Determination of monoamine and amino acid neurotransmitters and their metabolites in rat brain samples by UFLC–MS/MS for the study of the sedative-hypnotic effects observed during treatment with S. chinensis

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Abstract

\textit{Schisandra chinensis} (Turcz.) Baill. has been used as a sedative and hypnotic agent in traditional Chinese medicine for centuries. The purpose of this study was to reveal the influence of insomnia on the levels of the neurotransmitters: glutamate (Glu), \gamma-aminobutyric acid (GABA), noradrenaline (NE), dopamine (DA), serotonin (5-HT) and their metabolites (5-HIAA, DOPAC and HVA), and to study the role of \textit{S. chinensis} in the treatment of insomnia. To achieve this goal, an efficient, sensitive and selective method was developed and validated for the simultaneous determination of these five neurotransmitters and their metabolites in rat brain samples using ultra fast liquid chromatography/tandem mass spectrometry (UFLC–MS/MS). The analysis was performed on a Synergi Fusion-RP 80A ODS column (150 mm × 2.0 mm, 4.0/μm) using gradient elution, with the mobile phase consisting of acetonitrile and 0.05% formic acid in water. The method was validated using rat brain homogenate samples and showed a good linearity over a wide concentration range ($r^2 > 0.99$) with a lower limit of quantification (LLOQ) at 4–16 ng mL\textsuperscript{−1}. The intra and inter-day assay variability was less than 15% for all analytes. The results indicated that the condition of insomnia elevated GABA, NE, DA, DOPAC and HVA, and reduced 5-HT, 5-HIAA levels in rat brain. The oral administration of \textit{S. chinensis} (7.5 g kg\textsuperscript{−1} day\textsuperscript{−1}, eight days) influenced insomnia by significantly increasing or reducing the levels of the neurotransmitters parameters mentioned above. These results suggested that \textit{S. chinensis} could alter the levels of these brain neurotransmitters and their metabolites through its sedative-hypnotic effects.

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1. Introduction

Insomnia is a serious health problem affecting more than one-third of the world population. Enhancing sleep quality is an issue of significant importance to public health[1]. According to recent estimates, the prevalence of insomnia is approximately 10% in many countries and is considered a burden worldwide[2]. Therefore, a large number of natural products used in traditional Chinese medicine are increasingly being utilized[3–5]. Increasing evidence demonstrates that many Chinese medicinal products, which have been traditionally used for the treatment of insomnia, have therapeutic effects. These observations have been verified by a host of clinical studies.

\textit{Schisandra chinensis} (Turcz.) Baill., a traditional Chinese herbal remedy, has been widely used to efficiently treat insomnia and anxiety for thousands of years in Asia[6,7]. It is the most commonly prescribed Chinese herbal formulae with the effects of hypnotic, sedative and anti-convulsive and has also been used to treat insomnia and anxiety in the modern clinical practice as well[3,8]. Our previous research had been focused on the plasma and brain pharmacokinetics studies of lignans[9–13] to study their sedative-hypnotic effects. In recent years, many studies have demonstrated that the extract from \textit{S. chinensis} induces...
sedative-hypnotic effects by regulating neurotransmission such as in the GABAergic or serotonergic systems in the central nervous system (CNS) [14]. However, the mechanism involved in its sedative hypnotic effect remains unclear. There are no detailed reports on the exact functionality of *S. chinensis* and its impact on neurotransmitter levels in the CNS or nerve pathways.

Neurotransmitters (NTs) and their metabolites are widely distributed in the central nervous system and the peripheral body fluids of mammals [15,16]. They are known to play a significant role in the nervous system for numerous organisms. They consist of amino acid neurotransmitters (AANTs), such as glutamate (Glu) and its metabolite γ-aminobutyric acid (GABA), and monoamine neurotransmitters (MANTs) such as 5-hydroxytryptamine (5-HT), norepinephrine (NE), dopamine (DA), and their acidic metabolites 5-hydroxyindole-3-acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). The molecular structures of these chemicals are presented in Fig. 1. The monitoring of NTs and their metabolites is an essential tool for elucidating normal and pathological neural system functions. Trace-level measurements of NTs in biological specimens are especially important in studying the NT metabolism profile and the role of NTs and their metabolites in neurophysiology, behavioural effects, pathology, disease diagnosis and control. This is because changes in the metabolism and levels of these NTs have been associated with insomnia [17–19]. A change in the brain levels of NTs and their metabolites is the hallmark of neurotransmitter disorders. Analysis of NT levels could aid in disease diagnosis, prognosis and the monitoring of treatment. There are several HPLC or LC/MS methods that have been reported to determine the levels of various NTs [20,21].

