Preliminary identification of the absorbed bioactive components and metabolites in rat plasma after oral administration of Shaoyao-Gancao decoction by ultra-performance liquid chromatography with electrospray ionization tandem mass spectrometry

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ABSTRACT

Background: Shaoyao-Gancao decoction (SGD), a traditional Chinese medicine formula, has been used for the treatment of abdominal pain and dysmenorrhea disease in Asia over long period of time. Its effectiveness has been confirmed in clinic, but its active constituents remain unclear.

Materials and methods: In this paper, a rapid, sensitive and reliable ultra-performance liquid chromatography-electrospray ionization quadrupole-time-of-flight high-definition mass spectrometry (UPLC-ESI-Q-TOF-MS) in positive and negative ion mode were established to characterize the active constituents of SGD in vitro. The analysis was performed on a Waters UPLCTM HSS T3 (2.1 × 100 mm, 1.8 μm) using gradient elution system. Automated MetaboLynxTM technique was employed to screen for the potentially bioactive components in rat plasma after oral administration of SGD. MS/MS fragmentation behavior was proposed for aiding the structural identification of the components.

Results: Based on the developed method of fingerprint analysis, an injection run of the plasma sample was finished in 15.0 min. A total of 12 compounds including 9 prototype components such as gallic acid, albiflorin, liquiritin, pallidiflorin, liquiritigenin, isoLiquiritigenin, formononetin, isolicoflavonol, licoricone, C9H10O3 and 2 metabolites such as liquiritigenin-4’-O-glucuronide, formononetin glucuronide were identified or tentatively characterized. Of note, 3 ingredients were identified from Radix Paeoniae Alba, and 9 were from Radix Glycyrrhiza. Conclusion: The compounds found in dosed plasma could be the effective substances of SGD for treating dysmenorrhea, and may provide important experimental data for further pharmacological and clinical research of SGD. Furthermore, this work has demonstrated that the feasibility of the UPLC-ESI-Q-TOF-MS for rapid and reliable characterization of identification and structural elucidation of the chemical constituents and their metabolites from herbal medicines.

Key words: Constituents, herbal medicine, identification, metabolite, Shaoyao-Gancao decoction, UPLC-ESI-Q-TOF-MS

INTRODUCTION

Shaoyao-Gancao decoction (SGD) is a popular traditional Chinese medicine (TCM) and Shakuyaku-Kanzo-to in Japanese, firstly recorded in Shang Han Lun in Han Dynasty of Chinese history (202 BC-220 AD).[5] Its recipe is composed of two herbal medicines, Paeoniae Radix and Glycyrrhiza Radix, mainly used for the treatment of abdominal pain and dysmenorrhea disease.[5] Modern pharmacological studies have demonstrated that SGD carries various attractive biological activities.[3] Despite the popular use of SGD, little information is known about its metabolism after oral administration.[4] Several analytical methods have been reported for the measurement of major components in SGD preparations, but the
bioactive ingredients in vitro and metabolites of SGD are still not well understood. Recently, we had established the chemical fingerprint of SGD, and characterized 58 compounds by liquid chromatography coupled with mass spectrometry (LC-MS) and assigned their source herbs.\[^5\] 44 ingredients were identified from Glycyrrhiza radix; and 14 were from Paeoniae radix. Thus, it is of significance to systematically analyze the chemical constituents and in vitro metabolites of SGD, to interpret its material basis responsible for therapeutic effects.

It is necessary to develop a rapid and reliable analytical method for the identification of the constituents of TCM, to facilitate its clinical usage and quality control during production. Nowadays, LC-MS was widely applied for the analysis of herbal constituents in vitro and in vitro.\[^6-8\] However, there are many endogenous substances intervening the analysis of constituents absorbed into blood. Metabolynx\textsuperscript{TM} software could rapidly detect the peaks in LC/MS data and many papers employ this software to acquire information about the prototype and metabolites.\[^9\] Despite the popular usage of SGD, few reports are available on systematic analysis of its changes of metabolism after oral administration of SGD. Meanwhile, due to the complex chemical nature, metabolic study of SGD is more challenging and time-consuming than western drugs, UPLC and sub-2um particle size columns were used to increase chromatographic resolution and to shorten analysis time.\[^10,11\] Therefore, the present study was undertaken to investigate the chemical constituents and the metabolites in plasma, conducted via UPLC-ESI-Q-TOF-MS combined with Metabolynx\textsuperscript{TM} software.

