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Ultra-performance liquid chromatography–tandem mass spectrometry analysis of the bioactive components and their metabolites of Shaofu Zhuyu decoction active extract in rat plasma

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ABSTRACT

A rapid, sensitive and selective ultra-performance liquid chromatography–electrospray ionization tandem mass spectrometric (UPLC–ESI-MS/MS) method was developed for analysis and identification of the bioactive components and their metabolites in rat plasma following oral administration Shaofu Zhuyu decoction active extract. The analysis was carried out on an AcQuity™ UPLC chromatographic instrument and a QTOF mass spectrometer using positive and negative electrospray ionization (ESI), respectively. The results showed that sixteen peaks were detected and twelve peaks, including flavones, organic acids and terpene glycosides, were identified by comparing with reference compounds. Furthermore, nine metabolites, including quercetin glucuronide sulfates, quercetin diglucuronides, isorhamnetin sulfates, isorhamnetin glucosides, and isorhamnetin glucuronides were detected and identified in rat plasma based on the mass fragmentation behaviors and literature reports. These results provided a meaningful basis for evaluating the bioactive components and their action mechanisms of complex traditional Chinese medicines (TCMs).

1. Introduction

Shaofu Zhuyu decoction (SFZYD) is a famous ancient prescription in TCM, which was created by Qingren Wang of the Qing Dynasty of China and consisted of ten crude herbs, including *Radix Angelica sinensis*, *Rhizoma Ligusticum chuanxiong*, *Cortex Cinnamomum cassia*, *Fructus Foeniculum vulgare*, *Rhizoma Zingiber officinale*, *Resin Commiphora myrrha*, *Feces Trogopterus xanthipes*, *Pollen Typha angustifolia*, *Radix Paeonia lactiflora*, and *Rhizoma Corydalis yanhusuo*. In China, it is widely used in clinical practice to treat blood stasis syndromes in gynecology diseases, such as primary dysmenorrheal and menoxenia, and so on [1].

Traditional Chinese medicine (TCM) is a unique medical system used in Mainland China for centuries to treat many diseases. It is basically different from western medical practice. Many TCM formulae are able to regulate the holistic functions of human body, which has been attracting great interest. The recent study on SFZYD showed that it possessed significantly promoting blood circulation to remove blood stasis and alleviate pain [2], and inhibiting the constriction of uterine smooth muscles and showed anti-inflammatory activity [3].

In our previous study, it was also shown that SFZYD possessed multiple activities, such as the inhibition of isolated murine uterine contractions induced by Oxytocin, and the regulation of E_2 and P levels [2,4]. The activity and active components of SFZYD volatile oil have also been investigated [5]. The bioassay-guided separation of an aqueous extract of SFZYD led to the isolation of an active fraction, SF-11 which had obvious multiple activities of inhibition on isolated uterine contractions induced by Oxytocin, inhibition of platelet aggregation induced by ADP and PAF, and protection against HUVE cell damage induced by H_2O_2 [2,6].

The research on the chemical components of SFZYD showed that it contained numerous diverse compounds, including organic acids, alkaloids, flavonoids and polysaccharides [7–10]. However, the bioactive components are still not completely elucidated. We have analyzed and identified eleven compounds from SFZYD using HPLC–MS/MS and quantified seven compounds by HPLC from the SF-11 bioactive fraction. To the best of our knowledge, the absorption and metabolite components have never been reported. Drug metabolism research has played an important role in the drug discovery, drug design and drug clinical application. Therefore, fast and efficient ways to provide accurate information of drug metabolism on the target compounds and their major metabolites are required [11,12].

Ultra-performance liquid chromatography–electrospray ionization tandem mass spectrometry (UPLC–MS/MS) can provide a huge

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amount of information more rapidly and efficiently than other techniques. High selectivity and sensitivity, and rapid characteristics have allowed the wide application of UPLC–MS/MS for quantitative and qualitative analysis, as well as metabolite analysis and identification from bioassays of complex samples such as traditional Chinese medicines (TCMs) [13–16].

In the present study, an UPLC–QTOF-MS method was developed to analyze and identify the main bioactive components and their possible metabolites in rat plasma following administration of the active extract of SFZYD (SF-11). The results showed that twelve components and nine metabolites were identified in the SF-11 extract and the rat plasma samples, respectively. An UPLC with a photodiode array (PAD–UV) and MS^2 detector was used to analyze the components in the extract and in rat plasma after ingestion of the SF-11 fraction which contained flavones, organic acids and terpene glycosides.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile for UPLC analysis was of HPLC grade and was purchased from Tedia (Fairfield, OH, USA), formic acid was of AR grade and from the Shanghai Reagent Company (Shanghai, China). Water for UPLC analysis was purified by the Millipore water purification system (Millipore, Milford, MA, USA) and filtered with 0.22 μm membranes. Distilled water was used for the extract and for the preparation of samples.

