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Determination of five neurotransmitters in the rat brain for the study of the hypnotic effects of Ziziphi Spinosae Semen aqueous extract on insomnia rat model by UPLC-MS/MS

YAN Yan[1], LI Qiang[1], DU Hui-Zhi[1], SHEN Chen-Xi[1], LI Ai-Ping[1], PEI Xiang-Ping[2], DU Chen-Hui[2*], QIN Xue-Mei[1*]

[1] *Modern Research Center for Traditional Chinese Medicine, Shanxi University, Taiyuan 030006, China;*
[2] *School of Chinese Materia Medica, Shanxi University of Traditional Chinese Medicine, Taiyuan 030619, China*

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[ABSTRACT] Ziziphi Spinosae Semen (ZSS) has been used for treatment of insomnia in China for centuries. To reveal the influence of insomnia on the levels of the neurotransmitters including serotonin (5-HT), glutamic acid (Glu), γ-aminobutyric acid (GABA), noradrenaline (NE) and dopamine (DA), and to study the role of ZSS aqueous extract in the treatment of insomnia, an UPLC-ESI-MS/MS method was developed and validated for simultaneous determination of five neurotransmitters in the rat brain. The brain samples were pretreated by one-step direct protein precipitation with acetonitrile. The analytes were detected in positive mode with multiple reaction monitoring (MRM) and the procedure was completed in less than 10 min. The method showed a good linearity (R^2 > 0.9967) with the other validation parameters were within acceptance range. The results indicated that the concentration of 5-HT, GABA and DA is significantly lower (P < 0.01) in *para*-chlorophenylalanine (PCPA)-induced insomnia rat model group, while Glu and NE significantly higher than those in control group (P < 0.01). Treatment with ZSS aqueous extract (4 or 8 g·kg^{-1}·d^{-1} for seven days) could ameliorate the symptoms of insomnia by significantly changing the levels of the neurotransmitter parameters mentioned above. The data obtained in this study demonstrate that ZSS aqueous extract could ameliorate the symptoms of insomnia by modulating the levels of monoamines and amino acid neurotransmitters in the brain.

[KEY WORDS] Ziziphi Spinosae Semen; Neurotransmitters; UPLC-MS/MS; Hypnotic effects

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Introduction

Insomnia is characterized by sustained difficulties in initiating or maintaining sleep and causes significant impairment of daytime functioning [1]. As the pace of life becomes faster and work pressure gets heavier, the problem of sleep disorders has shown an upward trend year by year. About one-third of the general population worldwide experiences insomnia symptoms accompanied by daytime dysfunction [2]. Many chemosynthesized sedative-hypnotic drugs have been used in the clinic, but their therapeutic effects are not satisfying, and they have a series of side effects, such as dizziness, sleepiness, anxiety and even temporary amnesia [3]. Currently, a proportion of insomnia patients have resorted to various kinds of complementary and alternative medicine (CAM) [4]. Chinese herbal medicine is one of the most commonly used modalities of CAM [5].

Ziziphi Spinosae Semen (ZSS), the dried and ripe seed of *Ziziphus jujube* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou, has been used as a sedative in China for over a millennium. It was first listed in the *Divine Husbandman's Classic of the Materia Medica* [6]. The classical application of ZSS is supplementing the liver, quieting the heart, arresting the sweat and promoting the production of the body fluids. Alone or in combination with other herbs, ZSS is the most frequently used herb for treating insomnia, according to randomized controlled trials [3, 7-8]. The chemical composition of ZSS,

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[*Corresponding authors] Tel: 86-351-7011202, Fax: 86-351-3179979, E-mails: 13653412562@vip.163.com (DU Chen-Hui); qinxm@sxu.edu.cn (QIN Xue-Mei)
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including saponins, flavones, alkaloids and fatty oils, has been studied extensively [9-10]. Modern pharmacological studies have revealed that the methanol or ethanol extract of ZSS can improve sleep and treat people with anxiety [11-14]. FANG *et al*. reported that the water extract of ZSS could shorten sleep latency significantly, increase sleeping time and prolong movement convalescence time induced by sodium pentobarbital administration in mice [6]. Indeed, aqueous extraction (decoction), not alcohol extraction, is the main prescription form of traditional Chinese medicine (TCM), so it is much more meaningful to investigate the hypnotic effect of ZSS aqueous extracts.

According to the reported complex pathogenesis of insomnia, the disturbances in neurotransmitters in the brain are widely accepted to be associated with insomnia [15]. Various neurotransmitters can be classified into amino acids, monoamines, peptides and others. Among them, noradrenaline (NE), dopamine (DA), glutamic acid (Glu), gamma-aminobutyric acid (GABA) and serotonin (5-HT) play important roles in maintaining wakefulness and sleepiness. Thus, it is necessary to develop a reliable method to monitor these neurotransmitters to evaluate the therapeutic efficacies of ZSS aqueous extract by analyzing the concentration changes of neurotransmitters in the brain of insomnia animal models. Recently, several methods have been developed for the determination of these neurotransmitters, mostly based on liquid chromatography (LC) and capillary electrophoresis (CE), with various detection methods, such as ultraviolet, fluorescence, electrochemical and mass spectrometry (MS) [16-18]. Among these methods, LC-MS is the most frequently used method. However, previous quantitative methods of these polar endogenous neurotransmitters had limitations in tedious preparation or sensitivity.

