Comparative analysis of the multiple absorbed bioactive components in rat serum after oral administration of Xiexin Tang by HPLC-DAD coupled with HPLC-Q-TOF/MS

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Abstract: ***Background:*** Xiexin Tang (XXT) is a classic traditional Chinese medicine (TCM) formula administered in clinics for more than 1800 years. Recently, many studies have investigated the pharmacological effects and chemical composition of XXT. However, systematic studies on the material basis of its efficacy are lacking. In present study, the serum pharmacochemistry technique and high-performance liquid chromatography diode-array detection (HPLC-DAD) coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF/MS) was performed to screening and analysis of the multiple absorbed bioactive components and metabolites of orally dosed XXT in rat serum.

Methods: Bio-samples and herbal extracts were analyzed and detected by HPLC-DAD coupled with HPLC-Q-TOF/MS. Upon comparison of the chromatograms of the single-constituent decoctions with that of the XXT formulation, the peak number and intensity of the formulated decoction showed some variation from those of the single-constituent decoctions.

Results: Twenty-one serum-adsorbed constituents were identified after intragastric administration of herbal extracts, of which 8 originated from Rhei Radix et Rhizoma (RRR), 5 from Coptidis Rhizoma (CR), and 8 from Scutellariae Radix (SR). The results show that the main adsorbed constituents in the serum were anthraquinones, anthrones, chromones, and butyrophenones for RRR, alkaloids for CR, and flavonoids for SR.

Conclusion: The results demonstrate that an effective and reliable analytical method is set up for screening the bioactive components of Chinese herbal medicine, which provided a meaningful basis for further pharmacology and active mechanism research of XXT.

Keywords: Xiexin Tang, Serum pharmacochemistry, Bioactive components, HPLC-DAD, HPLC-Q-TOF/MS

1 INTRODUCTION

In recent years, the natural medicine and traditional Chinese medicine (TCM) have been widely researched and used in the world. Finding the active ingredients or leading compounds in traditional Chinese medicine is one of the important means of developing new drugs, such as resveratrol [1], tanshinone [2], ginseng saponin [3] and so on. The research of herbal extracts is also increasing gradually, such as Ginkgo biloba extract. However, these researches mostly focused on the study of natural medicine or one or some components of traditional Chinese medicine, which was equivalent to chemicals in research strategy rather than the practical application of traditional Chinese medicine with thousands of years history in China. The compatibility of traditional Chinese medicine prescription is one of the important characteristics of TCM in conventional sense, while the complexity of TCM compound ingredients has been become a bottleneck restricting the research of Chinese medicine. Serum pharmacochemistry of TCM, a combination of traditional pharmacochemical methods and modern analytical techniques, such as chromatography and mass spectrometry, has been used to identify the effective constituents of TCMs that migrate into the serum after absorption. This enables investigations of their efficacy and the identification of the active ingredients of TCM [3, 4]. Using this approach, Wang *et al* studied the bio-pharmacodynamics of Yin-Chen-Hao decoction and the active ingredients of Liu-Wei-Di-Huang pills, and achieved good results [5-8]. This method has been widely recognized and applied as a major method for studying the material basis of Chinese medicine.

Xiexin Tang (XXT), first recorded in the *Essential Prescriptions of the Golden Cabinet* (150-220 A.D., Han Dynasty) and employed in clinical settings for 1800 years, is composed of Rhei Radix et Rhizoma (RRR), Coptidis Rhizoma (CR) and Scutellariae Radix (SR) [9]. Modern pharmacological studies reveal that XXT has various properties including anti-inflammatory [10,11], antimicrobial [12], gastroprotective [13-15], and antihypotensive effects [16]. This prescription is particularly useful for treating patients with digestive tract disease, cardiovascular disease, etc. [17]. RRR, the radix and rhizoma of *Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf. and *Rheum officinale* Baill, a commonly used traditional Chinese medicine, is a sovereign