The reference compounds paeoniflorin, albiflorin, typhaneoside, isohamnetin-3-O-neohespeidoside, ferulic acid, vanillic acid, caffeic acid, and isohamnetin-3-O-rutinoside were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Materials

The crude drugs of *Radix Angelica sinensis*, *Rhizoma Ligusticum chuanxiong*, *Radix Paeonia lactiflora*, *Cortex Cinnamomum cassia*, *Fructus Foeniculum vulgare*, *Rhizoma Zingiber officinale*, *Resin Commiphora myrrha*, *Feces Trogopterus xanthipes*, *Pollen Typha angustifolia*, and *Rhizoma Corydalis yanhusuo*, were purchased from Minxian (Gansu), Pengzhou (Sichuan), Haigéer (Neimeng), Yulin (Guangsi), Yulin (Guangsi), Wuwei (Gansu), Chifeng (Neimeng), Songyang (Zhejiang) and Changzhi (Shanxi), respectively. All the raw materials were identified by the corresponding author. The voucher specimens (No. NJUTCM-20060818–20060827) were deposited in Jiangsu Key laboratory for TCM formulae Research of Nanjing University of Chinese Medicine.

2.3. Instrumentation

The UPLC–ESI-MS/MS system consisted of an AcQuity™ ultra-performance liquid chromatograph and an AcQuity Synapt Mass Spectrometer equipped with an electrospray ionization (ESI) source (Waters, Milford, MA, USA). An AcQuity Binary Solvent Manager system and an auto-sampler were used for the UPLC–MS/MS analysis. Data was performed with Masslynx V4.1 software (Waters).

2.4. Extract preparation

The 5.58 kg mixture of raw materials of *Radix Angelica sinensis*, *Rhizoma Ligusticum chuanxiong*, *Radix Paeonia lactiflora*, *Cortex Cinnamomum cassia*, *Fructus Foeniculum vulgare*, *Rhizoma Zingiber officinale*, *Resin Commiphora myrrha*, *Feces Trogopterus xanthipes*, *Pollen Typha angustifolia*, and *Rhizoma Corydalis yanhusuo* at a weight ratio of 3:1:2:1:0.5:2:1:3:1:1 (1080, 360, 720, 360, 180, 720, 360, 1080, 360, and 360 g) were crushed into small pieces. The mixture was refluxed with 55.8 L of water for 2 h. The filtrates were collected and the residues were then refluxed twice in 55.8 L of water for 1.5 h. Two batches of filtrate were combined. The solvent was removed below 70 °C to obtain a certain volume at the ratio of 1:1 (w/w, weight of all constituting herbs and the extract filtrates) under vacuum. 95% ethanol was added to the extract filtrates until the concentration of ethanol was adjusted to 80%. Ethanol was removed below 70 °C to obtain certain volumes of the filtrates. The final filtrates were separated by gradient elution with different concentrations of ethanol (10–90%) from macroporous adsorptive resins and the different fractions were obtained. The active fraction (SF-11), 40% ethanol elution fraction, was obtained by evaluating its efficacy in treating dysmenorrhea [3–8]. The active fraction was dissolved in 5 mL methanol for UPLC–MS/MS analysis. And the fraction was dissolved in water at the concentration of 42 mg/mL and administrated to rats to study the metabolites that appeared in rat plasma.

2.5. Chromatographic conditions

An AcQuity UPLC™ BEH C_{18} column (1.7 μm, 100 mm × 2.1 mm i.d.) (Waters) was used for the analysis. The column temperature was maintained at 30 °C. The standards and extracts were analyzed by UPLC chromatography using a gradient mobile phase consisting of 0.1% formic acid (HOOCH) in water as solvent A and acetonitrile (ACN) as solvent B. The gradient conditions of the mobile phase were: 0 min 95% A, 5.0 min 90% A, 8.0 min 88% A, 10.0 min 85% A, 15.0 min 85% A, 18.0 min 45% A, and 20.0 min 10% A. The flow rate was 0.40 mL min^{-1}. The mobile phase was passed through a 0.22 μm Millipore filter. The injection volume was 5 μL.

For operation in MS/MS mode, a mass spectrometer fitted with an orthogonal Z-spray ion interface was used for all analyses. Ionization was achieved using electrospray. For analysis, the electrospray source parameters were fixed as follows: electrospray capillary voltage was 4.0 kV for positive ionization mode and was 3.0 kV for negative ionization mode, source temperature was 100 °C and desolvation temperature was 250 °C. The cone voltage was set at 30 V. Nitrogen was used as cone and collision gases and the cone and desolvation gas flows were 50 and 700 L h^{-1}, respectively. The collision energy trap CE was set at 6.0 and the transfer CE was set at 4.0. The mass range was set from m/z 100 to 1000.

