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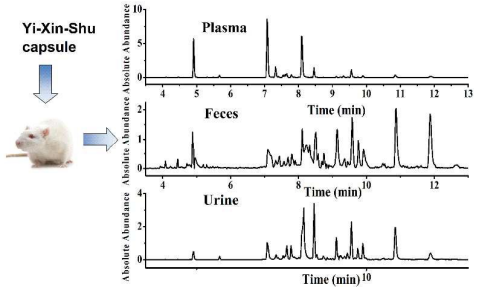

In this study, the metabolite profiles of YXS in different biofluids of rats were investigated.

Journal Name

ARTICLE

Metabolic profile of Yi-Xin-Shu capsule in rat by ultra performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry analysis

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Yi-Xin-Shu capsule (YXS), a Chinese Patented medicine, is widely used for treating coronary heart disease. However, due to lack of the studies on chemical and systematic metabolic profiling, the active substances and metabolic fate of YXS were still unknown, which became a huge obstacle for the clinical-safe medication administration and the quality control of YXS. To solve this problem, investigation of the metabolic profiles of YXS in rat plasma, urine and feces was conducted. In order to completely identify the predictable and unpredictable metabolites, a combined data mining strategy based on ultra performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF MS), was employed. As a result, total 184 compounds were tentatively identified in rat biological fluids after oral administration of YXS, including 143 compounds in plasma, 82 compounds in urine and 116 compounds in feces. Among them, 11 lignan-related metabolites were firstly identified and the metabolite pathways were tentatively proposed in this study. Except that, based on the identified tanshinone-related metabolites, the metabolite pathways of tanshinones were tentatively proposed firstly. In addition, the results indicated that ginsenosides, lignans and tanshinones were the major absorbed chemical components of YXS. From the total numbers of compounds detected in urine and feces, we considered that much more compounds were cleared through feces. Oxidation reactions as well as hydrolysis reactions were the major metabolic reactions *in vivo*. This study firstly reported the description of YXS metabolism *in vivo*. The metabolite profile analysis of YXS in different biological fluids provided a comprehensive understanding of the in *vivo* metabolic fates of constituents in YXS.

1. Introduction

Yi-Xin-Shu capsule (YXS), a Chinese Patented medicine, consists of seven herbal medicines including *Panax ginseng* (*Panax ginseng*, C. A. Meyer), *Radix ophiopogonis* [*Ophiopogon japonicus* (L.f) Ker-Gawl], *Astragalus membranaceus* (Fisch.) Bunge, *Radix Salviae Miltiorrhizae* (*Salvia miltiorrhiza* Bge.), *Fructus Crataegi* (*Crataegus pinnatifida*), *Rhizoma Chuanxiong* (*Ligusticum chuanxiong* Hort.) and *Fructus Schisandrae Chinensis* [*Schisandra chinensis* (Turcz.)]. Both clinical and modern pharmacological studies have demonstrated that YXS carries excellent efficacy in treating coronary heart diseases.[1-6] Therefore, it has been widely used by the patients with coronary heart diseases in China. The sales of YXS have broken through two hundred and twenty million in recent years, so it has become the core strength to support the traditional Chinese medicine industry.

Up to now, many researchers have taken up studies in the field of clinical trials[1-6] and quality control[7-10]. However, few reports are available on its *in vivo* metabolism, leading to the bioactive substances as well as the metabolic fate of YXS are still unknown, which entails a huge obstacle for clinical-safe medication administration and quality control of YXS. Therefore, clarification the therapeutic materials as well as the metabolic fate of YXS has a very important practical significance. Although the metabolites research of several single herbs composed of YXS[11-12] or the characteristic compounds[13-16] has been carried out, which greatly

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aided in the characterization of metabolites of YXS, the complex drug-drug interactions were usually unpredictable. Therefore, a holistic *in vivo* metabolism research of YXS is indispensable.

Owing to low concentration and interference from endogenous components, identification of metabolites presents a great challenge. High performance liquid chromatography coupled with mass-spectrometry (HPLC-MS),[17-20] especially ultra high performance liquid chromatography (UPLC) coupling with quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS),[21-22] has become a routine tool for the detection and identification of the predictable metabolites, because of its high-resolution and can provide molecular formula as well as exact molecular mass. But for unpredictable metabolites, which were formed by multi-step biotransformation, just relying on high resolution data makes the structure elucidation much more difficult. Thus, in order to identify the predictable and unpredictable metabolites, a combination data mining strategy based on the high-resolution data, including mass defect filter (MDF), extraction ion chromatography (EIC), isotope pattern matching (IPM) and modifier formula of the biotransformation (MFB) based on high-resolution LC-MS data was employed in this study.

Using the combined data mining strategy, we successfully identified 184 prototype components and metabolites in rat plasma, urine and feces after oral administration of YXS. The significance of our study is that it clarified the potential curative substances of YXS and described their metabolic fate *in vivo* for the first time. The results provided a meaningful basis for the clinical application of YXS and a comprehensive understanding of the in *vivo* metabolic fates of constituents in YXS.

2. Experimental

2.1. Materials and reagents

LC-MS grade acetonitrile was purchased from J. T. Baker (Phillipsburg, USA), and LC-MS grade formic acid was obtained from Fisher-Scientific (Fair Lawn, USA). Ultra-pure water was obtained from Burdick & Jackson (Honeywell, USA). Other reagents were of analytical grade.

