Simultaneous Quantification of Six Constituents in Qing-Huo-Zhi-Mai Tablet by High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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Received 8 October 2013; revised 24 November 2013

A novel sensitive and specific high-performance liquid chromatography–tandem mass spectrometry method was established for the simultaneous determination of six constituents including geniposide, andrographolide, dehydroandrographolide, ophiopogonin D, methylphiliopogonanone A and methylphiliopogonanone B in Qing-huo-zhi-mai (QHZM) tablet, a well-known Chinese herbal preparation. The chromatographic separation was performed on a C₁₈ column, and the mobile phase was composed of 0.04% acetic acid and acetonitrile with gradient elution. The detection of analytes was carried out by multiple reaction monitoring scanning with switching electrospray ion source polarity between positive and negative modes in a single run. The total run time was 15 min. The calibration curves were linear with all correlation coefficients >0.9979 in the tested ranges. The intra- and interday variations were no >7.0%, and the average recoveries were in the range of 93.2–108.5% with the relative standard deviations no >5.4%. The developed method was successfully employed to analyze five batches of QHZM tablet samples. This is the first time for the determination of ophiopogonin D, methylphiliopogonanone A and methylphiliopogonanone B in QHZM tablets.

Introduction

Qing-huo-zhi-mai (QHZM) tablet, a well-known Chinese herbal preparation, is composed of three herbs: Andrographis paniculata, Fructus Gardeniae and Radix Ophiopogonis. As recorded in China Pharmacopoeia (ChP, Version 2010), it has the effect of eliminating heat and venom, cooling blood and mitigating swell. In clinic, it has been commonly used for the treatment of sore throat, fever, toothache and bloodshot eyes caused by lung–stomach heat.

Phytochemical studies have showed that diterpenoids were the major constituents in A. paniculata, among which andrographolide and dehydroandrographolide were the main active components (1, 2). Fructus Gardeniae contained abundant iridoid glycosides, and geniposide was usually chosen as the chemical marker (3–6). In Radix Ophiopogonis, two classes of constituents including ophiopogonins and ophiopogonones were generally considered as the characteristic components (7, 8). Therefore, simultaneous quantification of these constituents in the three herbs could be significant for the quality control of QHZM tablet.

In ChP (Version 2010), two different high performance liquid chromatography (HPLC) systems were employed to assay geniposide, andrographolide and dehydroandrographolide in QHZM tablet, respectively. There were also some reports on quantitative analysis of QHZM tablet, in which high performance thin layer chromatography (HPTLC) (9–11) or HPLC-UV (12–14) methods were used frequently. However, most of these researches focused on the individual determination of 1, 2 or 3 constituents by different methods. Even though a simultaneous quantification method was developed in one literature (14), only three compounds in A. paniculata and Fructus Gardeniae were determined. Up to now, no components of Radix Ophiopogonis in QHZM tablet have been controlled.

Owing to the high specificity, high sensitivity and strong versatility, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS-MS) is particularly appropriate for the analysis of complex mixtures. It possesses tremendous advantages over HPLC-UV and HPLC-evaporative light scattering detector (ELSD), especially when analyzing substances such as ophiopogonins from Radix Ophiopogonis that have no obvious characteristic ultraviolet absorption. Therefore, this technology has been widely used for quantification analysis of multicomponents in traditional Chinese herbs and their preparations (15–19). Although this method has been developed to analyze QHZM tablet, it was only used for recognition and identification of chemical constituents (20). So far, there is no research concerning quantification of the constituents in QHZM tablet by HPLC–MS.

The aim of this study was to establish an HPLC–MS-MS method for the simultaneous determination of six constituents, including geniposide, andrographolide, dehydroandrographolide, ophiopogonin D, methylphiliopogonanone A and methylphiliopogonanone B, in QHZM tablet. Their chemical structures are shown in Figure 1. This present work would provide an alternative method to the current method in ChP for the quality control of QHZM tablet.