2.6. Sample preparation of plasma

Sprague–Dawley (SD) rats (180–220 g) were obtained from Nanjing University of Chinese Medicine and the animals had free access to food and water for 12 h before the experiments. The SF-11 bioactive fraction was administered orally to four rats at a single dose of 1.5 g kg^{-1}, and the blood samples (1.5 mL) were collected in heparinized tubes pre-dose (0 min) and at 5, 15, 30, 60, 120, 240, 360 min post-dose. The plasma samples were immediately separated by centrifugation at 3000 rpm for 10 min. Each plasma sample (0.2 mL) was placed in a 1.5 mL polypropylene tube, and 1 mL methanol was added to the tube. The tube was vortexed for 30 s. The precipitated protein was removed by centrifugation at 13,000 rpm for 10 min. The organic layer was transferred to another tube, and evaporated to dryness by N_2 and stored at −20 °C until analysis. For UPLC–MS/MS analysis the residue was dissolved in 150 μL methanol, and an aliquot of 5 μL was injected into the column. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (The project was approved by the Laboratory Animal Center of Nanjing University of Chinese Medicine).

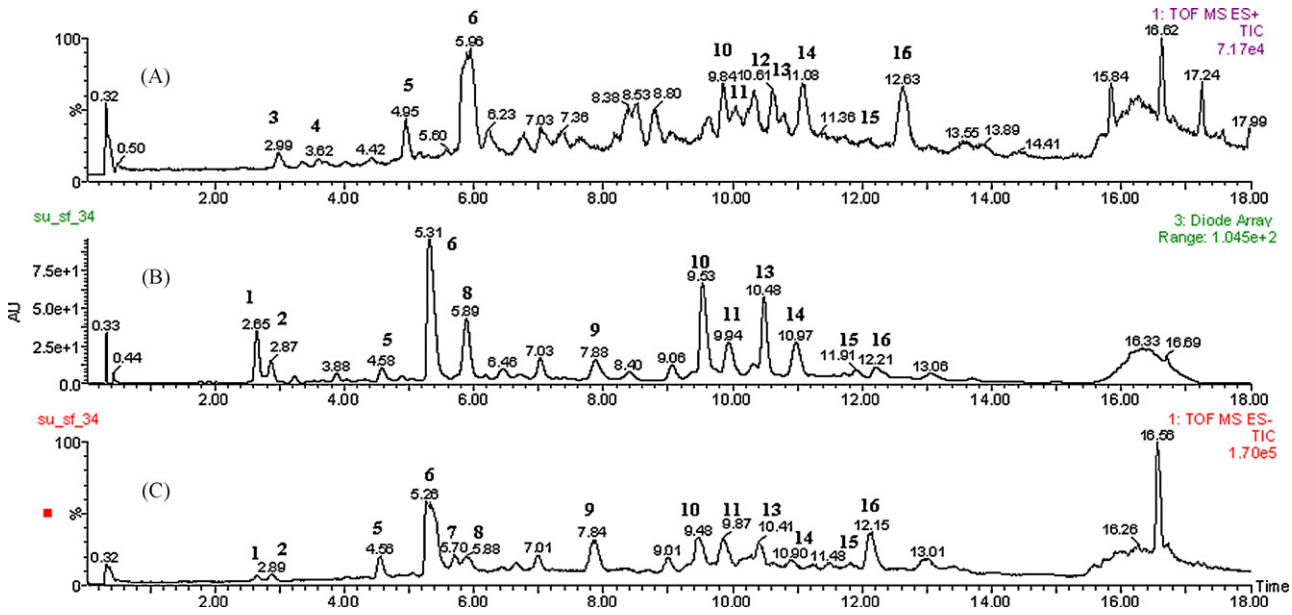

Fig. 1. UPLC–ESI-MS total ion current chromatograms at the positive (A) and negative (C) ion mode and UPLC–DAD-UV chromatograms (B) of SFZYD active extract.

3. Results and discussion

3.1. UPLC–MS/MS analysis and identification of the components in active fraction SF-11

Both negative and positive ESI modes were applied to analyze and identify the chemical components in the bioactive fraction of SFZYD. The total current chromatograms at the two ES modes are shown in Fig. 1. Sixteen peaks were detected and twelve components were identified by comparing the t_R values, UV λ_{max} values, and the MS fragments characteristics of the compounds. The analyzed and identified compounds are listed in Table 1.

Three flavonoid glycosides were identified by comparing the t_R values, UV λ_{max} values, and the MS fragments characteristics of the standard substances, as well as MS the fragments behaviors of the flavonoid. All three flavonoid glycosides contained fragment ion m/z 317 (Fig. 2). The MS/MS of m/z showed fragments including m/z 287, 273, 153, and 123. These data are consistent with those in the literatures [17–19]. Thus, the aglucone was identified as Isohamnetin. The three flavonoid glycosides were identified as typhaneoside, isohamnetin-3-O-neohesperidoside, and isohamnetin-3-O-rutinoside. The MS findings are shown in Fig. 3.