Here, a rapid and accurate UPLC-MS/MS method with higher sensitivity and shorter chromatographic run time was developed for the simultaneous determination of five neurotransmitters in the rat brain tissues. In addition, a simple direct protein precipitation (PPT) method was applied in the extraction procedure. Meanwhile, the determination method was successfully applied to analyze the neurotransmitters in a rat insomnia model induced by *Para*-chlorophenylalanine (PCPA), which interpreted the hypnotic effect of ZSS aqueous extracts against insomnia.

Materials and Methods

Reagents, chemicals and materials

HPLC-grade methanol and acetonitrile were obtained from Dikma (Tianjin, China). Ultra-pure water was produced by a Milli-Q water purification system (Milford, MA, USA). Formic acid (MS grade) was purchased from Fisher Scientific (Shanghai, China). All other reagents were of analytical grade.

PCPA and 5-HT (purity > 97%) were purchased from Tokyo Chemical Industry (Shanghai, China). GABA (purity > 99%) was purchased from Aladdin (Shanghai, China). Glu (purity > 99%) was purchased from Sangon Biotech (Shanghai, China). NE (purity > 99%) was purchased from National Institute for Drug Standard Material Inspection (Beijing, China). DA (purity > 97%) was purchased from Perfemiker (Shanghai, China). 3, 4-Dihydroxybenzylamine hydrobromide (DHBA, purity > 97%) was obtained from Sigma-Aldrich (USA) and used as internal standard (IS). Diazepam (DZP) tablet was supplied by Anhui Chengshi pharmaceutical Co., Ltd. (Anhui, China). The chemical structures of the five neurotransmitters and DHBA are shown in Fig. 1.

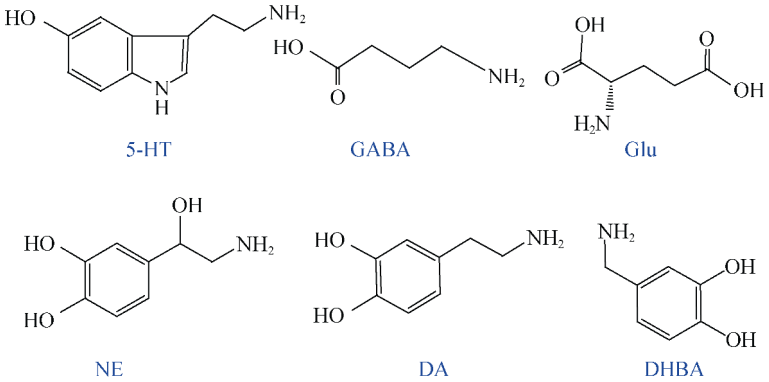

Fig. 1 Chemical structures of 5-hydroxytryptamine (5-HT), gamma-aminobutyric acid (GABA), glutamic acid (Glu), norepinephrine (NE), dopamine (DA) and 3, 4-dihydroxybenzylamine (DHBA, IS)

The reference standards of coclaurine, magnoflorine, vicenin II, spinosin, kaempferol-3-*O*-rutinoside, 6‴-feruloylspinosin and jujuboside A were purchased from Baoji-technology Co., Ltd. (Shaanxi, China). Swertisin was purchased from Weikeqi-technology Co., Ltd. (Chengdu, China). Jujuboside B was obtained from Chunqiu-technology Co., Ltd. (Nanjing, China).

ZSS was provided by Zhendong Chinese herbal development Co., Ltd. (Shanxi, China) and was authenticated by Dr. DU Chen-Hui to be the dried seeds of *Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou according to Chinese Pharmacopoeia (2015 version). The voucher specimens were preserved at the Modern Research Center for Traditional Chinese Medicine, Shanxi University, Taiyuan, China.

Preparation of standardized ZSS aqueous extract

ZSS (1 kg) was pulverized into suitable powder, immersed in 8 L water, and extracted twice by refluxing with boiling water for one hour per time. After filtration through eight layers of gauze, the supernatant was mixed, condensed and freeze-dried to powder (yield: 28.12%). For quality control, the contents of coclaurine, magnoflorine, vicenin Ⅱ, spinosin, swertisin, kaempferol-3-*O*-rutinoside, 6‴-feruloylspinosin, jujuboside A and jujuboside B were quantified according to our published method [19], and the contents of coclaurine, magnoflorine, vicenin Ⅱ, spinosin, swertisin, kaempferol-3-*O*-rutinoside, 6‴-feruloylspinosin, jujuboside A and jujuboside B in ZSS aqueous extract were 0.020%, 0.29%, 0.013%, 0.12%, 0.00070%, 0.0039%, 0.063%, 0.038% and 0.023%, respectively.