herb, which purges fire and toxins, clears the viscera and guides the descending of fire. The researches have shown that RRR has the regulating effects on gastrointestinal functions, anti-inflammatory, anti-pathogen, cardiovascular protective, antitumor, hepatoprotective, choleretic and anti-ageing effects as well as other pharmacological actions. CR, the rhizoma of *Coptis chinensis* Franch., *Coptis deltoidea* C. Y. Cheng et Hsiao and *Coptis teeta* Wall. CR has a long history of usage for clearing heat, eliminating dampness, purging fire and detoxification inTCM. CR, also called goldthread, was frequently used for the treatment of bacillary dysentery, typhoid, tuberculosis, epidemic cerebrospinal meningitis, empyrosis, pertussis, and other diseases. SR, the radix of *Scutellaria baicalensis* Georgi, has been used for thousands of years in traditional Chinese medicines, which was firstly cited in Shennong Bencao Jing (200-300 a.d., Han Dynasty), a classical masterpiece of TCM [18]. SR has been long used for antibacterial, antiviral, anti-inflammatory, antioxidation, protecting liver and protect cardiovascular system, etc. Although many studies have investigated the pharmacological effects and chemical composition of XXT, but, systematic studies on the material basis of its efficacy are lacking.

It is believed that the analysis of serum pharmacochemistry of traditional Chinese medicine will play an important role as an effective tool in terms of high-throughput elucidation of metabolic phenotypes of XXT. Thus, in this paper, a serum pharmacochemistry of traditional Chinese medicine approach based on a combination of high-performance liquid chromatography diode array detection (HPLC-DAD) coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF/MS) was adopted to screen and analyze the multiple absorbed bioactive components and metabolites of XXT in *vivo*.

2 MATERIAL AND METHODS

2.1 Reagents and Materials

Reference standards of aloeemodin, rhein, coptisine, epiberberine, jatrorrhizine, palmatine, berberine, wogonin, wogonoside, baicalin and baicalein were purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China); emodin and chrysophanol were purchased from Chengdu Institute of Biology, Chinese Academy of Sciences (Chengdu, China). Ultra-pure water was obtained by using a Milli-Q reference super purification system (Merck Millipore, Germany). Distilled water was used for the extraction and preparation of samples. All the solvents (HPLC grade) were acquired from Fisher Scientific (Massachusetts, America) and other chemicals (analytical grade) were obtained from Tianjin Fuchen Chemical Research Factory (Tianjin, China) unless otherwise stated.

2.2 Sample Preparation

Rhei Radix et Rhizoma, Coptidis Rhizoma and Scutellariae Radix were purchased from Fengyuan Tongling Crude Drug Company (Bozhou, China) and were identified by Professor Yu-qing Wang (Nanyang Medical College, Nanyang, China). A total of 400 g mixed herbal slice of RRR, CR, and SR (2:1:1, w/w/w) were extracted once with boiling water (1:10) for 90 min and filtered through gauze. The residue was refluxed with boiling water (1:8) for 60 min and again filtered through gauze. The two filtrates were merged and evaporated with rotary evaporator under vacuum, thus XXT extract was obtained. Then XXT extract was further vacuum-dried. The herbs RRR (200 g), CR (100 g), and SR (100 g) were individually extracted through the same procedure, and then the dried extracts of RRR, CR, and SR could also be obtained. The yield of dry extracts from XXT, RRR, CR, and SR were 31%, 43%, 26%, and 38% (3.2 g, 2.32 g, 3.85 g and 2.63 g crude herbs per 1 g extract) respectively. Each dry extract was weighed and placed in a 10 mL volumetric flask. Methanol was added to the extract and the resulting solution was left in an ultrasonic bath for 30 min. After this, the final volume was adjusted to 10 mL with methanol for final crude drug concentrations of 0.32 g/mL, 0.16 g/mL, 0.08 g/mL and 0.08 g/mL of XXT, RRR, CR, and SR, respectively.

2.3 Preparation of Standard Solutions

Stock solutions were separately prepared separately by dissolving the accurately weighed eight chemical standards with methanol. A mixed stock solution was obtained by mixing coptisine, epiberberine, jatrorrhizine, palmatine and berberine, and giving a final concentration of 102.0, 30.0, 44.6, 79.8 and 371.5 μg/mL, respectively. Then another mixed stock solution was obtained by mixing aloeemodin, rhein, wogonin, wogonoside, baicalin, baicalein, emodin and chrysophanol, and giving a final concentration of 27.5, 8.8, 164.0, 55.3, 718.1, 24.0, 6.0 and 10.0 μg/mL, respectively. All solutions prepared from the plant materials and standard compounds were stored at 4°C for later use.

2.4 In vivo Experiments Protocol

Male Sprague-Dawley rats (200 ± 20 g) were provided by Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). The rats were allowed to acclimatize for seven days in a well-ventilated room at a temperature of 24 ± 2°C and a relative humidity of 50 ± 5%, with a 12 h light/dark cycle. Food and tap water were provided ad libitum. The protocol was approved by the Animal Experimental Ethical Committee of Nanyang Medical College. All efforts were made to ameliorate suffering of animals.