The YXS sample (Batch No. 20131209) was manufactured by Guizhou Xinbang Pharmaceutical Co., LTD. 36 reference standards, including ginsenoside Re_3 (**43**), 20-Glc-Rf (**46**), Re (**52**), Rg_1 (**53**), Rf (**68**), Ra_2 (**71**), Ra_3 (**72**), Rb_1 (**73**), Rg_2 (**75**), Ra_1 (**76**), Rc (**77**), (*R*)-Rg_2 (**78**), Ro (**79**), Rh_1 (**80**), Rb_2 (**82**), Rb_3 (**83**), Rs_2 (**87**), Rd (**88**), Ro methyl ester (**94**), Rg_3 (**105**), (*R*)-Rg_3 (**106**), Rh_2 (**111**), (*R*)-Rh_2 (**112**), Notoginsenoside R_1 (**49**), Notoginsenoside R_2 (**70**), Quinquenoside R_1 (**84**), Chikusetsusaponin IVa (**103**), Astragaloside IV (**122**), Schizandrol A (**2**), Schisantherin A (**18**), Schisandrin A (**22**), Schisandrin B (**24**), Tanshinone I (**38**), Cryptotanshinone (**39**), Tanshinone IIA (**41**) and Vitexin (**113**), were either purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) or gifts from professor Xiuwei Yang, the State Key Laboratory of Natural and Biomimetic Drugs, Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University.

2.2. Sample preparation of YXS for animal treatment

9.5 g of the contents of YXS were weighed and then extracted ultrasonically with 95 mL of 70% methanol for 30 min at room temperature. The extracted solution was then filtered. This extraction was repeated two additional times. The combined filtrate was evaporated to dry using a rotatory evaporator at 40°C. Subsequently, the residue was dissolved in 25 mL of deionised water and the final concentration was 0.378 g/mL.

2.3. Animal and drug administration

Fourteen male Sprague-Dawley (weighting from 220 to 250 g) were obtained from the Vital River Laboratory Animal Technology Co. Ltd. The animals were acclimatized to laboratory conditions for 7 days and then fasted with free access to water for a 12 h period prior to the experiment. The rats were randomly divided into experiment group (n = 10) and blank group (n = 4). The extracted solution of YXS was administrated by oral administration at a single dose of 3.78 g/kg body weight to the experiment group. An equivalent of de-ionized water with no YXS extracted solution was given to the blank groups. All experiments were performed in compliance with the relative laws and institutional guidelines and the institutional committees have approved the experiments.

2.4. Biological samples collection

For the collection of plasma samples, the rats (n = 6) were anesthetized by intraperitoneal injection of 10% aqueous chloral hydrate after oral administration of the YXS preparation. The blood samples were collected from hepatic portal vein in heparinized tube at 0.5h, 1h, 2h, 4h, 6h and 8h, respectively. The collected samples were then centrifuged at 4000 rpm for 15 min at 4°C and then mixed to obtain a pooled plasma. The blank plasma was collected in the same way (n = 2).

For the collection of urine and feces samples, the rats (n = 4) were housed in separate metabolic cages with free access to deionised water. After oral administration of the YXS preparation, urine samples and feces samples were collected over 0-24 h period. Blank urine samples and blank feces samples were collected in the same way (n = 2).

2.5. Samples pretreatment

A volume of 4 mL plasma sample was immediately treated with 12 mL acetonitrile and then was vor-texed for 3 min to precipitate plasma proteins. After centrifuged at 12000 rpm for 15 min at 4°C, the supernatants were evaporated to dryness under nitrogen gas at room temperature. The residue was reconstituted in 200 μL methanol and centrifuged at 12000 rpm for 15 min at 4°C, followed by the injection of 1μL into the analysis system.

The feces sample was immediately extracted ultrasonically three times with 10 times of 70% methanol for 30 min at room temperature. The combined filtrate was evaporated to dryness using a rotatory evaporator at 40°C. The residue was dissolved in 5 mL of deionised water, and then subjected to the preconditioned solid phase extraction (SPE) column (Waters Oasis HLB SPE column, 60 mg, 3mL). After being washed off with 6 mL of deionised water, the SPE column was washed with 6 mL of methanol. The methanol elate was collected and evaporated to dryness at 40°C using nitrogen gas. The

residue was reconstituted in 200μL methanol, and centrifuged at 12000 rpm for 15 min at 4°C. A 1μL aliquot was injected into the analysis system. The urine sample (6 mL) was immediately loaded onto the preconditioned SPE column and the elution as well as concentration, preparation procedures was the same as the feces. A 1μL aliquot was injected into the analysis system.

2.6. Chromatographic and mass spectrometric conditions

Chromatographic analysis was performed on an Agilent 1290 UPLC system, equipped with a binary pump, an online vacuum degasser, an autosampler and a thermostatted column compartment. Chromatographic separation was performed on an Agilent ZORBAX RRHD Eclipse Plus C18 column (100 × 3 mm, 1.8 μm) connected to a Phenomenex Security GuardTM ULTRA Cartridge. The mobile phase consisted of 0.1% formic acid water (A) and 0.1% formic acid acetonitrile (B). A gradient elution program was optimized as follows: 0-7 min, 10-40% B; 7-9.5 min, 40-55% B; 9.5-12 min, 55-55% B; 12-17 min, 55-88% B. The data rate was set at 10 Hz, and the flow rate was 0.8 mL/min with a split ratio of 1:1. The multiple wavelengths were set at 203 nm, 260 nm and 286 nm, and the temperature was set at 45°C. The inject volume was 1 μL.

Detection was performed by an Agilent Q-TOF 6540, equipped with Agilent Jet Stream (AJS) ESI source. The instrument was performed in both positive and negative ion modes. The MS parameters were set as follows: gas temperature: 325°C, gas flow: 5 L/min, nebulizer pressure: 35 psi, sheath gas temperature: 400°C, sheath gas flow: 12 L/min, capillary voltage: 4000 V (ESI+) and 3500 V (ESI-), nozzle voltage: 500 V (ESI+) and 1500 V (ESI-), fragmentor voltage: 120 V (ESI+) and 280V (ESI-), collision-energy voltage: 30 V (ESI+) and 40 V as well as 60 V (ESI-). Internal references (Purine and HP-0921) were adopted to modify the measured masses in real time, and the reference masses in positive ion mode were at *m/z* 121.0509 and 922.0098, while in negative ion mode, they were at *m/z* 119.0363 and 1033.9881. The mass spectrometer was in full scan range of *m/z* 100-1700 for MS and MS/MS.

