0.2, 0.1 μg·mL^{-1}), MHPG (10, 5, 2.5, 1, 0.5, 0.2, 0.1 μg·mL^{-1}), HVA (50, 25, 12.5, 5, 2.5, 1, 0.5 μg·mL^{-1}), 5-HTP (5, 2.5, 1.25, 0.5, 0.25, 0.1, 0.05 μg·mL^{-1}), 5-HIAA (50, 25, 12.5, 5, 2.5, 1, 0.5 μg·mL^{-1}), GLN (100, 50, 25, 10, 5, 2, 1 μg·mL^{-1}), and GLU (100, 50, 25, 10, 5, 2, 1 μg·mL^{-1}) were prepared by diluting the maximum concentration mixed standard solution. High-, medium-, and low-concentration quality control (QC) solutions were DA (0.3, 4, 8 μg·mL^{-1}), NE (0.3, 4, 8 μg·mL^{-1}), 5-HT (1.5, 20, 40 μg·mL^{-1}), DOPAC (0.3, 4, 8 μg·mL^{-1}), MHPG (0.3, 4, 8 μg·mL^{-1}), HVA (1.5, 20, 40 μg·mL^{-1}), 5-HTP (0.15, 2, 4 μg·mL^{-1}), 5-HIAA (1.5, 20, 40 μg·mL^{-1}), GLN (3, 40, 80 μg·mL^{-1}), and GLU (3, 40, 80 μg·mL^{-1}) prepared independently with neurotransmitters standards.

Preparation of actual biological samples

Serum samples

Serum (100 μL) was mixed with 10 μL IS and 10 μL methanol, protein precipitated with 300 μL 0.1% formic acid-acetonitrile, vortexed for 5 min, and centrifuged at 4°C 10,010 *g* for 15 min, the supernatant was dried under nitrogen flow. The residue was re-dissolved in a 100 μL mobile phase, vortexed, centrifuged, and 3 μL supernatant was injected into the LC-MS system. QC solution (10 μL) instead of methanol were added to 100 μL biological matrix to prepare QC samples.

Limbic system samples

Each brain region of the limbic system was weighed, and the precooled saline was added at the ratio of 1:9 (*w/v*). The brain region homogenate was prepared by the ultrasonic cell crusher (Scientz, Zhejiang, China) under ice-cooling. Homogenate (100 μL) was mixed with 10 μL IS and 10 μL methanol, and the rest of operation was the same as the serum samples.

LC-MS Method validation

Method validation was performed according to the Food and Drug Administration guiding principles *Guidance for Industry, Bioanalytical Method Validation.*[22]