After one week of acclimatization, the rats were randomly divided into 5 groups with 6 rats in each: the control, XXT, RRR, CR, and SR groups. The rats in XXT, RRR, CR, and SR groups were intragastrically given XXT, RRR, CR, and SR extracts at a dose of 18 g/kg, 9 g/kg, 4.5 g/kg, and 4.5 g/kg (18 g, 9 g, 4.5 g and 4.5 g crude herbs per 1 kg rat body weight) dissolved and dispersed homogeneously in ultrapure water, respectively. The animal dose of XXT, RRR, CR, and SR extracts was extrapolated from the human daily dose, using the body surface area normalization method. The formula for dose translation was as follows: human dose of crude herbs in clinic × 0.018/200 × 1000 × the multiple of clinical equivalency dose (Li *el at*, 2012). The dose of XXT, RRR, CR, and SR extracts was equivalent to ten times of the adult daily dose XXT, RRR, CR, and SR (from XXT (20 g) in which RRR, CR, and SR were 10 g, 5 g and 5 g, respectively) crude herbs based on the TCM prescription. Model groups were intragastrically given the same volume of saline solution. All animals were administered by oral gavage two times each day for continuous 6 times. Blood samples (0.5 mL) of rats were collected by posterior

orbital venous plexus after the last dose into 1.5 mL Eppendorf centrifuge tubes. After keeping for 2 h in still position, the blood samples were centrifugated at 3000 r for 10 min. Then, the serum samples were separated and stored at −80 °C until analysis.

2.5 Samples Preparation

Serum samples were thawed at room temperature before preparation. Acetonitrile (600 μL) was added into each serum sample (200 μL) to precipitate protein. Afterwards, the mixture was vortexed for 30 s and centrifuged at 13,000 rpm for 10 min. Then, 450 μL supernatants of the serum samples were transferred into new tubes and evaporated to dryness under vacuum, respectively. The residues of serum samples were dissolved in 200 μL of 70% acetonitrile solution, and the mixtures were vortexed for 1 min, and centrifuged at 13,000 rpm for 10 min at 4°C. Finally, a 5 μL aliquot of supernatant was injected for HPLC-MS analysis.

In addition, the serum samples were randomly selected from each group and mixed together as the quality control (QC) samples, respectively. This pooled sample was used to provide a representative "mean" sample containing all analytes that was encountered during the analysis, and it was used to validate stability of HPLC-Q-TOF/MS system. The QC samples were injected five times at the beginning of the run in order to condition or equilibrate the system and then every ten samples to further monitor the stability of the analysis. The acquired QC data were used to investigate the analytical variability in the whole run. This was necessary in order to evaluate whether the analytical system had changed (and to what extent) over the time course of the analysis, and essential for evaluating the variation in the analytical results and therefore the reliability of the metabolite profiling data [19-22].

2.6 HPLC-DAD and HPLC-Q-TOF/MS Conditions

The HPLC analysis was performed on an Agilent 1260 HPLC system (Agilent, USA). Samples were separated on an Agilent Poroshell 120 EC-C_{18} column (3 × 150 mm, 2.7 μm) protected by a 5 mm guard column using gradient elution. The mobile phase consisted of two solvents: solvent A was a mixture of 0.02 mol/L ammonium acetate in water-acetic acid (pH = 3.5) and solvent B was 100% acetonitrile. Gradient elution was performed as follows: 0–4 min, 2%–24.5% B; 4-14 min, 24.5%-26.3% B; 14-17 min, 26.3%-30% B; 17-20 min, 30%-90% B;

20-26 min, 90% B. An aliquot of 5 μL of sample solution was injected into the Poroshell 120 EC-C_{18} column. The flow rate was 0.5 mL/min, and column was maintained at 40ºC. UV-Vis chromatograms were recorded at 260 nm by diode-array detection (DAD). MS was performed on a G6530 Q-TOF/MS (Agilent, USA). The instrument was operated by using electrospray ionization (ESI) source in positive and negative mode. The ionization source conditions were as follows: gas temperature of 320°C, drying gas flow rate of 8 L/min, nebulizer pressure of 40 psig, sheath gas temperature of 300°C, sheath gas flow rate of 12 L/min, and capillary voltage of 3.5 kV. An acquisition time of 500 ms was used throughout with an acquisition rate of 2 spectrals. The skimmer voltage was set at 65 v and Oct/RF Vpp was 750 v. Recalibration reference mass parameters were as follows: detection window of 50 ppm and minimum height of 1000 counts. The MS data were collected from *m/z* 100 to 1700 Da in positive and negative ion modes, and stored in centroid mode.