However, to the best of our knowledge there is no reported LC–MS method for the simultaneous determination of all these NTs and their metabolites in the brain, with higher sensitivity and shorter chromatographic run time. Therefore, this study was conducted to develop an efficient, sensitive and selective UPLC–MS/MS method for the simultaneous determination of eight NTs and their metabolites in rat brain samples to investigate the sedative-hypnotic effects of *S. chinensis* extract on insomnia rats.

2. Experimental

2.1. Materials and reagents

*S. chinensis* (Turcz.) Baill. was purchased from the TCM shop of Tianyitang (Shenyang, China) and identified by the associate professor, Ying Jia (Department of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang, China). DA, HVA, NE, 5-HT, 5-HIAA and GABA were purchased from Sigma (St. Louis, MO, USA). DOPAC, Glu and the internal standard (IS) 3,4-dihydroxybenzylamine (DHBA) were purchased from Alfa Aesar (Company Inc., USA). 4-chloro-DL-phenylalanine (PCPA) was supplied by Sigma (Company Inc., USA). Diazepam was purchased from Xinyi Pharmaceutical Ltd. (Shanghai, China).

Acetonitrile and formic acid of HPLC grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water prepared with demineralised water was used throughout the study. All the other reagents were of analytical grade.

2.2. Preparation of *S. chinensis* decoction

The dosing solutions used for all animal studies were prepared by extracting the crude powder (250 g) of *S. chinensis* with 95% ethanol at reflux for 2 h (three times) followed by concentration under vacuum. The ethanol crude extract was then dissolved in 500 mL water, which served as the intragastric solution for further use. The decoction was stored in the refrigerator at 4 °C.

2.3. Animals and treatment

Male pathogen-free Sprague-Dawley rats (220–250 g) were kindly provided by the Experimental Animal Center of Shenyang Pharmaceutical University. The animal study was carried out in accordance with the Guideline for Animal Experimentation
of Shenyang Pharmaceutical University and the protocol was approved by the Animal Ethics Committee of the institution.

The animals were allowed to acclimatise to the environment for one week before the experiment. Thirty-two rats were randomly divided into four groups, with eight animals each: the normal control group, the blank insomnia model control group, the diazepam (2.0 mg kg\(^{-1}\)) group and the S. chinensis group (7.5 g kg\(^{-1}\)). The experimental groups received an oral administration of the S. chinensis decoction in distilled water at a concentration of 0.5 g ml\(^{-1}\), whereas the normal control group and the insomnia model group received distilled water. Diazepam was dissolved in distilled water at a final concentration of 0.2 mg ml\(^{-1}\). The diazepam and S. chinensis decoction solutions were administered orally, once daily, for eight consecutive days.

Insomnia was induced in the rats by the chemical reagents method, as described previously [22]. In brief, PCPA was suspended in 0.5% gum acacia/physiological saline. The rats in the blank insomnia model control group, the diazepam group and S. chinensis group were given intraperitoneal injection of PCPA (300 mg kg\(^{-1}\)), whereas the normal control group was administered the same volume of saline once daily lasting two days, respectively. After the injection of PCPA, the rats in the model group lost their circadian rhythm and were sleepless all day which proves the success of the model. Two hours after the final administration, the rats were sacrificed by decapitation and the brains were rapidly removed, frozen and stored at \(-80^\circ\)C until extraction.

### 2.4. Instruments and UFLC–MS/MS conditions

The controls and samples were analysed on a Q TRAP\textsuperscript{TM} 4000 MS/MS system from Applied AB Sciex (Foster City, CA, USA) coupled to a Prominence\textsuperscript{TM} UFLC system (Shimadzu, Japan). Separations were accomplished on a Synergi Fusion-RP 80A ODS column (150 mm \(\times\) 2.0 mm, 4.0 \(\mu\)m) (Phenomenex, USA) with a guard cartridge, at temperature of 30 \(^\circ\)C. The mobile phase, consisting of 0.05% formic acid in water (solvent A) and acetonitrile (solvent B) was delivered at a flow rate of 0.2 mL min\(^{-1}\). The linear gradient elution program was as follows: (1) held at 2% B in 0.1–2.5 min; (2) from 2% B to 30% B in 2.5–8.0 min; (3) from 30% B to 2% B in 8.0–9.0 min; and (4) held at 2% B for 2.0 min. The injection volume was 2 \(\mu\)L and the total time taken for the chromatographic run was 11.0 min per sample.