Effects of ZSS aqueous extract on PCPA-induced insomnia model rats

Animals and treatments

Seventy-two male Sprague-Dawley rats (180−200 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (SCXK (Jing) 2012-0001, Beijing, China). The animals were allowed access to water and food *ad libitum* and maintained at a constant temperature 25 °C and humidity (45% ± 5%) under a 12 h light/dark cycle for one week before the experiment. The animal study was performed according to the International Rules Concerning Animal Experiments and the Internationally Accepted Ethical Principles for Laboratory Animal Use and Care. After one week of environmental adaptation, the rats were randomly divided into two groups, i.e., normal control (NC, n = 12) group and PCPA group (n = 60). For the PCPA group, rats were intraperitoneally injected (i.p.) with PCPA suspended in 0.5% physiological saline at a dose of 400 mg·kg^{-1} per day. After the injection of PCPA for three days, the concentration of 5-HT in the serum of rats was detected, and then rats in the PCPA group whose level of 5-HT was lower than that in the normal rats were regarded as insomnia rats (data not shown), which was consistent with the previous study [20]. In addition, the rats in the PCPA group lost their circadian rhythm and were sleepless all day, which also confirmed the success of the insomnia model. The insomnia rats were randomly subdivided into five groups, i.e., insomnia model (IM) group, diazepam (DZP) group and three drug treatment groups. The treatment groups were fed the low dosage (2 g·kg^{-1}) of ZSS aqueous extract (SL), the middle dosage (4 g·kg^{-1}) of ZSS aqueous extract (SM), the high dosage of ZSS aqueous extract (8 g·kg^{-1}) of ZSS (SH), and 1 mg·kg^{-1} of DZP (all dissolved in physiological saline), respectively, for seven days. For the NC group, rats received an equal volume of physiological saline.

Sample collection and preparation

At the end of the study, rats were anesthetized with 10% chloral hydrate. The brain was quickly removed, put on ice, and frozen with liquid nitrogen. All biological samples were stored at −80 °C until analysis.

At analysis, 0.1 g brain tissue samples were weighed and homogenized in a 10-fold (*W/V*) volume of ice-cold acetonitrile. The homogenized mixture was centrifuged at 9000 *g* for 20 min at 4 °C. Then, 800 μL of the supernatant was spiked with 50 μL of DHBA and 50 μL of water−acetonitrile (8 : 2, *V/V*) containing 0.2% formic acid, followed by vortexing for 5 min. Subsequently, these samples were centrifuged at 9000 *g* for 10 min at 4 °C. The supernatants were quantitatively transferred to a 10 mL glass tube and evaporated to dryness at 37 °C under a slight stream of nitrogen. Finally, the residue was redissolved with 100 μL of initial mobile phase, and the aliquot of 3 μL was injected into the LC-MS/MS system for analysis.

UPLC-ESI-MS/MS analysis

Quantitative analysis was performed on an Agilent 1290 Infinity II RRLC (Agilent, Malaysia) system coupled to a Q-Trap^{TM} 3200 MS/MS system (AB SCIEX, Singapore). Chromatographic separation was performed on an ACQUITY UHPLC^{TM} BEH C_{18} column (100 mm × 2.1 mm I.D., 1.7 μm, Waters, USA) at 30 °C. The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile with the following gradient: 0–2.5 min, 5% B; 2.5–4 min, 5%−20% B; 4–7 min, 20%−90% B. The mobile phase returned to the initial condition within 0.5 min and then maintained the starting conditions for 1.5 min. The flow rate was 0.2 mL·min^{-1}.

The Q-Trap system equipped with a turbo ion spray source (Foster City, CA, USA) in the multiple reaction monitoring (MRM) mode was operated in positive ion mode, and source-dependent parameters were optimized as follows: vaporizer temperature, 500 °C; nebulizing gas (GS1), 50 psi; drying gas (GS2), 50 psi; curtain gas, 40 psi. Nitrogen gas was used in all analyses. Data acquisition and processing were performed using Analyst software version 1.6.2 (AB SCIEX, Singapore). The MRM parameters are outlined in Table 1.

Table 1 The multiple reaction monitoring (MRM) transitions and parameters for the detection of the five analytes and IS. (5-HT: 5-hydroxytryptamine, GABA: gamma-aminobutyric acid, Glu: glutamic acid, NE: norepinephrine, DA: dopamine, DHBA: 3, 4-dihydroxybenzyl amine)

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Delustering Potential (V)	Collision energy (V)
5-HT	177.1	160.1	21.6	14.6
GABA	104.1	87.1	20.3	13.5
Glu	148.1	130.1	22.9	13.6
NE	170.1	152.1	15.2	10.3
DA	154.1	137.0	22.0	13.8
DHBA	140.1	123.1	14.9	13.4

Stock solution, calibration standards and quality control samples

Stock solutions of 5-HT, GABA, Glu, DA, NE and DHBA were prepared in water-acetonitrile (8 : 2, *V/V*) containing 0.2% formic acid at the concentration of 0.99, 10.01, 10.12, 1.03, 1.02 and 1.02 mg·mL^{-1}, respectively. The mixed stock solution containing each of the above five standard neuro-

transmitters was prepared in the final concentration of 1.8 μg·mL^{-1} for 5-HT, 3.6 mg·mL^{-1} for GABA and Glu, 4.5 μg·mL^{-1} for DA, and 225.0 μg·mL^{-1} for NE. Then, the working solution of DHBA (36 μg·mL^{-1}) was prepared by diluting this stock solution with methanol. A series of mixed working solutions for 5-HT, DA, GABA, Glu and NE were obtained by serial dilutions of mixed stock solution.