**MATERIALS AND METHODS**

**Chemicals and materials**

Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Methanol was HPLC grade (Fisher, USA). Distilled water was purchased from Watson’s Food and Beverage Co., Ltd. (Guangzhou, China). Leucine enkephalin was purchased from Sigma-Aldrich (MO, USA). Formic acid was purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Estradiol benzoate injection was purchased from (Animal pharmaceutical Hangzhou, China (batch number 120529); oxytocin injection was purchased from Furen Huaiqing Tang Pharmaceutical Co., Ltd., Henan, China (batch number 1206241). Other reagents and chemicals were of analytical grade. The two crude drugs, Radix Paeoniae Alba and Radix Glycyrrhizae were purchased from Harbin Tongrentang Drug Store (Harbin, China), and authenticated by Prof. Xijun Wang, Department of Pharmacognosy of Heilongjiang University of Chinese Medicine.

**Preparation of SGD samples**

According to the instructions recorded in ‘Shanghanlun’, SGD was prepared in the following procedure. Radix Paeoniae Alba (12g) and Radix Glycyrrhizae (12g) were crushed to power (40 mesh size) and immersed in 600ml deionized water for 1 h and then decocted to boiling for 30 min until the volume of water reduced to about 300mL. Supernatant of the extractive solution was filtered via 6 layer gauzes and made to a concentration of 1 g crude drug per milliliter, and the decoction was transformed into the freeze-dried powder. 100 milligrams of the freeze-dried powder were extracted with 10 mL methanol for 30 min under ultrasonics. The methanol extraction was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was filtered via a 0.22 μm membranes, the aliquot was subjected to UPLC analysis.

**Animal experiments**

14 Wistar rats (220-250g body weight) were purchased from the laboratory animal center of Heilongjiang University of Chinese Medicine (Harbin, China) and divided into two groups (group A, drug group for dosed rat plasma, n = 7; group B, control group for blank rat plasma, n = 7) and kept in a breeding room. The animals were fasted for 12 h with free access to water before the experience. Group A was dysmenorrhea model induced by estradiol benzoate injection combined with oxytocin. The dry powder of SGD was immersed in distilled water overnight. SGD (0.22 g/ml) was administered orally to the rats of group A at a dose of 1 ml/100g body weight. Equal dose of distilled water was orally administered to the rats of group B. After 30 min, the rats were anaesthetized by intraperitoneal injection of 3% pentobarbital sodium (0.15 mL per 100g body weight). Animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use.

**Preparation of serum samples**

The blood samples were collected from hepatic portal vein at 30 min after administration (n = 7 for each group) and the rats were sacrificed. Then, the serum was separated immediately by centrifuging at 13000 rpm for 10 min at 4°C. 40 uL phosphoric acid was added to 2.0 mL of the above supernatant and ultrasonicated for 1 min, and then vortexed for 30s. The mixed solution was applied to pre-activated Waters Oasis HLB solid phase extraction C\textsubscript{18} columns (Waters Corporation, USA). Before that, the column was washed with 3 mL of methanol and 3 mL of water, successively. Then, 2ml of 100% methanol elutes and the elutes were collected and dried under nitrogen gas at 45°C. The residues were re-constituted in 100 uL methanol, centrifuged at 13 000 rpm for 15 min at 4°C. The sample was filtered through a 0.22 μm membrane.
filter unit before analysis, and a 8ul aliquot was injected for UPLC-MS analysis.

**Chromatographic analysis system**

Chromatographic analysis was performed in a Waters Acquity ultra performance LC system (Waters Corporation, Milford, USA) controlled with Masslynx (V4.1), consisting of a binary pump solvent management system, an online degasser, and an autosampler. Separation was performed on an ACQUITY UPLCHSS T3 Column (2.1 × 100 mm, 1.8 μm, Waters Corporation, Milford, USA) held at 40°C, and the flow rate was 0.4 mL min⁻¹, respectively. The optimal mobile phase was composed of water (A) and acetonitrile (B) both containing 0.1% (v/v) formic acid. The linear elution gradient program was used as follows: 0-4 min, 5-15% A; 4-6 min, 15-25% A; 6-6.5 min, 25-30% A; 6.5-13 min, 30-58% A; 13-15 min, 58-100% A; 15-17 min, 100% A; 17-17.5 min, 100-5% A; 17.5-19 min; 5% A.