The mass spectrometer was operated in the positive ion mode with a TurbolonSpray source. Table 1 represents the optimised MRM parameters for the analytes and the IS. The Q1, Q3, declustering potential (DP) and collision energy (CE) values were based on Analyst software 1.5 from Applied Biosystems/MDS Sciex. The

### Table 2

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear range (ng mL(^{-1}))</th>
<th>Regression equation (y = ax^2 + bx + c)</th>
<th>Correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>8.0–4000</td>
<td>(y = 0.0004 + 0.0058x)</td>
<td>0.9923</td>
</tr>
<tr>
<td>Glu</td>
<td>16–8000</td>
<td>(y = 0.0235 + 0.0102x)</td>
<td>0.9917</td>
</tr>
<tr>
<td>NE</td>
<td>4.0–2000</td>
<td>(y = 0.0003 + 0.0023x)</td>
<td>0.9943</td>
</tr>
<tr>
<td>DA</td>
<td>4.0–2000</td>
<td>(y = 0.0016 + 0.0014x)</td>
<td>0.9910</td>
</tr>
<tr>
<td>5-HT</td>
<td>4.0–2000</td>
<td>(y = 0.0009 + 0.0027x)</td>
<td>0.9952</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>4.0–2000</td>
<td>(y = 0.0005 + 0.0012x)</td>
<td>0.9967</td>
</tr>
<tr>
<td>DOPAC</td>
<td>8.0–4000</td>
<td>(y = 0.0023 + 0.0086x)</td>
<td>0.9923</td>
</tr>
<tr>
<td>HVA</td>
<td>8.0–4000</td>
<td>(y = 0.0008 + 0.0039x)</td>
<td>0.9950</td>
</tr>
</tbody>
</table>
other ionisation parameters were as follows: curtain gas (CUR), 20 (arbitrary units); ion source gas 1 (GS1), 50 (arbitrary units); ion source gas 2 (GS2), 50 (arbitrary units); and source temperature (TEM), 500 °C; entrance potential (EP), 10 V. The dwell time of each MRM transition was 50 ms. The mass spectrometer and the UFLC system were controlled by Analyst 1.5.1 software from Applied Biosystems/MDS Sciex.

2.5. Standard solution and quality control samples

The standard stock solutions (100 μg mL\(^{-1}\)) of the six analytes (DA, NE, 5-HT, DOPAC, HVA and 5-HIAA) were prepared by dissolving the appropriate amount of the chemical reference in acetonitrile. The standard stock solutions of Glu and GABA were prepared in ultrapure water, at 100 μg mL\(^{-1}\). The standard stock solution of the IS (DHBA) was prepared in acetonitrile-water solution (60:40, v/v). The stock solutions of the analytes were further diluted with water to prepare working standard solutions at the desired concentrations.

The brain tissue samples were homogenised in a four-fold excess volume of methanol. The brain tissue standards for each of the eight analytes were prepared GABA, at concentrations of 8.0, 40, 200, 600, 1000, 2000 and 4000 ng mL\(^{-1}\), Glu at concentrations of 16, 80, 400, 1200, 2000, 4000 and 8000 ng mL\(^{-1}\), DA, NE, 5-HT and 5-HIAA at concentrations of 4.0, 20, 100, 300, 500, 1000 and 2000 ng mL\(^{-1}\), DOPAC and HVA at concentrations of 8.0, 40, 200, 1000, 2000 and 4000 ng mL\(^{-1}\). These standards were prepared by adding the appropriate amount of the standard working solutions to the blank brain tissue homogenates. Quality control samples were prepared in the same fashion. A working solution for the IS (200 ng mL\(^{-1}\)) was also prepared.