The calibration standards were made by spiking 50 μL of the above mixed working solutions and 50 μL of IS into 800 μL of blank brain homogenates to obtain five calibration standards in the desired concentrations (1, 2, 10, 20, 50, 79 and 99 ng·mL^{-1} for 5-HT, 1998, 3996, 19 980, 39 960, 99 900, 159 840 and 199 800 ng·mL^{-1} for GABA and Glu, 2.5, 5, 25, 50, 125, 200 and 250 ng·mL^{-1} for DA, 122, 244, 1219, 2438, 6094, 9750 and 12 187 ng·mL^{-1} for NE, respectively). The quality control (QC) samples were prepared in the same way as the calibration solution, with the final dilutions of 1, 2, 10, and 79 ng·mL^{-1} for 5-HT; 1 998, 3 996, 19 980 and 159 840 ng·mL^{-1} for GABA and Glu; 122, 244, 1219 and 9750 ng·mL^{-1} for NE; 2.5, 5, 25, and 200 ng·mL^{-1} for DA.

Method validation

The mean value of analytes-to-IS peak area ratios of blank matrix samples was set as Y_0, and analytes-to-IS peak area ratio of calibration standards was set as Y_n (n = 1, 2, …, 7). More especially, six replicates of the brain homogenate were prepared as a blank matrix sample. Then calibration curves for five neurotransmitters were established using the least-square linear regression of the differences between Y_0 and Y_n against the nominal concentrations with weighting factor 1/concentration^2.

The lower limit of detection (LLOD) was determined as signal-to-noise ratio > 3, and the lower limit of quantification (LLOQ) was measured as signal-to-noise ratio > 10. The intra- and inter-day accuracy and precision were carried out by determining five QC samples at four different concentrations on the same day and on three consecutive days, respectively. Accuracy was expressed as relative error (RE, %), and precision was described as relative standard deviation (RSD, %). The acceptability criteria for accuracy and precision were ± 15% (± 20% for the LLOQ), according to the guidelines of the FDA. The extraction recovery and matrix effects of the five neurotransmitters were evaluated by comparing the peak area of every analyte in post-extraction spiked samples (A) with those in pre-extraction spiked samples (B), and the peak area of every analyte in post-extraction spiked samples (A) with those obtained from the pure reference standard solutions (C) at three QC levels at the same concentration, respectively. The stability of all analytes in blank brain tissue samples was tested by analyzing five replicate QC samples at three different concentrations during sample collection and handling. The freeze-and-thaw stability was determined after three freeze-thaw cycles (from –20 to 20 °C) on consecutive days. Long-term stability was studied by storing QC samples at –70 °C for 30 days. Short-term stability was assessed by analyzing QC samples kept at room temperature for 4 h. The post-preparation stability was determined in the extracted QC samples kept in the autosampler at 4 °C for 24 h.

Determination of neurotransmitters in rat brain samples

This validated method was applied to determine the concentrations of 5-HT, GABA, Glu, DA and NE in rat brain tissue. The concentrations of these five analytes in the NC, IM, DZP, SL, SM and SH groups were calculated from their corresponding calibration curves.

Data analysis

The five neurotransmitters were compared between different experimental groups using one-way ANOVA followed by the LSD test of variance homogeneity and Dunnett's T3 test of variance heterogeneity after a normal distribution test, and $P < 0.05$ was considered statistically significant.

Results and Discussion

UPLC-MS/MS optimization

In this study, the BEH C_{18} column (Waters, USA), Kromasil C_{18} column (Kromasil, Sweden) and Venusil MP C_{18} column (Agela, China) were tried. The Kromasil C_{18} column and Agela MP C_{18} column did not have good retention for all analytes. The BEH C_{18} column, prepared using ethyl-bridged hybrid organic/inorganic materials, provided better resolution and symmetric peaks as well as suitable retention for all the analytes. In addition, the other important factors, such as the composition of the mobile phase and the elution program, were systematically explored to obtain the better peak shape. For example, the different mobile phases consisting of acetonitrile and water with formic acid, acetic acid or ammonium at different concentrations were examined. As a result, the mobile phase system consisting of 0.1% aqueous formic acid and acetonitrile provided symmetric peaks, higher responses and lower background noise of GABA, Glu, DA and NE (data not shown). In addition, the flow rate and column temperature were optimized. Another important aspect of the analytical method is analysis time. Since DA is somewhat unstable because of its high tendency to oxidate [17], the analysis time should be as short as possible. In this study, the sample analysis time was 7 minutes plus another two minutes for re-equilibration owing to gradient conditions, which were shorter than those described elsewhere (10 to 35 min) [17, 21].

Sample preparation optimization

The procedures of brain sample pretreatment were focused on minimizing the matrix effect and increasing the extraction recovery. Some neurotransmitters are rapidly degraded by endogenous enzymes when brain tissue is homogenized in water only [22]. Thus, the solvents used to homogenize the brain samples, such as 1.89% formic acid in water, 0.2% formic acid in water, 0.2% formic acid in acetonitrile and pure acetonitrile, were examined. When brain tissue was homogenized in 0.2% formic acid/water or 1.89% formic acid/water and then extracted by protein precipitation with acetonitrile, it was found that 5-HT, DA and Glu showed poor peak shapes. Eventually, high extraction recovery and good peak shape

were obtained by direct protein precipitation (PPT) with ice-cold acetonitrile in our experiment. The supernatants were quantitatively transferred to a 10 mL glass tube, evaporated to dryness, and redissolved in this work, which was not agreement with previous literature where the extract samples were directly injected without evaporation and reconstitution [20].