Experimental

Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Center Valley, PA, USA), and HPLC grade acetic acid was obtained from Dikma Technologies Inc. (Lake Forest, CA, USA). Methanol used in sample preparation was of analytical grade and purchased from Yongda Chemical Reagent Co., Ltd. (Tianjin, China). Purified water was supplied by Wahaha Group Co., Ltd. (Hangzhou, China).

Geniposide, andrographolide and dehydroandrographolide were purchased from National Institutes for Food and Drug Control (Beijing, China). Methylphiliopogonanone A and
methyllophiopogonanone B were obtained from Shanghai Shifeng Biological Technology Co., Ltd. (Shanghai, China). Ophiopogonin D was supplied by Shanghai Yilin Biological Technology Co., Ltd. (Shanghai, China). The purities of all standards were ≥98% according to HPLC analysis.

Five batches of QHZM tablet samples produced by different manufacturers were purchased from local drug stores.

Sample preparation
The coatings of tablet samples were first removed completely, and the remains were smashed into powder. An amount of 0.06 g of the powder was weighed accurately and settled to a volumetric flask of 10 mL, and then was ultrasonically extracted using 9 mL methanol at 30°C for 60 min. After standing at room temperature, the extraction solution was diluted to 10 mL and then was filtered through filter paper. Subsequent filtrate was centrifuged at 14,000 rpm for 10 min. Afterwards, 0.5 mL of supernatant was diluted by equivalent purified water. The diluted solution was analyzed by HPLC–MS–MS.

The negative control samples, lacking A. paniculata, Fructus Gardeniae and Radix Ophiopogonis, were prepared, respectively, according to the prescription proportion and processing of the QHZM tablet. The negative control solutions were then prepared from the negative control samples according to the above sample preparation method.

Standard solution preparation
Appropriate amounts of geniposide, andrographolide, dehydroandrographolide, ophiopogonin D, methyllophiopogonanone A and methyllophiopogonanone B were separately weighed and dissolved in methanol to prepare six individual stock solutions. A series of standard working solutions were then prepared by appropriately mixing and diluting six stock solutions with 50% methanol. Their concentration ranges are given in Table 1. All solutions were stored at 4°C before analysis.

Figure 1. The chemical structures of six constituents in QHZM tablet.
Instrumental and chromatographic conditions
An Agilent 1200 liquid chromatography system (Agilent, Palo Alto, CA, USA) equipped with a quaternary solvent delivery system, an autosampler and a column compartment was used. The chromatographic separation was carried out on an Agilent Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm) at 35 °C. The mobile phase consists acetonitrile (A) and 0.04% acetic acid (B), with a linear gradient elution at a flow rate of 0.8 mL/min. The gradient program was as follows: 0–3 min, 16% A; 3–4 min, 16–60% A; 4–10 min, 60% A; 10–12 min, 60–95% A; 12–15 min, 95% A. The injection volume was 10 μL.

A 3200 QTRAP™ system from Applied Biosystems/MDS Sciex (Applied Biosystems, Foster City, CA, USA), a hybrid triple quadrupole–linear ion trap mass spectrometer equipped with Turbo V sources and a Turbolonspray interface, was employed for detection. The instrument was operated using electrospray ionization source in switching polarity between positive and negative modes. The switching program was as follows: Period 1: 0–9 min, negative mode for the determination of geniposide, andrographolide and dehydroandrographolide; Period 2: 9–10.8 min, positive mode for the determination of ophiopogon D; Period 3: 10.8–15 min, negative mode for the determination of methyllophiopogonanone A and methyllophiopogonanone B. The ion spray voltage was set to 5,500 and 4,500 V, respectively. The turbo spray temperature was maintained at 650 °C. Nebulizer gas (gas 1) and heater gas (gas 2) were set at 60 and 65 pounds per square inch (psi), respectively. The curtain gas was kept at 30 psi, and interface heater was on. Nitrogen was used in all cases. Multiple reaction monitoring (MRM) mode was employed for quantitation. The MS–MS fragment ions, declustering potentials (DP) and collision energies (CE) of six analytes are described in Table II, and the product ion scan spectra are shown in Figure 2. All instrumentation were controlled and synchronized by Analyst (versions 1.5.2) from Applied Biosystems/MDS Sciex.