2.6. Sample preparation

Male pathogen-free Sprague-Dawley rats (250–280 g) were used. The brains were rapidly removed, frozen and stored at −80 °C until extraction. The frozen brain tissue samples were dissected and homogenised in a four-fold excess volume of methanol. The homogenates were centrifuged at 18,000 × g for 20 min at 4 °C. Then, 1 mL of the supernatant was transferred to a 10 mL centrifuge tube, along with 10 μL of the IS solution and 10 μL water, followed by vortexing for 5 min and shaking for 5 min. Next, these samples were centrifuged at 3000 × g for 10 min at 4 °C. The supernatants were quantitatively transferred to a 5 mL glass tube and evaporated to dryness at 35 °C under a slight stream of nitrogen. Finally, the dried extract was reconstituted in 100 μL water, followed by the injection of a 2 μL aliquot into the UFLC–MS/MS system for analysis.

2.7. Statistical data analysis

All values are represented as the mean ± S.D. The data obtained were analysed by ANOVA using the SPSS (Statistical Package for the Social Science) 16.0 statistical software, using the Independent Samples T-test after their natural logarithmic transformation or the Mann–Whitney test. p < 0.05 was considered statistically significant for all the tests.

3. Results and discussion

The aim of this work was to develop a multiclass reliable UFLC–MS/MS method for the analysis of five neurotransmitters and their three metabolites, in the rat brain, during the treatment of insomnia using S. chinensis.

3.1. Determination of MS/MS parameters

The standard solution (100 ng mL\(^{-1}\)) for each analyte was infused into the ESI source at positive ion mode for MS/MS optimisation. In all the cases, [M+H]+ ions were selected as the precursor ions. The most abundant transition was selected for quantitation, and the second most abundant transition was used for confirmation. The collision energy was optimised to achieve the highest sensitivity. Table 1 shows the MS/MS transitions used for quantification and confirmation, along with the optimised cone voltages.
Table 4: Stability of the eight analytes in rat brain (n = 3).

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Concentration (ng mL(^{-1}))</th>
<th>24 h, room temperature</th>
<th>30 days, (-20^\circ\text{C})</th>
<th>3 freeze-thaw cycles</th>
<th>8 h, 4(^\circ\text{C})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RE (%)</td>
<td>RSD (%)</td>
<td>RE (%)</td>
<td>RSD (%)</td>
<td>RE (%)</td>
</tr>
<tr>
<td>GABA</td>
<td>40</td>
<td>3.2</td>
<td>5.1</td>
<td>-3.2</td>
<td>6.3</td>
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<tr>
<td></td>
<td>600</td>
<td>7.2</td>
<td>5.2</td>
<td>-4.1</td>
<td>6.2</td>
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<tr>
<td></td>
<td>2800</td>
<td>-6.5</td>
<td>7.2</td>
<td>-7.8</td>
<td>4.3</td>
</tr>
<tr>
<td>Glu</td>
<td>80</td>
<td>5.9</td>
<td>10.3</td>
<td>-1.9</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>2.1</td>
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<td>-6.1</td>
<td>4.5</td>
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<td>7.1</td>
<td>5.2</td>
<td>2.1</td>
<td>6.2</td>
</tr>
<tr>
<td>NE</td>
<td>20</td>
<td>-4.4</td>
<td>2.3</td>
<td>5.7</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6.9</td>
<td>5.2</td>
<td>-2.1</td>
<td>4.4</td>
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<tr>
<td></td>
<td>1400</td>
<td>-4.1</td>
<td>3.8</td>
<td>7.1</td>
<td>1.4</td>
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<td>DA</td>
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<td>5-HT</td>
<td>20</td>
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<td>5-HIAA</td>
<td>20</td>
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<tr>
<td>DOPAC</td>
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<td>-9.7</td>
<td>8.3</td>
</tr>
<tr>
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<tr>
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<tr>
<td>HVA</td>
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<td>7.4</td>
<td>1.8</td>
<td>2.1</td>
<td>7.7</td>
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</table>

The chromatographic variables were optimised to achieve adequate separation between the analytes without interference from the background, and with short retention times and good peak shapes. For this purpose, the compositions of the chromatographic column and the mobile phase were investigated. DA, NE and their metabolites are polar molecules with poor retention in a regular reversed phase column. In this study, the use of a less organic mobile phase in the chromatographic column could retain the analytes. The best results were obtained when acetonitrile was used as the organic solvent, with the addition of formic acid (0.05% (v/v)) to improve the resolution and efficiency.

