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Global identification and quantitative analysis of chemical constituents in traditional Chinese medicinal formula Qi-Fu-Yin by ultra-high performance liquid chromatography coupled with mass spectrometry

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ABSTRACT

Qi-Fu-Yin (QFY), a classical traditional Chinese medicine formula, is proven to have significant neuroprotective effects by modern pharmacological studies. However, the chemical constituents of QFY have not been fully explored. In this study, an ultra-high performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UHPLC-QTOF MS) was developed for comprehensive analysis of chemical constituents in QFY. By using characteristic ions and fragmentation rules, a reliable identification of 156 compounds was described here, including 69 triterpene saponins, 23 oligosaccharide esters, 22 flavanoids, 9 alkaloids, 9 phenolic acids, 8 phthalides, 7 phenylethanoid glycosides, 3 xanthones, 3 sesquiterpene lactones, 2 ionones and 1 iridoid glycoside. Twenty-six major compounds were then determined in a single run by UHPLC coupled with triple quadrupole tandem mass spectrometry (QQQ MS) with fast positive/negative polarity switching. It allows for the acquisition of MS data in both ionization modes from a single run. The proposed method was then validated in terms of linearity, accuracy, precision and recovery. The overall recoveries for 26 analytes ranged from 91.35% to 109.58%, with RSDs ranging from 0.82% to 4.83%. In addition, the content of 26 analytes in QFY prepared by five batches of herbal materials was also analyzed. These results demonstrated that our present method was effective and reliable for comprehensive quality evaluation of QFY. Meanwhile, the study might provide the chemical evidence for revealing the material basis of its therapeutic effects.

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1. Introduction

Qi-Fu-Yin (QFY), a classical complex formula of traditional Chinese medicine (TCM), was listed in 51st volume of "Jingyue Encyclopedia" authored by Jing-yue Zhang of the Ming Dynasty. QFY comprises seven herbs, namely Ginseng Radix et Rhizoma (*Renshen*), Rehmanniae Radix Praeparata (*Shudi*), Angelicae Sinensis Radix (*Danggui*), Atractylodis macrocephalae Rhizoma Praeparata (*Chaobaizhu*), Glycyrrhizae Radix et Rhizoma Praeparata cum Melle (*Zhigancao*), Ziziphi Spinosae Semen (*Suanzaoren*) and Polygalae Radix Praeparata (*Zhiyuanzhi*). Modern pharmacological studies revealed that QFY have the effect of nootropic, memory improvement and neuroprotection, especially for treatment of Alzheimer's disease (AD) [1–9]. Although pharmacological effects on QFY have been well investigated in previous literatures, the chemical analyses of QFY are still limited.

In recent years, liquid chromatography coupled with mass spectrometry (LC–MS) method has been used to characterize the chemical composition in complex matrix without isolating individual compound [10–15]. High resolution MS can provide chemical structural information with high mass accuracies and high selectivity. [16–20]. It is expected to be an efficient and powerful tool for qualitative and quantitative analysis of complicated compounds in TCM, especially for unknown compounds.

In this study, an ultra-high performance liquid chromatography coupled with coupled with quadrupole time-of-flight tandem mass spectrometry (UHPLC-QTOF MS) method was developed for rapidly separation and reliably identification of the multiple components in QFY. A total of 156 compounds were unambiguously identified or tentatively characterized based on retention time, MS and MS/MS spectra data in both negative and positive ion modes. A validated UHPLC coupled with triple quadrupole tandem mass spectrometry (QQQ MS) method was then established for quantitative analysis of twenty-six marker compounds with widely known bioactivity or relative high content in QFY decoction. This study will facilitate

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the comprehensive quality control, the pharmacological research and the clinical applications of QFY.

2. Experimental

2.1. Materials and reagents

Herbal materials of *Renshen*, *Shudi*, *Danggui*, *Chaobaizhu*, *Zhigancao*, *Suanzaoren* and *Zhiyuanzhi* were collected or purchased from genuine producing areas of these herbs and morphologically authenticated by Professor Ping Li. The voucher specimens were deposited in State Key Laboratory of Natural Medicines (China Pharmaceutical University), Nanjing, China.