Results
Optimization of sample pretreatment
In order to optimize the extraction conditions, extraction solvent and time were investigated so as to obtain satisfactory extraction efficiency and quantitative results. Different concentrations of solvents such as 100, 75, 50 and 25% methanol were investigated. The results (Figure 3) demonstrated that the extraction efficiency of geniposide increased with the reduction of methanol proportion. However, the highest extraction efficiency of other four analytes was obtained when 100% methanol was employed. Considering that the amounts of the other four analytes were less than that of geniposide in QHZM tablet, 100% methanol was chosen as the extraction solvent. Then, the samples were extracted with methanol by ultrasonic extraction for 15, 30, 45 and 60 min, respectively, to screen optimal extraction time. It was indicated that geniposide, andrographolide, dehydroandrographolide, methyllophiopogonanone A and methyllophiopogonanone B could be extracted completely within 60 min. Therefore, ultrasonic extraction for 60 min was selected to prepare the sample solutions.

Solvent effect of geniposide was observed clearly (Figure 4a) when 100% methanol was used as the solvent of sample solutions due to the mismatch of sample solvent and mobile phase. In order to eliminate solvent effect, dilution of sample solutions with purified water so as to decrease the proportion of methanol was attempted. From the extract ions chromatograms of geniposide in sample solution of 50% methanol (Figure 4b), it could be obviously seen that solvent effect was not detected any more. Therefore, methanol extraction solution was diluted by equivalent purified water before injected to HPLC–MS–MS system.

Selection of MS monitoring mode
With regard to the six standards, negative ion mode offered better sensitivity than positive ion mode. Therefore, the six analytes should be analyzed in the negative ion mode. However, during our preliminary experiment, it was found that ophiopogonin D and its isomer, ophiopogonin D’, had the same fragment ions in negative ion mode, and the separation of them was difficult. Nevertheless, they had different characteristic fragment ions in positive ion mode. Considering that the response of ophiopogonin D did not decrease evidently in positive ion mode compared with that in negative ion mode, the positive ion mode was ultimately selected to analyze ophiopogonin D, while other five constituents were detected in negative ion mode. Therefore, a
polarity switching liquid chromatography (LC)–MS-MS method that was the electrospray ionization source was operated in different modes during different time, was employed in order to simultaneously detect six constituents in one analysis run.

Method validation

Specificity
The specificity of this method was evaluated by comparison of negative control solutions with mixed standard solution and sample solution. The total ion chromatograms of them are shown in Figure 5. The retention times of the six analytes were 4.2, 7.4, 8.3, 9.9, 12.0 and 12.8 min, respectively. No interference peak was observed in three negative control solutions.

Linearity, limit of detection and limit of quantification
The linearity of the method was examined through the series of standard working solutions. Calibration curves were obtained by plotting the peak areas versus its concentrations of each analyte. Regression equation, weight, linear range and correlation coefficient of each analyte are shown in Table I. Good linearity with a correlation coefficient exceeding 0.9979 was observed for each analyte. Limit of detection (LOD) and limit of quantification (LOQ) based on the signal-to-noise ratio of 3 and 10, respectively, were obtained by diluting mixed standard solution with 50% methanol. The results are also listed in Table I.

Figure 2. The product ion scan spectra of six standards.

Figure 3. Effect of the proportion of methanol on extraction efficiency of five constituents. (1) Geniposide, (2) andrographolide, (3) dehydroandrographolide, (4) methylophiopogonanone A and (5) methylophiopogonanone B. Ophiopogonin D was not detected.
Accuracy and precision

Accuracy was evaluated by adding six stock solutions with three different amounts (high, middle and low) to known amounts of QHZM tablet samples (Huiren, Jiangxi, 1210003). Then the result-ant samples were extracted and analyzed with the proposed method, and triplicate experiments were performed at each level. The average recoveries were estimated by the following equation: (total detected amount - original amount)/added amount × 100%. All of the average recoveries were in the range of 93.2–108.5% with the relative standard deviations (RSD) of <5.4%. The recovery results of each analyte are summarized in Table III.