Method validation

The chromatograms of the blank brain tissue and blank brain tissue spiked with the five mixed neurotransmitters standards and IS were graphed in Fig. 2. No significant and direct interferences from the brain sample were observed at the retention times of 5-HT, DA, NE, Glu, GABA and IS, which indicated that the procedure was specific.

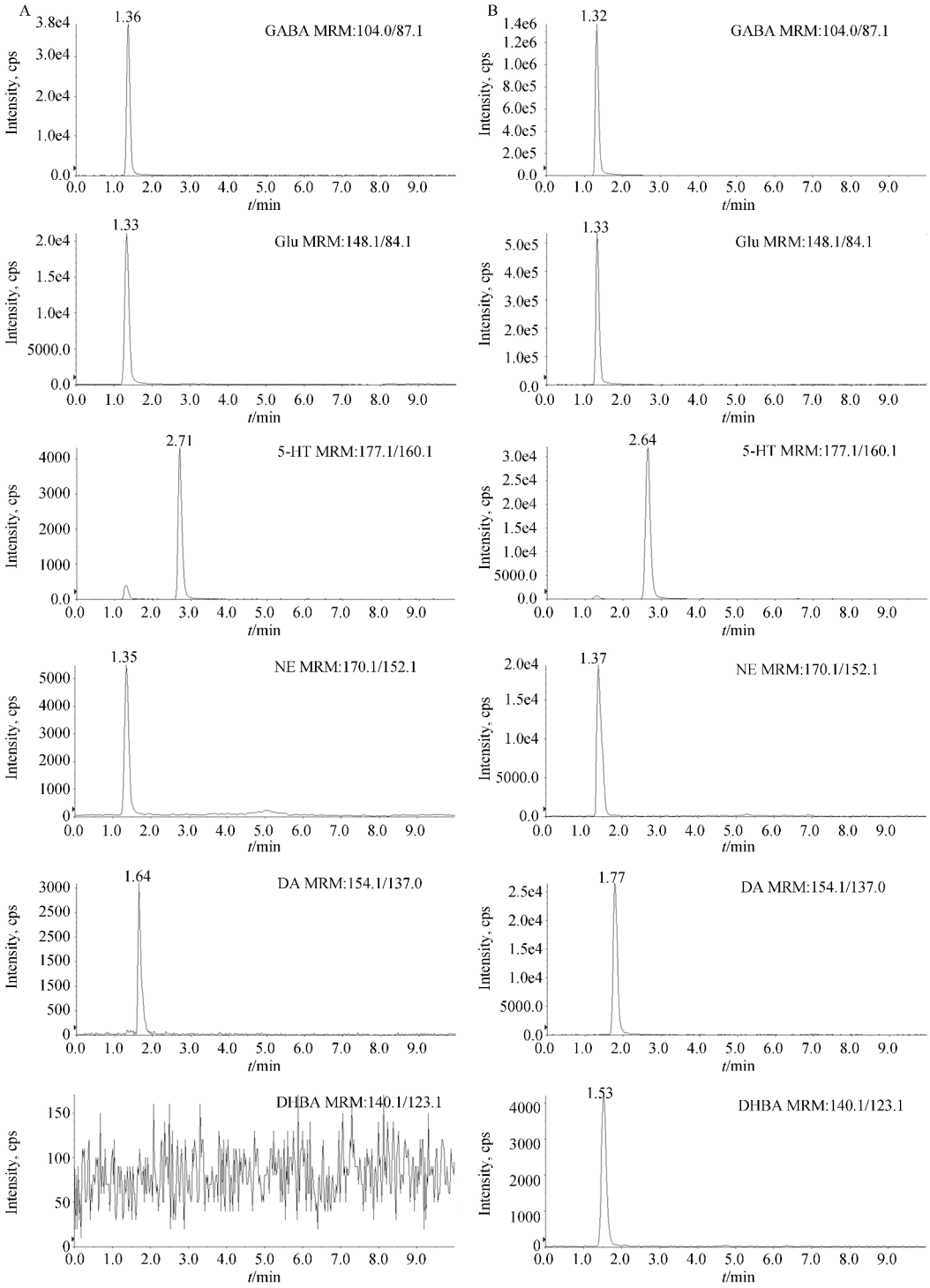

Fig. 2 Representative multiple reaction monitoring (MRM) chromatograms of (A) blank brain sample; (B) blank brain sample spiked with the analytes at LLOQs and IS

Because of the presence of endogenous analytes in brain tissue, the peak areas of the spiked standard were constructed by subtracting the corresponding areas derived from the blank matrix samples. Thus, blank matrix values must be subtracted from each calibration standard or QC. As shown in Table 2, all calibration curves had good correlation coefficient (R^2) values (> 0.9967). The intra-day, inter-day precisions and accuracies of QC samples are presented in Table 3. Intra-day precision was optimal, with RSDs between 0.64% and 9.41% and accuracy between –8.1% and 12.5% for four different concentrations of QC samples. The inter-day precision values also varied less than 15%, with RSDs between 3.97% and 11.81% and accuracy between –3.7% and 13.1% for all the QCs except the LLOQ. The recovery ranged from 73.34% to 114.46%, and the CVs ranged from 1.68% to 13.28%, which indicated that the one-step PPT method could ensure acquisition of accurate and consistent data. In addition, the mean matrix effects were ranged from 74.02% to 125.02%, and the CVs were all less than 15%. These results indicate that no significant matrix effects were obtained (Table 4). The results of stability experiments showed that no significant degradation occurred, as summarized in Table 5.