**Mass spectrometry conditions**

The UPLC was directly interfaced with a Waters Synapt™ High Definition TOF Mass (HDMS) system (Waters Corporation, Milford, USA) equipped with an electrospray ion source operating in either positive or negative ESI mode. The optimal conditions of MS analysis were designed as follows: ESI + mode, capillary voltage of 3.0kV, sampling cone voltage was 40.0 V, extraction cone voltage was 2.0 V; ESI mode, capillary voltage of 2.4kV, sampling cone voltage was 40.0V, extraction cone voltage was 2.0V. The temperature was set at 110°C, desolvation gas temperature was 300°C, desolvation gas flow was 800L h⁻¹. The mass spectrometer was calibrated using a solution of sodium formate before the experiment. The full-scan MS data were produced across the mass range of 100-1500 Da with collision energy of 30-55eV in positive and 25-50eV negative mode. Scans were of 0.30s duration. Data were collected in centroid mode and mass was corrected during acquisition using an external reference (Lock-Spray™) comprising a 200pg mL⁻¹ solution of leucine-enkephalin via a locksspray interface, generating a reference ion at 556.2771 Da ([M + H⁺]⁺) for positive ESI mode and while at m/z 554.2615 Da ([M-H⁻]) in negative ion mode.

**Data processing**

All operations, acquisition, and analysis of data were monitored by Metabolynx XS version 4.1 (Waters Corp., Milford, USA) for peak detection and peak alignment. The method parameters for data processing were set as follows: retention time range 0.1-20.5 min, mass range 100-1500 Da, retention time tolerance 0.2 min, mass tolerance 0.05 Da, noise elimination level 6; peak intensity threshold 50. The peak width at 5% height and the peak-to-peak baseline noise were automatically determined. Data-dependent acquisition was applied and the most intense ions detected in each MS scan were selected for determination of their fragmentation patterns. For further confirmed the structure and the source of the metabolites, the raw data were processed by a Metabolynx 4.1 (Waters Corp., Milford, USA).

**RESULTS AND DISCUSSION**

**Optimization of chromatographic condition**

To obtain LC chromatograms with good separation and peak shape, we used a low mobile phase flow rate of 0.4mL/min. On the other hand, a low LC flow rate was compatible to our ESI spray tip, and yielded maximum ionization efficiency. In the performance of gradient optimization, gradient time, gradient shape and initial composition of the mobile phase were taken into consideration, and the optimized gradient elution was presented in Section 2.5. Three column temperatures, 20°C, 30°C, and 40°C, were tested and 40°C gave the best result. Two columns, a Waters UPLC™ HSS T₃ (2.1 × 100 mm, 1.8 μm) and an ACQUITY BEH C18 (100 × 2.1 mm, 1.7 μm), were investigated and found that the ACQUITY HSS T3 had a higher peak capacity, better resolution and stronger retention ability. The representative base peak intensity (BPI) chromatograms of SGD in positive mode and negative mode are shown in Figures 1 and 2, respectively. In our study, a 15 min UPLC/MS profiling significantly shortens the time of sample analysis.

**Characterization of chemical constituents from SGD**

To characterize the chemical constituents of SGD, a UPLC-QTOF-MS method was established. All known compounds were identified by comparing with reference standards. For unknown compounds, the structures were tentatively characterized based on their chromatographic and spectrometric data, referring to previous literatures. The component herb from which each compound was derived was confirmed by individually analyzing the herbs with the same UPLC-QTOF-MS method. Finally, a total of 12 compounds were identified or tentatively characterized [Figure 1 and Table 1], including allicic acid, alibiflorin, liquiritin, pallidiflorin, liquiritigenin, isoLiquiritigenin, formononetin, isoflavanone, licoricone, C₆H₅O₂, and two metabolite liquiritigenin-4’-O-glucuronide, formononetin glucuronide were identified or tentatively characterized. Of note, 3 ingredients were identified from Radix Paeoniae Alba, and 9 were from Radix Glicyrrhiza.
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Table 1: Characterization of chemical constituents in vivo and metabolites of Shaoyao-Gancao decoction by UPLC-ESI-Q-TOF-MS