2.7. Data mining strategy

In order to distinguish the prototype compounds from the metabolite compounds, we designed compounds detected in both drug-related biofluids and YXS preparation as prototype components, and compounds detected only in drug-related biofluids but not in YXS as metabolites. When screening the prototype compounds, constituents, previously isolated and identified from each single herb composed of YXS, were extracted in the metabolic profiles of plasma, urine and feces samples, following with the MS/MS experiments to elucidate the structures. It seems not to be a difficult work. But when screening the metabolites in biofluids, it becomes much more difficult. Thus, a data mining strategy including EIC, MDF, IPM and MFB techniques was employed in this study. The EIC and IPM techniques can extract the metabolites with expected *m/z* and isotope pattern. The MDF technique, used for detecting drug metabolites via post-acquisition processing of high-resolution LC-MS data, enables high-resolution mass spectrometers to be utilized to detect both predictable and unpredictable metabolites through defining narrow ranges of changes in the mass defect dimension of high-resolution LC-MS data regardless of the molecular masses as well as fragmentation patterns. The MFB technique exhibits the modifier formula of the biotransformation from the parent compound to its metabolites, which greatly aided in the identification of the metabolites. Metabolite ID (Agilent) software, merging the multiple data processing technologies, has been proved to be an extremely useful tool in metabolites identification and was introduced in this study.

In general, the combined data mining strategy mainly involved three steps:

(Ⅰ) Establishment of chemical components database. The compounds previously isolated and identified from each single herb composed of YXS were retrieved to establish the chemical components database. All of them were sorted by compound name, molecular formula, accurate mass and chemical structure.

(Ⅱ) Screening prototype compounds. The compounds listed in chemical components database were detected via extraction their ion chromatograms from the high-resolution LC-MS data, followed by targeted MS/MS experiments for further structure elucidation.

(Ⅲ) Screening predictable and unpredictable metabolites. The compounds listed in the chemical components database were input into the Metabolite ID software with a proper mass defect value, and a series of YXS-related metabolites were generated. All YXS-related metabolites underwent targeted MS/MS analysis for further structure elucidation.

3. Results and discussion

Because different categories of compounds exhibited significantly different ion responses in the positive- and negative-ion modes, the screening identification and further characterization of the metabolites of YXS were performed by UPLC-Q-TOF MS in positive- and negative-ion mode. The metabolite profiling of plasma, urine and feces samples in positive- and negative-ion modes were shown in Fig. 1, respectively. Using the data mining strategy, a post-acquisition data processing method, a total of 184 compounds were identified from rat biofluids.

3.1. Identification of components in biofluids

For prototype compounds, all the compounds previously isolated and identified from each single herb composed of YXS were detected in EICs mode of YXS and rat biofluids. As a result, total 122 compounds were confirmed as prototype compounds, including 24 lignans, 17 tanshinones, 71 ginsenosdies, 2 flavonoids, 2 lactones, 3 phenolicacids as well as triterpenoid acid, and 3 astragalus saponins (shown in Table S1, ESI†). Always some isomers of known compounds were detected and identified. Among the identified compounds, 36 constituents were unambiguously identified by comparison with the reference standards, and other structures were further characterized by accurate mass and corresponding MS/MS fragmentation ions.

For metabolites, a total of 62 YXS-related metabolites were tentatively identified in rat biofluids. According to the origins of parent compounds and structural types, all these metabolites were categorized as lignans-related, tanshinone-related and ginsenoside-related compounds (shown in Content S1, ESI† and Table S2, ESI†).

The typical MS/MS spectrums of **M2** (A), **M38** (B) and **M60** (C) were shown in Fig. 2. Total 37 lignans-related metabolites were tentatively identified in rat biofluids by comparison with the literature[13] or the fragmentation ions obtained from their MS/MS spectra. All of them were mainly generated from three parent compounds, including Schisandrin A, Schisandrin B and Schisantherin B. Metabolites **M1**, **M2**, **M4**, **M6-M8**, **M10**, **M12**, **M24**, **M26** and **M29** were firstly identified in this study. Total 17 tanshinone-related metabolites were tentatively identified in rat biofluids. The detected result suggested that the tanshinone-related metabolites were mainly generated from three parent compounds, including Cryptotanshinone, Tanshinone IIA and Tanshinone I. In addition, 10 ginsenoside-related metabolites were detected from the drug-containing plasma, feces samples as well as urine sample. The results suggested that almost all of the detected metabolites were secondary glycosides, which were formed by sequential deglycosylation metabolites in intestine. In addition, compound X in Fig. 1 A3 was an endogenous component, which was confirmed by comparison with the blank urine sample.

3.2. Analysis of metabolic and dispositional pathway

The prototype and related metabolites identified in this work provided a global view of metabolite profiles of YXS in rat plasma, urine and feces. In this study, the metabolite profiling in different biological fluids were also investigated. From the results, we discovered that lignans, tanshinones and ginsenosides comprised of majority of compounds in rat plasma, urine as well as feces.