Linearity and LLOQ

Because the biological sample contained the endogenous

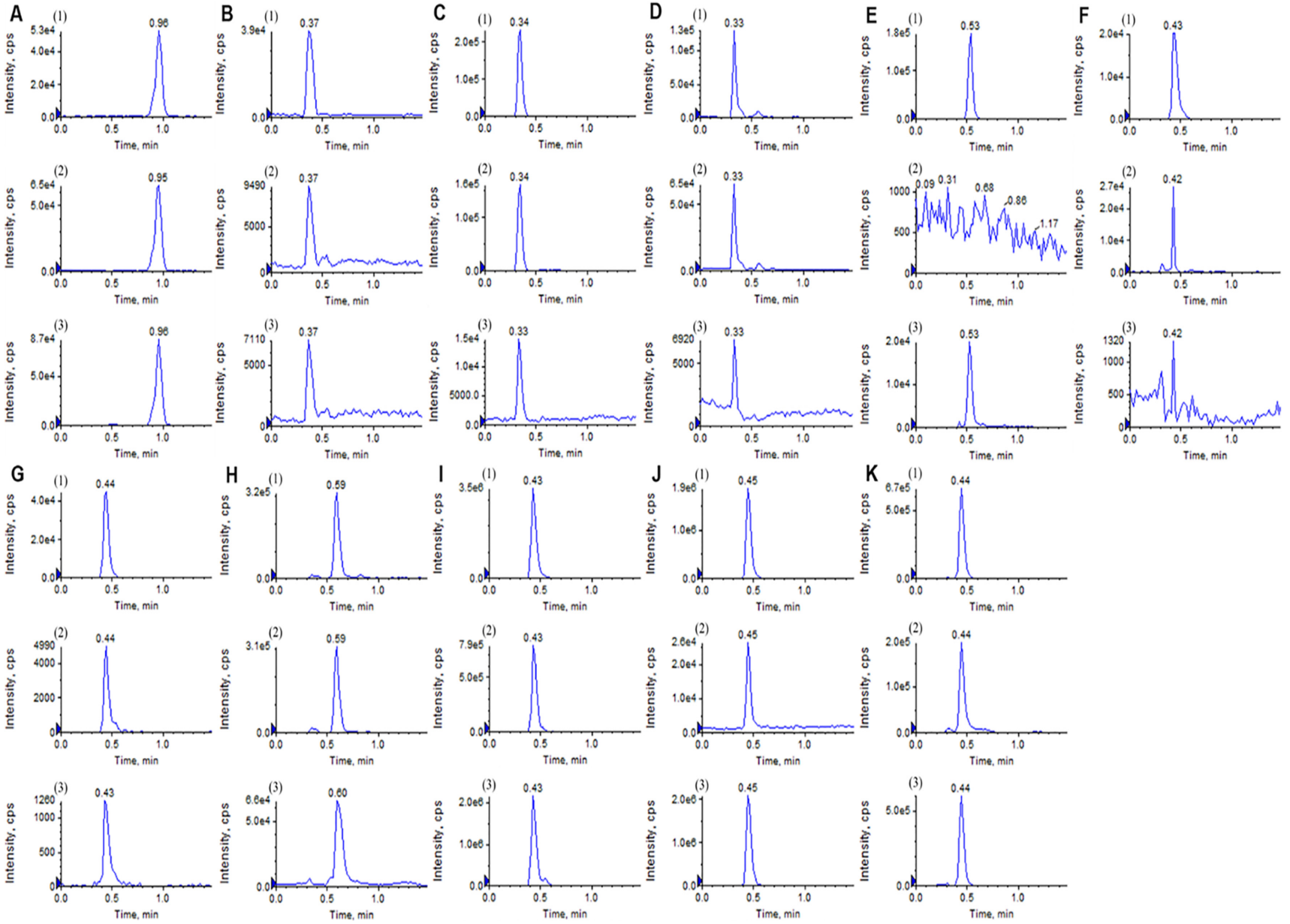

Figure 3. Representative MRM chromatograms of neurotransmitters and [^{2}H5]-L-Glutamic Acid: (1) standard solutions, (2) rat serum, (3) rat brain. (A) DA, (B) NE, (C) 5-HT, (D) DOPAC, (E) MHPG, (F) HVA, (G) 5-HTP, (H) 5-HIAA, (I) GLN, (J) GLU, (K) IS.

compound to be tested, the calibration curve in this method, which contained seven standards of each analyte, was prepared by using the alternative matrix instead of the biological matrix to assess the basic concentration range for each analyte. Taking into account the mass spectrometry system, the alternative matrix was methanol-H_2O (1:1, *v/v*). IS was used to correct the response and ensure exact quantification performance. The calibration curve of each neurotransmitter was determined by plotting the peak area ratio of the analyte to IS (y-axis) and the nominal value (x-axis) of the analyte, constructed by the least-squares regression analysis, then a correlation coefficient (r) was gained. According to the guidelines, the method is considered linear for the non-zero calibrators are ±15% of nominal concentrations, except the lower limit of quantification (LLOQ) which is ± 20% in each validation run, and at least six non-zero calibration levels meet the above criteria in each validation run. The quantitative range of the calibration curve was determined based on the basal concentration of a specific matrix. And the calibration samples were dual. The specificity of the method was evaluated by obtaining chromatograms of real biological samples and alternative matrix samples. The chromatogram was shown in Figure 3. The LLOQ is defined as the concentration level with the signal-to-noise ratio of more than 10, which could be evaluated with an intra- and inter-day accuracy between 80% and 120% and imprecision less than 20%.

Accuracy and precision

QC samples are used to assess the precision and accuracy of the assay and the stability of the samples. Because of the inherent analytes in the biological matrix, the actual biological matrix samples were always analyzed with QC samples together to calculate spiked concentrations. Each concentration was formulated into six samples and analyzed for three days to assess precision and accuracy.

Carryover

Carryover was tested by injecting a drug-free alternative matrix sample following an injection of sample spiked at the highest calibrator of the calibration curve. If carry over was less than 20% of the LLOQ and less than 5 % of IS, the residue was considered negligible.

Extraction recovery and matrix effect

The recovery values of the extraction of neurotransmitters and IS were ascertained by comparing the peak areas of biological samples spiked analytes before protein precipitation with those from the samples spiked after the pretreatment. The matrix effect was to evaluate the matrix factor of L-theanine and IS by calculating the ratio of the peak area under the presence or absence of the matrix. The matrix effects were evaluated by evaluating the relative standard deviation (RSD) of the matrix factor normalized by IS at low- and high-concentration QC levels and the actual biological matrix samples were analyzed with QC samples together.

Stability

Stability was evaluated by comparing the changes in the concentration difference between the simulated QC samples and the actual matrix samples on different days and at beginning under the same conditions. The samples were placed at room temperature for 8 h, -80°C refrigerator for 20 days, three freeze-thaw cycles, and the post-preparative samples placed for 24 h at 4°C.