Table 2 The equations, linear range, correlation coefficient and LLOQ of neurotransmitters in rat brain

Analyte	Regression equation	Linear range (ng·mL^{-1})	Correlation coefficient (R^2)	LLOQ (ng·mL^{-1})	LOQ Accuracy (%)
5-HT	$y = 0.0765x + 0.0486$	1−99	0.9967	0.99	6.02
GABA	$y = 0.0038x + 23.77$	1998−199 800	0.9971	1998	–11.3
Glu	$y = 0.0011x + 4.1126$	1998−199 800	0.9987	1998	–2.1
NE	$y = 0.0011x + 0.0046$	122−12 187	0.9982	121.88	7.2
DA	$y = 0.0348x + 0.0505$	2.5−250	0.9973	2.5	8.51

Table 3 Intra-day and inter-day precisions and accuracies for the determination of the five analytes from the assay samples (mean ± SD, $n = 5$)

Analyte	Nominal concentration. (ng·mL^{-1})	Intra-day			Inter-day		
		Observed concentration (ng·mL^{-1})	Precision RSD%	Accuracy RE%	Observed concentration (ng·mL^{-1})	Precision RSD%	Accuracy RE%
5-HT	1	1.03 ± 0.074	7.23	4.29	0.964 ± 0.11	11.81	–2.28
	2	1.89 ± 0.18	9.41	–3.97	1.95 ± 0.17	8.64	–1.31
	10	9.67 ± 0.70	7.25	–1.97	10.58 ± 0.79	7.48	7.31
	79	86.43 ± 4.25	4.91	9.57	85.88 ± 3.82	4.46	8.88
GABA	1998	1837.00 ± 21.37	1.16	–8.06	1828.32 ± 73.21	4.00	–8.50
	3996	3802.55 ± 84.76	2.23	–4.84	4142.40 ± 292.57	7.06	3.66
	19 980	22 380.44 ± 139.81	0.64	12.01	22 601.40 ± 288.6	1.28	13.12
	159 840	173 673.21 ± 7209.66	4.15	8.65	162 462.16 ± 14703.11	9.05	1.64
Glu	1998	1844.035 ± 123.23	6.68	–7.43	1847.26 ± 92.90	5.03	–7.27
	3996	4153.97 ± 128.43	3.09	4.27	3871.64 ± 281.16	7.26	–2.82
	19 980	22 413.97 ± 812.34	3.62	12.52	21 842.59 ± 833.62	3.81	9.65
	159 840	150 924.99 ± 2307.43	1.53	–5.29	158 944.58 ± 10590.41	6.66	–0.26
NE	122	117.72 ± 8.44	7.17	–3.41	117.47 ± 9.49	8.08	–3.62
	244	240.21 ± 10.20	4.25	–1.45	234.72 ± 17.29	7.37	–3.70
	1219	1192.98 ± 20.54	1.72	–2.11	1275.83 ± 71.79	5.63	4.68
	9750	10 502.63 ± 514.23	4.90	7.7	10 773.77 ± 427.59	3.97	10.50
DA	2.5	2.50 ± 0.19	7.90	0.07	2.44 ± 0.20	8.21	–2.32
	5	4.74 ± 0.18	3.85	–5.31	5.02 ± 0.31	6.22	0.34
	25	23.91 ± 1.32	5.52	–4.38	24.42 ± 2.58	10.56	–2.34
	200	219.47 ± 8.15	3.71	9.74	212.07 ± 9.67	4.56	6.03

Determination of neurotransmitters in rat brain

Regulation of sleep and wakefulness may be a complex process involving different neurotransmitters, such as 5-HT, GABA, Glu, DA and NE. PCPA, an inhibitor of tryptophan

hydroxylase, which selectively depletes 5-HT in the brain peripheral tissues and blood, leads to a state of almost complete insomnia [23]. In the present study, the five neurotransmitters of the rat brain samples in the NC group, IM group, DZP group and three ZSS-treatment groups were quantified by the validated assay. The concentrations of all five analytes were calculated from their corresponding calibration curves. Box-whisker plots that demonstrate the concentration changes of these five analytes are shown in Fig. 3 and the corresponding results were listed in Table 6. They revealed that the levels of Glu and NE in the rat brain tissues were significantly increased ($P < 0.01$), whereas the levels of 5-HT, GABA and DA were significantly decreased ($P < 0.01$), in the PCPA-induced insomnia rats compared to control rats.