For lignans, total 61 prototype compounds and metabolites were detected in rat plasma, urine and feces. An interesting thing was observed that, almost all the lignans detected in urine were not identified in feces. Total 23 prototype compounds were identified in urine while only 18 prototype compounds were identified in feces. From the numbers of prototype lignans detected in urine and feces, we considered that much more prototype lignans were cleared through urine. With respect to the prototype lignans, 8 components, including **2-3**, **14-16**, **18**, **22** as well as **24**, detected in this study have been previously reported.[23] In addition, 16 prototype lignans, including **1**, **4-13**, **17**, **19-21** and **23**, were detected in the biofluids for the first time. Most of them were isomers of known compounds. The metabolites of Schisandrin A have been reported, and total 51 metabolites were identified in rat biofluids.[13] Parts of the reported metabolites were detected in our study, which can partly validated the results of the previous study on rat biological metabolite profile.[13] Except that, 11 lignan-related metabolites, including **M1-M2**, **M4**, **M6-M8**, **M10**, **M12**, **M24**, **M26** and **M29**, were firstly identified. All of them were derived from Schisandrin A, Schisandrin B or Schisantherin B. The metabolic reactions of the lignans were also investigated in this study. The metabolic pathways of the reported metabolites were shown in Fig. S1.The metabolic pathways of the newly identified metabolites were firstly proposed (shown in Fig. 3). From Fig. S1, ESI† and Fig. 3, we observed that the metabolic sites for lignans were mainly on the methoxy group and the biphenyl cyclooctene. Always, losing CH_2 or OCH_2 was the first biotransformation step, followed by further transformation through oxidation, including mono-oxidation, di-oxidation and carboxylic acid reactions. In addition, direct oxidation metabolites of Schisandrin B, including **M4**, **M8**, **M10** and **M12**, were also detected.

For tanshinones, total 17 prototype compounds and 15 metabolites were identified both in rat plasma and rat feces, but all of them were present from rat urine. This indicated that feces excretion may be the main route for clearance of tanshinone-related compounds, which can relieve kidney burden when taking YXS. In the past few years, several reports on the metabolic profiles of *Radix Salviae Miltiorrhizae*[11, 12] as well as characteristic compounds [24-26] have been published. 9 prototype compounds, including **25-26**, **32-33**, **35**, **37-39** as well as **41**, and all 15 metabolites detected in this study have been reported in the literatures. However, a few tanshinone-related metabolites previously reported have not been detected in this study, [12] probably because the administration dosage chosen in this study was greatly different from that in the literature. Luckily, 8 compounds (**27-31**, **34**, **36** and **40**) were detected in plasma and feces for the first time. Although the tanshinone metabolites have been reported previously,[11-12] the metabolic pathways have not been proposed up to now. Therefore, emphasis was put on the metabolic reactions and pathways based on the identified tanshinone-related metabolites. The proposed metabolic pathways were shown in Fig. 4. We observed that hydroxylation reactions, including mono-hydroxylation, di-hydroxylation and tri-hydroxylation, were the major metabolic reactions in the metabolism of tanshinones. The metabolic sites for tanshinone were mainly on the methoxy group and the saturated carbon ring. When the metabolic site occurred on the methoxy group, the fragmentation ion of loss of CH_2OH can be observed in the MS/MS spectra and when the metabolic site occurred on the saturated carbon ring, the fragmentation ions of successive losses of H_2O can be observed.

Several reports focused on the metabolic study of characteristic compounds such as ginsenoside Rg_1 [27-28], Rb_1 [29] and Rb_2 [30] have been published. A series of metabolites formed mainly via sugar degradation or oxidation reaction. However, few reports focused on the metabolic study of the single herb *panax ginseng*. In fact, *panax ginseng* was deemed as monarch drug in YXS prescription and the ginsenosides from *panax ginseng* were the major components in YXS. After oral administration of YXS extraction in rats, saponins were detected and characterized as various parent forms and metabolites in rat biological fluids with relatively high amounts. This result may provide a comprehensive understanding on the metabolic profile of total saponins of *panax ginseng in vivo*. In this study, total 71prototype compounds were detected in the rat plasma, urine and feces. Among them, 37 components have been previously isolated from the stem and radix of *panax ginseng*. However, the remaining 34 compounds including **42**, **44**, **47**, **51**, **54-55**, **57-62**, **64-67**, **69**, **74**, **81**, **85**, **90-93**, **98**, **100-104** and **107-110** were firstly identified *in vivo* and most of them were the isomers of known compounds. A total of 49 prototype compounds and 7 metabolites were detected in plasma indicating that they were the main circulating forms of YXS saponins. Whether they were directly absorbed into blood or indirectly absorbed after hydrolysis via intestinal bacteria was still unclear. Total 48 compounds were detected in urine while 57 compounds were detected in feces, indicating that most of the ginsenosides were cleared through both urine and feces excretion and most of them were cleared mainly through prototype form. A small amount of metabolites **M54-M62**, the hydrolysis products of ginsenosides, were detected in rat plasma indicating that some ginsenosides encountered hydrolysis reactions in intestine. 7 metabolites including **M55-M57** and **M59-M62** were

detected for the first time. **M53**, the Rb_1 oxygenated metabolite, identified in the urine sample was not detected in feces sample collected from 0 to 24 h after the oral dosing suggesting that after being absorbed in blood, Rb_1 encountered oxidation and the metabolite was excreted via urine. That is, hydrolysis as well as oxidization was the major metabolic pathway *in vivo*.

Compared to the literatures, much more prototype compounds of lignans, tanshinones and ginsenosides were detected, which probably mainly owned to the high-resolution and sensitivity of the UPLC-Q-TOF MS. Both of the chromatographic and mass spectrometric conditions were optimized to be suitable for the detection of components in YXS. Not only the major compounds but also the minor or trace components can be detected via extraction ion chromatogram. It is notable that some isomers of known compounds can also be extracted and their structures were characterized by comparing the fragmentation pathways with those of the known compounds. In addition, although with low concentration and interference from endogenous components, 11 lignan-related metabolites as well as 7 ginsenoside-related metabolites formed by single-step or multi-step biotransformation were firstly detected and identified. That is, the proposed data mining strategy plays an important role in the metabolic study of YXS *in vivo*.