| Rt   | Identification                | Negative ion (m/z) | positive ion (m/z) | Element composition                  | mv (Da) | MS/MS (m/z) | Source |
|------|-------------------------------|-------------------|-------------------|--------------------------------------|---------|-------------|--------|
| 1.50 | Gallic acid                   | 169.0134          | -1.2              | C_4H_8O_5                             | 170.022 | 169[M-H]^−; 125[M-H-CO]^- | RPA    |
|      |                               |                   |                   |                                      |         |             |        |
| 3.45 | Not identified                | 165.0549          | -4.4              | C_4H_8O_5                             | 166.1739| 165[M-H]^−; 137[M-H-C_2H_4]^+ | RPA    |
| 5.10 | Albiflorin                    | 479.1550          | 0.7               | C_2H_4N_5O_2                          | 480.1632| 479[M-H]^−; 449[M-H-CH_3O]^-; 357[M-H-C_2H_4O_2]^+; 327[M-H-CH_2O-C_2H_4O_2]^+ | RPA    |
| 6.05 | Liquritigenin-4’-O-glucuronide| 431.0911          | -3.4              | C_2H_2O_4                              | 432.1056| 431[M-H]^−; 255[M-H-(GluA-H_2O)]^-; 175[[GluA-H_2O-H_2O]^-; 433[M-H]+; 257[M-H-(GluA-H_2O)]^- | RG     |
| 6.11 | Liquiritin                    | 417.1172          | 1.5               | C_2H_2O_9                              | 418.126 | 417[M-H]^−; 255[M-H-(Glc-H_2O)]^-; 135[C_4H_8O_3]^+ | RG     |
| 7.50 | Formononetin gluturonide      | 443.0986          | 0.8               | C_2H_3O_9                              | 444.1056| 443[M-H]^−; 267[M-H-(GluA-H_2O)]^-; 175[[GluA-H_2O-H_2O]^- | RG     |
| 7.65 | Pallidiflorin                 |                   |                   |                                      |         |             |        |
| 7.85 | Liquiritigenin                | 255.0645          | -1.3              | C_2H_3O_9                              | 256.0735| 255[M-H]^−; 135[C_2H_2O_5]^+; 91[C_2H_2O_3]^+; 257[M-H]+; 137[C_2H_2O_5]^+ | RG     |
| 9.71 | Isoliquiritigenin             | 255.0679          | 2.1               | C_2H_3O_9                              | 256.0735| 255[M-H]^−; 135[C_2H_2O_5]^+; 119[C_2H_2O_3]^+ | RG     |
| 9.99 | Formononetin                 | 267.0653          | -0.5              | C_2H_3O_9                              | 268.0735| 267[M-H]^−; 252[M-H-CH_3]^-; 195[M-H-CH_2-HCO-CO]^-; 269[M-H]+; 254[M-H-CH_3]^-; 226[M-H-CH_2-HCO-CO]^- | RG     |
| 12.51| Isolicoflavonol               | 353.1007          | -1.8              | C_2H_5O_9                              | 354.1103| 353[M-H]^−; 338[M-H-CH_3]^-; 163[M-H-C_2H_4O_2]^+ | RG     |
| 12.60| Licoricone                    | 381.1353          | 1.5               | C_2H_2O_9                              | 382.1419| 381[M-H]^−; 366[M-H-CH_3]^-; 353[M-H-C_2H_4]^+; 339[M-H-C_2H_4]^+; 383[M-H]+; 369[M-H-CH_3]^-; 355[M-H-C_2H_4]^+ | RG     |

Note: *: Metabolite; RPA: Radix paeoniae alba; RG: Radix glycyrrhizae

Figure 1: Representative BPI chromatograms of dosed rat plasma samples obtained at 30 min after oral administration of Shaoyao-Gancao decoction and blank rat plasma samples in positive mode. (a), controlled serum; (b), dosed serum in positive ion mode

Figure 2: UPLC-MS BPI chromatograms of dosed rat plasma samples after oral administration of Shaoyao-Gancao decoction and blank rat plasma samples in negative mode. (a), controlled serum; (b): dosed serum in negative ion mode

UPLC/MS conditions. By examining total ion current chromatograms, the absorbed components and metabolites failed to be discovered due to their low concentrations. In order to improve the detection sensitivity, the extracted ion chromatograms with Metabolynx were adopted. By comparing these obtained extracted ion chromatograms, peaks that were appeared both in dosed rat plasma but not in blank rat plasma were considered as the components absorbed into plasma in prototype. According to the method described above, 9 compounds were absorbed into rat plasma in prototype and identified as gallicacid, albiflorin, liquiritin, pallidiflorin, liquiritigenin, isoLiquiritigenin,
formononetin, C\text{9}H\text{10}O\text{3} (not identification), isoloflavonol, licoricone [Figure 3]. From the result, it is clear that 12 absorbed compounds are the major constituents of SGD, while the other minor components were not absorbed into rat plasma or their concentrations were too low to be detected.