Table 4 Recoveries and matrix effects for the analytes in rat brain (Mean ± SD, $n = 3$)

Analyte	Spiked concentration (ng·mL^{-1})	Recovery (%)	Matrix effect (%)
5-HT	2	90.86 ± 3.63	74.02 ± 7.53
	10	97.18 ± 5.17	88.03 ± 2.27
	79	99.99 ± 1.47	107.93 ± 4.59
GABA	3996	73.34 ± 0.72	125.02 ± 6.34
	19 980	86.83 ± 3.56	110.15 ± 2.61
	159 840	99.78 ± 1.60	100.49 ± 2.11
Glu	3996	90.48 ± 3.55	110.30 ± 2.78
	19 980	92.87 ± 3.31	100.68 ± 4.16
	159 840	114.46 ± 5.81	90.84 ± 2.51
NE	244	109.11 ± 4.01	72.13 ± 5.49
	1219	108.60 ± 5.21	91.62 ± 8.16
	9750	99.96 ± 3.90	110.34 ± 1.76
DA	5	93.21 ± 3.59	103.03 ± 4.72
	25	92.05 ± 2.31	95.30 ± 6.85
	200	99.88 ± 2.55	104.51 ± 7.83

Table 5 The stability of the five analytes in rat brain under different storage conditions

Analyte	Nominal Conc. (ng·mL^{-1})	Three-freeze-thaw cycles		Autosampler (24 h)		25 °C (4 h)		Frozen for 30 days	
		Precision RSD%	Accuracy RE%	Precision RSD%	Accuracy RE%	Precision RSD%	Accuracy RE%	Precision RSD%	Accuracy RE%
5-HT	2	4.76	5.38	2.47	7.31	2.91	8.31	5.19	5.07
	10	7.67	–0.94	4.75	7.65	1.34	11.27	2.43	0.42
	79	4.24	–2.12	4.05	8.84	4.27	3.11	4.06	–0.74
GABA	3996	0.86	–1.40	1.36	–5.91	2.97	–0.08	7.06	–2.67
	19 980	0.70	12.3	0.71	12.3	0.47	11.02	0.87	11.64
	159 840	3.39	5.16	3.19	11.15	1.26	–0.16	0.70	3.67
Glu	3996	2.00	14.43	2.24	3.11	1.92	7.76	0.57	13.58
	19 980	0.75	9.41	3.16	11.34	2.46	9.12	0.50	9.48
	159 840	1.10	–2.72	1.66	–6.05	2.27	–3.12	1.29	–4.98
NE	244	4.45	–8.36	1.46	9.16	2.0	–1.46	1.88	–4.80
	1219	2.36	10.28	1.61	8.71	2.74	3.38	1.20	10.45
	9750	3.20	12.02	3.11	7.12	4.2	4.15	3.26	10.31
DA	5	6.69	–6.52	2.63	–8.31	4.18	–9.29	2.07	–5.41
	25	9.92	–1.70	4.14	–3.07	5.44	–2.32	8.86	–2.44
	200	1.54	12.73	2.75	10.44	1.95	12.32	0.82	13.50

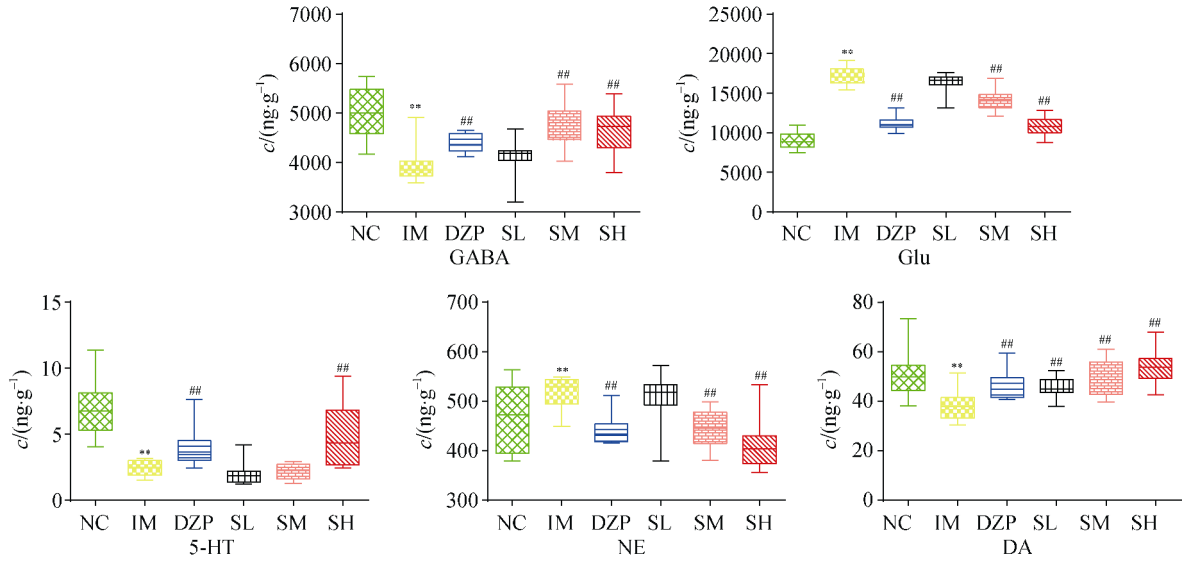

Fig. 3 The box plots showing the levels of the five differential neurotransmitters among NC, IM, DZP, SL, SM and SH groups. The statistical significance of differences between the model group and three doses of *Ziziphi Spinosae Semen* (ZSS) treatment groups (n = 12) are indicated as $^{\#\#}P < 0.01$ *vs* model group; $^{**}P < 0.01$ *vs* control group