3.3. Correlative analysis of metabolite profiling in rat biological fluids

It is widely assumed that only the components absorbed into the blood have the chance of showing bioactivity after oral administration of herbal medicine, except for compounds directly exerting effects on the gastrointestinal tract.[31] Therefore, in order to screen the active substances of YXS, it is extremely significant to confirm which compounds were absorbed into blood by rats after oral administration of YXS. Total 143 prototype compounds and metabolites, including 54 lignans, 32 tanshinones, 54 ginsenosides, 2 lactones and 1 astragalus saponin were identified in the plasma. In positive ion mode, the total content of lignans in plasma was up to 58.868% indicating that lignans were one of the major kinds of compounds *in vivo*. Compounds **2**, **3**, **16**, **18**, **19**, **22**, **M1**, **M7**, **M17** as well as **M27** had the relative high abundances, and their relative contents were in the range of 1.719% ~ 5.987% (shown in Table S3, ESI†). It was reported that lignans own many pharmacological effects, such as protection liver, the central nervous system, cardiovascular system, urogenital system and digestive system.[32-33] Studies have showed that lignans have blood vessel dilatation, coronary artery expanding, promoting coronary blood flow effects and can enhance the ability of scavenging free radicals.[32] Thus, they were closely related to the treatment of coronary heart disease. In the positive ion mode, tanshinones were another major kind of compounds *in vivo* due to the total content of the tanshinones was up to 40.326%. The relative contents of compounds **38**, **39**, **41**, **M40** and **M44** were in the range of 3.617% ~ 10.453% (shown in Table S3, ESI†). It is well known to us all that tanshinones exhibit diverse biological effects.[34-35] Both experimental and clinical studies suggested that tanshinones possess protection of cardiac muscle, dilatation of blood vessel, inhibition of arteriosclerosis and thrombus, improvement of microcirculation, regulation of restoration and regeneration of tissue.[35] To some extent, these detected 32 tanshinones *in vivo* played an important role in treating coronary heart disease. Ginsenosides, as the major components, were detected *in vivo*. In negative ion mode, the total content of ginsenosides was up to 99.159%. Ginsenoside Re, Rf, Rb_1, Chikusetsusaponin IVa, Rc, Rd, F_2 and Rg_3 were identified with high intensity and their relative contents were in the range of 2.719% ~ 20.768% in the plasma (shown in Table S3, ESI†). Many researchers showed that ginsenosides from *panax ginseng* exerted satisfactory therapeutic effects in cardiovascular disease.[36-40] Studies have shown that ginsenoside Rg_2 can obviously prolong the formation of blood clots and inhibit the platelet aggregation rate induced by adenosine diphosphate (ADP) in rats.[36] Ginsenoside Rg_1 can stimulate angiogenesis and ischemic cardiac function recovery.[37-38] Ginsenoside Rb group can protect ischemic myocardium and obviously reduce blood viscosity and platelet aggregation of the acute blood stasis model in rats.[39-40] The pharmacological effects study may clarify the mechanism of treating cardiovascular disease of ginsenosides. In addition, much more ginsenosides were identified in plasma, which greatly aided to explain the mechanism of YXS for treating cardiovascular disease.

4. Conclusions

In this study, the metabolites profiles of YXS in rat plasma, urine and feces samples were qualitatively described by UPLC-Q-TOF MS method. To avoid the endogenous interference and completely identify the predictable and unpredictable metabolites, a data mining strategy was employed. Using the data mining strategy, total 184 compounds were identified *in vivo*. Besides, the metabolic reactions and pathways were investigated and proposed. The metabolites were generated via various metabolic reactions, such as mono-oxidation, di-oxidation, tri-oxidation, hydrolysis, and so on. With respect to the clearance of metabolites, feces and urine excretion may be the main route. This work provided a theoretical foundation for elevating the scientific connotation of YXS and it will be helpful to reveal the action mechanism of YXS. In addition, this study further demonstrated the effectiveness of the UPLC Q-TOF MS approach and the data mining strategy in the *in vivo* metabolites identification of herbal medicine.

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References

1 Y.T. Tong and H.X. Wang, *Chin. J. Integr. Med. on Cardio/Cerebrovascular Dis.*, 2012, **10**, 472.
2 C.Y. Li and Z.X. Shi, *Chin. J. Integr. Med. on Cardio/Cerebrovascular Dis.*, 2013, **11**, 351.
3 H. Chen, *Chin. J. Integr. Med. on Cardio/Cerebrovascular Dis*., 2010, **8**, 779.

4 Y.J. Tao, B.H. Jiang, H. Qiy and C.R. Li, *Chin. J. Integr. Med. on Cardio/Cerebrovascular Dis*., 2009, **7**, 596.

5 X.D. Fu and G. Li, *Sh. J. TCM*, 2007, **41**, 25.

6 C.H. Chai, J.L. Wang and R.K. Lin, *Modern J. Integr. Tradit. Chin. West. Med*., 2008, **17**, 2269.

7 J.X. Du, Y.H. Gu and M. Li, *Chin. J. Experimental Tradit. Med. Formulae*, 2010, **16**, 88.

8 Q.L. Xu, X.J. Mao and H.L. Xiong, *Chin. J. Chin. Mater. Med*., 2005, **30**, 1462.

9 X.Q. Li, M.W. Li and L.P. Chen, *Asia-pacific Tradit. Med*., 2014, **10**, 12.

10 Z.K. Guo and C.L. Ta, *J. Changchun. Coll. Tradit. Chin. Med*., 2005, **21**, 37.

11 J.H. Sun, M. Yang, J. Han, B. R. Wang, X.C. Ma, M. Xu, P. Liu and D.A. Guo, *Rapid Commun. Mass Spectrom*., 2007, **21**, 2211.