Reference substances of ginsenoside Rg_1, ginsenoside Re, ginsenoside Rb_1, ginsenoside Rc, ginsenoside Ro, ginsenoside Rb_2, ginsenoside Rd, ginsenoside Rg_3, chlorogenic acid, caffeic acid, ferulic acid, atractylenolide III, atractylenolide II, liquiritin, liquiritigenin, glycyrrhizin, jujuboside A and jujuboside B were purchased from Must Biological Technology Co. Ltd. (Chengdu, China). Acteoside, *E*-Ligustilide, liquiritin apioside, magnoflorine, vicenin II, spinosin, polygalaxanthone III, 3,6′-disinapoyl sucrose, tenuifoliside A and tenuifolin were purchased from Phystandard Technology Co. Ltd. (Shenzhen, China). Licorice-saponin A3 and licorice-saponin G2 were isolated from *Glycyrrhiza uralensis* Fischer in the authors' laboratory by repeated silica gel column chromatography. The purity of each compound was determined to be higher than 98% by HPLC. Their chemical structures were identified by comparison of their ^{1}H NMR, ^{13}C NMR and high-resolution MS data with References

HPLC-grade acetonitrile and formic acid were purchased from ROE Scientific Inc. (USA). Deionized water (18 MΩ cm^{-1}) was purified using a Milli-Q water purification system from Millipore (Bedford, MA, USA). Methanol of HPLC-grade was purchased from Jiangsu Hanbon Sci. & Tech. Co. Ltd. (Nanjing, China).

2.2. Sample preparation and standard solutions

According to the original composition and preparation method recorded in "Jing Yue's encyclopedia", the seven ingredient herbs of QFY including *Renshen* (6 g), *Shudi* (9 g), *Danggui* (9 g), *Chaobaizhu* (5 g), *Zhigancao* (3 g), *Suanzaoren* (6 g) and *Zhiyuanzhi* (5 g) were accurately weighed and extracted by refluxing for 2 h with 400 mL of water, and cooled at room temperature. The water extraction was filtered, and the filtrate was diluted 2 times by water. Then the diluent was centrifuged (13,000 rpm, 10 min). Finally, the liquid supernatant was saved as the resultant sample in −80 °C for further LC–MS analysis.

Standard solution of chlorogenic acid (**S2**), caffeic acid (**S5**), magnoflorine (**Z2**), vicenin II (**Z3**), ferulic acid (**S6**), liquiritin (**L3**), polygalaxanthone III (**P6**), liquiritin apioside (**L4**), spinosin (**Z7**), acteoside (**R6**), 3,6′-disinapoyl sucrose (**P8**), tenuifoliside A (**P9**), liquiritigenin (**L12**), ginsenoside Rg_1 (**G1**), ginsenoside Re (**G2**), licorice-saponin A3 (**L21**), tenuifolin (**P31**), jujuboside A (**Z18**), ginsenoside Rb_1 (**G9**), ginsenoside Rc (**G10**), ginsenoside Ro (**G11**), licorice-saponin G2 (**L26**), ginsenoside Rb_2 (**G12**), jujuboside B (**Z19**), ginsenoside Rd (**G14**), glycyrrhizin (**L31**), atractylenolide III (**A2**), ginsenoside Rg_3 (**G20**), *E*-ligustilide (**S17**) and atractylenolide II (**A3**) was prepared in the concentration ranging from 0.12 to 1.04 mg/mL in 50% methanol and stored at 4 °C until use. Dihydromyricetin (**IS1**) was selected as an internal standard for the determination of **S2**, **Z2**, **Z3**, **S6**, **L3**, **P6**, **L4**, **Z7**, **R6**, **P8**, **P9** and **L12**, while saikosaponin A (**IS2**) was applied to determine **G1**, **G2**, **L21**, **P31**, **Z18**, **G9**, **G10**, **G11**, **G12**, **Z19**, **G14**, **L31**, **S17** and **A3**. A standard working solution of the mixtures of 26 analytes and 2 internal standards was obtained by diluting stock solutions to desired concentrations.