3.2. UPLC–MS/MS analysis and identification of the absorption and metabolite components in rat plasma

Gradient reversed phase UPLC with absorbance detection and full scan data dependent MS^2 were used to analyze and identify the absorption and metabolic components in rat plasma after oral administration of active fraction of SF-11. Plasma collected 0–6 h after ingestion of the active fraction of SF-11, and the plasma sample were analyzed by UPLC–QTOF-MS. The detected total ion current chromatography in negative ion mode and positive ion mode for plasma sample collecting 60 min after ingestion of the active fraction are shown in Fig. 4.

By comparing the t_R values, UV λ_{max} values, and the MS/MS ion fragments characteristics of the peaks with reference compounds and the literatures, the different classes of metabolites detected were summarized as follows:

Quercetin: The plasma of four rats contained traces of the aglycone, quercetin, which had a $[M-H]^-$ at m/z 301 and MS^2 fragment ions at m/z 179 and 151 at the negative ion mode, and had a $[M+H]^+$ at m/z 303 and MS^2 fragment ions at m/z 275 and 181 at the positive ion mode.

Isorhamnetin: The plasma of four rats contained traces of the aglycone, isorhamnetin, which had a $[M-H]^-$ at m/z 315 and MS^2 fragment ions at m/z 301, 287 and 273 at the negative ion mode, and had a $[M+H]^+$ at m/z 317 and MS^2 fragment ions at m/z 303, 289 and 275 at the positive ion mode.

Quercetin diglucuronides: One quercetin diglucuronide at peak 2 (11.95 min), was detected in the plasma in four rats plasma during 0–6 h and the mass spectra of the compound is summarized in Table 2. The data indicated that the two glucuronyl units were attached at different positions on the quercetin skeleton. If they had been linked at the same position it is unlikely that an M-176 fragment would have been produced at m/z 477 as it has been shown that disaccharides conjugate fragments with the loss of the intact disaccharide moiety [20].

Quercetin glucuronide sulfates: Peak 1 (9.87 min) was identified as quercetin glucuronide sulfates. The mass spectrum of the compound is summarized in Table 2.

Isorhamnetin sulfates: At the negative ion mode, peak 3 (15.28 min) had a $[M-H]^-$ at m/z 395 and MS^2 yielded a major ion at m/z 315 ($[M-H]^- - 80$), which was consistent with the fragmentation of isorhamnetin sulfate. There was also a $[M+H]^+$ at m/z 397 and MS^2 yielded a major ion at m/z 317 ($[M+H]^+ - 80$) at the positive ion mode. In accordance with the data on the characteristics of MS/MS, the compound at peak 3 was identified as isorhamnetin sulfate. However, the position of the sulfate group on isorhamnetin sulfate remains undetermined.

Isorhamnetin glucosides: The rats' plasma collecting during 0–6 h contained quercetin-glucoside, the mass spectra of the compound is summarized in Table 2.

Isorhamnetin glucuronide: The plasma of rats contained quercetin glucuronide, the mass spectra of the compound is summarized in Table 2.

Methyl-ferulic acid: Ferulic acid was identified in rat plasma as methylating metabolite, a methylated compound produced a $[M-H+CH_3]^-$ at m/z 207 and MS^2 yielded ions at m/z 193 ($[M-H-CH_3]^- - 14$, with the loss of CH_3) at the negative ion mode,

Table 1
The MS data of (+) ESI-MS spectra and (−) ESI-MS spectra, and the identification results of the constituents of SFZYD active extract.

Peaks no.	Retention time (min)	ES$^+$, m/z			ES$^-$, m/z			UV λ_{max} (nm)	Compounds identified
		[M+H]$^+$	[M+Na]$^+$	Other ion	[M−H]$^-$	[M+HCOO]$^-$	Other ion		
1	2.647				167			216	Vanillic acid
2	2.872				179			321	Caffeic acid
3	2.990			558, 543, 391, 375, 307, 264, 207, 151					Unknown
4	3.595	303		340, 285, 193, 181	301				Quercetin
5	4.58	481	503	362, 319, 301, 197, 179, 161, 133, 105	479		525, 515, 327, 283, 163, 119	230	Albiflorin
6	5.310		503	498, 463, 357, 339, 323, 301, 277, 197, 179, 151, 123, 105	479	525	449, 327, 241, 179. 197, 141	230	Paeoniflorin
7	5.726				197	241	179, 141	226	Protocatechuic acid
8	5.890	195			193		178, 141	321	Ferulic acid
9	7.840			525, 609					Unknown
10	9.870	771		625, 479, 342, 317	769	815	609, 509, 477, 463, 299, 431	252	Typhaneoside
11	10.330			566, 595, 650, 449, 287				246	Unknown
12	10.446	625		479, 317	623			252	Isohamnetin-3-O-neohesperidoside
13	11.080	225	247	207	223			276	Senkyunolide I
14	11.505	225	247	207	223			276	Senkyunolide H
15	12.386	625		499, 479,317, 207, 163	623		315, 251, 207, 179	252	Isohamnetin-3-O-rutinoside
16	12.635			679, 633, 317, 281					Unknown