Chronic unpredictable mild stress model

Rats in the normal control group ($n = 6$) were raised in combined cages, while the model control group ($n = 6$) was raised in solitary cages and carried out CUMS modes to establish depression model.[23] The model control group rats received continuous stimulation for 4 weeks, randomly stimuli involving tail-clamping for 2 min, heat stress at 45°C for 10 min, empty bottle placement for 6 h, co-cage rearing for 6 h, tilting the squirrel cage for 12 h, flash lighting for 12 h, wet litter for 12 h, water prohibition for 24 h, fasting for 24 h, and day and night upside-down for 24 h. Additionally, the same stimulus did not reappear within two days.

Behavioral testing

Before and during CUMS model duplicating, the sucrose preference test (SPT), body weight (BW), and food intake (FI) were measured on Monday, Wednesday, and Thursday each week, respectively, and the open field test (OFT) was measured before and after CUMS model duplicated, and forced swimming test (FST) was implemented after the model was established. The sucrose consumption and purified water consumption were measured to calculate the sucrose preference index in SPT. Recorded the immobility time of rats within 5 min in FST, separately. Record the number of times the rats stand upright, comb or cross the grid within 5 min to calculate the score in OFT. Standing upright once, combing or crossing a grid would count as one point.

Sample collection

After the behavioral test, about 0.3 mL of blood from the orbital venous plexus to prepare serum after centrifuging at 1110 *g* for 10 min after standing for 1 h at room temperature. Then the rats were sacrificed and the brains were immediately decapitated. Stripped off the AM, HIP, PFC, PO, and ST quickly on the ice, then placed in the EP tube and stored at -80°C for further analysis.

Statistical analysis

The data were presented as mean ± standard deviation (SD). Statistical analysis was completed via independent sample *T*-test with IBM SPSS Statistics 25 software (SPSS Inc., Chicago, IL, USA), and $^{*}P < 0.05$ was considered to be significant differences, $^{**}P < 0.01$ indicating very signif-

icant differences, while ***ns*** indicating no significant differences. The figures were presented by OriginPro 2021 (OriginLab, Northampton, UK).

Results and Discussion

Optimization of LC-MS/MS conditions

MRM mode was adopted to obtain enhanced selectivity and sensitivity for analytes. The CE and DP were obtained through tuning and optimization of mass spectrometry. This method used deuterated isotopes, which were similar in structure to analytes, as an internal standard. The acetonitrile and methanol were selected as organic phases. The results showed that using acetonitrile had a lower column pressure and smaller noise. Therefore, 0.1% formic acid, ammonium formate (5 mM) - H_2O (A) and 0.1% formic acid-acetonitrile (B) was used as a mobile phase. We found that the peak response was low, although it was enhanced using 0.1% formic acid- H_2O and 0.1% formic acid acetonitrile, the baseline was higher. Finally, 0.1% formic acid was used in the aqueous phase to obtain a satisfactory baseline and peak shape. Gradient elution was used first, and the similar retention time of each analyte was found at 90% - 45% A. The shape of column peaks was good, except 5-HTP chromatography peak which has a bifurcated phenomenon. When elution of 50% A was eluted, the analyte retention time and the gradient elution had no obvious difference, with good shape of column peaks. However, the peak shape of HVA was poor. The actual biological matrix was separately examined with two methods, and it was found that isocratic elution had a smaller matrix effect. Considering the ionization efficiency, isocratic elution was finally selected. During the preparation of the solution, formic acid was added to dissolve and preserve the stability of the analytes.

Method validation

Linearity and range

Neurotransmitters exhibited satisfactory linearity within their corresponding range are shown in Table 1. The LLOQ of DA, NE, 5-HT, DOPAC, MHPG, HVA, 5-HTP, 5-HIAA, GLU, and GLN in alternative matrix samples were 10 $ng{\cdot}mL^{-1}$, 10 $ng{\cdot}mL^{-1}$, 50 $ng{\cdot}mL^{-1}$, 10 $ng{\cdot}mL^{-1}$, 10 $ng{\cdot}mL^{-1}$, 50 $ng{\cdot}mL^{-1}$, 5 $ng{\cdot}mL^{-1}$, 50 $ng{\cdot}mL^{-1}$, 100 $ng{\cdot}mL^{-1}$, and 100 $ng{\cdot}mL^{-1}$ respectively, with an accuracy (Relative error, RE) within ± 20% and a precision (RSD) within 20%. Supporting information is shown in Table S2.

Accuracy and precision

The inter- and intra-day accuracy and precision of each neurotransmitter with this method were acceptable as shown in Table 2 (Table S2). The absolute values of RE and RSD were less than 15%.

Extraction recovery and matrix effect

The extraction recovery and matrix effect of each neurotransmitter were acceptable as shown in Table 2(Table S3), and the relative recovery rate in the samples was between 86.1 and 109.2%, while the RSD of the matrix factor was not more than 13.4%.

