Determination of Synephrine and N-Methyltyramine in Zhishi and Zhike (Immature Citrus Fruits) by High-Performance Liquid Chromatography with Electrochemical Detection

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High-performance liquid chromatography (HPLC) with electrochemical detection was used to determine synephrine (SYN) and N-methyltyramine (NMT) in Zhishi (Kijitsu 桔実) and Zhike (Kikoku 桔巻). This electrochemical detection method has not only specific selectivity but also very high sensitivity, thus facilitating the determination of SYN and NMT from pulverized Zhishi and Zhike samples. Zhishi and Zhike derived from Citrus aurantium produced in different districts in China were analysed. This method was found useful for evaluating the quality of a crude drug such as Citrus aurantium.

Keywords synephrine; N-methyltyramine; Citrus aurantium; Chinese crude drug; HPLC; electrochemical detection

Crude drugs derived from citrus fruits, whose Chinese names are Zhishi and Zhike (Japanese name: Kijitsu 桔実 and Kikoku 桔巻) are excellent Chinese medicinal materials considered to function as regulators of vital energy and to remove phlegm. In traditional Chinese medicine, they have been used as expectorants and antitussives. Zhishi is derived from unripe fruit and Zhike from the nearly-ripe fruit of Citrus aurantium. Both drugs contain synephrine (SYN) and N-methyltyramine (NMT) (Chart 1) which have numerous pharmacological activities such as raising blood pressure, increasing blood flow, and enhancing the contractive strength of heart muscle.1-3 At present, the content of SYN is employed as a criterion for evaluating the quality of these crude drugs.

SYN in citrus crude drugs has been determined by different methods such as high-performance liquid chromatography (HPLC) with UV detection4-6 and thin-layer chromatography (TLC)-densitometry detection.7,8 NMT has been determined by HPLC with UV detection5 and TLC.8

TLC requires development and coloration with a color reagent and this is time-consuming, even for semi-quantitative analysis. HPLC with UV detection requires the complex pretreatment of samples to avoid interference by other substances.

Recently, HPLC with electrochemical detection has been used to determine SYN in insect brain.9,10 This approach is characterized by specific selectivity with high sensitivity. NMT may be oxidized at a suitable potential, since it also possesses a phenolic hydroxyl group electrochemically oxidizable. HPLC with electrochemical detection was used in this study to simultaneously determine SYN and NMT in Zhishi and Zhike from different districts in China. This assay method should prove useful for measuring the content of NMT and SYN in various crude drugs derived from citrus fruits.

Experimental

Plant Materials Experimental materials of Citrus aurantium were collected at several locations in China in June-July, 1989.

Chemicals and Reagents NMT hydrobromide was obtained from the Academy of Medicinal and Pharmaceutical Industries of Hunan in China. SYN was purchased from Sigma Chemical Co., and norepinephrine (NE) hydrochloride from Aldrich Chemical Co., powders of Chinese medicine extract from Tsumura Co., and Kanebo Co., and other chemicals from Wako Pure Chemical Industries, Ltd.

Chromatography The HPLC system consisted of a Jasco-880PU pump (Jasco, Japan), 6125 injector fitted with a 50 µl injection loop (Rheodyne, U.S.A.), Kaseiorb LC-ODS-120-5 column (250 x 4.6 mm i.d., Tokyo, Kasei, Japan), and ED-1 type electrochemical detector (Kotaki, Japan). The mobile phase was prepared from 0.02 M citric acid-0.02 M NaH₂PO₄ (7:3, v/v) and adjusted to a final pH of 3.0 with citric acid. The system was operated at room temperature.

Sample Preparation Extraction Procedure A: Pulverized sample (1 mg) was extracted with 3 ml of methanol under ultrasonication for 30 min. After filtration, the residue was washed with 3 ml of methanol and the combined filtrate was evaporated to dryness. The residue was dissolved in 0.2 ml of 0.01 N HCl. To the solution, 0.5 ml of 2.0 x 10⁻¹⁰ M NE solution and 0.3 ml of the mobile phase were added to give a sample solution. Extraction Procedure B: Pulverized sample (200 mg) was extracted with water (10 ml) at 90°C for 6 h with occasional shaking. After filtration, the residue was re-extracted in the same manner. The combined filtrate was applied onto a column of Amberlite IRC-50 (H⁺ form) as reported by Kinoshita et al.11 and Namba et al.12 The column was washed with water and eluted with 5N NH₄OH. The eluate was evaporated to dryness in vacuum. The residue was dissolved in 10 ml of 0.01 N HCl. The solution was diluted with the mobile phase to give a volume of 50 ml. A 0.25 ml of this solution was taken, then 0.5 ml of 2.0 x 10⁻¹⁰ M NE solution and 0.25 ml of mobile phase were added to give a sample solution.