Precision was determined by adding stock solution of ophiopogonin D (≏60 μg ophiopogonin D) to the samples of QHZM tablet (Huiren, Jiangxi, 1210003). The resultant samples were extracted and analyzed by the abovementioned method. Six independent and parallel experiments were performed within 1 day to evaluate the intraday precision while interday precision was determined for three independent days. The concentration of each solution was determined by the calibration curve formed at the same day. The intra- and interday precisions expressed as RSD were not >6.8 and 7.0%, respectively (Table IV).

Repeatability and stability

The repeatability of the instrument was estimated by carrying out six independent measurements of a mixed standard solution, and the RSD values of six analytes were within the range of 0.88–4.6% (Table IV).

Stability of sample solutions was tested at 4°C. The sample solution was analyzed at 0, 2, 6 and 10 h, respectively, and the relative error values of six analytes were all within 4.7%, which indicated a good stability in 50% methanol solution within 10 h at 4°C.

Discussion

Extraction solvent and time are the two crucial factors that can affect the efficiency of ultrasonic extraction. Therefore, these two factors were investigated in our experiment. The results suggested that ultrasonic extraction for 60 min with 100% methanol could extract completely for six target components. Moreover, 50% methanol as the sample solvent could obviously eliminate the solvent effect of geniposide. In order to reduce the analysis time, a polarity switching LC–MS-MS method was employed, through which the six components were simultaneously analyzed within 15 min.

The results of samples analysis showed that all the contents of andrographolide, dehydroandrographolide and geniposide could meet the criterion of ChP (Version, 2010). However, the contents of methyllophiopogonanone A and methyllophiopogona-none B of Radix Ophiopogonis from different manufacturers were diverse greatly, which indicated that the qualities of commercial samples of QHZM tablet were different. Therefore, the components of Radix Ophiopogonis should also be controlled to guarantee the homogeneity and stability of the qualities of QHZM tablets from different batches and pharmaceutical factories.

LOQ of ophiopogonin D of the proposed method was 252.0 ng/mL, which was far lower than that of HPLC-UV (0.1 mg/mL) (21) and HPLC-ELSD (≥0.0315 mg/mL) (22–24) in literatures. However, ophiopogonin D, the main active constituent of Radix Ophiopogonis, could not be detected in all samples. Probably, it was because that the quantity control of ophiopogonin D was not regulated in the present criterion, which led that the extraction efficiency of ophiopogonin D was neglected. This suggested that the manufacturing process

Figure 4. The extract ions chromatograms of geniposide in QHZM tablet solutions with different solvents. (a) Methanol, (b) 50% methanol. Arrow shows the solvent effect.
of QHZM tablet should be further improved to ensure the extraction of ophiopogonin D.

**Conclusion**

In the present study, a novel HPLC–MS-MS method was established to simultaneously determine six constituents including geniposide, andrographolide, dehydroandrographolide, ophiopogonin D, methylophiopogonanone A and methylophiopogonanone B, in QHZM tablet. Ophiopogonin D, methylophiopogonanone A and methylophiopogonanone B were quantified for the first time in QHZM tablet. The method was employed to evaluate five commercial samples of QHZM tablet. The results showed that the constituents from *Radix Ophiopogonis* were obviously discrepant between different pharmaceutical factories, which indicated that the constituents from *Radix Ophiopogonis* should be controlled to guarantee the quality of QHZM tablet. The developed method proved specific, sensitive and reproducible and could be employed in comprehensively evaluating the quality of QHZM tablet.

**Funding**

We thank the financial supports from the Natural Science Foundation of Hebei Province of China (C2011206158, 08B031), the National Natural Science Foundation of China (81102412), the Ministry of Education Key Project of Science and Technology Foundation of China (211021).