2.3. Qualitative analysis by UHPLC-QTOF MS

Liquid chromatographic analysis was carried out on an Agilent 1290 UHPLC system (Agilent Corp., Santa Clara, CA, USA) equipped with a binary pump, a diode-array detector, an auto sampler and a thermostatically controlled column compartment. Samples were separated on an Agilent Zorbax Extend C_{18} column (2.1 mm × 150 mm, 5 μm). The mobile phase consisted of water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). A gradient program was used as follows: 0–5 min, 8% B; 5–15 min, 8-15% B; 15–25 min, 15–24% B; 25–40 min, 24-35% B; 40–48 min, 35–60% B; 48–60 min, 60–100% B; 60–65 min, 100% B. The flow rate was set as 0.5 mL/min. The column temperature was kept at 30 °C. Sample injection volume was 5 μL.

The LC system was coupled to a 6530 QTOF Mass Spectrometer (Agilent Corp., Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source. Q-TOF/MS analysis was performed in positive mode and negative mode using full scan mode. The conditions of the ESI source were as follows: drying gas (N_2) temperature, 350 °C; drying gas flow rate, 10.0 L/min; nebulizer gas (N_2) pressure, 35 psig; shealth gas (N_2) temperature, 350 °C; shealth gas flow rate, 11.0 L/min; capillary voltage, 3500 V; fragmentor, 135 V; skimmer, 65 V; OCT RF V, 750 V. The collision energy (CE) was set at 15–30 eV and the mass range recorded *m/z* 100–2000. To increase mass accuracy, the TOF mass spectrometer was calibrated every day before analysis, and, subsequently, using internal reference masses at *m/z* 112.9885 and *m/z* 1033.9881 in the negative ion mode, whereas at *m/z* 121.0508 and *m/z* 922.0098 in the positive ion mode. All operations, acquisition, and analysis of data were monitored by Agilent LC/MS MassHunter Acquisition Software Version B.05.01 and operated Workstation Qualitative Analysis Software Version B.06.00.

2.4. Quantitative analysis by UHPLC-MS

The UHPLC-QQQ MS quantification of QFY samples was performed on a Shimadzu LC-30AD UHPLC system (Kyoto, Japan) coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer (Kyoto, Japan). The separation of the 26 analytes and 2 internal standards was performed on the same column and mobile phase as the UHPLC-QTOF MS qualitative analysis under Section 2.3. The gradient program of the mobile phase was changed as follows: 0–5 min, 8–10% B; 5–8 min, 10–20% B; 8–15 min, 20–35% B; 15–20 min, 35–50% B; 20–24 min, 50–100% B; 24–27 min, 100% B, while the flow rate was 0.5 mL/min and the column temperature was set as 30 °C. Sample injection was 0.5 L. Simultaneous positive and negative ionization mode was applied to the determination of the 26 analytes, owing to high sensitivity. The conditions of MS analysis were as follows: drying gas (N_2) temperature, 300 °C; drying gas flow rate, 10.0 L/min; nebulizer gas (N_2) flow, 3 L/min; capillary voltage, 4000 V. The analyte confirmation was performed by using retention time and multi-reaction monitoring (MRM) mode, while the MRM conditions were optimized as Table S1. The dwell time of each ion pair was 40 ms. All the data of UHPLC-QQQ MS analysis were processed by using Shimadzu Labsolutions LCMS Version 5.65 (Kyoto, Japan).

For HPLC-Q MS quantification, chromatographic separation was performed on an Agilent 1200 LC system (Agilent Corp., Santa Clara, CA, USA), equipped with a quaternary pump, a diode-array detector, an auto sampler and a thermostatically controlled column compartment. The gradient program of the mobile phase was changed as follows: 0–5 min, 8% B; 5–8 min, 8–10% B; 8–15 min,

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