and [M+H+CH_3]$^+$ at m/z 209 and MS^2 yielded ions at m/z 195 ([M+H−CH_3]$^+$ − 14, with the loss of CH_3) at the positive ion mode.

Hydroxyl-paeoniflorin: Paeoniflorin is one of the essential components in the SFZYD active extract. This study showed that hydroxylation was the crucial metabolic transformation pathway. Peak 5 (18.97 min) produced a [M−H]$^-$ at m/z 496 and a major ion at m/z 540 ([M−H+HCOO]$^-$ + 44) at the negative ion mode. There was also a [M+H]$^+$ at m/z 498 and a major ion at m/z 521 ([M+H+Na]$^+$ + 23) at the positive ion mode.

The present study is the first to report on the use of multiple combined techniques (UPLC–QTOF-MS) to analyze and identify the absorbance and the metabolites appearing in rat plasma after administration of SF-11 extract. These findings provide much more detailed data for the components absorbed of TCMs than was previously achieved in earlier studies using HPLC analysis [21] or single stage MS in the selected ion monitoring mode [22]. We have studied the main chemical structures in the bioactive extract, such as organic acids, terpene glycosides and flavonoid glycosides, and so on. This study identified the possible metabolites in the SFZYD bioactive extract with MS^2 in the positive ion mode and negative ion mode, and showed that components with the same structure type may metabolize through the similar biotransformation pathway.

This investigation showed that ferulic acid was found in rat plasma as methyl-ferulic acid. Previous reports on the main metabolites of ferulic acid have demonstrated that ferulic acid

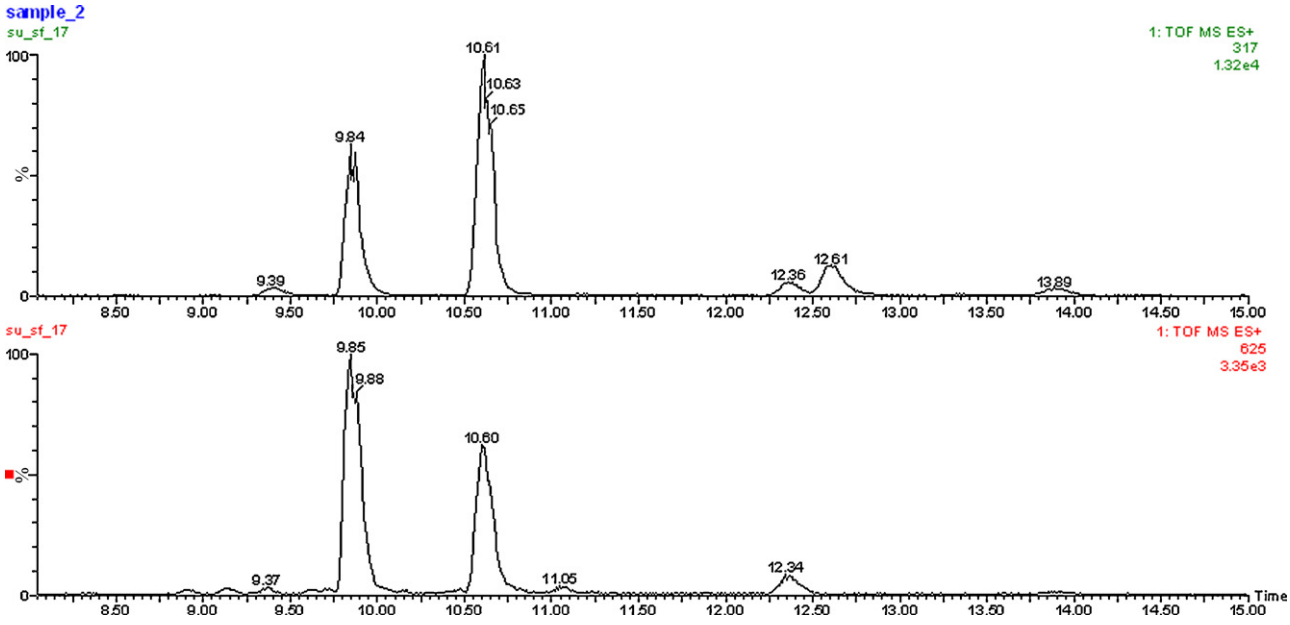

Fig. 2. The ion fragments m/z 625, 317 of three flavonoid glycosides.