Ten microliters of sample solution was injected into the HPLC column.

Results and Discussion

Hydrodynamic voltammograms are shown in Fig. 1. SYN and NMT each possess a hydroxyl group on the benzene nucleus, which is oxidized at a suitable potential. As seen in Fig. 1, NMT was oxidized at a potential above 0.8 V and SYN at above 0.9 V. Based on data from the hydrodynamic voltammograms, the detection potential for analysis of SYN and NMT was normally maintained at 1.2 V vs. Ag/AgCl. Ten microliters of standard solution mixture of NE as the internal standard compound, SYN and NMT were injected into the column followed by chromatography in the mobile phase pumped at a flow rate of 1 ml/min. The retention times of NE, SYN, and NMT were 3.4, 5.8 and 14.5 min, respectively (Fig. 2).
A series of solutions containing the internal standard and various amounts of SYN and NMT were analysed. Peak height ratios relative to standard SYN and NMT were plotted against amounts of SYN and NMT. SYN in 17-10700 ng/ml was found to have a good linear relationship to peak height ratio with a correlation coefficient of 0.9981. In 270 ng/ml, relative standard deviation was 1.38% (n = 15) and the detection limit (S/N = 3) was 13 ng/ml. NMT in 12-26600 ng/ml indicated a good linear relationship to peak height ratio with a correlation coefficient of 0.9994. In 720 ng/ml, relative standard deviation was 1.44% (n = 15) and the detection limit (S/N = 3) was 12 ng/ml.

In the solvent for extracting the sample, 85% ethanol, hot water (90–95 °C), and methanol have been used in some cases. SYN easily dissolves in methanol and thus methanol extraction of the sample by ultrasonication was conducted in this study. For evaluation of practical application, methanol extraction (extraction procedure A) was compared with water extraction (extraction procedure B). The recovery of SYN was essentially the same by both methods (Table I). Methanol was thus a more suitable solvent for sample extraction, and extraction procedure A, which is applicable to a small amount of sample, requires less time, and is convenient and very simple.

A typical chromatogram for the extracted sample solution is shown in Fig. 3. There was no interference with the peaks of SYN and NMT. SYN and NMT in Zhishi and Zhihe from different districts in China were analysed and the
Table III. Content of SYN in Powders of Chinese Medicine Extract Produced in Japan

<table>
<thead>
<tr>
<th>Name of powder</th>
<th>Content of SYN (%)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakuryoin-gou-hangekouboku-to</td>
<td>0.067 5%&lt;sup&gt;a&lt;/sup&gt;, 11%&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Chikujontan-to</td>
<td>0.047 7%&lt;sup&gt;a&lt;/sup&gt;, 7%&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Chouto-san</td>
<td>0.060 11%&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Daisakto-to</td>
<td>0.027 7%&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Juncho-to</td>
<td>0.022 8%&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Hei-san</td>
<td>0.078 22%&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Hochuekki-to</td>
<td>0.034 8%&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Keigairengyo-to</td>
<td>0.020 6%&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Kouso-san</td>
<td>0.037 19%&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mashin-in-gan</td>
<td>0.025 12%&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Seijouboufu-to</td>
<td>0.019 4%&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Sokenakketsu-to</td>
<td>0.025 5%&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tsudo-san</td>
<td>0.076 12%&lt;sup&gt;a&lt;/sup&gt;, 8%&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tokishigayaku-ka-goshuyu-shokyo-to</td>
<td>0.007 7%&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
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Content of a) Kijitsu (Immature Orange), b) Chinpi (Citrus Unshiu Peel), and c) Goshuyu (Evodia Fruit) in Chinese medicine used for the powders.

results are shown in Table II. Both were found to have anti-contraction activity and the effectiveness of the two drugs is indication of their amounts. Zhihi and Zhike usually contained SYN and NMT, and in most samples of Zhishi their amounts were higher than in Zhike. Their quantity in Chou Zhihi was higher than in other species.

The present method having high sensitivity was thus used to determine SYN in powders of Chinese medicine extracts available in Japan. The extraction of SYN was carried out by extraction procedure A and the results are shown in Table III. All samples contained extracts of Kijitsu, Chinpi (陳皮) and/or Goshuyu (吳萸萸), the content of each of which is shown in Table III. It has been reported that these three crude drugs usually contain synephrine of about 0.2%,<sup>3,4,13</sup>

In conclusion, HPLC with electrochemical detection makes possible the rapid, interference-free assay of SYN and NMT. It has high sensitivity and specific selectivity and can be conducted quite easily. It is suitable for determining SYN and NMT in immature citrus fruits as well as assessing the quality of Zhishi and Zhike.

References