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Qualitative and quantitative analysis of chemical constituents in traditional Chinese medicinal formula Tong-Xie-Yao-Fang by high-performance liquid chromatography/diode array detection/electrospray ionization tandem mass spectrometry

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ABSTRACT

Tong-Xie-Yao-Fang (TXYF), a famous traditional Chinese medicine formula, has efficient effects on treatment of the diarrhea-predominant irritable bowel syndrome (D-IBS), a disease with high incidence worldwide. However, the active principles for this complex formula have not been fully explored so far. In this paper, high-performance liquid chromatography coupled with diode array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS/MS) was applied for the qualitative and quantitative analysis of major chemical constituents in TXYF. Two monoterpene glycosides, one chromone and five polymethoxylated flavones were tentatively characterized based on the retention times, UV spectra and MS data. Fifteen compounds were unambiguously identified by comparison with reference standards. Constituents such as lactone and steroidal, which could not be found by single HPLC method due to the low content in the formula, were identified in this paper. Seven compounds (gallic acid, prim-*O*-β-D-glucosylcimifugin, paeoniflorin, cimifugin, naringin, hesperidin and 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol) were quantified by HPLC-DAD using a C18 column and gradient elution with acetonitrile and water–0.1% formic acid. The method exhibited intra- and inter-day precision of less than 2.35% and 3.14%, respectively. The LODs and the LOQs for the analytes were less than 0.47 and 1.82 μg ml^{-1}, respectively. The overall recoveries ranged from 96.82% to 102.47%, with the R.S.D. ranging from 1.17% to 3.94%. These results demonstrated that our present method was effective and reliable for comprehensive quality evaluation of TXYF. Meanwhile, the study might provide the chemical evidence for revealing the material basis of its therapeutic effects.

1. Introduction

There is an increasing use and research of Traditional Chinese medicine (TCM) because of its unique diagnostic methods, systematic approach and abundant historical literature. Although well-accepted in China, it is considered an alternative medical system in much of the Western world. Meanwhile, the relationship between the compounds and the pharmacological effects of TCM is still unclear because of the extreme complexity in TCM preparations [1,2]. Thus, analysis of the active principles in Chinese medicinal herbs is a key to reveal the secret of their effectiveness.

Tong-Xie-Yao-Fang (TXYF) is one of famous traditional Chinese formulas consisting of four crude drugs: Rhizome *atractylodis macrocephalae* (Bai-Zhu), Radix *Paeoniae Alba* (Bai-Shao), Pericarpium *Citri Reticulatae* (Chen-Pi) and Radix *Saposhnikovia* (Fang-Fen). The prescription has been widely used for clinical treatment of diarrhea-predominant irritable bowel syndrome (D-IBS) in China [3]. However, the actual bioactive components of TXYF have remained unclear. Recently, some active ingredients related to pharmacological functions are gradually being revealed. Among them, monoterpene glycosides, flavonoids, and chromones are the most representative components of TXYF. For instance, paeoniflorin played a key role in the anti-inflammatory effect of TXYF, which could suppress PMNs' activity [4]. Naringin and hesperidin were the main flavonoids highly contained in TXYF with their antioxidant and inhibitory activities reported in literature [5]. Prim-*O*-β-D-glucosylcimifugin and 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol showed significant analgesic, antipyretic and anti-inflammatory activities [6].

Although so many beneficial effects have been shown, there are no published reports about the comprehensive quality evaluation of TXYF. Therefore, it is necessary to develop a rapid and sensitive

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method to identify and quantify the components in TXYF, which is helpful for controlling the quality and searching the bioactive substances of this formula.

High-performance liquid chromatography coupled with diode array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS/MS) has been widely used as a powerful means for the analysis of multi-component herbal medicines, which can provide a UV chromatogram and comprehensive MS data about the compounds in complex mixtures [7–10]. This technology facilitates the identification of unknown components in the herbal system remarkably with high sensitivity and accuracy, especially for which standards are unavailable. In our study, an HPLC-DAD-ESI-MS/MS method was developed to identify and quantify the major constituents of TXYF for the first time. A total of twenty three compounds in the formula were identified or tentatively characterized. Furthermore, quantification of seven bioactive components was carried out with HPLC-DAD and ten batches of samples were analyzed, which would contribute to the quality control of TXYF preparations.

2. Experiment

2.1. Reagents and materials

The acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Formic acid and methanol (analytical reagent) were purchased from Shanghai Chemical Reagent Factory (Shanghai, China). Pure water for HPLC analysis was purified using a Milli-Q water purification system (Millipore, MA, USA). Other reagents and chemicals were of analytical grade.