3 RESULTS

3.1 The Establishment of Methodology of Serum Sample

Representative base peak intensity chromatograms of the serum samples are shown in Fig. 1. The extracted ion chromatographic peaks of 21 ions (547.1453, 547.1451, 409.0772, 461.1089, 445.0775, 459.0930, 338.1389, 336.1227, 320.0912, 459.0928, 352.1546, 336.1228, 431.0975, 227.0714, 283.0248, 269.0451, 269.0457, 283.0609, 283.0614, 269.0452 and 253.0506 in serum sample) were selected for method validation [19, 20, 22]. The repeatability of method was evaluated by using five replicates of QC sample. The relative standard deviations (RSD%) of peak areas, retention times and *m/z* were 1.11-5.95%, 0-0.28% and 0.00014-0.0026%, respectively. The post-preparation stability of samples was tested by analyzing QC sample kept in auto sampler at 4°C for 0, 4, 8, 12 and 24 h. The relative errors of peak areas were less than 6.87%, which demonstrated that the method had excellent repeatability and stability.

3.2 Identification of Absorbed Bioactive Components

Through the analysis of the serum samples at multiple blood collection time points, the serum samples of XXT were provided with Rhei Radix et Rhizoma, Coptidis Rhizoma and Scutellariae

Radix for 1 h, with the highest quality fraction. The quantity and quality fraction of the serum samples decreased significantly after ig 2 h, so the serum samples were analyzed with 1 h.

Using HPLC, XXT, single-constituent decoctions, drug-containing serum, and blank serum were analyzed and compared to identify the source of the absorbed constituents. A total of 21 transitional constituents were identified in the rat serum samples after intragastric administration of XXT (Fig. 1), of which peaks **3**, **4**, **13**–**15**, **17**, **20** and **21** originated from DH (Fig. 2), peaks **7**–**9**, **11**, and **12** from HL (Fig. 3), and peaks **1**, **2**, **5**, **6**, **10**, **16**, **18**, and **19** from HQ (Fig. 4).

The chromatograms of the DH decoction and its corresponding serum samples are shown in Fig. 2. After comparing the retention time, UV absorption spectra, and mass spectral data of each compound with that of the control, we preliminarily identified peak **3** as 2-methyl-5-carboxymethyl-7-hydroxychromoglucuronide, peak **4** as 2-*O*-cinnamoyl-glucogallin, peak **13** as emodin glucoside, peak **14** as resveratrol, peak **15** as rhein, peak **17** as aloe aloeemodin, peak **20** as emodin, and peak **21** as chrysophanol (Table 1).

The chromatograms of the HL decoction and its corresponding serum samples are shown in Fig. 3. After comparing the retention time, UV absorption spectra, and mass spectral data of each compound with the that of the control, we preliminarily identified peak **7** as jatrorrhizine, peak **8** as epiberberine, peak **9** as coptisine, peak **11** as palmatine, and peak **12** as berberine (Table 1).

The chromatograms of the HQ decoction and its corresponding serum samples are shown in Fig. 4. After comparing retention time, UV absorption spectra and mass spectral data of each compound with that of the control, we preliminarily identified peak **1** as 6-*C*-arabinosyl-8-*C*-glucosyl-chysin, peak **2** as 6-*C*-glucosyl-8-*C*-arabinosyl-chrysin, peak **5** as baicalin, peak **6** as oroxylin glucoside, peak **10** as wogonoside, peak 16 as baicalein, peak **18** as wogonin, and peak **19** as oroxylin (Table 1).