Stability

Stability investigation showed the RSD was not more than 11.6% and RE values were within -10.3% to 11.2% after the samples were frozen for 20 days at -80°C, placed at room temperature for 8 h, subjected to a freeze-thaw cycle thrice, and placed for 24 h in auto-sampler vials, which met the requirements for biological samples. The information is shown in Table 3.

Carryover

The effect of carryover on the drug-free alternative matrix samples following the highest calibration sample was assessed. The average carryover was not more than 13.26%, demonstrating no significant carryover effect in the method.

Behavioral tests

We observed an insignificant variance among various

Table 1. Data for linearity range and calibration curves.

Analytes	Slope ($\times 10^{-1}$)	Intercept	r^2	Linear range ($ng{\cdot}mL^{-1}$)
DA	1.716	0.041	0.9910	10 - 1000
NE	12.588	0.018	0.9908	10 - 1000
5-HT	1.936	0.145	0.9938	50 - 5000
DOPAC	7.870	0.041	0.9892	10 - 1000
MHPG	2.317	0.005	0.9908	10 - 1000
HVA	0.248	0.060	0.9912	50 - 5000
5-HTP	14.030	0.017	0.9942	5 - 500
5-HIAA	14.655	0.024	0.9888	50 - 5000
GLN	2.648	0.065	0.9870	100 - 10000
GLU	8.132	0.761	0.9926	100 - 10000

Table 2. Accuracy, precision, extraction recovery and matrix effect data of neurotransmitters at low- QC concentrations (Table S1 and table S2, $n = 6$).

Analytes	Added concentration ($ng \cdot mL^{-1}$)	Intra-day (%)		Inter-day (%)		Serum		Limbic system	
		Precision (RSD)	Accuracy (RE)	Precision (RSD)	Accuracy (RE)	Extraction recovery (%, Mean ± SD)	Matrix effect (%)	Extraction recovery (%, Mean ± SD)	Matrix effect (%)
DA	30	4.9	-2.9	3.7	-0.3	88.0 ± 6.6	5.3	86.1 ± 7.9	6.1
NE	30	7.6	-4.0	9.7	-5.3	94.3 ± 5.8	11.3	99.0 ± 8.2	7.5
5-HT	150	3.3	2.6	2.8	2.0	99.9 ± 2.8	3.4	98.4 ± 6.3	3.1
DOPAC	30	1.0	-2.9	2.3	-1.3	96.6 ± 2.0	6.3	98.6 ± 2.1	8.7
MHPG	30	2.5	-2.0	2.4	-1.6	100.9 ± 2.3	2.1	100.3 ± 2.0	12.1
HVA	150	2.4	-2.6	1.9	-2.7	97.9 ± 5.0	2.7	96.8 ± 12.5	11.1
5-HTP	15	2.6	-1.4	1.9	1.1	101.3 ± 3.9	3.4	98.9 ± 3.7	3.7
5-HIAA	150	2.3	-2.4	1.9	-2.0	100.6 ± 4.1	8.7	103.9 ± 7.5	6.9
GLN	300	3.8	-2.2	2.9	5.1	100.1 ± 3.1	2.1	100.3 ± 2.4	4.1
GLU	300	3.9	-1.6	4.4	-4.0	95.7 ± 3.4	7.2	96.6 ± 5.4	7.8

Table 3. Stability of analytes in serum and brain under different conditions ($n = 5$).

Analytes	Added concentration ($ng \cdot mL^{-1}$)	Serum								Limbic system							
		Short-term stability (8 h)		Long-term stability (-80°C for 20 d)		Three freeze-thaw cycles stability		Post-preparative samples (24 h in 4°C)		Short-term stability (8 h)		Long-term stability (-80°C for 20 d)		Three freeze-thaw cycles stability		Post-preparative samples (24 h in 4°C)	
		RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)
DA	30	-6.3	6.5	7.2	8.7	-7.1	7.5	-4.3	6.1	4.7	5.4	4.7	7.7	5.1	8.1	-7.2	7.3
NE	30	3.5	3.5	-6.6	5.6	-6.5	7.3	5.8	7.1	7.6	5.7	7.7	8.2	4.5	8.4	2.0	3.5
5-HT	150	6.4	5.2	9.7	9.5	4.6	3.2	7.1	3.1	9.7	4.8	-6.9	9.0	7.9	4.6	-4.4	4.1
DOPAC	30	6.8	10.3	5.4	8.5	-6.2	7.2	3.3	3.6	-10.3	7.7	9.0	5.5	-7.2	9.1	6.3	7.3
MHPG	30	5.1	6.1	6.5	8.0	3.2	6.3	1.3	4.3	8.3	8.1	5.6	4.6	2.2	6.2	7.1	6.4
HVA	150	6.5	5.4	7.6	3.5	-6.2	7.2	8.0	3.8	3.8	3.5	-6.6	8.9	7.2	7.3	-4.5	7.1
5-HTP	15	-3.4	4.4	10.0	4.9	11.2	8.3	-7.1	8.2	-7.7	8.4	6.5	7.1	-8.3	9.1	-6.3	5.3
5-HIAA	150	8.1	6.5	-8.6	6.1	8.5	5.5	6.5	5.4	7.4	4.7	8.9	4.2	5.1	5.6	-7.1	6.1
GLN	300	-4.3	3.3	8.4	8.7	-7.2	3.5	8.1	7.3	5.8	8.3	5.5	11.6	8.2	9.5	6.2	3.6
GLU	300	3.8	5.2	-4.4	4.9	5.7	5.9	9.2	8.2	6.3	2.4	-6.5	8.1	3.2	4.7	9.5	3.5