**Detection and identification of conjugated metabolites in rat plasma**

The compounds absorbed into rat plasma were further metabolized by various drug metabolizing enzymes. These metabolic reactions can be divided into two cases called phase I and phase II reactions. The phase II reactions were focused on in this section, which occurred by conjugation with endogenous molecule (glucuronic acid, amino acid, etc.) to form conjugated metabolites. These reactions can lead to structural changes and certain shifts of exact masses. New peaks discovered in dosed rat plasma samples, but not in blank rat plasma samples should be considered as the probable conjugated metabolites. Finally, these selected peaks were analyzed by MS, MS/MS spectra in product ion mode. In this way, a total of 2 conjugated metabolites were tentatively identified as glucuronic acid-related metabolites, including liquiritigenin-4'-O-glucuronide, formononetin glucuronide.

TCM is consisted of multiple medicinal plants (called ‘formula’) that can regulate homeostasis of the body by acting on multiple targets or systems. The therapeutic effects of herbal medicine are due to the synergistic contribution of multiple-constituent, developing a sensitive analytical method for the identification of the constituents in vitro is very necessary and valuable to ensuring the reliability in clinic and enhancing QC. However, their specific active constituents and corresponding metabolites are still under investigation. Furthermore, whether the bioactive components can be absorbed into the plasma, and how they changed in formula still uncertain. Fortunately, serum pharmacochemistry approach has been proved to be helpful to investigate the bioactive constituents and metabolites of TCM.

SGD, a well-known formula containing *Paoniae Radix* and *Glycyrrhiza Radix*, Shakuyaku-Kanzo-to in Japanese, has attracted increasingly much attention as one of the most popular and valuable herbal medicine in clinic. We had identified 58 compounds in SGD by UPLC-MS analysis and assigned their source herbs, and had basically elucidated the chemical composition of SGD preparation. However, the systematic analysis of chemical constituents in vitro of SGD is difficult to determine and still largely remain unknown. Recently, because of the high speed of analysis, sensitivity and confirmation of structural information, rapid resolution UPLC/MS with automated MetaboLynx analysis were established to characterize the chemical constituents and their metabolites of SGD. The analysis was performed on a Waters UPLC HSS T3 (2.1 × 100 mm, 1.8 μm) using gradient elution system. MS/MS fragmentation behavior was proposed for aiding the structural identification of the components. With the optimized conditions, a total of 12 peaks were tentatively characterized by comparing the retention time and mass spectrometry data and retrieving the reference literatures. The deriving herb for each compound was also assigned. Of note, 9 ingredients were identified from Glycyrrhizae Radix, and 3 were from Paoniae Radix.

Considering that in vitro metabolism study is closely related to the bioactivity of SGD, with the help of MetabolynxTM, the constituents can be extracted easily with no previous knowledge of the compound structure. It is of significance to systematically analyze the chemical constituents and in vitro metabolites in SGD formula. Today, tandem mass spectrometry is used as a routine basis for structure elucidation of metabolites. More recently, hybrid instruments mass analyzers are used. In this work we used a triple-quadrupole instrument for the characterization of SGD metabolite. It is concluded that a rapid and robust platform based on UPLC-ESI-Q-TOF-MS was successfully developed for globally identifying multiple-constituent of traditional Chinese medicine prescriptions. To the best of our knowledge, this is the first report on systematic analysis of chemical constituents and in vitro metabolites of SGD.

**CONCLUSIONS**

An efficient and rapid UPLC-ESI-MS/MS method was successfully established to identify the absorbed components and metabolites in dysmenorrheal model rat plasma after oral administration of SGD, a well-known traditional Chinese medicine prescription. As a result, in vitro, 12 SGD-related
compounds (parent compounds and metabolites) in biological matrices were absorbed into rat plasma in prototype and simultaneously identified, which may be directly related to its therapeutic effect. In addition, by scanning all possible metabolites in EIC mode, 2 conjugated metabolites were detected and tentatively identified from the drug-containing plasma samples, provided a comprehensive understanding of the in-vitro metabolic fates of constituents in SGD. Further study was required to elucidate the pharmacokinetics and pharmacodynamics properties of the major SGD-related plasma compounds and clarify their biological activity, which will facilitate its clinical usage and quality control during production. Based on these results, this work improve our understanding on the chemical constituents of SGD and their metabolic profiling, and help to classify the material basis responsible for the therapeutic effects of SGD. It should be further investigated the bioactivity and specific property of the multiple-constituent that have been identified in SGD. Furthermore, the high-speed and sensitive UPLC-ESI-MS/MS was a useful strategy which was extended to investigate the bioactive ingredients and metabolites of other TCM.

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