3.3 Identification of Absorbed Bioactive Components from DH

3.3.1 *Anthraquinones*

The retention time of compound **13** was 15.022 min and its quasi-molecular ion peak was *m/z* 431.0975 $[M-H]^-$. With tandem MS, the parent ion was cleaved into fragment *m/z* 269.0448 after losing a glucose molecule. Fragment *m/z* 269.0448 was then further cleaved into *m/z* 241.0502 and

m/z 225.0551, which followed the same cleavage pathway as that of emodin. Therefore, it was concluded that compound **13** was emodin glucoside [23]. The retention time of compound **15** was 18.713 min and its quasi-molecular ion peak was *m/z* 283.0248 $[M-H]^-$. It was then fragmented into *m/z* 257.0452 $[M-H-COH_2]^-$ after losing CO, and each of the two adjacent carbons bonded with a hydrogen. Fragment *m/z* 239.0344 $[M-H-CO_2]^{\backslash-}$ was the major product after the molecular ion lost one CO_2. After comparing with the control samples, compound **15** was identified as rhein [24]. The cracking pathway is shown in Fig. 5. The retention time of compound **17** was 21.824 min. The quasi-molecular ion peak, *m/z* 269.0457 $[M-H]^-$, lost one uncharged CO molecule and an oxygen atom, forming *m/z* 225.0555 $[M-H-CO-O]^-$. Fragment *m/z* 223.0393 $[M-H-CO-H_2O]^-$, was the major product of the molecular ion and formed by losing H_2O and CO. Fragment *m/z* 240.0425 $[M-H-COH]^-$, was inferred to be the product of the molecular ion after losing CO and one hydrogen atom. Additional cross-referencing with the control samples revealed that compound **17** was aloeemodin [25]. The retention time of compound **20** was 22.752 min. After losing an uncharged CO molecule, the quasi-molecular ion peak, *m/z* 269.0452 $[M-H]^-$, became fragment *m/z* 241.0505 $[M-H-CO]^-$. The *m/z* 225.0554 was the product of *m/z* 241.0505 losing one oxygen atom. After comparing with control samples, compound **20** was identified as emodin [26]. The retention time of compound **21** was 23.882 min. Its quasi-molecular ion peak was *m/z* 253.0506 $[M-H]^-$, which fragmented to form *m/z* 239.0342 $[M-H-CH_2]^-$ after losing CH_2, and the fragment *m/z* 225.0551 $[M-H-CO]^-$ was formed after the quasi-molecular ion lost an uncharged CO. After cross-referencing with the control samples, compound **21** was identified as chrysophanol [27]. The cracking pathway is shown in Fig. 6.

3.3.2 *Stilbene*

The retention time of compound **14** was 15.951 min and its quasi-molecular ion peak was *m/z* 227.0714 $[M-H]^-$, which fragmented into *m/z* 185.0606 after losing C_2H_2O, and *m/z* 143.0496 after losing $C_4H_4O_2$. Fragment *m/z* 143.0496 was then further cleaved into *m/z* 117.0342 after losing $C_2H_3^-$. After cross-referencing with the literature, compound **14** was identified as resveratrol [28]. The cracking pathway is shown in Fig. 7.

3.3.3 *Chromone*

Under the negative ion mode, the full scan MS revealed that the quasi-molecular ion was *m/z* 409.0772 $[M-H]^-$, with a retention time of 8.212 min for compound **3**. Additional full-scan tandem MS showed main fragment ions of *m/z* 365.0869, 233.0452 and 189.0554, which were identified as fragments of the quasi-molecular ion without CO_2, $C_6H_8O_6$ and CO_2+$C_6H_8O_6$, respectively. Fragment ions of *m/z* 175.0241, 149.0452 and 131.0346,were identified as characteristic ion peaks of glucuronic acid. After cross-referencing with the literature, compound **3** was preliminarily identified as 2-methyl-5-carboxymethyl-7-hydroxyl chromoglucuronide [29]. The cracking pathway is shown in Fig. 8.

3.3.4 *Tannins*

The retention time of compound **4** was 8.422 min, and its quasi-molecular ion peak was *m/z* 461.1089 $[M-H]^-$. After losing $C_6H_{12}O_6$ and $C_9H_8O_2$, fragments of *m/z* 299.0555 $[M-H-C_6H_{12}O_6]^-$ and *m/z* 331.0662 $[M-H-C_9H_8O_2]^-$ were observed. Fragment *m/z* 299.0555 was further cleaved into fragment *m/z* 169.0135 after losing $C_9H_8O_2$, while fragment *m/z* 331.0662 was further cleaved into fragment *m/z* 169.0135 after losing $C_6H_{12}O_6$. After cross-referencing with the literature, this compound was identified as 2-*O*-cinnamoyl-glucogallin [30]. The cracking pathway is shown in Fig. 9.