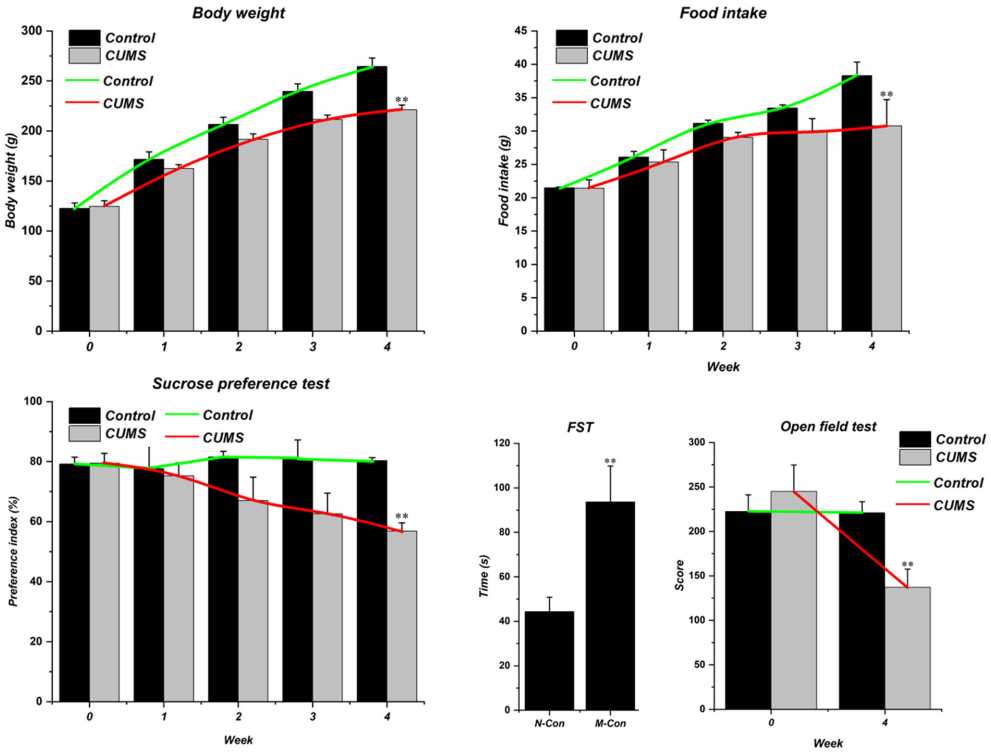

Figure 4. Behavioral differences of rats between normal control group and model control group. Values were calculated as mean ± SD (n = 6). **P < 0.01, very significant differences. FST: Forced swimming test. *Control*, normal control group, *CUMS*, model control group.

groups ($P > 0.05$) before modeling, while very significant differences were presented in normal control group rats and model control group rats after modeling in BW, FI, SPT, and OFT in Figure 4. SPT is a reward-based behavioral detection method, and during the experiment, there was a stable preference index in normal control group rats, while the model control group rats decreased significantly ($^{**}P < 0.01$). The scores of normal control group rats remained at a steady level, while the model control group rats decreased significantly with the procedure of CUMS in OFT, studies have shown the more depressed rats are, the less score in the field because of the less interest in exploration. FST is a desperate behavioral test method, which has good predictive validity through immobility time. Compared with the normal control group rats, the immobility time in FST was significantly increased ($^{**}P < 0.01$) after CUMS was built. To conclude, the results of behavioral tests showed that the behavioral status and anhedonia symptoms of the model group were consistent with the characteristics of depression and the model was established successfully.