12 X. Zhao, D.H. Yang, F. Xu, S. Huang, L. Zhang, G.X. Liu and S.Q. Cai, *Biomed. Chromatogr*., 2015, **29**, 285.

13 M.Y. Liu, S.H. Zhao, Z.Q. Wang, Y.F. Wang, T. Liu, S. Li, C.C. Wang, H.T. Wang and P.F. Tu, *J. Chromatogr. B*, 2014, **949-950**, 115.

14 J.M. Yang, S.P.P. IP, H.K.J. Yeung and C.T. Che, *Acta Pharmaceutica Sinica B*, 2011, **1**, 46.

15 J.R. Wang, L.F. Yau, T.T. Tong, Q.T. Feng, L.P. Bai, J. Ma, M. Hu, L. Liu and Z.H. Jiang, *J Agric Food Chem*., 2015, **63**, 2689.

16 L. Yang, S. Xu, C. Liu and Su Z, *Anal Bioanal Chem*.**,** 2009, **395**, 1441.

17 J.Z. Chen, G.X. Chou, C.H. Wang, L. Yang, S.W.A. Bligh and Z.T. Wang, *J. Pharm.Biomed. Anal*., 2010, **52**, 687.

18 M.Y. Liu, S.H. Zhao, Z.Q. Wang, H.T. Wang, X.W. Shi, Z.M. Lv, H.H. Xu, H.R. Wang, Y.F. Du and L.T. Zhang, *J. Sep. Sci*., 2011, **34**, 3200.

19 Y.H. Wang, C. Qiu, D.W. Wang, Z.F. Hu, B.Y. Yu and D.N. Zhu, *J. Pharm. Biomed. Anal*., 2011, **54**, 1110.

20 H.Y. Zhao, M.X. Fan, L. Fan, J.H. Sun and D.A. Guo, *J. Chromatogr. B*, 2010, **878**, 1113.

21 Y.Y. Chen, J.M. Guo, Y.P. Tang, L. Wu, W.W. Tao, Y.F. Qian and J.A. Duan, *J. Pharm. Biomed. Anal*., 2015, **112**, 60-69.

22 J.L. Geng, Y. Dai, Z.H. Yao, Z.F. Qin, X.L. Wang, L. Qin and X.S. Yao, *J. Pharm. Biomed. Anal*., 2014, **96**, 90.

23 H. Sun, F.F. Wu, A.H. Zhang, W.F. Wei, Y. Han and X.J. Wang, *Biomed. Chromatogr*, 2013, **27**, 1511.

24 J.H. Sun, M. Yang, X.M. Wang, M. Xu, A.H. Liu and D.A. Guo, *J. Pharm.Biomed. Anal*., 2007, **44**, 564.

25 L. Guo, L. Duan, X. Dong, L.L. Dou, P. Zhou, P. Li and E.H. Liu, *J. Pharm.Biomed. Anal*., 2015, **107**, 473.

26 M.M. Wang, H.X. Dai, X.R. Li, Y.H. Li, L.J. Wang and M. Xue, *J. Chromatogr. B*, 2010, **878**, 915.

27 Y. Takino, T. Odani, H. Tanizawa and T. Hayashi, *Chem. Pharm. Bull.,* 1982, **30**, 2196.

28 T. Odani, H. Tanizawa and Y. Takino, *Chem. Pharm. Bull.,* 1983, **31**, 292.

29 T. Odani, Y. Takino, *Chem. Pharm. Bull.,* 1983, **31**, 1059.

30 M. Karikura, H. Tanizawa, T. Hirata, T. Miyase, Y. Takino, *Chem. Pharm. Bull.,* 1992, **40**, 2458.

31 X.J. Wang, H. Sun, A.H. Zhang, G.Z. Jiao, W.J. Sun and Y. Yuan, *Analyst*, 2011, **136**, 5068.

32 X.D. Huang and K. Ren, *J. Jilin Med. Coll*., 2010, **31**, 46.

33 L.H. Yu and G.T. Liu, *Prog. In Chem*., 2009, **21**, 66.

34 X.R. Chen, J.B. Lu and H.P. Shi, *Chin. Hosp. Pharm. J*., 2001, **21**, 44-45.

35 X.J. Pu and K.L. Xu, *Clin. Eng*., 2009, **16**, 154-155.

36 J.M. Tian, L.J. Song, H. Li, J.M. Ye and L.Y. Li, *Shanghai J. Tradit. Chin. Med*., 2009, **43**, 79.

37 M. Yang, G.L. Chen, C. Chen, Y. Zhang and C.Y. Yan, *J. Emerg. Tradit. Chin. Med*., 2009, **18**, 403.

38 Y. Jin and G.N. Liu, *J. Chin. Pharm. Univ*., 2007, **36**, 517.

39 X.X. He, H.L. Xu, X.F. Yu, S.C. Qu and D.Y. Sui, *Chin. Pharmacol. Bull*., 2007, **23**, 1259.

40 X.M. Cui, L.S. Xu and Q. Wang, *Chin. Tradit. Pat. Med*., 2006, **28**, 1526.

Figure legends

Fig. 1 Extracted ion chromatograms (EICs) of YXS metabolites in rat biofluid samples: Plasma (A1), Feces (A2) and Urine (A3) in positive ion mode; Plasma (B1), Feces (B2) and Urine (B3) in negative ion mode.

Fig. 2 The mass spectra of typical metabolites, M2 (A), M38 (B) and M60 (C).

Fig. 3 The proposed metabolic pathways of lignans (* metabolic sites).

Fig. 4 The proposed metabolic pathways of tanshinones (* metabolic sites).