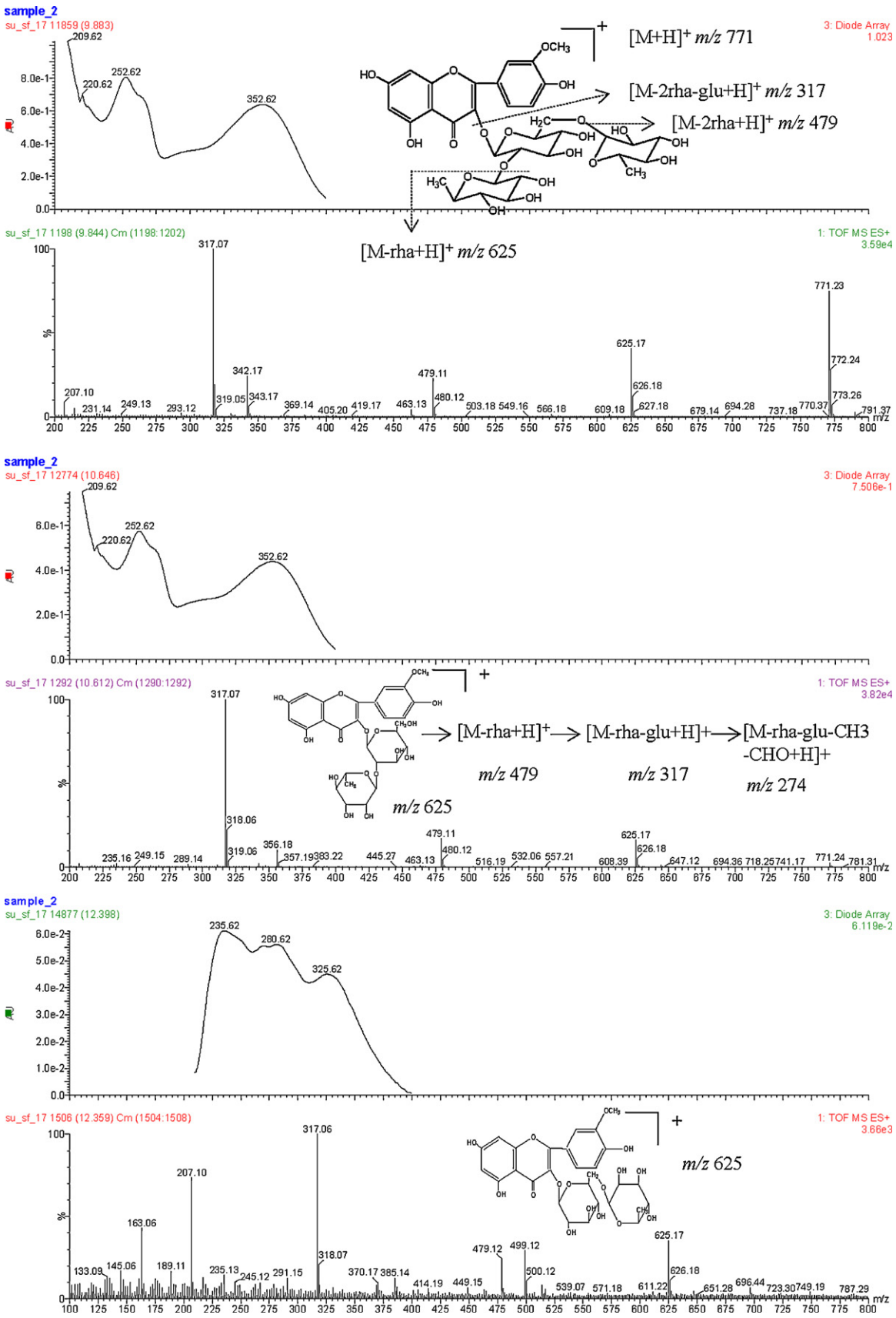

Fig. 3. The MS spectrometry characteristics of three flavonoid glycosides.