3.2. Optimisation of chromatographic separation

The method described in this study was applied for the determination of neurotransmitters in the rat brain homogenate were validated and the results were within the acceptable criteria (RSD%: <15%; RE%: ±15%). These results were represented in Table 3, and they indicate that the method has acceptable precision and accuracy.

3.3. Method validation

The validation of linearity, accuracy (precision and trueness), sensitivity and selectivity was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [23].

3.3.1. Linearity and LLOQ

Calibration curves for the neurotransmitters and their metabolites in the rat brain were generated by plotting the peak area ratio (y) of the analytes to the IS against the nominal concentrations (x) of the analytes in the standards by 1/\(x^2\) weighted least square linear regressions. The standard calibration curves for the spiked brain tissue homogenates showed good linearity for the analytes. The results are shown in Table 2.

The lower limits of quantification for each analyte in the brain tissue homogenates were 8.0 ng mL\(^{-1}\) for GABA, DOPAC and HVA, 4.0 ng mL\(^{-1}\) for DA, NE, 5-HT and 5-HIAA and 16 ng mL\(^{-1}\) for Glu, respectively.

3.3.2. Precision and accuracy

The intra-day precision, inter-day precision and accuracy of the eight analytes in the rat brain homogenate were validated and the results were within the acceptable criteria (RSD%: <15%; RE%: ±15%). These results were represented in Table 3, and they indicate that the method has acceptable precision and accuracy.

3.3.3. Extraction recovery and matrix effect

The mean extraction recoveries of the eight analytes were more than 85.0% at different concentration levels (Table 3), and the average extraction recovery of the IS was 88.7%, which are acceptable values.

The matrix effect of the analytes ranged from 90% to 110% at three concentration levels (Table 3); whereas the matrix effect of the IS was 89.4%. These findings indicate that there was no significant matrix effect for the analytes and IS.

3.3.4. Stability

The concentration measured for the eight analytes at each QC level were deviated by 12.0%, which demonstrated that they were stable in the biosamples at room temperature for 24 h, at \(-20^\circ\text{C}\) for at least 15 days, after three freeze and thaw cycles, and at \(4^\circ\text{C}\) in the autosampler for 8 h after preparation. This result is well within the acceptable limit and is described in Table 4.

3.4. Determination of neurotransmitters in rat brain

The method described in this study was applied for the determination of neurotransmitters in the rat brain. Fig. 3 indicates the chromatograms corresponding to the eight analytes and the IS. The results of the four groups, i.e., the normal control group, the blank insomnia model control group, the diazepam group and the S. chinensis group are shown in Table 5. The PCPA-induced insomnia rat

and collision energy values. The optimum values for the other parameters are indicated in Section 2. MS/MS spectra of the analytes and the IS (DHBA) are presented in Fig. 2.
Fig. 3. Typical chromatograms of (A) standards plus IS. Note that the signals of MAMTs and their metabolites, together with DHBH. (B) Identification of NTs and their metabolites in rat brain samples. Representative MBM chromatograms of GABA, Glu, NE, DA, 5-HT, 5-HIAA, DOPAC, HVA and DHBH (IS).
model showed a significant increase in the brain levels of GABA, NE, DA and the metabolites DOPAC and HVA (p < 0.05), as well as a significant decrease in the 5-HT and 5-HIAA levels (p < 0.05). However, the administration of diazepam and S. chinensis significantly reduced the elevation of GABA, NE, DA, DOPAC and HVA, and increased the levels of 5-HT and 5-HIAA (p < 0.05). We inferred that the sedative-hypnotic effect of S. chinensis was presumably acting via the noradrenergic, dopaminergic GABAergic and/or serotonergic systems in the brain [24–26]. Taking these results into consideration, the effects of S. chinensis on the monoamines and amino acid neurotransmitters levels in the brain may be an important mechanisms involved in fighting insomnia.

4. Conclusion

In this paper, we conclude that S. chinensis could ameliorate the symptoms of insomnia. The mechanisms of its action may be associated with its sedative-hypnotic effects by modulating the levels of monoamines and amino acid neurotransmitters and their metabolites in the brain. To the best of our knowledge, this is the first report to thoroughly study the sedative-hypnotic mechanism of S. chinensis and simultaneously analyse eight analytes in rat brain samples. The data obtained in this study would make a great contribution to the application and development of S. chinensis decoction for the treatment of insomnia.

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References

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