Neurotransmitter levels

This study measured the levels of ten neurotransmitters related to depression in five brain regions of the limbic system in the central and serum in the peripheral system as shown in Figure 5. The measured catecholamine neurotransmitters include DA, NE, DOPAC, MHPG and HVA; indoleamine neurotransmitters include 5-HT, 5-HTP and 5-HIAA; amino acid neurotransmitters include GLU and GLN, and due to the concentration of endogenous substances in vivo and matrix effects, some metabolites or precursors had not been detected via this method. It could be seen from the measurement results that compared with normal control group rats, the levels of 5-HT, 5-HIAA, DA, NE, DOPAC, HVA, etc. in the peripheral and limbic system of rats induced by CUMS were significantly reduced, which were consistent with the findings of decreased levels of monoamine neurotransmitters in the cerebrospinal fluid of patients with depression.[24] In the pathological condition of neurological disease, circulating levels of neuroactive compounds may be deregulated, suggesting peripheral neurochemical changes may be involved in pathological processes directly or indirectly. Therefore, the measurement of peripheral neurotransmitter levels is as important as the central. Compared with normal control group rats, the content of DA, 5-HT, DOPAC, and 5-HTP in the serum of model control group rats were very significantly reduced ($^{**}P < 0.01$) and NE, HVA, 5-HIAA were significantly reduced ($^{*}P < 0.05$), while GLN and GLU significantly increased ($^{**}P < 0.01$).

5-HT can regulate the activity of emotions and neural circuits, especially the medial PFC and AM areas, and dopa-

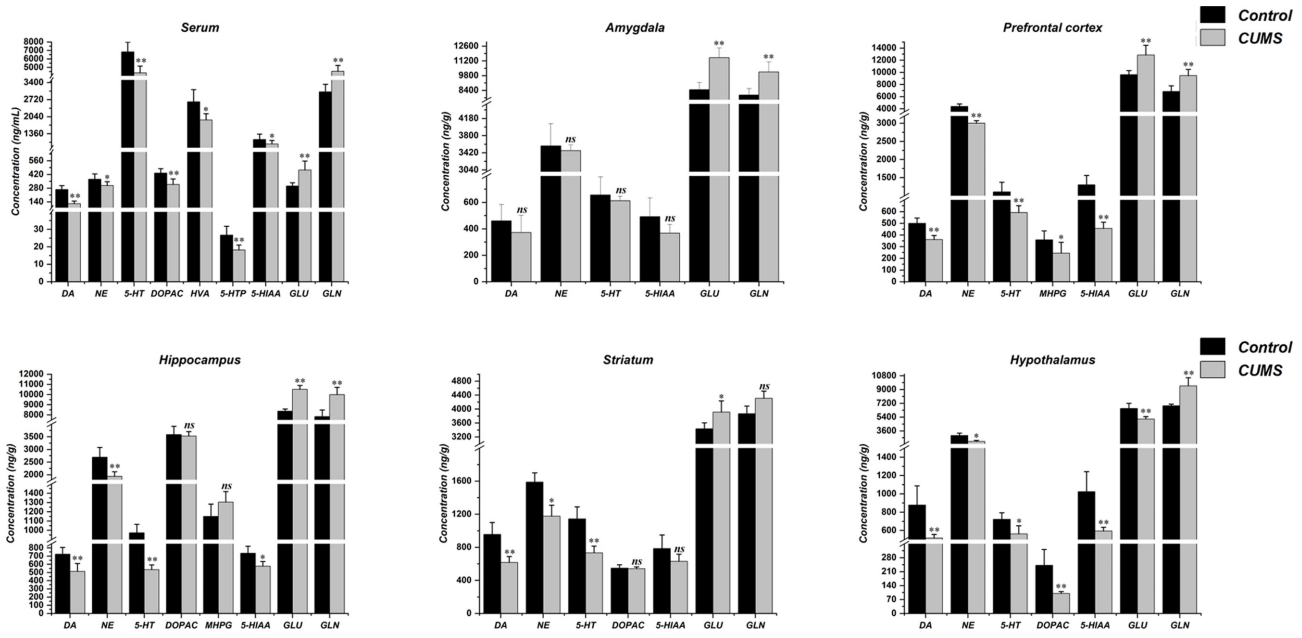

Figure 5. The content of various neurotransmitters in serum and limbic system between normal control group and model control group. Values were calculated as mean ± SD (n = 6). **P < 0.01, very significant differences, *P < 0.05, significant differences, *ns*, no significant differences. DA: dopamine, NE: norepinephrine, 5-HT: Serotonin, DOPAC: 3,4-Dihydroxyphenylacetic acid, MHPG: 3-methoxy-4-hydroxyphenylglycol, HVA: homovanillic acid, 5-HTP: 5-hydroxytryptophan, 5-HIAA: 5-hydroxyindole acetic acid, GLU: L-glutamic acid, GLN: L-glutamine. *N-Con*, normal control group, *M-Con*, model control group.