3.4 Identification of Absorbed Bioactive Components HL

3.4.1 *Alkaloids*

The retention time of compound **7** was 10.543 min. Its quasi-molecular ion peak was *m/z* 338.1389 M^+, which fragmented into 323.1154 $[M-CH_3\cdot]^+$, 322.1076 $[M-CH_3\cdot-H\cdot]^+$, 308.0925 $[M-CH_3\cdot-CH_3\cdot]^+$, 294.1128 $[M-CH_3\cdot-H\cdot-CO]^+$, 280.0971 $[M-CH_3\cdot-CH_3\cdot-CO]^+$ daughter ions. Comparison with the control samples revealed compound **7** to be jatrorrhizine. The retention time of compound **8** was 10.822 min and its quasi-molecular ion peak was *m/z* 336.1227 M^+, which fragmented into *m/z* 321.1005 after losing $CH_3\cdot$, and *m/z* 306.0763 after losing $2CH_3\cdot$. Fragment *m/z* 321.1005 was then further cleaved into 320.0920 $[M\text{-}CH_3\cdot\text{-}H\cdot]^+$, 292.0977 $[M-CH_3\cdot-H\cdot-CO]^+$ and 278.0821 $[M-2CH_3\cdot-CO]$ after losing $H\cdot$, $H\cdot$+CO, $CH_3\cdot$+CO, respectively. After comparison with control samples, compound **8** was identified as epiberberine. The retention time of compound **9** was 10.982 min and its quasi-molecular ion peak was *m/z*

320.0912 M^+. The parent ion was fragmented to form *m/z* 318.0763 $[M-2H\cdot]^+$, and *m/z* 292.0971 $[M-CO]^+$ ions after losing 2H·, and uncharged CO, respectively. Cross-referencing with the control samples revealed that compound **9** was coptisine. The cracking pathway is shown in Fig. 10. The retention time of compound **11** was 13.952 min and its quasi-molecular ion peak was *m/z* 352.1546 M^+. The parent ion was cleaved into *m/z* 337.1317 $[M-CH_3\cdot]^+$, 322.1076 $[M-2CH_3\cdot]^+$ and 308.1285 $[M-CHO-CH_3\cdot]^+$ ions after losing $CH_3\cdot$, $2CH_3\cdot$, and C_2H_4O, respectively. Fragment *m/z* 337.1317 was further fragmented into *m/z* 336.1239 $[M-CH_3\cdot-H\cdot]^+$ after losing H, respectively. Fragment *m/z* 308.1285 lost an additional $CH_3^{\cdot}$ to form *m/z* 293.1055 $[M-CHO-2CH_3\cdot]^+$. Cross-referencing with the control samples revealed compound **11** to be palmatine. The retention time of compound **12** was 14.442 min and its quasi-molecular ion peak was *m/z* 336.1228 M^+. The parent ion was fragmented to form *m/z* 321.1006 $[M-CH_3\cdot]^+$, 306.0771 $[M-2CH_3\cdot]^+$ and 292.0976 $[M-C_2H_4O]^+$ ions after losing $CH_3\cdot$, $2CH_3\cdot$ and C_2H_4O, respectively. Fragment *m/z* 321.1006 was further cleaved to form *m/z* 320.0925 $[M-CH_3\cdot-H\cdot]^+$, 292.0976 $[M-CH_3\cdot-CHO]^+$ and 278.0822 [M-2$CH_3\cdot$-CO] after losing H·, CHO and $CH_3\cdot$+CO, respectively. After cross-referencing with the control samples, compound **12** was identified as berberine [31]. The cracking pathway is shown in Fig. 11.

3.5 Identification of Absorbed Bioactive Components from HQ

3.5.1 *Flavonoids*

The feature fragmentation approach of flavonoids is shown in Fig. 12. For compound **1**, under negative-ion mode, the full-scan MS detected a quasi-molecular ion of *m/z* 547.1453, with a retention time of 7.182 min. Full-scan tandem MS identified the main fragment ions *m/z* 415.1032, 253.0505 and 225.0550 as the quasi-molecular ion without $C_5H_{10}O_4$, $C_6H_{10}O_5$ and CO, respectively. After cross-referencing with the relevant literature, compound **1** was identified as 6-*C*-arabinosyl-8-*C*-glucosyl-chysin. Compound **2** was identified under negative ion mode, and the full scan MS revealed a quasi-molecular ion at *m/z* 547.1451, with a retention time of 8.151 min. Full-scan tandem MS show the major fragment ions of *m/z* 385.0925 $[M-H-C_6H_{10}O_5]^-$, 253.0501 $[M-H-C_6H_{10}O_5-C_5H_8O_4]^-$ and 225.554 $[M-H-C_6H_{10}O_5-C_5H_8O_4-CO]^-$. By

cross-referencing the literature, compound **2** was determined to be 6-*C*-glucosyl-8-*C*-arabinosyl-chrysin [32].