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**Table III**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Original (µg)</th>
<th>Addition (µg)</th>
<th>Total (µg)</th>
<th>Average recovery (%)</th>
<th>RSD (%)</th>
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<tr>
<td>Geniposide</td>
<td>327.2</td>
<td>191.4</td>
<td>518.6</td>
<td>103.5</td>
<td>1.5</td>
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<td></td>
<td>385.5</td>
<td>237.2</td>
<td>622.7</td>
<td>107.5</td>
<td>2.5</td>
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<tr>
<td></td>
<td>575.9</td>
<td>393.1</td>
<td>969.0</td>
<td>106.8</td>
<td>4.3</td>
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<tr>
<td>Andrographolide</td>
<td>124.5</td>
<td>310.1</td>
<td>434.6</td>
<td>100.2</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>161.3</td>
<td>294.0</td>
<td>455.3</td>
<td>97.7</td>
<td>4.8</td>
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<tr>
<td>Dehydroandrographolide</td>
<td>148.0</td>
<td>326.7</td>
<td>474.7</td>
<td>100.2</td>
<td>5.0</td>
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<tr>
<td>Ophiopogonin D</td>
<td>–</td>
<td>16.1</td>
<td>17.1</td>
<td>106.2</td>
<td>4.4</td>
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<tr>
<td>Methylophiopogonanone A</td>
<td>0.0566</td>
<td>0.00266</td>
<td>0.0593</td>
<td>101.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Methylophiopogonanone B</td>
<td>0.0303</td>
<td>0.00103</td>
<td>0.0313</td>
<td>100.4</td>
<td>4.9</td>
</tr>
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</table>

–, not being detected.

**Table IV**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precision (%)</th>
<th>Repeatability (%)</th>
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<tr>
<td></td>
<td>Intraday</td>
<td>Interday</td>
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<tr>
<td>Geniposide</td>
<td>4.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Andrographolide</td>
<td>5.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Dehydroandrographolide</td>
<td>4.4</td>
<td>6.9</td>
</tr>
<tr>
<td>Ophiopogonin D</td>
<td>5.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Methylophiopogonanone A</td>
<td>6.8</td>
<td>3.7</td>
</tr>
<tr>
<td>Methylophiopogonanone B</td>
<td>5.7</td>
<td>7.0</td>
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</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Geniposide (mg/g)</th>
<th>Andrographolide (mg/g)</th>
<th>Dehydroandrographolide (mg/g)</th>
<th>Ophiopogonin D (mg/g)</th>
<th>Methylophiopogonanone A (µg/g)</th>
<th>Methylophiopogonanone B (µg/g)</th>
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<tbody>
<tr>
<td>Jinhaitang, Guangxi (20120601)</td>
<td>1.728</td>
<td>0.3229</td>
<td>0.6806</td>
<td>5.4</td>
<td>0.006872</td>
<td>0.009208</td>
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<tr>
<td>Jiliangshan, Jiangxi (20120907)</td>
<td>2.557</td>
<td>1.059</td>
<td>0.3916</td>
<td>3.4</td>
<td>0.004461</td>
<td>0.007325</td>
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<tr>
<td>Banzhou Tianlong, Guangxi (120701)</td>
<td>1.447</td>
<td>0.4902</td>
<td>0.5148</td>
<td>6.8</td>
<td>0.007202</td>
<td>0.011000</td>
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<tr>
<td>Huiren, Jiangxi (1210003)</td>
<td>2.178</td>
<td>0.7194</td>
<td>0.9725</td>
<td>3.7</td>
<td>0.05635</td>
<td>0.30376</td>
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<td>Huiren, Jiangxi (1302002)</td>
<td>2.173</td>
<td>0.8399</td>
<td>0.9798</td>
<td>7.0</td>
<td>0.3793</td>
<td>0.2026</td>
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</table>

–, not being detected.
References