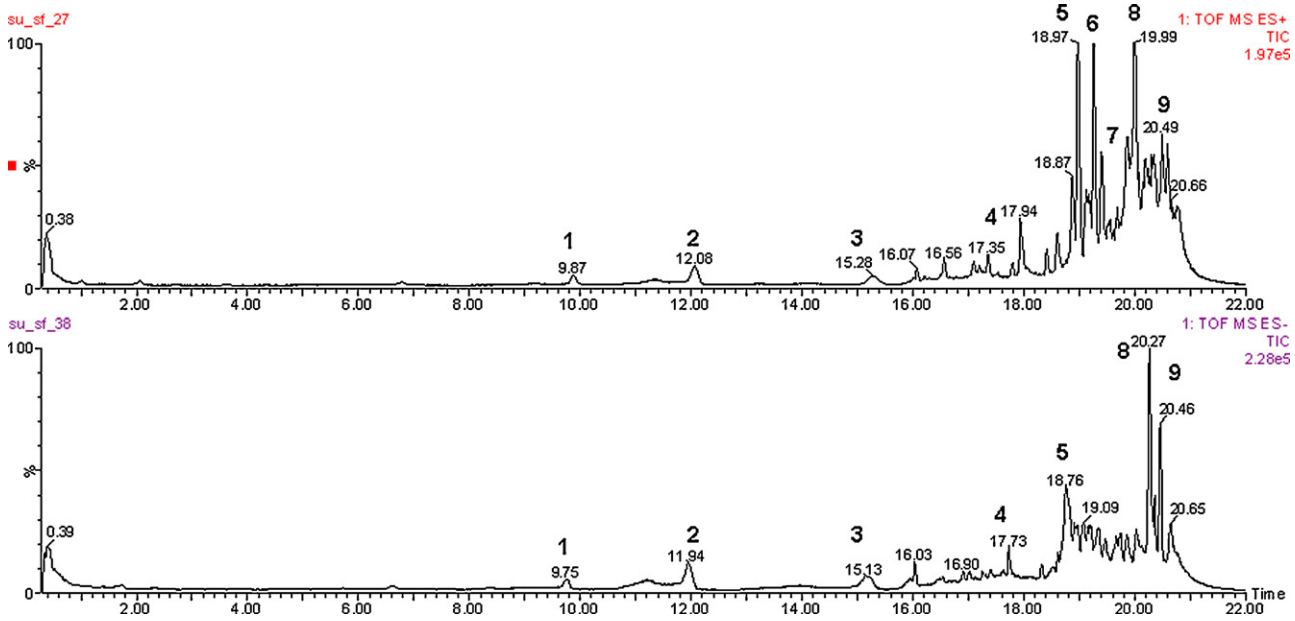

Fig. 4. UPLC–ESI-MS total ion current chromatograms of the metabolites in rat plasma sample collecting 60 min after ingestion of the SF-11 fraction extract at the negative and positive ion mode.

glucuronide, ferulic acid sulfates, and methyl-ferulic acid detected [23]. It was implied that the other chemical components influenced the metabolites of ferulic acid probably.

Paeoniflorin found in Paeoniae Radix is one of the main monoterpene glucoside bioactive components of active extract. Following oral administration of the active extract in rats, a hydroxyl-paeoniflorin metabolite of paeoniflorin in rat plasma was identified based on UPLC–MS/MS. Previous studies have reported that the deglucosylated derivative and paeonimetabolin I, II, III were also identified [24,25]. The results also approved that the other chemical components may influence the metabolites again.

The flavonoid glycosides, including typhaneosid, isohamnetin-3-O-neohesperidoside, isohamnetin-3-O-rutinoside, and the aglucone of isohamnetin and their metabolites of phase I and II were also detected but not the original compounds. The metabolites included isorhamnetin sulfates, isorhamnetin glucosides and isorhamnetin glucuronides. These results showed that the flavonoid glycosides were absorbed as the aglycone obtained through hydrolysis. These data were consistent with the reports on biotransformation pathway of flavonoid glycosides [26–33].

3.3. Possible metabolic pathway of flavonoid glycosides after oral administration of SFZYD extract

Biotransformation of drug proceeds in two distinct steps at least. During first step (phase I), compounds are functionalized by oxidation, hydrolysis or reduction, leading to the introduction of, e.g. hydroxyl, amino, carboxyl or thiol groups into the molecule (primary metabolites). The phase I transformation is a necessary step for subsequent conjugation. In the second step (phase II), primary metabolites undergo conjugation reactions to form secondary metabolites. The phase II transformation leads to not only an inactivation of primary metabolites, but also an increased hydrophilicity, hence secondary metabolites except acetylation and methylation showed enhanced excretion [34–36]. In this study, the bioactive components and their possible metabolites of SFZYD active were analyzed. These data may provide guidance for investigat-

Table 2
UPLC–QTOF-MS/MS identification the metabolites in rat plasma after the ingestion of SFZYD active extract ($n = 4$).