Compound **5** eluted at 8.591 min, and the full-scan MS under negative ion mode showed a quasi-molecular ion at *m/z* 445.0775. Full-scan tandem MS revealed that fragment ion, *m/z* 269.0454, was 176 Da smaller than the quasi-molecular ion, due to the loss of $C_6H_8O_6$. HPLC-MS/MS analysis was performed on a baicalin standard solution, exhibiting the same retention time and fragment ions as compound **5**. Therefore, compound **5** was confirmed to be baicalin. The retention time of compound **6** was 10.133 min and its quasi-molecular ion peak was detected at *m/z* 459.0930 $[M–H]^-$. The main fragment ions included *m/z* 283.0603 $[M–H–C_6H_8O_6]^-$, 269.0453 $[M–H–C_6H_8O_6–CH_2]^-$, 241.0495 $[M–H–C_6H_8O_6–CO]^-$, and 197.0601 $[M–H–C_6H_8O_6–CO_2–CO]^-$. The main fragment ions of this compound were identical to those of compound **10**, but with different retention times. This suggests that compounds **6** and **10** were isomers. Compound **10** was confirmed to be wogonoside after comparison with control samples. After an additional literature search, compound **6** was identified as oroxyloside [33, 34].

The retention time of compound **10** was 11.161 min and its quasi-molecular ion peak was *m/z* 459.0928 $[M–H]^-$. The parent ion was fragmented to form *m/z* 283.0609 $[M–H–C_6H_8O_6]^-$ after losing one molecule of glucuronic acid. Fragment ion *m/z* 283.0609 was further cleaved into the *m/z* 269.0449 $[M–H–CH_2]^-$ after losing CH_2. Fragment *m/z* 269.0449 formed the *m/z* 241.0498 ion after losing CO at the C-4 position of the C-ring. The *m/z* 197.0605 ion was the fragmentation product of *m/z* 269.0449 after losing a CO and a CO_2 molecule. After comparison with the control samples, compound **10** was identified as wogonoside [33].

In negative ion mode, the retention time of compound **16** was 21.013 min, and its quasi-molecular ion was *m/z* 269.0451. The tandem MS scan revealed the main fragments to be *m/z* 251.0346, 241.0403, 223.0391 and 197.0605, which were products of the quasi-molecular ion losing H_2O, CO, H_2O+CO and CO+CO_2, respectively. After comparison with the control samples, compound **16** was identified as baicalein [34].

The retention time of compound **18** was 21.926 min and its quasi-molecular ion peak was *m/z* 283.0609 $[M–H]^-$, which formed *m/z* 268.0375 $[M–H–CH_3{\cdot}]^-$ after losing a methyl radical.

Fragment *m/z* 268.0375 became the *m/z* 240.0421 ion after losing CO at the C-4 position of the C-ring, and became fragment ion *m/z* 224.0476 after losing CO at C-4 and O at the C-1 position of the C-ring. The base peak ions *m/z* 239.0341 and *m/z* 223.0392 were fragmentation products of *m/z* 268.0375 with the loss of $COH^{\cdot}$ and $CO_2H^{\cdot}$, and the *m/z* 212.0473 ion was the fragmentation product upon the loss of two uncharged CO. Cross-referencing with the control samples indicated that compound **18** was wogonin [33, 34]. The retention time of compound **19** was 22.093 min and its quasi-molecular ion peak was *m/z* 283.0614 $[M–H]^-$, which fragmented into *m/z* 268.0375 $[M–H–CH_3^{\cdot}]^-$, 265.0503 $[M–H–H_2O]^-$ and 255.0659 $[M–H–CO]^-$ after losing $CH_3^{\cdot}$, H_2O and CO, respectively. After cross-referencing with the literature, compound **19** was preliminarily identified as oroxylin A [35].