minergic regulation with the ventral ST and medial PFC as the center.[25] Therefore, there was a higher content in the limbic system and the periphery. In the pathogenesis of depression, serotonergic neurotransmission was weakened[4] and the content was reduced shown in Figure 5. 5-HT would quickly metabolize by the mitochondrial enzyme MAO to 5HIAA, which could be used for the analysis of serotonin levels in the brain. Therefore, 5-HIAA is considered as a biomarker of neurological diseases.[26] It could be seen that 5-HIAA and 5-HT had the same changing trend in the peripheral and central systems, and 5-HIAA content decreased after CUMS induction in Figure 5, which was consistent with previous reports.[24] 5-HTP is converted from the essential amino acid tryptophan by tryptophan hydroxylase, and further into serotonin, which has been used in alternative medicine as an effective auxiliary means in depression.[27] In depression mesolimbic DA pathway hypofunctioning has been reported, especially associated with motivation and anhedonia.[28] The main receptor for DA release in the forebrain is the striatum, and DA can be transmitted through the striatum to play its basic role in motor control, cognitive behavior, and neuropsychiatric symptoms.[29] NE is mainly synthesized and secreted by sympathetic postganglionic neurons and noradrenergic neurons in the brain, and in the blood, NE mainly comes from the adrenal medulla. GLU and GLN are the most abundant amino acids in the brain and in the body respectively, widely distributed in the cerebral cortex, hippocampus, cerebellum and striatum,[30,31] therefore, there was a higher concentration in both the periphery and the center. The increase in glutamine content was speculated to be related to the damage of astrocytes caused by chronic stress stimulation.[32]

These brain areas in the limbic system are all involved in emotion regulation and its neurotransmitter levels were altered to varying degrees in CUMS-induced rats. The amygdala is the central part of generating, recognizing, and regulating emotions, especially fear. Experiments[33] showed rats with amygdala injury have decreased food intake and have behaviors such as anxiety and depression. The excessive activation of the amygdala and the increase of glial cell density are the key factors leading to suicide in depressed patients.[34] Except for 5-HIAA in the amygdala, other metabolites of monoamine neurotransmitters were not detected. The levels of DA, NE, 5-HT, GLU, and GLN are shown in Figure 5. The prefrontal cortex is an important high-level nerve center that regulates cognition and behavior. Studies have shown that in the early stages of neurodevelopment, the reduction in the thickness of the ventromedial prefrontal cortex could lead to depression in children.[35] The levels of DA, NE, 5-HT, MHPG, and 5-HIAA in model control group rats were significantly reduced ($^{**}P < 0.01$, $^{*}P < 0.05$), while GLU and GLN were significantly increased ($P < 0.01$). The hippocampus is mainly responsible for learning and memory and its role in emotional behavior is complicated, which is the main structure affected by psychiatric illness and a key regulator of

