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Online identification of the antioxidant constituents of traditional Chinese medicine formula Chaihu-Shu-Gan-San by LC–LTQ-Orbitrap mass spectrometry and microplate spectrophotometer

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ABSTRACT

Chaihu-Shu-Gan-San (CSGS), a traditional Chinese medicine (TCM) formula containing seven herbal medicines, has been used in treatment of gastritis, peptic ulcer, irritable bowel syndrome and depression clinically. However, the chemical constituents in CSGS had not been studied so far. To quickly identify the chemical constituents of CSGS and to understand the chemical profiles related to antioxidant activity of CSGS, liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap (LC–LTQ-Orbitrap) mass spectrometry has been applied for online identification of chemical constituents in complex system, meanwhile, antioxidant profile of CSGS was investigated by the fraction collecting and microplate reading system. As a result, 33 chemical constituents in CSGS were identified. Among them, 13 components could be detected both in positive and in negative ion modes, 20 constituents were determined only in positive ion mode and 2 components were only detected in negative ion mode. Meanwhile, the potential antioxidant profile of CSGS was also characterized by combination of 96-well plate collection of elutes from HPLC analysis and microplate spectrophotometer, in which the scavenging activities of free radical produced by DPPH of each fraction could be directly investigated by the analysis of microplate reader. This study quickly screened the contribution of CSGS fractions to the antioxidant activity and online identified the corresponding active constituents. The results indicated that the combination of LC–MS^n and 96-well plate assay system established in this paper would be a useful strategy for correlating the chemical profile of TCMs with their bioactivities without isolation and purification.

1. Introduction

Traditional Chinese Medicines (TCMs) have been proved to have a significant effect in treatment of chronic and systematic diseases with fewer side effects. In Chinese herbal therapy, the most widely used medicines are combined by many herbs and prepared according to TCM formulation concepts. It is acknowledged that complex interactions could produce synergistic effects and reduce possible side effects from some of the herbs. However, the extreme complexity of TCM formulas containing many poorly characterized chemical constituents makes standardization of herbal products and understanding of their action mechanisms challenging. In order to discern the chemical compositions of TCM formulas, many techniques, such as GC–MS [1], LC–MS [2,3] and LC–NMR [4] have been used to develop specific analytical methods for comprehensively describing and identifying the chemical components of TCMs. As a powerful analytical tool, liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC–ESI-MS^n) has been widely applied to directly identify known compounds and recognize unknown compounds from the complex mixtures [5,6].

Chaihu-Shu-Gan-San (CSGS) is one of the most widely used TCM formulas in China for treatment of gastritis, peptic ulcer, irritable bowel syndrome and depression. Pharmacological studies have proved that CSGS had prominent effects in kinds of anti-inflammatory, antidepression, anti-ulcer and prevention of liver injury [7]. CSGS involves seven commonly used Chinese herbs, i.e. the roots of *Bupleurum chinense* DC. (*Chai-Hu*), the pericarps of *Citrus reticulata* Blanco (*Chen-Pi*), the roots of *Paeonia lactiflora* Pall. (*Bai-Shao*), the fruits of *Citrus aurantium* L. (*Zhi-Qiao*), the roots of *Cyperus rotundus* L. (*Xiang-Fu*), the roots of *Ligusticum chuanxiong* Hort. (*Chuan-Xiong*) and the roots of *Glycyrrhiza uralensis* Fisch. (*Gan-Cao*). Major constituents in these single herbs have been well studied, for instance, *Chai-Hu* and *Gan-Cao* mainly contain triterpenoid saponin compounds such as saikosaponin A, saikosaponin D [8], glycyrrhizic acid and licorice-saponinG_2 [9]. Different kinds of flavonoids such as naringin, narirutin, hesperidin and neohes-

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peridin are found in *Chen-Pi* [10] and *Zhi-Qiao* [11]. Monoterpene glycosides such as paeoniflorin, benzoylpaeoniflorin and lactiflorin are usually recorded as the active substances of *Bai-Shao* [12]. However, to the best of our knowledge, the profile of chemical constituents in CSGS has not been investigated so far.

In the present study, LC–ESI-MS^n analysis was developed to identify the main constituents of CSGS, which gave the accurate molecular weights by orbitrap analyzer and the fragmentation patterns acquiring from multi-stage mass fragmentation in linear trap quadrupole (LTQ) for comprehensive understanding of chemical structures in complex mixture. Our previous study indicated that the antioxidant activity of CSGS may play a key role for its antidepressive effect [13]. To explore the active fractions responsible for antioxidant activity of CSGS, the antioxidant profile of CSGS was investigated by combination of 96-well plate collection of elutes from HPLC analysis and microplate spectrophotometer, in which the scavenging activities of free radical produced by DPPH of each fraction could be directly investigated by the analysis of microplate reader.

2. Experimental

2.1. Solvents and chemicals

The HPLC grade acetonitrile and methanol from Fisher (NJ, USA) were used for chromatography. Analytical-grade ethanol was purchased from Beijing Reagent Company (Beijing, China). Water was purified by Milli-Q academic water purification system (Millipore, France).

Synephrine, ferulic acid, naringin, hesperidin, neohesperidin, saikosaponin A, and glycyrrhizic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Paeoniflorin (isolated and purified from *Paeonia lactiflora* Pall.), nobiletin and tangeretin (isolated and purified from pericarps of *Citrus aurantium* L.) were provided by our group. Saikosaponin A was detected with purity of 95.0% and others were determined with purity more than 98%. DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Sigma–Aldrich (Shanghai, China).