4 DISCUSSION AND CONCLUSIONS

In this study, chromatographs were compared among single-constituent decoction, formulated decoction, serum samples and controls using the same chromatographic parameters. Retention times were used to analyze and identify the chromatographic peaks upon comparison to those of controls and standards. Serum samples collected at different time points were analyzed, and it was found that 1 h after the intragastric administration of XXT, the rat serum samples contained many constituents in the highest mass fraction. Therefore, serum samples collected after 60 min were selected for analysis.

By analyzing the transitional constituents absorbed into the blood, serum pharmacochemical research can identify the material basis for the efficacy of TCM. Using the approach of serum pharmacochemistry in TCM research, the transitional constituents in rat serum were analyzed after oral administration of XXT using HPLC-DAD and HPLC-Q-TOF/MS. The results show that the main adsorbed constituents in the serum were anthraquinones, chromones, stilbenes and tannins from RRR, alkaloids from CR, flavonoids from SR. Many papers suggest that, anthraquinones display the beneficial effects of antibacteria, anti-inflammation, antivirus, anticancer, etc.[36]. Chromones have anti-inflammatory, analgesia, antihypertensive effects, etc. [37]. Stilbenes possesses a wide range of biological properties including potent anti-tumor, anti-inflammatory and antioxidative effects, etc. [38, 39]. In recent years, the pharmacological action of tannins have

been extensively studied, mainly including bacteriostasis, hemostatic action, anticoagulant action, improving renal function, etc.[40]. In recent pharmacological studies, alkaloids, main bioactive components of CR have been reported to exhibit various pharmacological effects, such as anti-bacterial, anti-inflammatory, anti-oxidative, anti-tumor, anti-diabetic, hypolipidemic and neuroprotective activities [41-44]. Pharmacological studies have indicated that flavonoids share many beneficial activities with RS with respect to anti-virus, anti-inflammatory, anti-allergic, anti-oxidant and hepatoprotective properties [45-47]. Therefore, it is reasonable for anthraquinones, chromones, stilbenes, tannins, alkaloids and flavonoids to be confirmed as bioactive components.

Based on the serum pharmacochemistry of traditional Chinese medicine and HPLC/DAD coupled with HPLC-Q-TOF/MS, a method for screening and analysis of the multiple absorbed bioactive components and metabolites of XXT in orally dosed rat plasma has been established. As a result, 21 of the constituents were separated and characterized by the methodological system, which enhanced the speed and targeting of bioactive components in TCM.

The present study, compared with the previous studies, showed differences or improvements as follows. First of all, it is that an integrated method for screening the bioactive components in rat plasma after oral administration of XXT has been developed. Furthermore, according to the literature, most of the identified components in dosed rat plasma possess pharmacological activities; therefore, serum pharmacochemistry of traditional Chinese medicine is suitable for the study of real bioactive components in XXT, and their pharmacological mechanism could be well understood accordingly. In addition, the application of the method to XXT not only provided chemical support for the chromatographic fingerprint technology, but also provided valuable information for a comprehensive pharmacokinetics investigation of this famous formula. Regrettably, due to limitations of the extraction techniques and detection tools, only a fraction of the components in the formula were tentatively identified. Therefore, identification of multiple absorbed bioactive components originated from the formula still needs to be done for complete understanding of its mechanism.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by State Committee of Science and Technology of the People's Republic of China.

HUMAN AND ANIMAL RIGHTS

The animal experiment was carried out according to the Regulations of Experimental Animal Administration.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Figure Legends

Fig. 1 The total ion chromatogram of blank serum (A), XXT (B), and serum after XXT treatment (C)

Fig. 2 The total ion chromatogram of blank serum (A), RRR (B), and serum after RRR treatment (C)

Fig. 3 The total ion chromatogram of blank serum (A), CR (B), and serum after CR treatment (C)

Fig. 4 The total ion chromatogram of blank serum (A), SR (B), and serum after SR treatment (C)

Fig. 5 Mass spectrometric fragmentation pathway of rhein

Fig. 6 Mass spectrometric fragmentation pathway of chrysophanol

Fig. 7 Mass spectrometric fragmentation pathway of resveratrol

Fig. 8 Mass spectrometric fragmentation pathway of 2-methyl-5-carboxymethyl-7-hydroxychromoglucuronide

Fig. 9 Mass spectrometric fragmentation pathway of 2-*O*-cinnamoyl-glucogallin

Fig. 10 Mass spectrometric fragmentation pathway of coptisine

Fig. 11 Mass spectrometric fragmentation pathway of berberine

Fig. 12 The feature fragmentation approach of flavonoids.