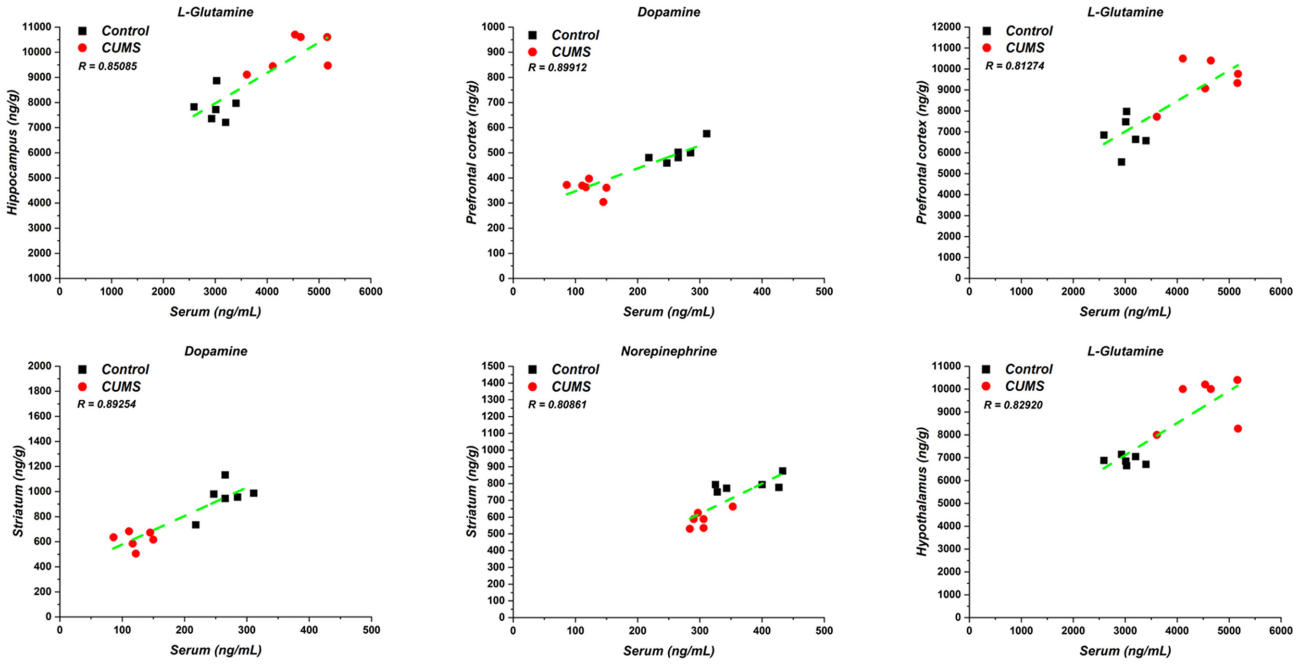

Figure 6. Correlation analysis of neurotransmitters which correlation coefficient > 0.8 between Control and CUMS. (A) Level of L-glutamine between serum and hippocampus, level of (B) dopamine and (C) glutamine between serum and prefrontal cortex, level of (D) dopamine and (E) norepinephrine between serum and striatum, (F) level of L- glutamine between serum and hypothalamus.

the stress response.[36] The levels of DA, NE, 5-HT, and 5-HIAA were significantly reduced ($^{**}P < 0.01$, $^{*}P < 0.05$) in model control group rats, while GLU and GLN were significantly increased ($^{**}P < 0.01$). The striatum composes the cortex-thalamic-striatal neural circuit, playing a role in regulating voluntary movement, emotions, and rewards. Studies have found the blood oxygen level in the ventral striatum of patients with depression was significantly reduced and the size of the striatum became smaller.[37] Compared with normal control rats, the content of DA, NE, 5-HT in model control group rats was significantly reduced ($^{**}P < 0.01$, $^{*}P < 0.05$), while the content of GLU was significantly increased ($^{**}P < 0.01$). The hypothalamus is the center that regulates physiological balance and neuroendocrine. The neuroendocrine hypothesis believes hypothalamic-pituitary-adrenal axis dysfunction is the key to the onset of depression.[38] From the result, we could see in the model group rats the levels of DA, NE, 5-HT, DOPAC, 5-HIAA and GLU increased significantly ($^{**}P < 0.01$, $^{*}P < 0.05$), while GLN decreased significantly ($^{**}P < 0.01$).

According to the measurement results, it could be seen the changes in neurotransmitter levels in the peripheral and central regions of the rats induced by CUMS are basically consistent. Correlation analysis of neurotransmitter changes is shown in Table S4 and the neurotransmitters had a good level of relevance (R > 0.8) as shown in Figure 6. The results suggested changes in peripheral neurotransmitters may be directly related to the onset of neurological diseases. In the future, the peripheral transformation of neurotransmitters will surely become the goal of the diagnosis and treatment of neurological diseases.

Conclusions

In this study, we developed and validated an efficient and sensitive LC-MS/MS method for determination of ten neurotransmitters in rat serum, amygdala, prefrontal cortex, hippocampus, striatum, and hypothalamus simultaneously. The results of method verification met the bioanalytical requirements in terms of specificity, sensitivity, accuracy and precision, etc. The method has been successfully used to quantify neurotransmitters of normal rats and CUMS-induced depression rats. The test results showed the levels of catecholamines, indoleamine and excitatory neurotransmitters and their metabolites in the peripheral and central system of rats after CUMS induction had changed significantly, and the results of correlation analysis indicated peripheral neurotransmitters may be involved in neurological diseases. The established method could promote the targeted metabolomics research of depression and other neurological diseases and provide better strategies for the study of the pathogenesis of depression.

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Abbreviations

AM: amygdala; BW: body weight; CE: collision energy; CUMS: chronic unpredictable mild stress; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; DP: declustering potential; FI: food intake; FST: forced swimming test; GLN: glutamate; GLU: glutamine; HIP: hippocampus; HVA: homovanillic acid; IS: internal standard; LC-MS: liquid chromatography - mass spectrometry; LC-MS/MS: liquid chromatography - tandem mass spectrometry; LLOQ: lower limit of quantification; MHPG: 3-methoxy-4-hydroxy-phenylglycol; MRM: multiple reaction monitoring; NE: norepinephrine; OFT: open field test; PFC: prefrontal cortex; PO: hypothalamus; QC: quality control; RE: relative error; RSD: relative standard deviation; SD: standard deviation; SPT: sucrose preference test; ST: striatum; 5-HT: serotonin; 5-HTP: 5-hydroxytryptophan; 5-HIAA: 5-hydroxyindole acetic acid.

Conflict of Interest

The authors have declared no conflict of interest.

Supplementary Information

Supplementary information is available at https://docs.google.com/document/d/1WOqTAa183YLxQpkdyNvVwx2c4zCMmhY_/edit?usp=sharing&ouid=111353140014732050956&rtpof=true&sd=true.

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