Reference compounds of paeoniflorin and naringin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Gallic acid, cimifugin, prim-*O*-β-D-glucosylcimifugin, hesperidin, 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, nobiletin and tangeretin were purchased from Nanjing Zelang Biological Technology Co. Ltd. (Nanjing, China). Standard compounds of oxypaeoniflorin, (+)-Catechin, albiflorin, benzoylpaeoniflorin, β-sitosterol and atractylenolide II were obtained from Chengdu Mansite Pharamacetical Co. Ltd. (Sichuan, China). Their structures were fully characterized by NMR spectroscopy and mass spectrometry (MS). The purity of each compound was determined to be higher than 98% by HPLC.

Herbal medicines samples of Radix *Paeoniae Alba*, Rhizome *atractylodis macrocephalae*, Pericarpium *Citri Reticulatae* and Radix *Saposhnikovia* were purchased from Anhui, Zhejiang and Jiangsu provinces in China and were identified by Professor Zhunan Gong.

2.2. Standard solutions

Standard stock solutions of the 7 reference standards (gallic acid, prim-*O*-β-D-glucosylcimifugin, paeoniflorin, cimifugin, naringin, 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol and hesperidin) were prepared by dissolving them in methanol. They were then diluted to different concentrations for construction of calibration plots in the ranges of 0.50–20.00, 2.20–88.00, 5.65–226.0, 1.40–56.00, 6.15–246.0, 3.60–144.0, 9.95–398.0 μg ml^{-1}. All the solutions were stored at 4 °C and brought to room temperature before use.

2.3. Sample solutions

Rhizome *atractylodis macrocephalae* (6 g), Radix *Paeoniae Alba* (4 g), Pericarpium *Citri Reticulatae* (3 g) and Radix *Saposhnikovia* (4 g) were immersed in 200 mL ethanol–water (70:30, v/v) for 1 h and extracted in a reflux water bath for 1 h. The operations were repeated for two times, and the total extracts were combined together and evaporated to dryness under reduced pressure with a rotary evaporator at 60 °C. One gram powder of evaporated residue was dissolved with methanol–water (70:30, v/v) into a 50 ml volumetric flask and filtered through a 0.22 μm nylon filter for HPLC analysis. All sample solutions were stored at 4 °C and used at room temperature.

2.4. HPLC-DAD-ESI-MS system

2.4.1. Chromatographic analysis

HPLC analysis was performed on an Agilent 1290 series HPLC system equipped with a binary pump, on-line degasser, auto plate-sampler, column oven and diode array detector (DAD). A number of preliminary experiments were performed to optimize mobile phase composition, elution conditions and detection wavelength. Finally, analysis was carried out at 35 °C on a Krosmasil C18 (250 mm × 6 mm, 5 μm) from Hanbang Science & Technology (Jiangsu, China). The mobile phase system was acetonitrile (A) and water–0.1% formic acid (B). Gradient programmer was performed in linear gradient (8–20% A at 0–12 min, 24–60% A at 35–37 min, 64–70% A at 47–55 min, 75–100% A at 55–70 min). The flow rate was kept at 0.6 ml min^{-1} and the sample volume injected was set at 5 μL. The monitored wavelength was set at 280 nm, and UV spectra from 190 to 400 nm were also recorded for peak characterization.

2.4.2. Mass spectrometry

The above HPLC system interfaced with an Agilent 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, MA, USA) was used for carry out the HPLC/DAD/ESI-MS/MS analysis, with the same column, elution program and flow rate of HPLC analysis. The conditions of ESI source were as follows: source voltage, 3500 V; drying gas (N2) flow rate, 11.0 L min^{-1}; drying gas temperature, 350 °C; nebulizer, 45 psi. The mass spectrometric data was acquired from *m*/*z* 100 to 1000 in both positive and negative ion modes.

2.5. Qualitative analysis

Identification of constituents in TXYF extract was carried out by HPLC-DAD and LC/ESI/MS analysis. In the full scan mass spectra, most of the constituents exhibited their quasi-molecular ions $[M+H]^+$, adduct ions $[M+Na]^+$ and $[M+K]^+$ in positive ion mode, while exhibited $[M-H]^-$ and $[M+HCOO]^-$ in negative ion mode under the soft electrospray ionization condition. Precursor ions were subjected to collision-induced dissociation (CID) to generate the fragment ions and the fragmentation patterns were proposed for the structural identification of constituents.

2.6. Validation of the quantification method

The method was validated for linearity, limits of detection and quantification (LODs and LOQs), precision (inter-day and intra-day precision), reproducibility, and stability following the International Conference on Harmonization (ICH) guideline [11].

3. Results and discussion

3.1. Optimization of HPLC/DAD/ESI-MS conditions

In the present study, different kinds of mobile phases, such as acetonitrile and methanol with a variety of modifiers were tested. It was found that mobile phase with acetonitrile had better resolutions and shorter duration of analysis than those with methanol. Formic acid was added to improve the ionization responses and restrain the peak tailing of compounds 1, 6 and 8. And a low concentration of formic acid (0.1%, v/v) was preferred as it produced the best sensitivity, efficiency and peak shape. A similar tailing-reduced effect was found with phosphoric acid, but we avoided its use