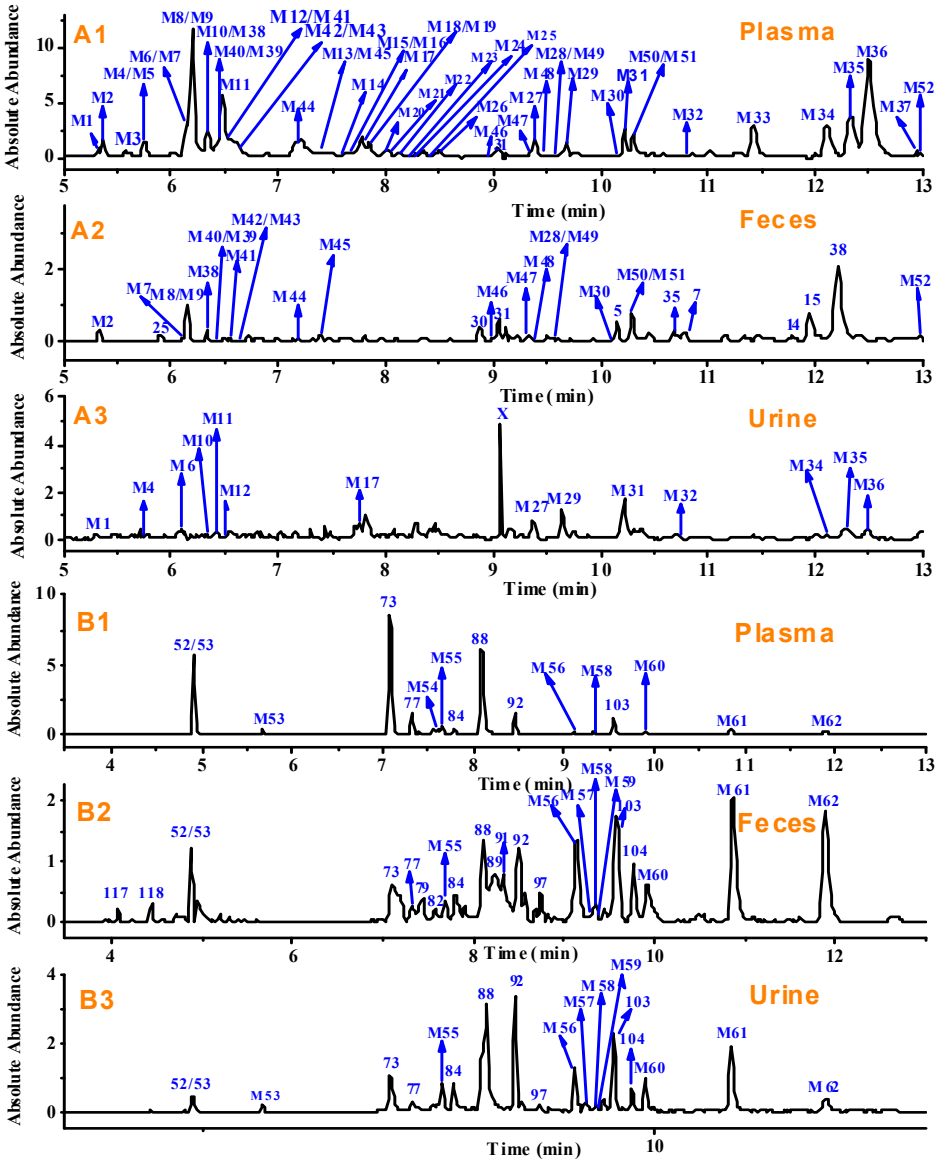

Fig.1

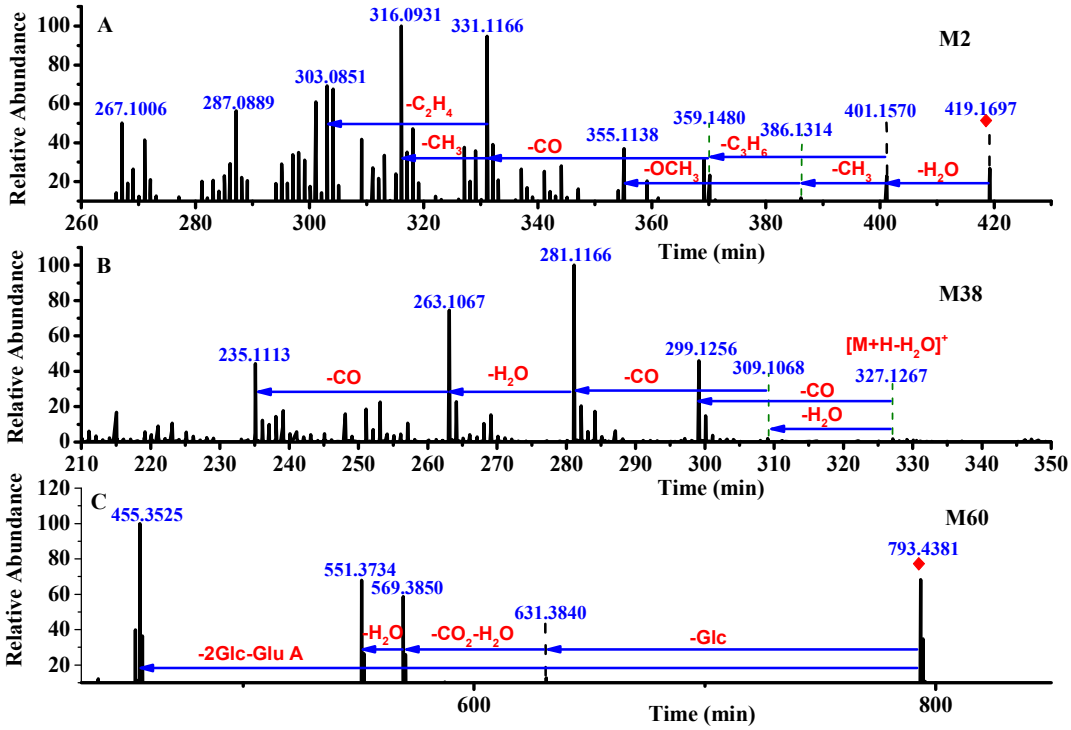

Fig. 2