Table 6 Concentration of neurotransmitters in rats from different groups (Mean ± SD, ng·g^{-1}, n = 12)

Analyte	NC	IM	DZP	SL	SM	SH
GABA	5012.50 ± 473.19	3926.04 ± 344.01**	4389.11 ± 188.40$^{\#\#}$	4117.52 ± 322.42	4766.89 ± 400.59$^{\#\#}$	4654.19 ± 419.11$^{\#\#}$
Glu	9059.85 ± 981.96	17 169.32 ± 1057.93**	11 175.35 ± 783.08$^{\#\#}$	16 375.51 ± 1107.25	14 188.89 ± 1283.11$^{\#\#}$	10 902.72 ± 1136.00$^{\#\#}$
5-HT	7.10 ± 2.19	2.47 ± 0.56**	3.85 ± 1.36$^{\#\#}$	1.97 ± 0.76	2.17 ± 0.55	4.78 ± 2.22$^{\#\#}$
NE	467.72 ± 65.30	517.64 ± 31.48**	440.48 ± 28.80$^{\#\#}$	506.22 ± 51.34	444.80 ± 35.95$^{\#\#}$	410.82 ± 48.14$^{\#\#}$
DA	50.37 ± 8.63	37.83 ± 5.61**	46.67 ± 6.11$^{\#\#}$	45.61 ± 3.73$^{\#\#}$	50.47 ± 6.76$^{\#\#}$	53.62 ± 6.52$^{\#\#}$

$^{**}P < 0.01$ *vs* the normal control group, $^{\#\#}P < 0.01$ *vs* the insomnia model group; NC, normal control group; IM, insomnia model group; DZP, Diazepam group; SL, low-dose ZSS treated group; SM, middle-dose ZSS treated group; SH, high-dose ZSS treated group

ZSS is the most commonly used single herb as a sedative and hypnotic medicine for its action on insomnia and anxiety [24]. DZP, the positive control for this study, is efficient in the treatment of symptoms of insomnia. Several studies have shown that the action of DZP is mostly mediated by the modulation of GABA action at $GABA_A$ receptors [25]. Glu and GABA, which act as excitatory and inhibitory amino acid neurotransmitters, respectively, play important roles in regulating sleep and wakefulness. Glu, known as a suppressor of sleep, exerts its actions in the central nervous system (CNS) through two principal types of receptors, ionotropic Glu receptors and metabotropic Glu receptors [15]. In contrast, GABA, a sleep-promoting factor, is derived from Glu under the action of glutamate decarboxylase [26] and inhibits arousal systems to promote sleep by binding to the $GABA_A$ receptor [27]. Consistent with a previous survey [16], the rats in the IM group showed significantly higher level of Glu ($P < 0.01$) in comparison with the NC group, which could be reduced after treatments with ZSS at SM and SH doses, while there was no significant change in SL group. In addition, the concertation of GABA in the brain of IM rats was significantly decreased compared with NC rats. After treatment with ZSS, the levels of GABA were strengthened at the SM and SH doses ($P < 0.01$) as compared to the IM group. However, there was no significant change in SL group. The effect of SM and SH doses was most noticeable, which not only significantly restored the levels of GABA and Glu but also showed a similar regulation effect of DZP. These results suggest that the hypnotic effect of ZSS aqueous extract might be associated with changes in amino acid metabolism participating in neurotransmitter synthesis.

5-HT, DA and NE are also important monoaminergic neurotransmitters involved in regulating sleep and waking. Decreased 5-HT at central sites has been correlated with worse sleep. In this study, the concentration of 5-HT in the IM group observed was significantly lower than that in the NC group, which suggests that deficiency in 5-HT is involved in the pathophysiology of insomnia. The level of 5-HT was reversed by treatment with SH or DZP, which may have restored serotonin synthesis and thus restored sleep. In addition, DA and NE are collectively known as catecholamines. DA is synthesized by tyrosine in the blood and is further metabolized to NE and HVA [15]. Compared to the NC group, a notable decrease in DA was found ($P < 0.01$), while NE

significantly increased in the IM group ($P < 0.01$). However, previous literature reported that DA was increased in brain samples of PCPA-induced insomnia rats [21], which was opposite to the current results. It was reported that the concentrations of DA in various brain regions of PCPA-induced mice were different and were depressed in the hypothalamus, corpus striatum and brain stem [15]. In contrast, the concentration of DA in all our drug-treated groups showed a significant increase ($P < 0.01$) in a dose dependent manner, while NE in the SM and SH groups showed an obvious decrease ($P < 0.01$) compared with the IM group. The above results demonstrate that the traditional decoction method by boiling ZSS powdered crude drug was effective for insomnia associated with monoaminergic metabolism participating in neurotransmitter synthesis.

Conclusion

In the present study, an LC-MS/MS method with a simple preparation procedure, higher sensitivity and shorter chromatographic conditions was developed and used for the determination of the levels of five neurotransmitters to evaluate the hypnotic effect of ZSS aqueous extract. The data obtained in this study demonstrate that ZSS aqueous extract (4 and 8 $g \cdot kg^{-1}$ for seven days) could ameliorate the symptoms of insomnia by modulating the levels of monoamines and amino acid neurotransmitters in the brain. Meanwhile, this study makes a great contribution to the application and development of ZSS decoction for the treatment of insomnia.

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