Peaks	Retention time (min)	ES$^+$, m/z		ES$^-$, m/z		Compound
		$[M+H]^+$	MS^2	$[M-H]^-$	MS^2	
1	9.87	559	479 ([M+H]–SO_3), 375 ([M+H]–GlcUA), 303 ([M+H]–SO_3–GlcUA)	557	477 ([M−H]–SO_3), 371 ([M−H]$^-$–GlcUA), 301 ([M−H]$^-$–SO_3–GlcUA)	Quercetin glucuronide sulfates
2	11.95	657	481 ([M+H]–GlcUA), 303 ([M+H]–GlcUA–GlcUA)	653	477 ([M−H]–GlcUA), 301 ([M−H]–GlcUA–GlcUA)	Quercetin diglucuronides
3	15.28	397	317 ([M+H]–SO_3)	395	315 ([M−H]–SO_3)	Isorhamnetin sulfates
4	17.35	209	195 ([M+H]–CH_3)	207	193 ([M−H]–CH_3)	Methyl-ferulic acid
5	18.97	498	521 ([M+H+Na]+23)	496	540 ([M−H]+HCOO)	Hydroxyl-paeonimetabolin
6	19.25	479	317 ([M+H]–Glc)	477	315 ([M−H]–Glc)	Isorhamnetin glucosides
7	19.29	303	181 ([M+H]–122) 153 ([M+H]–122-CO)	301	179 ([M−H]–122), 151([M−H]–122-CO)	Quercetin
8	19.89	493	317 ([M+H]–GlcUA)	491	315 ([M−H]–GlcUA)	Isorhamnetin glucuronides
9	20.27	317	195 ([M+H]–122), 167 ([M+H]–122-CO)	315	193 ([M−H]–122), 165([M−H]–122-CO)	Isorhamnetin

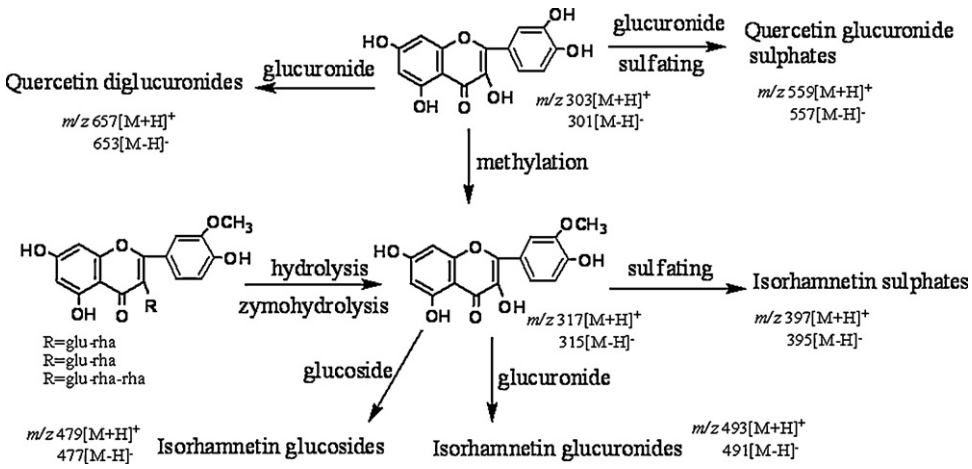

Fig. 5. The possible metabolic biotransformation pathway and the metabolites of three flavonoid glycosides.

ing the absorption and metabolism of complex active components of TCMs.

The metabolites of the three flavonoid glycosides, isohamnetin-3-O-neohesperidoside, isohamnetin-3-O-rutinoside, and typhaneosid were first reported of SFZYD in this paper. The presumed metabolic pathway including phase I and II, are shown in Fig. 5.

The data in this paper demonstrated that the feasible methods of UPLC coupled with QTOF–MS/MS were established to analyze the trace levels of natural products in impure extracts or biosamples. By using reference compounds, available trace quantities of analysis can be identified. While in the absence of standards, MS^2 could facilitate a degree of structural identification, such as that obtained with quercetin glucuronide sulfates, isohamnetin glucuronides and quercetin diglucuronides (Table 2), which would not be possible with traditional single stage MS. It has been discussed in some detail with specific reference to quercetin metabolites in earlier publications [19,20].

Further information on the position and orientation of substituent groups would be required the use of NMR. However, this would involve not only extensive sample purification, but also a requirement for hypersensitive analysis to the low nanogram quantities of metabolites. In addition, the technique of multi-tandem mass spectrometry may be more important for the identification of trace levels of metabolites in the future, especially for complex systems of TCMs.

4. Conclusions

In this paper and based on our previous research, we developed the UPLC coupled with QTOF-MS methods for analysis and identification the main bioactive components and their possible metabolites in plasma. The results showed that twelve components and nine metabolites were identified. It is the significant characteristics of flavonoid glycosides that the aglycones of isohamnetin and quercetin were absorbed into blood and their conjugations of glucuronide, sulfates and glucosides were also detected but not the parent compounds. The developed method was simple, reliable and sensitive, which revealed that it will be appropriate for rapid analysis and identification the characterization of main bioactive components and their metabolites in biosamples. Though the structures of some metabolites could not be determined conclusively by $LC–MS^n$, the present method will be helpful to a better understanding for the *in vivo* metabolism of SFZYD and it is valuable and dependable for the further study of the metabolism of complex systems such as herbal extracts or TCM formulae.

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