All raw herbs were purchased from Beijing Tongren Tang Pharmaceutical Co. Ltd. (Beijing, China) and identified as the roots of *Bupleurum chinense* DC., the roots of *Paeonia lactiflora* Pall., the pericarps of *Citrus aurantium* L., the fruits of *Citrus reticulata* Blanco, the roots of *Cyperus rotundus* L., the roots of *Ligusticum chuanxiong* Hort. and the roots of *Glycyrrhiza uralensis* Fisch. by Associate Professor Yulin Lin of the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences and Peking Union Medical College. The voucher specimens are deposited in our laboratory of IMPLAD.

2.2. Sample preparations

The CSGS extract was prepared based on the traditional method used in TCM practice. Briefly, 8.4 g of mixed crude herbs, *Chai-Hu*, *Chen-Pi*, *Bai-Shao*, *Zhi-Qiao*, *Xiang-Fu*, *Chuan-Xiong* and *Gan-Cao* in the proportions of 4:4:3:3:3:3:1 by weight were crushed into small pieces. The mixture of the herbs was soaked together in 200 ml of water for 1 h at room temperature and thereafter refluxed for 2 h. The filtrate was collected and the residues were then refluxed twice in 200 ml of water for 1.5 h. The three filtrates were combined and concentrated under vacuum to give 0.655 g extract. The extracts of the individual herbs, *Chai-Hu* (**1**, 0.144 g), *Chen-Pi* (**2**, 0.12 g), *Bai-Shao* (**3**, 0.12 g), *Zhi-Qiao* (**4**, 0.156 g), *Xiang-Fu* (**5**, 0.096 g), *Chuan-Xiong* (**6**, 0.084 g) and *Gan-Cao* (**7**, 0.10 g) were prepared using procedures identical to that for CSGS. 66.5 mg of CSGS extract (equivalent to 0.84 g of raw herbs in the proportions listed above) was dissolved in 10 ml of deionized water. 10 μl of the resulting solution was injected into the HPLC system for LC–MS^n analysis. An amount of extract of the single herb equal to the same amount of raw herb in the 66.5 mg of CSGS extract was prepared and analyzed identically to CSGS. All samples were analyzed in triplicate.

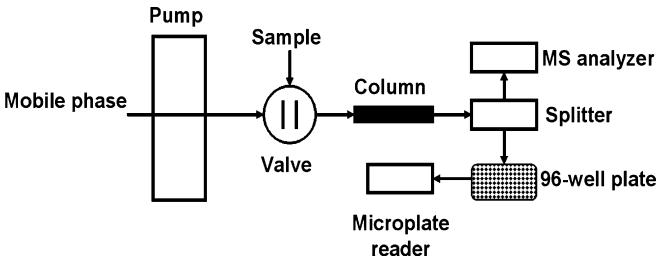

Fig. 1. Schematic diagram of high-performance liquid chromatography coupled to orbitrap analyzer and analytical system of antioxidant activities profiling.

2.3. Chromatography

The LC system consisted of a Finnigan Surveyor LC system with a built-in degasser and autosampler. HPLC analysis was performed on a Waters SunFire™ (2.1 mm × 150 mm, 5 μm) C_{18} column together and the column temperature was set at 30 °C. A mixture of aqueous with 0.1% formic acid (A) and acetonitrile (B) was used as the mobile phase. Gradient chromatography was performed in linear gradient (8:92 at 0–3 min, 8:92–31:79 at 3–30 min, 31:69–95:5 at 30–50 min and 95:5–100:0 at 50–60 min, v/v). Re-equilibration duration was 10 min between individual runs and the flow rate was 0.2 ml/min.

2.4. Mass spectrometry

Mass spectra were analyzed on a Finnigan LTQ-Orbitrap XL instrument with an ESI source (Thermo Electron, Bremen, Germany). Nitrogen and helium were used as the sheath and auxiliary gas and the collision gas, respectively. Values of auxiliary gas flow rate and capillary voltage were set at 5 arbitrary units and 40 V in positive ion mode and 8 arbitrary unit and −45 V in negative ion mode, respectively.

The scan event cycle used a full scan mass spectrum at resolution of 15,000 (at *m/z* 400) and three corresponding data-dependent MS/MS events acquired at a resolving power of 7500. The most intense ions detected in full scan MS were selected for data-dependent scanning. MS/MS activation parameters were set at isolation width of 2 Da, normalized collision energy of 35%, and an activation time of 30 ms. An external calibration for mass accuracy was performed the day before the test. The mass spectrometric data was collected from *m/z* 100 to 1000 in positive and negative ion mode.

2.5. Characterization of antioxidant profile by investigating scavenging activity of CSGS on DPPH radicals in 96-well plates

The fractions eluted from chromatographic column were splitted at a ratio of 1:1 (same length of pipelines between splitter to MS detector and splitter to 96-well plate), in which 50% was flew into MS detector and another 50% was collected in a 96-well plate (COSTAR, Corning Inc.) with time interval of 1 min (Fig. 1). 200 μl of a DPPH solution in 70% methanol (0.06 mM) was added directly to each well while totally 60 fractions were gathered and placed in the dark at room temperature for 40 min. The absorbance was measured with Microplate Spectrophotometer (MQX200 uQuant,