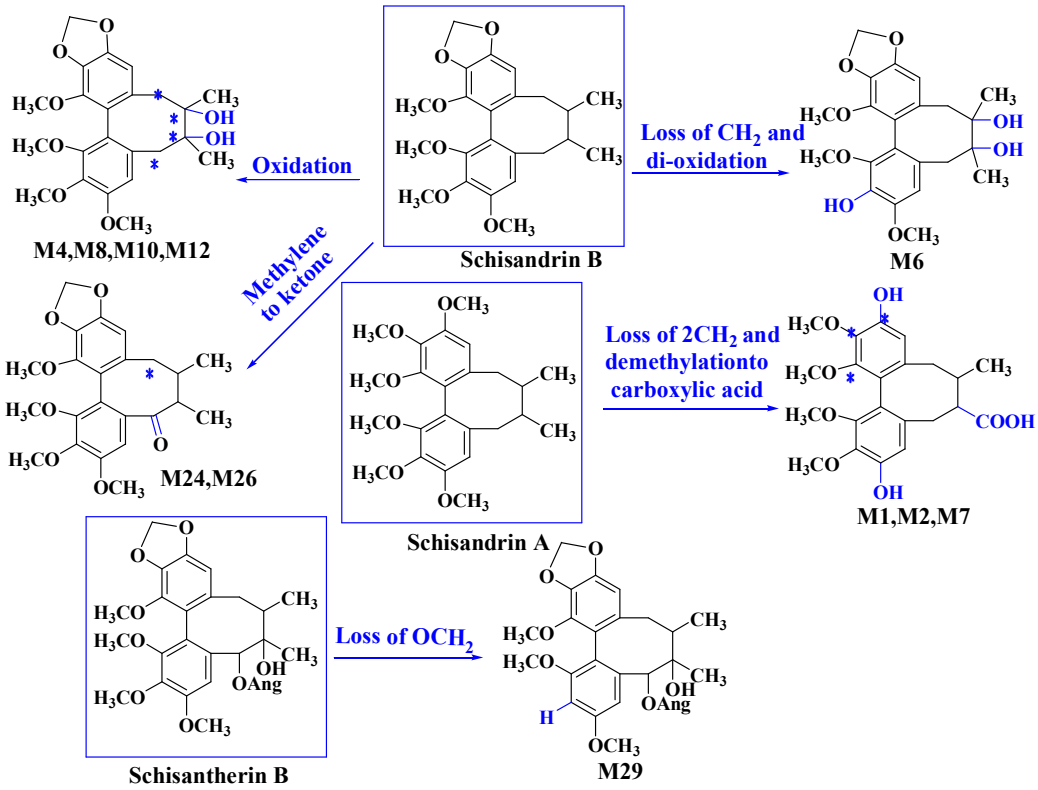

Fig. 3

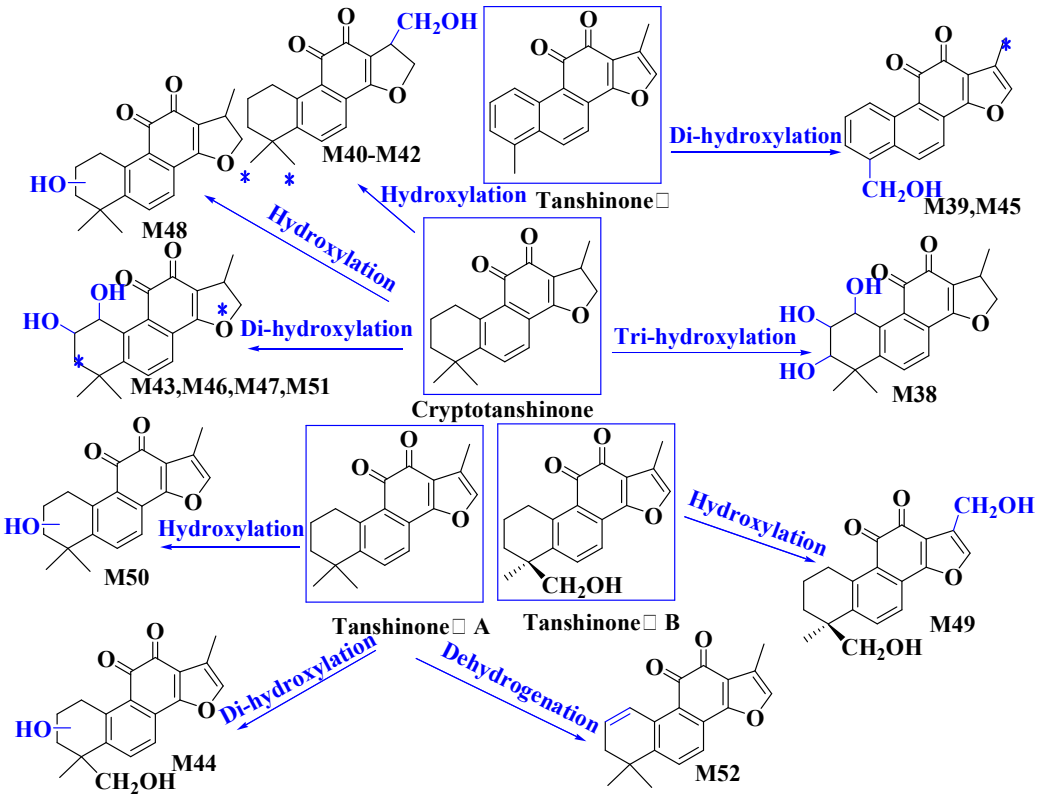

Fig. 4